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Alterations in Allergenic Potential of *Ambrosia Artemisiifolia* L. Pollen by Abiotic (temperature and NO₂) and Biotic (plant-associated fungi) Factors

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II. ABBREVIATIONS

Acronym	Definition
AgNO$_3$	Silver nitrate
AlCl$_3$	Aluminum chloride
AllFam	Database for classifying allergens into protein families
ANOVA	Analysis of variation
BLAST	Basic local alignment search tool
BPB	Bromophenol blue
DAS	Days after sowing
DDT	DL-Dithiothreitol
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assays
EtBr	Ethidium Bromide
HCl	Hydrogen chloride
IgE	Immunoglobulin E
IPG	Immobilized Ph Gradient
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
Kan$^+$	With kanamycin sulfate
KCl	Potassium chloride
KH$_2$PO$_4$	Potassium phosphate monobasic
KNO$_3$	Potassium nitrate
LB	Luria-Bertani
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MEA	Malt extract agar
MgCl$_2$·6H$_2$O	Magnesium chloride hexahydrate

MgSO$_4\cdot$7H$_2$O	Magnesium sulfate heptahydrate
Na$_2$CO$_3$	Sodium carbonate
Na$_2$HPO$_4$•7H$_2$O	Sodium monohydrogen phosphate heptahydrate
Na$_2$S$_2$O$_3$	Sodium thiosulfate
NaCl	Sodium chloride
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NaH$_2$PO$_4$	Monosodium phosphate
NaH$_2$PO$_4$•H$_2$O	Sodium dihydrogen phosphate monohydrate
NaNO$_2$	Sodium nitrite
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
NCBI	National Center for Biotechnology Information
NH$_4$HCO$_3$	Ammonium bicarbonate
NO$_2$	Nitrogen dioxide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
rAmb a	Recombinant *Ambrosia* allergen in common ragweed
RCP	Representative Concentration Pathways
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SNA	Synthetic nutrient agar
SOD	Superoxide dismutase
TAE	Tris-Acetate-EDTA
TBS	Tris-buffered saline
Tris-HCl	Tris(hydroxymethyl)aminomethane
UPLC-MSMS	Ultra-performance liquid chromatography-tandem mass spectrometry

III. SUMMARY

The spread of common ragweed (*A. artemisiifolia*) due to global warming poses a health threat through its allergenic pollen, yet there is limited research on how rising temperatures and their interactions with air pollution affect its allergenic potential. This study examines the impact of elevated temperature ($+4\ ^{\circ}C$) and elevated NO_2 in two Bavarian regions with different climates, simulating future conditions. Ragweed plants were grown under controlled conditions to assess the effects on plant growth, pollen release phenology, pollen allergenicity of aqueous pollen and the production of allergenicity related compounds in pollen. Additionally, this study explores the proteomic changes in pollen, focusing on stress responses, metabolic processes, and expression of allergenic proteins.

The findings included that 1), warmer temperatures boosted plant growth, reduced pollen release per male inflorescence while extended the release period, but beyond certain limits, the plant growth promotion diminished. Elevated NO_2 promoted pollen release, especially under higher temperatures. 2), both elevated temperature and elevated NO_2 affected pollen flavonoid content, with temperature having a greater impact on flavonoid composition. Elevated temperature reduced the ability of pollen flavonoids to inhibit allergen-IgE binding. 3), elevated temperature and NO_2, independently and synergistically, increased the allergenicity of aqueous protein extracts and the levels of NADPH and NADH oxidases in pollen, with the highest levels observed under combined treatments. Synergistic effects between elevated temperature and elevated NO_2 on increasing H_2O_2 were also observed. 4), Amb a 1 and its isoforms contributed the most to the detected allergenicity in aqueous protein extracts. Proteins such as peptidyl prolyl cis trans isomerase, calmodulin, and calreticulin, may also contribute to ragweed pollen allergenicity. 5), proteomic analysis revealed that in the relatively warmer climate, elevated temperature and its combination with elevated NO_2, significantly induced Amb a 1 and its isoforms while reduced several other allergenic proteins involved in cell wall biogenesis, and degradation. 6), *in vitro* nitration experiments implied possible structural conformational changes in allergens, potentially altering the allergenicity of ragweed pollen.

In conclusion, the combination of high temperatures and air pollutants like NO_2 exacerbate ragweed pollen's health risks, particularly in warmer regions. Strategies to reduce NO_2 levels and curb ragweed spread are vital for mitigating its allergenic impacts.

As biotic factor fungi associated with leaves of outdoor-grown ragweed plants were assessed by ITS barcoding and to get isolates for examining their impact on ragweed pollen allergenicity. The fungal community on ragweed leaves comprised Ascomycota and Basidiomycota, and certain fungal taxa, such as *Podospora*, *Bipolaris*, and *Sporobolomyces*, exhibited preferences for specific leaf layers, suggesting spatial niche differentiation within the plant. Among the tested fungal isolates, *Beauveria bassiana* appeared to contain allergenic proteins that interact with commercial ragweed pollen to enhance allergic responses, underscoring the need to identify specific allergenic components within *B. bassiana*. Other fungal isolates showed no obvious IgE binding, highlighting variability in allergenic potential and the need for further research under diverse conditions.

IV. LIST OF FIGURES AND TABLES

1: Chapter - INTRODUCTION

1.1 Impacts of common ragweed (*Ambrosia artemisiifolia* L.)

1.1.1 Common ragweed as invasive and allergenic plant

1.1.1-1 Characteristics of common ragweed

Common ragweed (*Ambrosia artemisiifolia* L., Asteraceae), is an annual herbaceous plant of high environmental and health concern. As a serious weed in many areas world-wide it causes yield losses in agricultural land (Alberternst et al., 2016; Essl et al., 2015; Makra et al., 2015; Montagnani et al., 2017; Simard et al., 2020), and, moreover, poses a health risk because of its high pollen allergenicity (Gard, 2012; Gentili et al., 2018; McGoey and Stinchcombe, 2021).

Ragweed plants are characterized by a high reproductive capacity with 70,000 and 100,000 seeds per plant annually. The seeds are persistent for up to 30 to 40 years and are able to germinate in a wide range of soil conditions even under nutrient-deficient soils or barren wastelands (Baskin and Baskin, 1980; Karrer, 2016; Toole and Brown, 1946). Consequently, ragweed plants are able to thrive in various habitats, including wastelands, roadsides, arable fields (Burge and Rogers, 2000; Claude et al., 2007; Essl et al., 2009; MacKay and Kotanen, 2008; Milakovic et al., 2014; Pinke et al., 2013; Webster and Nichols, 2012), railway embankments, landfill sites, construction sites (Bullock et al., 2012), and urban ruderal habitats. Notably, these locations are typically in close proximity to human living environments, and include urban areas and high traffic sites with prominent air pollution (Ziska et al., 2003).

It has been known that common ragweed is a thermophilic plant, with a wide temperature range of growth starting at 0.9 °C and an upper limit at 40 °C (Deen et al., 1998; Deen et al., 2001). Moreover, an increase in temperature within the range of 8 to 31.7 °C stimulates the growth of ragweed plants (Bazzaz, 1974; Gentili et al., 2019). This characteristic of ragweed plants may imply a favorable response to global warming, as suggested by previous studies indicating that thermophilic plants tend to exhibit increased growth rates in warmer conditions.

1.1.1-2 Invasion of common ragweed in Europe

Since the introduction of ragweed to Europe from North America during the second half of the 19th century (Járai-Komlódi and Juhász, 1993; Makra et al., 2005) it rapidly spread in the late 20th century in over 30 European countries (Bullock et al., 2012; Carosso and Gallesio, 2000; Comtois, 1998; Hrabovský et al., 2016; Járai-Komlódi and Juhász, 1993; Makra et al., 2005; Makra et al., 2014; Reznik, 2009; Rodinkova et al., 2012).

In Germany, the main expansion of the invading ragweed populations from eastern and southern Europe occurred between 2000 and 2007 (Alberternst and Nawrath, 2008; Buters et al., 2015; Hall et al., 2020; Nawrath and Alberternst, 2014). Control of ragweed has only partly been successful. For example, in Bavaria continuous monitoring and eradication measures resulted only in a reduced but not a full control of ragweed expansion (Alberternst et al., 2016; Buters et al., 2015; DPA and Local, 2020; Höflich et al., 2016).

1.1.2 Impacts of common ragweed on human health

The most direct danger to humans is the ragweed pollen emitted during flowering. Upon inhalation of airborne ragweed pollen, the immune system of individuals sensitized to ragweed pollen reacts by releasing chemical mediators, including histamine and cytokines, in significant quantities (Schindler, 1993) (Figure 1 - 1). As a result, affected individuals show a range of allergic reactions, including hay fever, running nose and itchy eyes, even severely allergic asthma and rhinitis (Boulet et al., 1997; DellaValle et al., 2012; Jones et al., 2019).
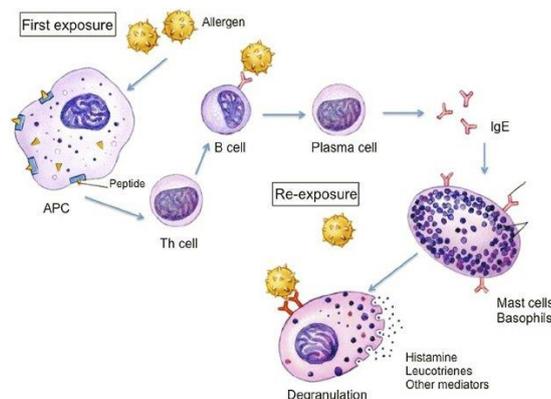
Figure 1 - 1: Schematic diagram showing type 1 hypersensitivity reactions (Pedrosa et al., 2014). APC = antigen-presenting cell; Th cell = helper T cell; B cell = bursa-derived cell; IgE = Immunoglobulin E.

Ragweed pollen allergy has increasingly garnered global attention. Within its native North American range, air quality reports from 1994 to 2010 revealed a discernible pattern indicating earlier and extended pollen seasons and increased airborne pollen concentrations of common ragweed (Breton et al., 2006; Chan-Yeung et al., 2010; Frenz, 1999; White and Bernstein, 2003; Zhang et al., 2015), and also an increase in ragweed sensitized patients jumping from 15 to 26% (Arbes-Jr et al., 2005; Bousquet et al., 2008).

Also in certain Asian countries, the prevalence of ragweed pollen allergy has been documented, with observed frequencies of 11.8% among 170 Nepali patients (Monika et al., 2020) and 8.66% among 4203 Chinese children (Wang et al., 2020). Thus world-wide, the human population sensitized to ragweed pollen is rising (Lake et al., 2017). In Europe, ragweed pollen allergy affects over 10 million individuals, leading to annual economic losses of approximately Euro 7.4 billion (Ariano et al., 2015; Heinzerling et al., 2009; Schaffner et al., 2020). The prevalence of *Ambrosia* pollen sensitization varies by country, ranging from the highest value reached 53.8% in Hungary to 2.3% in Finland (Ariano et al., 2015). Moreover, the clinical significance of ragweed pollen has risen, establishing it as a major contributor to hay fever (Beggs, 2015; Burbach et al., 2009; Celenk and Malyer, 2017; Oh, 2018; Schmidt, 2016). For instance, the frequency of ragweed pollen sensitization in Austria increased from 8.5% to 17.5% between 1997 and 2007 (Hemmer et al., 2011). In addition, a consistent annual rise in the number of patients with ragweed pollen allergies in Romania from 2014 to 2018 was observed, accompanied by an expanding geographic range (Leru et al., 2019). Furthermore, the rate of sensitization to ragweed pollen in Germany witnessed a significant rise, surging from 21.4% between 1998 and 2007 to 36.3% between 2008 and 2017 (Forkel et al., 2020). Collectively, these observations highlight the widespread occurrence and severity of ragweed pollen allergies (Bocsan et al., 2019; Leru et al., 2022; Leru et al., 2019). It is noteworthy that Bavaria, situated in southern Germany, possesses one of the most substantial recorded ragweed populations in the country (Bundesamt, 2021), with 20 - 30% of allergy patients already exhibiting sensitivity to ragweed pollen (Buters et al., 2015; Ruëff et al., 2012).

The increasing prevalence of ragweed pollen allergies is not only linked to the unrestrained spread of ragweed plants but also to the long-distance transport of ragweed pollen to regions

devoid of ragweed plants (Lorenzo et al., 2006; Menzel et al., 2021). Notably, pollen remains active after undergoing long-distance transport and can lead to sensitization (Celenk, 2019). The exceptionally high concentrations of *Ambrosia* pollen in Leicester (United Kingdom) and Leiden (Netherlands) on September 4 and 5, 2014, were potentially caused by pollen transported from source areas on the Pannonian Plain and Ukraine (de Weger et al., 2016). Similar long-distance transport of *Ambrosia* pollen has been extensively documented, including routes from Eastern Europe to Italy (Cecchi et al., 2007), from Western Europe to Hungary (Makra et al., 2007), and from southern regions like Ukraine to Poland (Kasprzyk et al., 2011; Stach et al., 2007; Stępalska et al., 2020).

Therefore, the locally established ragweed populations and those in neighboring countries collectively suggest that the severity and incidence of ragweed allergies for example in Bavaria continue to increase (DPA and Local, 2020).

1.2 Allergenic potential of ragweed pollen

1.2.1 Increased pollen allergic symptoms correlate with pollen exposure

Prior studies have established a positive correlation between the immune response and the quantity of inhaled pollen (Jäger, 2000). This suggests a positive association between the concentration of allergenic pollen, and the severity of symptoms at a population level (Ariano et al., 2015; Schmidt, 2016; Ziska et al., 2019). As mentioned above, pollen amounts are closely linked to expansion of ragweed populations. Additionally, enhanced plant growth including longer male inflorescences contribute to an increase in pollen release (Alberternst et al., 2016; Essl et al., 2015; Gentili et al., 2019; Montagnani et al., 2017; Zhao et al., 2017b). Moreover, a promotion of its reproductive growth leading to a moderate increase in pollen release has also been shown, when environmental factors (such as NO_2) impede plant growth (Zhang et al., 2020a; Zhao et al., 2016; Zhao et al., 2017b). Hence, a strong correlation exists between environmental factors, responses of ragweed plants and the quantity of pollen production as well as pollen exposure.

Beyond the impact of pollen release on pollen allergy symptoms, an early onset and prolonged duration of the pollen season can increase pollen exposure, potentially worsening

allergy symptoms (D'Amato et al., 2007; Gentili et al., 2019; Rasmussen et al., 2017; Ziska et al., 2011). Clinical and epidemiological data establish associations between ragweed pollen exposure and patterns of allergic sensitization (Ariano et al., 2015; Bocsan et al., 2019; Chen et al., 2018a; Leru et al., 2019).

1.2.2 Allergenicity related compounds in ragweed pollen

In addition to the impacts of temporal and spatial variations in pollen exposure, the physicochemical properties of ragweed pollen can directly determine its allergenicity. Ragweed pollen, a major contributor to fall pollen allergies, contains water-soluble proteins that can be rapidly released upon contact with humans, eliciting allergic hypersensitivity reactions (Oh, 2018; Volcheck, 2009). Allergy sufferers exhibit varying reactions to minor allergens, and thus, immunoblotting assays, employing multiple antibodies within pollen-specific patient sera mixtures, are utilized in diagnostics for a comprehensive evaluation of potential allergenicity to pollen allergens (Chen et al., 2012; Gómez-Esquivel et al., 2021; Grijincu et al., 2023; Morales et al., 2012a; Vakili-Moghaddam et al., 2019; Wopfner et al., 2008), particularly to all ragweed allergens (Cheng et al., 2023; Gentili et al., 2019; Oberhuber et al., 2008; Zhao et al., 2016). Alternatively, increased expression of transcripts or quantities of major allergens, such as Bet v 1 for birch pollen (Ahlholm et al., 1998; Beck et al., 2013; Buters et al., 2008; Seutter-Von Loetzen et al., 2014) and Amb a 1 for ragweed pollen (Choi et al., 2018; El-Kelish et al., 2014; Kanter et al., 2013; Pasqualini et al., 2011; Singer et al., 2005; Zhao et al., 2017a) have been considered as indicators of potential allergenicity.

In addition to allergenic proteins, immunomodulatory compounds have been identified in pollen, participating in the allergic inflammatory response. These include substances that stimulate and enhance allergic inflammation responses, such as reactive oxygen species (ROS)-related compounds (Bacsi et al., 2005; Boldogh et al., 2005; Pasqualini et al., 2011; Pazmandi et al., 2012), lipids (Bashir et al., 2012; Dahl, 2018), pollen-derived adenosine (Gilles et al., 2012; Mueller et al., 2016; Wimmer et al., 2015), and non-allergenic pollen-derived substances (Buhner et al., 2023; Codina and Lockey, 2017; Gilles et al., 2012). Additionally, compounds that mitigate or suppress allergic reactions, like flavonoids, have been identified (Reinmuth-Selzle et al., 2017).

1.2.2-1 Allergens in ragweed pollen

Pollen serves as a significant source of proteins (Komosinska-Vassev et al., 2015), constituting one of the primary sources of respiratory allergens in humans (Howlett et al., 1981; Negrini, 1992). The latest information from Allfam (http://www.meduniwien.ac.at/allergens/allfam/) identifies the top five families of pollen allergens as profilin, prolamin, expansins, EF-hand domains containing proteins, and Ole e 1 – related proteins (Radauer et al., 2014). These proteins play indispensable roles in the growth and development of pollen (Radauer et al., 2008; Songnuan, 2013; Stanley, 1971), particularly in the crucial processes of germination and fertilization of pollen tubes (Chae and Lord, 2011; Ischebeck et al., 2014). However, within the same protein family, not all members elicit allergic reactions, indicating variations in their allergenicity. These differences arise from structural variations, including distinct amino acid sequences or conformational changes, as well as disparities in the composition and accessibility of their allergenic epitopes (Dall'antonia et al., 2014; Ivanciuc et al., 2009; Seidler et al., 2024; Songnuan, 2013; Stadler and Stadler, 2003; Traidl-Hoffmann et al., 2009). Moreover, numerous allergens, for instance, Amb a 1 in ragweed pollen (Wolf et al., 2017; Wopfner et al., 2009), Bet v 1 in birch pollen (Grutsch et al., 2017; Kofler et al., 2012; Smole et al., 2010; Wagner et al., 2008), and some other respiratory allergens (Caraballo et al., 2020; Park et al., 2002), exist in multiple isoforms that exhibit variations in allergenicity, despite sharing comparable molecular sizes, similar or identical biological functions, and similar amino acid sequences (Pomés et al., 2018; Radauer et al., 2014). The production of protein isoforms may be caused by alternative mRNA splicing, variable promotor usage, as well as post translational modifications (Smith and Kelleher, 2013, 2018). To date, a total of 11 well-characterized allergens, featuring diverse isoforms present in ragweed pollen, have been identified and described in the WHO/IUIS allergen nomenclature database (Table 1 - 1) (Organization and Societies, 1994). The identification of new allergens is still ongoing.

The IgE reactivity, serving as an indicator of allergenicity, varies greatly among the 11 identified ragweed pollen allergens. For instance, Amb a 1, the major ragweed allergen, elicited a response in over 90% of the ragweed-sensitized population (Adolphson et al., 1978; Pichler et al.,

2015). Additionally, Amb a 11 (Bouley et al., 2015) and Amb a 12 (Zhao et al., 2016), two other allergens, demonstrated sensitization rates as high as 66% and 68%, respectively, suggesting their crucial role as allergenic components in pollen. The remaining 8 allergens were considered minor allergens, with sensitization rates ranging from 30% - 50% for Amb a 3 (Adolphson et al., 1978), 20% - 40% for Amb a 4 (Léonard et al., 2010), 10% - 15% for Amb a 5 (Ghosh et al., 1993; Metzler et al., 1992), Amb a 9 and Amb a 10 (Wopfner et al., 2008), 20% - 35% for Amb a 6 (Roebber et al., 1983) and Amb a 8 (Wopfner et al., 2008), 15% - 20% for Amb a 7 (Roebber and Marsh, 1991), respectively.

Table 1 - 1: Known allergens in common ragweed pollen (Adolphson et al., 1978; Bouley et al., 2015; Ghosh et al., 1993; Groeme et al., 2016; Léonard et al., 2010; Metzler et al., 1992; Pichler et al., 2015; Roebber et al., 1983; Roebber and Marsh, 1991; Wopfner et al., 2008; Zhao et al., 2016)

Allergens	IgE-reactivity (%)	Molecular Weight (kDa)	Description
Amb a 1	> 90	38	Pectate lyase, with 5 main isoforms; Involved in the pectin catabolic process
Amb a 3	30 - 50	11	Plastocyanin; Copper-binding proteins;
Amb a 4	20 - 40	30	Defensin-like protein linked to polyproline-rich region
Amb a 5	10 - 15	5	Cystein-rich protein
Amb a 6	20 - 35	10	Non-specific lipid transfer protein type 1; Involved in wax or cutin deposition in the cell walls; Panallergen
Amb a 7	15 - 20	12	Plastocyanin; Copper-binding proteins; partly known sequence
Amb a 8	20 - 35	14	Profilin; Actin-binding proteins; Panallergen
Amb a 9	10 - 15	9	Polcalcin; with 2 EF-hand domains; Calcium-binding proteins; Panallergen
Amb a 10	10 - 15	17	Polcalcin-like protein, 4 EF-hand domains; Calcium-binding proteins; Panallergen
Amb a 11	50 - 66	37	Cysteine protease
Amb a 12	41 - 68	48	Enolase; Involved in the glycolytic process

The IgE reactivity for a specific allergen is driven by various factors, such as its amino acid sequence or structure (Dall'antonia et al., 2014; Ivanciuc et al., 2009; Ivanciuc et al., 2003; Stadler and Stadler, 2003), indicating potential variations in allergenicity among distinct isoforms of the same allergen. For instance, to date, there are five isoforms of Amb a 1, with their amino acid

sequence identities ranging from 63% to 86% (Organization and Societies, 1994). Immunological characterization showed varying levels of IgE binding activities among these isoforms, with Amb a 1.01 displaying the highest allergenic potential (Wolf et al., 2017). In addition, the timing of allergen expression and release influences the quantity of allergen, subsequently impacting the intensity of the allergic reaction and, ultimately, the allergenic potential of the allergen (Oh, 2022; Tegart et al., 2021; Zhang and Steiner, 2022).

Generally, the substantial expression and release of allergenic proteins in pollen are associated with vital physiological functions essential for pollen. For instance, Amb a 1, a 38 kDa protein belonging to the pectate lyase protein family (Adolphson et al., 1978; Oberhuber et al., 2008; Wopfner et al., 2005). Pectate lyases are enzymes involved in plant cell wall organization by degrading pectines, which are important for pollen growth (El-Kelish et al., 2014; Hugouvieux-Cotte-Pattat et al., 2014). Moreover, the minor allergens, including Amb a 6 (10 kDa), Amb a 8 (14 kDa), Amb a 9 (9 kDa), and Amb a 10 (17 kDa), pertain to the families of non-specific lipid transfer proteins, profilins, and polcalcins, respectively. These protein families, are recognized as pan-allergens, which are ubiquitous allergenic proteins in all plant families, playing essential roles in fundamental processes across organisms (Hauser et al., 2010; McKenna et al., 2016; Wopfner et al., 2008). For example, non-specific lipid transfer proteins could play roles in depositing wax or cuticle in the cell walls, contributing to the ability of plants to withstand stress stimuli, including drought and heat stress (El-Kelish et al., 2014; Gao et al., 2022; Liu et al., 2015). Additionally, the secretion of this protein was found to guide pollen tubes down the transmitting tract (Radauer and Breiteneder, 2006). Regarding profilins, the binding of this protein to actin regulates actin microfilament polymerization and depolymerization (Kandasamy et al., 2007). This regulation is essential for the polar growth of pollen tubes, which necessitates the circulation of vesicles in large numbers along actin microfilaments (Songnuan, 2013). As for polcalcins with EF-hand domains, this calcium-binding protein mediates calcium signaling pathways that are necessary for pollen tube growth (Steinhorst and Kudla, 2013). Furthermore, Amb a 12, a 48 kDa protein, is assigned to the enolase family (Zhao et al., 2016), a potential pan-allergen in pollen and plant foods (Grijincu et al., 2023). Enolase plays a crucial role in the glycolytic pathway, a pivotal route for energy production in organisms (Avilán et al., 2011), and it can mitigate the elevated energy

requirements during both pollen development and pollen tube growth (Chen et al., 2018b; Selinski and Scheibe, 2014; Yue et al., 2014).

1.2.2-2 Allergic inflammation enhancers

ROS-related substances, such as NADPH oxidase and hydrogen peroxide (H_2O_2), released during pollen hydration have been shown to increase ROS levels in epithelial cells and mouse conjunctival cells, thereby inducing allergic airway inflammation (Bacsi et al., 2005; Boldogh et al., 2005; Pasqualini et al., 2011; Smiljanic et al., 2017; Swindle and Metcalfe, 2007; Van-Der Vliet, 2011; Wang et al., 2009). It is known that ROS usually refers to a reactive form of oxygen, such as superoxide anion ($\cdot O_2^-$), H_2O_2, and hydroxyl radical ($\cdot OH$) (Krumova and Cosa, 2016; Madkour, 2019). It is generally suggested that the basal levels of ROS generated under favorable environmental conditions are not only non-damaging to cells (Foyer and Noctor, 2005), but also play a limited role in plant developmental processes (Mhamdi and Van-Breusegem, 2018) or are linked to the regulation of morphogenetic processes associated with phytohormones such as cytokinins and auxins (Xia et al., 2015). Extensive research has shown that ROS serve as signaling molecules in plants, regulating various physiological processes such as pollen tube growth (Kaya et al., 2014; Kim et al., 2021b; Potocký et al., 2007), root development (Eljebbawi et al., 2021; Manzano et al., 2014; Swanson and Gilroy, 2010), and flower senescence in plants (Cavaiuolo et al., 2013; Rogers and Munné-Bosch, 2016; Rogers, 2012).

However, stressful environmental conditions, such as drought, heat, and salt stress (Sachdev et al., 2021; Singh et al., 2022; You and Chan, 2015), can excessively accelerate cellular ROS concentrations, ultimately leading to oxidative stress (Gill and Tuteja, 2010). This oxidative stress can cause damage to membrane lipids, proteins, and nucleic acids, and eventually results in cell death in plants (Baxter et al., 2014; Huang et al., 2019; Jaleel et al., 2009). To counteract the excess production of ROS, plants trigger adaptive responses to environmental challenges. These responses involve the utilization of antioxidant enzyme systems such as superoxide dismutase (SOD) (Hayyan et al., 2016; Wang et al., 2018), catalase (CAT), and peroxidase (POD) (Almagro et al., 2009; Nokthai et al., 2010), as well as non-enzymatic antioxidants like glutathione (GSH) (Khanna-Chopra and Chauhan, 2015; Kumar et al., 2012; Vaidyanathan et al., 2003; Xu et al.,

2006) and flavonoids (Basu et al., 2010; Chutipaijit et al., 2009; Gorgini-Shabankareh et al., 2021; Xu et al., 2008), to neutralize ROS. Under stress conditions such as drought (Basu et al., 2010; Gorgini-Shabankareh et al., 2021; Qayyum et al., 2021; Sharma and Dubey, 2005; Zlatev et al., 2006), heat (Chakraborty and Pradhan, 2011; Kaur et al., 2018; Khanna-Chopra and Chauhan, 2015; Kim et al., 2020; Kumar et al., 2012; Xu et al., 2006), or elevated O_3 (Eltayeb et al., 2007; Rao et al., 1996), these antioxidants are significantly increased alongside the level of intracellular ROS, promoting the conversion of ROS to harmless substances and thereby reducing oxidative damage to cells. Notably, during these reactions, the oxidative activity of NADPH oxidase produces superoxide ($-O_2^-$) ions, a major ROS, which are subsequently converted to H_2O_2 by superoxide dismutase (Marino et al., 2011; Sheng et al., 2014; Vignais, 2002). Furthermore, at the transcriptional level, ROS play a pivotal role in regulating gene expression in plants. They mediate the activation or suppression of transcription factors, thereby modulating the expression of genes necessary for adaptation to environmental challenges (Zandi and Schnug, 2022), such as the activation of defense genes (Exposito-Rodriguez et al., 2017) and the jasmonic acid pathway (Zhang et al., 2020c) by H_2O_2. Additionally, ROS regulate the expression of cell wall-related genes, facilitating cell wall remodeling, which encompasses both stiffening and loosening, to bolster plant tolerance to stress (Mase and Tsukagoshi, 2021).

Environmental conditions are known to influence the formation of ROS-related compounds in many plant species, including common ragweed, which showed increased levels of ROS-related compounds like NAD(P)H oxidase and H_2O_2 in pollen when exposed to elevated O_3 (Pasqualini et al., 2011). Furthermore, ragweed pollen from Metropolitan region in Madrid with high levels of NO_2 and SO_2 had higher levels of NADPH oxidase activity and H_2O_2 content than pollen from a less polluted area (Lucas et al., 2019). However, it remains unclear whether changes in NAD(P)H oxidase or H_2O_2 levels in ragweed pollen occur at elevated temperatures or at elevated temperatures combined with NO_2, and how these changes might impact individuals suffering from ragweed allergies.

In addition to ROS-related substances, lipids in ragweed pollen may also promote allergic inflammatory reactions (Bashir et al., 2012). Pollen lipids were shown to be ligands to allergenic proteins and induce a type 2 reaction in effector cells in the mammal immune system (Dahl, 2018;

Traidl-Hoffmann et al., 2002). Moreover, the presence of pollen-derived adenosine in ragweed pollen extracts significantly enhanced ragweed pollen-induced allergic airway inflammatory responses (Gilles et al., 2011; Wimmer et al., 2015). In addition, the presence of pollen-derived non-allergenic substances in ragweed pollen extracts, including the low-molecular-weight compound and phytoprostane E1, may regulate IgE-mediated allergy responses by inducing IgE production in Th2-primed B cells (Oeder et al., 2015). Future in-depth studies of pollen-derived adenosine and pollen-derived non-allergic substances will facilitate the development of therapeutic strategies to limit these allergen-independent pathways.

1.2.2-3 Allergic inflammation reducers

Flavonoids, a class of plant secondary metabolites, exhibit widespread occurrence in nature and are prevalent in various plant organs, with pollen standing out as a particularly rich source (Campos et al., 1997; El-Ghouizi et al., 2023). These compounds are known for their diverse biological functions, including antioxidant activity (Sina et al., 2015), anti-inflammatory effects (Hämäläinen et al., 2007; Muhlemann et al., 2018), and anti-allergic properties (Gentili et al., 2019; Grutsch et al., 2014). These properties lead to speculation that flavonoids may mitigate the allergenic potential of pollen.

There are more than 4000 different compounds that make up flavonoids (Dugo et al., 2005). The content and composition of flavonoids in pollen are sensitive to a wide range of environmental factors, including but not limited to: temperature (Gentili et al., 2019; Mohammadrezakhani et al., 2018), photoperiod (Taylor, 1965), air pollutants (SO_2, NO_2, CO, hydrocarbons, and atmospheric particulate matter) (Rezanejad, 2009; Robles et al., 2003), ozone (O_3) (Dixon and Paiva, 1995), drought (Ballizany et al., 2012; Kang et al., 2011; Yuan et al., 2012), and CO_2 (El-Kelish et al., 2014).

Recent research indicates two mechanisms through which flavonoids modulate allergic responses. On one hand, flavonoids potentially diminish the intensity of allergic reactions by reducing IgE production, mainly inhibiting the release of histamine and cytokines from mast cells (Kimata et al., 2000; Mlcek et al., 2016; Wei et al., 2012; Zhang et al., 2018). On the other hand, flavonoids potentially disrupt the binding of IgE to pollen allergens (Romano et al., 1996),

consequently diminishing allergenicity. This hypothesis gains further support from several studies indicating that glycosylated quercetin- and kaempferol-type flavonoids, along with naringenin, act as physiological ligands for Bet v 1, the major birch allergen (Grutsch et al., 2014; Kofler et al., 2012; Seutter-Von Loetzen et al., 2014). The flavonoid-Bet v 1 complex formed may cover allergen epitopes.

In ragweed pollen, it is known that within a certain temperature range, the content of flavonoids decreases with increasing temperature (Gentili et al., 2019). Additionally, drought, CO_2, and NO_2 impact the contents of some flavonoid derivatives (El-Kelish et al., 2014; Zhao et al., 2017b). Moreover, a direct role for rutin in pollen allergenicity modulation was tested, showing an inverse relationship between the total flavonoid concentration and overall allergenicity (Gentili et al., 2019). However, it has not been studied whether there is a direct interference of flavonoids with IgE binding to specific ragweed allergens. Furthermore, it remains unclear whether elevated temperature and NO_2 modify flavonoid amounts and composition in a way that alters allergen-IgE binding in ragweed.

1.3 Environmental factors influencing plant growth traits and allergenic potential of ragweed pollen

Environmental changes affect the growth of ragweed plants through various factors related to climate change, including warming, and precipitation (IPCC, 2013, 2022). Notably, changes in the composition of ambient air, such as rising carbon dioxide (CO_2) levels, and increases in air pollutants like nitrogen oxides (NO_X), sulfur oxides (SO_X), and O_3, also make significant contributions to climate change (Hasanuzzaman et al., 2016; Hasanuzzaman et al., 2017; Zhang and Steiner, 2022). These environmental changes have been shown in ragweed plants to affect seed yield, weight, and germination (Bae et al., 2016; Farooq et al., 2019; Leskovsek et al., 2012; Zhao et al., 2017b), as well as survival tolerance ranges for seedling growth (Deen et al., 1998; Deen et al., 2001; Gentili et al., 2019; Leskovsek et al., 2012; Onen et al., 2017). Phenology-related traits of ragweed plants such as the duration of pollen release (Deen et al., 1998), pollen production and metabolism in ragweed pollen (El-Kelish et al., 2014; Kanter et al., 2013; Zhao et

al., 2017b) are also affected. Among these effects, alterations in the duration of pollen release, along with changes in pollen production, may influence pollen allergenicity by affecting pollen inhalation and exposure in individuals allergic to ragweed pollen (D'Amato et al., 2007; Gentili et al., 2019; Leiblein-Wild and Tackenberg, 2014; Rasmussen et al., 2017; Ziska et al., 2011; Ziska et al., 2019). Moreover, enviromental changes can modify pollen allergenicity by upregulating allergen expression in pollen (Choi et al., 2018; Kanter et al., 2013; Singer et al., 2005; Zhao et al., 2017a; Ziska et al., 2011) or chemically altering the protein structure of translated allergens (Kim et al., 2014; Lucas et al., 2019; Poschl and Shiraiwa, 2015; Zhao et al., 2016).

1.3.1 Climate change and relation to pollen allergenicity

1.3.1-1 Effects of climate change on allergenic plants

Climate change refers to long-term shifts in temperature and precipitation (IPCC, 2013, 2022), which are the main drivers of plant growth and pollen development (Gray and Brady, 2016; Hatfield and Prueger, 2015; Zhang and Steiner, 2022). A previous study indicated that herbaceous plants, such as grasses and ragweed, are highly sensitive to climate, especially ambient temperature and water availability, compared to other taxa (Matyasovszky et al., 2015). However, the response to elevated temperatures and drought during the growing season varied among different grass species. This has been seen in the case that elevated temperatures and drought have been observed to lead to lower total pollen weight, allergen content, and protein content in pollen extracts of two timothy grasses, while these changes were not consistent across different species of perennial ryegrass (Jung et al., 2021).

Climate change exerts significant effects on the prevalence and severity of allergy by not only modulating the temporal (such as onset and duration of pollen release) and spatial (such as pollen load) magnitude of subject exposure to pollen (Choi et al., 2021; Levetin, 2021; Van-Vliet et al., 2002), but also affecting the allergenic potency of the pollen itself (Choi et al., 2021; Gentili et al., 2019; Zhao et al., 2016). For example, drought often associated with climate change, can significantly impact allergenic plants, including negatively affecting plant growth (Gusmao et al., 2012). For drought-treated ragweed plants, the risk of pollen exposure for pollen-sensitized

patients was reduced due to the suppression of ragweed pollen (El-Kelish et al., 2014). However, allergic symptoms may not be alleviated, due to increased levels of both, the major pollen allergen Amb a 1, and oxidative stress indicators (El-Kelish et al., 2014).

In addition, CO_2 stands prominent among major greenhouse gases, with its escalating concentrations intricately linked to global warming and climate change (NOAA, 2023; Organization, 2022). Climate monitoring revealed that elevated CO_2 levels in the atmosphere extended the duration of allergenic pollen seasons and augmented pollen loads, consequently amplifying pollen allergenicity (Kim et al., 2021a; Schmidt, 2016). Chamber experiments demonstrated that elevated CO_2 concentrations, exceeding 600 ppm, increased Amb a 1 levels in ragweed pollen (Choi et al., 2018). At 700 ppm CO_2, increasing pollen yield (Kelish et al., 2014) and transcripts encoding allergenic ragweed proteins (Amb a) were found (El-Kelish et al., 2014). A follow-up *in vivo* study in mouse indicated that pollen from ragweed plants cultivated under 700 ppm CO_2 induced more pronounced allergic lung inflammation compared to those grown under 380 ppm CO_2. Moreover, increased allergic symptoms correlated with the interplay of multiple metabolites in the pollen (Rauer et al., 2021).

Moreover, ozone (O_3) ranks among major contributors to air pollution and global warming (EPA, 2023; Organization, 2022). Elevated environmental ozone levels enhanced the allergenicity of birch pollen, evident in escalated allergen content (Beck et al., 2013). Under controlled conditions, elevated levels of O_3 fumigation during the plant growing season did not induce Amb a 1 content but upregulated stress-related transcripts in ragweed pollen (Kanter et al., 2013). Conversely, an elevation in allergen content was observed in rye (*Secale cereal*) pollen (Eckl-Dorna et al., 2010). *In vitro* O_3 treatment of ragweed pollen for seven consecutive days did not elevate Amb a 1 levels but stimulated ROS-generating NAD(P)H oxidase, potentially heightening ragweed pollen allergenicity (Pasqualini et al., 2011).

1.3.1-2 Effects of temperature on pollen allergenicity

In addition to the aforementioned factors associated with climate change, global warming—referring to sustained increase in global temperatures—has the potential to trigger a greater health problem with increasing individuals in the European region in the forthcoming decades

(Rasmussen et al., 2017). However, the effects of elevated temperature on alterations in pollen allergenicity are intricate.

From the aspect of pollen season, analysis of long-term pollen data from 60 North American stations spanning from 1990 to 2018 reveals widespread advancements and prolongation of pollen seasons (+ 20 days) along with an increase in pollen concentrations (+ 21%) across North America. These changes are strongly correlated with observed warming trends (Anderegg et al., 2021). In addition, the temperature increase over the past two decades has already resulted in an earlier start to the ragweed pollen season in the northern hemisphere (Ziska et al., 2019). These monitoring results find validation in common garden experiments involving 38 European ragweed populations across Europe, highlighting a flowering season that starts up to 5 weeks earlier in warmer regions (Leiblein-Wild and Tackenberg, 2014).

Moreover, regarding the pollen load, a study revealed that the continuous warming trends observed across various climate regions of the United States between 2000 and 2010 contributed to an elevation in both the average peak daily and annual totals of airborne pollen counts during the same period (Seth and Bielory, 2021). A comparable outcome emerged in southern Germany, where a decade-long observation in Bavaria demonstrated that rising temperatures, particularly during the spring seasons, correlated with an escalation in pollen loads (Rojo et al., 2021).

Furthermore, at the molecular levels, pollen samples from monitoring research utilizing volumetric traps have shown a positive correlation between temperature and the concentration of Phl p 5 for the Poaceae family (Alan et al., 2018; Scevkova et al., 2020). In controlled field studies, birch pollen sampled from two common gardens with different daily mean temperatures during the growing season, demonstrated that higher temperature significantly induced the amount of Bet v 1 (Ahlholm et al., 1998). However, the absolute allergen content (primarily Phl p 1 and Phl p 5) of the timothy cultivars was consistently and significantly lower in the warmer treatments (Jung et al., 2021). In chamber experiments, ragweed plants grown under three constant thermal regimes revealed that an increase in temperature can enhance pollen sensitization by modulating Amb a 1-IgE binding activity (Gentili et al., 2019).

One of the reasons for the diverse responses of plant pollen to elevated temperatures is the challenge to isolate the interaction of other environmental factors with temperature in outdoor plant growth (Ahlholm et al., 1998; Buters et al., 2008; Morales et al., 2012b). Moreover, plant species and cultivars differ in temperature requirements and tolerances (Bita and Gerats, 2013; Chaudhary et al., 2020; Wahid et al., 2007). Furthermore, plant reactions to elevated temperatures are contingent upon factors such as the maximum temperature reached, the frequency of high-temperature events, and the diurnal timing of their incidence (Stella et al., 2021; Sun et al., 2019).

Elevated temperatures can accelerate metabolic processes and photosynthesis (Bernacchi et al., 2023; Lippmann et al., 2019; Nievola et al., 2017). However, despite their preference for warmer temperatures, thermophilic plants may still experience stress during periods of extreme heat (Bita and Gerats, 2013). Specifically, when subjected to temperatures at least 5 °C above their optimal growing conditions, higher plants manifest a distinct set of cellular and metabolic responses necessary for survival under such conditions (Choi et al., 2016; Guy, 1999), and this may also be reflected in pollen composition (Hinojosa et al., 2019).

1.3.2 Air quality indicator of NO_2 and its relation to pollen allergenicity

1.3.2-1 General information of NO_2

NO_2 or NO_x (a mixture of different gaseous nitrogen oxides including NO_2) pollution is one of the major pollutants in the atmosphere, created mostly by the direct mixing of air oxygen and nitrogen in combustion processes including transportation, industrial production, and energy production in thermal power stations (Energy and Change, 2013; EPA, 2023).

The current annual guideline value for atmospheric NO_2 set by the World Health Organization (WHO) is 40 µg m^{-3} (approximately 21.3 ppb) (Organization, 2022). NO_2 levels exceeding this threshold on an annual basis are deemed detrimental to health, and exposure beyond this limit should not occur more than 18 times in any calendar year. Additionally, the hourly limit for nitrogen dioxide is capped at 200 µg m^{-3} (approximately 106 ppb) (Parliament and Union, 2008). The Federal States and the German Environment Agency (UBA) have conducted an air quality assessment, utilizing data available since 2001 and from around 400 automatic

measuring stations across Germany (Agency, 2001). In 2018 and 2019, nearly 42% and 20% of measuring stations situated near road traffic recorded NO_2 concentrations exceeding the annual mean limit of $40\,\mu g/m^3$, respectively, signaling an overall decrease in NO_2 pollution across Germany (Nasr et al., 2020). However, in 2022, the annual mean value measured at the Munich station in Bavaria, close to road traffic, was $49\,\mu g/m^3$—a level significantly surpassing the WHO recommendation (Agency, 2023). Furthermore, point measurements in Munich during May and July 2016, the pre-pollination season of *Ambrosia*, registered NO_2 peaks of up to 106 ppb in the central city area of Munich (Zhu et al., 2020).

1.3.2-2 Impact of NO_2 on humans and plants

Nitrogen dioxide is associated with negative impacts on human health, as elevated levels of nitrogen dioxide may contribute to airway inflammation and impaired lung function, increasing susceptibility to respiratory infections (Hesterberg et al., 2009; Kelly, 2014). A dose-response study indicated that exposing asthma patients to high concentrations of nitrogen dioxide for short periods multiple times a day can increase allergic inflammation (Barck et al., 2005). Additionally, a case study conducted in mountainous regions suggested a positive correlation between cumulative exposure to nitrogen dioxide and influenza incidence (Zeng et al., 2020).

The impact of NO_2 exposure on plant growth is twofold, depending on the concentration and exposure time of NO_2 treatment, as well as the different plant species and growth stages studied. When the NO_2 concentration is within an optimal range, it can confer benefits to plant growth (Takahashi and Morikawa, 2014). For instance, *Arabidopsis* seedlings exposed to an environment with 10 - 50 ppb of NO_2 for several weeks exhibited more than a twofold increase in shoot biomass, total leaf area, and major mineral element content per seedling compared to unexposed seedlings (Takahashi and Morikawa, 2019a). Likewise, enhancements in fruit yield, flower quantity, biomass, major mineral element content, and cellular contents were observed in various horticultural plants subjected to 50 ± 10 ppb of NO_2 for 96 days (Takahashi et al., 2011), 50 - 200 ppb of NO_2 treatment for 5 - 6 weeks (Adam et al., 2008), or treated with 150 ± 50 ppb of NO_2 for 10 weeks (Takahashi et al., 2005), respectively. Conversely, if the NO_2 concentration or exposure time is excessive, oxidative stress is induced in plants, rendering NO_2 detrimental to their well-

being. The primary mechanisms through which nitrogen dioxide negatively impacts plants involve inducing oxidative stress, leading to cell membrane rupture, protein oxidation, and other cellular injuries (Takahashi and Morikawa, 2019b; Zhao et al., 2016).

1.3.2-3 Effects of NO_2 on pollen allergenicity

In comparison to temperature, the investigation into the influence of NO_2 on pollen allergenicity is predominantly centered on its potential to induce alterations in the expression of allergenic proteins in pollen, or to impact the structure of these proteins, thereby modifying the allergenicity of pollen (Barck et al., 2005; Cuinica et al., 2014; Zhao et al., 2016; Zhou et al., 2021).

In investigations concerning the impacts of environmental pollutants on pollen allergenicity, numerous pollen samples were gathered from outdoor locations characterized by elevated levels of air pollution. For instance, in a study of *Cassia siamea* pollen, pollutants, including NO_2, led to protein extracts from *Cassia siamea* pollen triggering increased production of total white blood cells and lymphocytes in animals, in parallel with elevated IgE antibody production and enhanced type I hypersensitivity reaction (Hinge et al., 2017). Similarly, in *Canna indica* (Majd et al., 2004) and *Platanus* (Zhou et al., 2021) pollen, air pollutants such as NO_2 heightened pollen sensitization by influencing pollen structure, protein release, and protein structure in pollen. Meanwhile, studies on ragweed pollen revealed that air pollutants, including high concentrations of NO_2, and SO_2, enhance pollen sensitization by simultaneously modifying allergen expression and expression patterns (Ghiani et al., 2012). However, the focus of these studies has been on exploring the influence of nitrogen dioxide, a major air pollutant, on pollen allergenicity, acknowledging that the impact of additional pollutants on pollen allergenicity cannot be disregarded.

In support of chamber experiments assessing the singular impact of NO_2, NO_2-exposed *Acer negundo* pollen exhibited higher protein content and greater allergen-IgE binding compared to non-exposed pollen (Sousa et al., 2012), a trend similarly observed for *Betula pendula*, *Ostrya carpinifolia*, and *Carpinus betulus* pollen (Cuinica et al., 2014). In ragweed pollen, application of 40 ppb or 80 ppb NO_2 throughout the plant growth season resulted in a significant increase in allergen-encoding transcripts with higher NO_2 concentration (Zhao et al., 2017a). Additionally,

under identical experimental conditions, two-dimensional immunoblots and LC-MS/MS detected more Amb a 1, demonstrating higher allergenic potential under elevated NO_2 conditions (Zhao et al., 2016), providing evidence of an augmented risk to human health *in vitro*.

1.3.2-4 Effects of NO_2 on protein structure

In terms of changes in protein structure, nitration of proteins, a post-translational modification (PTM) occurring through reactions with nitric oxide (NO) and other nitrogen oxides, has been shown to modify protein structure and function, indicating its involvement in diverse oxidative stresses (Chaki et al., 2011; Takahashi and Morikawa, 2019b) and inflammatory conditions (Corpas et al., 2009). Particularly noteworthy is its potential to augment the ability of proteins to induce immune responses (Ackaert et al., 2014; Gruijthuijsen et al., 2006; Karle et al., 2012). The alteration in the allergenic potential of a protein due to tyrosine nitration is a complex phenomenon influenced by several factors, including the specific protein, the degree of nitration, and individual immune responses (Corpas et al., 2009). Chemically nitrated Bet v 1 exhibited increased immunogenicity and allergenicity compared with untreated Bet v 1, primarily attributed to enhanced presentation of allergen-derived HLA-DR-associated peptides (Karle et al., 2012), structural changes with oligomerization (Ackaert et al., 2014), and the proliferation of specific T-cell lines which was considered as immune effects (Gruijthuijsen et al., 2006).

It has been shown that this PTM occurs typically involving the nitration of tyrosine residues (Radi, 2004), and can be predicted by computational predictors (Nilamyani et al., 2021; Xie et al., 2018). However, protein tyrosine nitration exhibits a selective nature, as evidenced by several studies indicating that not all exposed tyrosine residues or proteins undergo nitration (Ischiropoulos, 2003; Souza et al., 1999). Moreover, the abundance of tyrosine residues within a specific protein does not reliably determine its susceptibility to nitration (Bayden et al., 2011; Ischiropoulos, 1998).

Thus, this study aims to clarify which ragweed pollen allergens are susceptible to nitration and to investigate the subsequent alterations in allergenicity following nitration. Specifically, the research seeks to determine whether nitration enhances, diminishes, or results in no significant change in the allergenic properties of the ragweed pollen allergens.

1.3.3 Effects of combined elevated temperature and elevated NO$_2$ on pollen allergenicity

So far, limited research exists on pollen allergenicity governed by multiple factors in greenhouse conditions. It is known that in an outdoor study, in urban areas with elevated air pollutant concentrations and ambient daytime temperatures, ragweed plants increased in height and released pollen earlier and in greater quantities (Ziska et al., 2004). Additionally, in a chamber experiment, elevated CO$_2$ concentrations, combined with drought, resulted in heightened levels of specific flavonoid metabolites and transcripts encoding allergenic ragweed proteins (Amb a) (El-Kelish et al., 2014). Conversely, drought alleviated the increase in pollen load induced by elevated CO$_2$ concentrations (El-Kelish et al., 2014). Regarding the two single factors of elevated NO$_2$ and increased temperature, previous studies have shown that each of them could stimulate ragweed pollen production (Gentili et al., 2019; Zhao et al., 2017b). However, the implications of future warming conditions on ragweed plants remain uncertain, including both potential benefits and detriments. Furthermore, it remains unclear whether the growth of ragweed plants is synergistically promoted by elevated temperature and NO$_2$, or if these factors mutually inhibit each other's effects when combined.

Taken together, considering the most pessimistic scenario of RCP 8.5, the pathway with the highest greenhouse gas emissions in the absence of climate change policies among the entire set of RCPs for the next 50 years, a projected 4-degree temperature rise is anticipated in the Bavarian region by the year 2070 (Hausfather, 2019; IPCC, 2013; Loucks, 2021; Riahi et al., 2011). Given the existing knowledge about nitrogen dioxide, particularly the elevated levels in the Munich area (Agency, 2023; Zhu et al., 2020) and its recognized role as a pollen allergenicity enhancer (Zhao et al., 2017a; Zhao et al., 2016), coupled with Munich being heavily affected by ragweed plants (Buters et al., 2015; Hall et al., 2020) and ragweed hay fever disease (Brandes and Nitzsche, 2006; Ruëff et al., 2012), there is an urgent need to comprehend the changes in ragweed pollen allergenicity over the next 50 years in Bavaria. This understanding becomes crucial in the context of warmer temperatures and elevated nitrogen dioxide levels. Furthermore, Würzburg, situated in the northwest of Bavaria, presents a distinct climate from Munich in the northern Pre-Alps region

(Falk and Mellert, 2011; Rubel et al., 2017), exhibiting an average summer temperature 1.5 K higher than that of Munich during the climatological period of 1981 - 2010 (Wetterdienst, 2016).

Therefore, it is imperative to assess the effects of NO_2 and future temperatures on the seasonal abundance of pollen, the duration of the pollen season, as well as the pollen allergenicity for a comprehensive risk evaluation of ragweed pollen, which holds significant importance for the management, prevention, and control of ragweed plants and the associated pollen allergy.

1.4 Plant-associated fungi influencing plant growth and allergenic potential of ragweed pollen

1.4.1 Plant-associated fungi and their interactions with plants

Biotic factors include all living organisms that interact with plants within their ecosystem (Orcutt and Nilsen, 2000). Fungi, in particular, are integral biotic components of terrestrial ecosystems (Fairs et al., 2010; Marinkovich, 2004), fulfilling diverse and critical roles in nutrient cycling, decomposition, and symbiotic relationships with plants (Finlay et al., 2006; Giri and Saxena, 2017; Mohan et al., 2014).

Plant-associated fungi, form different relationships with plants, exerting influence over their growth, health, and survival (Berg, 2009; Motaung et al., 2020; Poudel et al., 2021). Based on fungal-plant interactions, these plant-associated fungi can be categorized into several types: 1) Pathogenic fungi, capable of causing plant diseases. Infection by these fungi may lead to plant wilting and stunted growth (EI-Khallal, 2007; Karagiannidis et al., 2002; Luo et al., 2014). Additionally, it can impact pollen characteristics (Bastia et al., 2000; Ma et al., 2000; Pontaroli et al., 2000) and the allergenic potential of the pollen (Gayatri S et al., 2012; O'riordain et al., 2002; Sinkevičienė et al., 2023). Upon pathogenic fungal infection, many plant species produce defense-related proteins, predominantly classified as pathogenesis-related proteins (PRs) (Midoro-Horiuti et al., 2001; Midoro-Horiuti et al., 2000; Sinha et al., 2014; Van-Loon et al., 2006). Previous studies have demonstrated that approximately 25% of pollen allergens belong to plant PRs and their homologs (Traidl-Hoffmann et al., 2002; Van-Loon and Van-Strien, 1999).

In addition, 2) fungi forming symbiotic relationships with plants, such as mycorrhizal fungi, collaborate with the plant root system to enhance nutrient supply and promote plant growth (Artursson et al., 2006; Diagne et al., 2020; Mohammadi et al., 2011). 3) A less well defined group of fungi are endophytes which grow inside plant tissues with neutral, positive or negative effects on plants growth and biochemical traits (Chaudhary et al., 2022; Obersteiner et al., 2016). Moreover, 4) plant-associated fungi may also be saprophytic, with some decomposing plant residues, affecting soil nutrient cycling and, consequently, plant growth (Almonacid et al., 2015; Fracchia et al., 2000; Saldajeno et al., 2008).

1.4.2 Biotic factors inducing allergen expression in pollen

In addition to affecting pollen production and thus altering pollen allergenicity, plant-associated fungi can affect the expression of pollen allergens that determine pollen allergenicity. Several major allergens, such as Bet v 1 in birch pollen (Ipsen and Løwenstein, 1983; Swoboda et al., 1994), Ole e 1 in olive pollen (Asturias et al., 1997), Aln g 1 in alder pollen (Breiteneder et al., 1992), Cor a 1 in hazelnut pollen (Pastorello et al., 2002), Que a 1 in white oak pollen (Niederberger et al., 1998), and Fag s 1 in European beech pollen (Moraes et al., 2018), share homology with group 10 of PRs. PR 10 belongs to a family of intracellular acidic proteins with a molecular weight range of 16 to 19 kDa, involved in plant defense responses (Somssich et al., 1988; Van-Loon et al., 1994; Warner et al., 1993). The expression of one gene encoding group 10 of PRs was up-regulated in yellow lupine root nodules in response to *Pseudomonas syringae* (Sikorski et al., 1999). Similarly, expression of PR 10 was induced in soybean during *Phytophthora sojae* infection (Xu et al., 2014), and in *Lilium regale* Wilson during *Fusarium oxysporum* infection (He et al., 2014).

In addition, certain minor allergens such as Art v 2 in mugwort (Nilsen and Paulsen, 1990), and Pru p 9 in peach pollen (Blanca et al., 2020), belong to the PR 1 family consisting of acidic and small proteins with a molecular weight between 14 and 17 kDa (Breen et al., 2017; Van-Loon and Van-Strien, 1999). Genes encoding group 1 of PRs were activated in *Solanum tuberosum* upon treatment with *Phytophthora infestans* (Taylor et al., 1990; Zaynab et al., 2021), as well as in *Solanum lycopersicum* during *Ralstonia solanacearum* infection (Chen et al., 2023). PR 1 protein

accumulated in *Nicotiana langsdorfii* leaves upon tobacco mosaic virus infection (Gordon-Weeks et al., 1997).

Moreover, one pollen allergen, Ole e 9, belonging to group 2 of PRs, has been identified in olive pollen (Huecas et al., 2001). PR 2 is a member of the β-1,3-glucanases family, consisting of monomeric enzymes with a molecular weight between 20 and 30 kDa (Sinha et al., 2014). Expressions of β-1,3-glucanase genes were induced in *Arachis hypogaea* seedlings against *Macrophomina phaseolina* (Iwuala et al., 2020), in *Triticum aestivum* against *Bipolaris sorokiniana* (Aggarwal et al., 2011), and in rice seedlings against *Magnaporthe oryzae* (Wang et al., 2021).

1.4.3 Fungal colonization and its impact on pollen allergenicity

In addition to pathogenic fungi inducing plant stress and the subsequent expression of PRs in pollen, the pollen-associated microbiome can also impact pollen allergenicity through various ways. Fungi and bacteria are the predominant microbial colonizers of airborne pollen (Grewling et al., 2019; Magyar et al., 2022; Manirajan et al., 2018). A positive correlation was observed between the diversity of the colonized microbiome and pollen allergenicity (Manirajan et al., 2019; Obersteiner et al., 2016). In addition, pollen has the potential to serve as a carrier for compounds secreted by colonizing bacteria and fungi, responsible for inducing lung inflammation and allergic sensitization responses (Oteros et al., 2019).

Moreover, once fungi colonize, they cannot be separated from pollen, leading to the inhalation of both pollen and colonizing fungi into the airways together (Magyar et al., 2022). However, it is known that fungal spores (Burge and Rogers, 2000; Denning et al., 2006; Kaur, 2021), fungal fragments, and proteins from inside fungal cells (Górny et al., 2002) are relevant causative agents for fungal allergies, ranking as the second most important cause of outdoor airborne allergies, with up to 80% of asthmatics sensitized (Lopez and Salvaggio, 1985). Additionally, various allergen surveys have reported that many individuals suffer from sensitization to multiple allergens (polysensitized) (Bousquet et al., 2010; Luo et al., 2019; Salo et al., 2014). Hence, when inhaling pollen and fungi simultaneously, polysensitized individuals are

likely to experience allergic reactions to both pollen and fungal allergens, thereby amplifying the index of allergic symptoms related to pollen.

Furthermore, certain fungal proteins exhibit homologies to specific pollen allergens thus sharing the capacity to bind specific antibodies due to their similar molecular structures. This phenomenon results in cross-reactivity with pollen allergens (Aalberse, 2007; Ghosh et al., 2014; Porter et al., 2015; San Segundo-Acosta et al., 2019; Sircar et al., 2020). In such cases, the allergenicity reflected by both pollen and colonizing fungi is further amplified.

1.4.4 Homology induced cross-reactivity

IgE cross-reactivity were widely observed within aeroallergens and/or food allergens, (Popescu, 2015), and within fungal allergens (Morales-Amparano et al., 2021; Simon-Nobbe et al., 2008). According to the Official Allergen List by the WHO/IUIS Allergen Nomenclature Subcommittee (http://www.allergen.org), fungal airway allergens share sequence similarities with 2 of the 11 reported of ragweed pollen allergens, namely enolase and pectate lyase.

As highlighted in Table 1 - 1, Amb a 12 is a member of the enolase family and exhibits homology with Cla h 6 (*Cladosporium herbarum*) (Simon-Nobbe et al., 2000), Asp f 22 (*Aspergillus fumigatus*), Pen c 22 (*Penicillium citrinum*) (Lai et al., 2002), Alt a 6 (*Alternaria alternata*) (Breitenbach and Simon-Nobbe, 2002), Cur l 2 (*Curvularia lunata*) (Sharma et al., 2006), and Rho m 1 (*Rhodotorula mucilaginosa*) (Chang et al., 2002). Additionally, Amb a 1 (Table 1 - 1), belonging to the pectate lyase family, shares homology with Pen c 32 (*Penicillium citrinum*) (Chiu et al., 2008).

Considerable evidence supports cross-reactivity among enolase allergens within aeroallergens. In the range of fungal allergens, cross-reactivity have been identified between enolase allergens, such as Asp f 22 and Pen c 22 (Chang et al., 2002), and Asp f 22 and Alt a 6 (Lai et al., 2002). In addition, cross-reactivity of enolase allergens has been demonstrated between fungal and pollen allergens, including Hev b 10 and Asp f 6 (Crameri, 1998; Rihs et al., 2001; Wagner et al., 2001), Hev b 9 and Alt a 6 (Simon-Nobbe et al., 2000; Wagner et al., 2000) and Hev b 9 and Cla h 6 (Simon-Nobbe et al., 2000). Additionally, reports indicated cross-reactivity

between pectate lyase allergens within pollen allergens, such as between Amb a 1 and its mugwort homologue Art v 6 (Jahn-Schmid et al., 2012), and between Art v 1 and Art v 4 (Léonard et al., 2010; Oberhuber et al., 2008).

1.5 Objectives of the present work

Based on the open questions and complexity of environmental interactions, plant growth and the relation of both to pollen allergenicity (Figure 1 - 2), this work addressed three new aspects of ragweed pollen allergenicity.
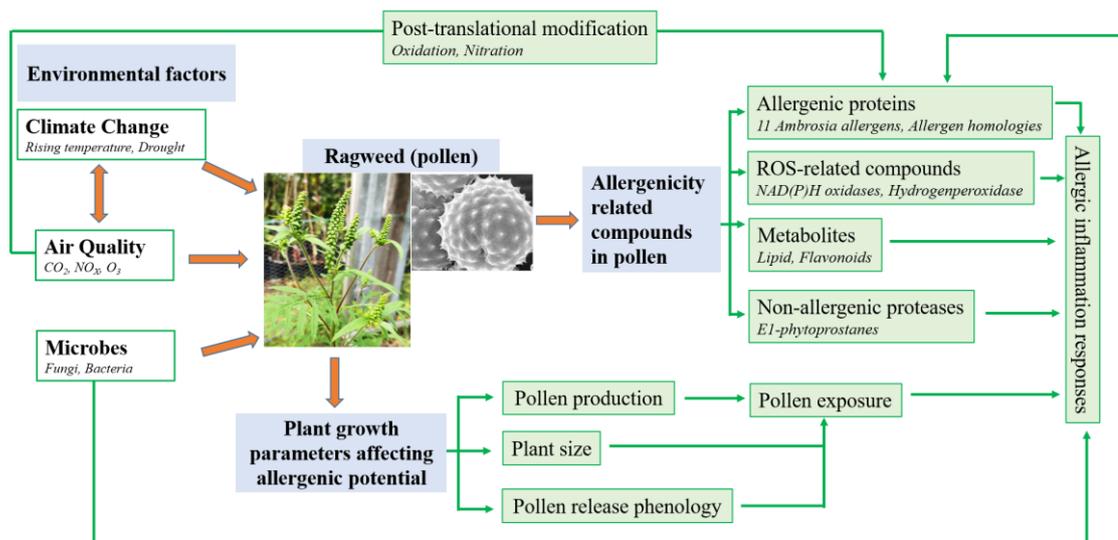
Figure 1 - 2: Research clues showing environmental factors, including climate change, air quality, and microbes, can alter allergic inflammation responses by influencing plant pollen exposure and the production of allergenicity-related compounds in ragweed pollen, which have an unclear effect on human health.

1.5.1 What is the influence of elevated temperature on the allergenic potential of ragweed plants with and without nitrogen dioxide in the ambient air?

The primary objectives of the first part of this study were to assess the impact of current and future anthropogenic environmental influences (elevated temperature, presence/absence of elevated NO_2, and their combination) on an allergenic plant associated with high health risks. Two factors (temperature and nitrogen dioxide) were applied in controlled experiments representing modeled temperature regimes in two distinct urban areas in Bavaria now and under future conditions i.e. assuming a 4 °C temperature increase as well as NO_2 as air pollutant. The analysis

encompassed plant traits (growth, phenology) and pollen characteristics. Pollen characteristics were further characterized by proteome sequencing, and further identification of proteins eliciting IgE reactions beyond the 11 known ragweed pollen allergens.

In addition, factors modulating pollen allergenicity (enhancers such as oxidative stress indicators and tyrosine nitrated residues, and attenuators like total flavonoid compounds) were included. Specifically, among these factors, questions remained regarding which ragweed pollen allergens can be nitrated and how allergenicity changes after nitration. Hence, a preliminary inquiry using recombinant allergens (rAmb a) expressed in *Escherichia coli* (*E. coli*) bacteria was conducted and the allergenicity of TNM-treated rAmb a was further assessed. Allergenicity was tested using dot blot and two dimensional immunoblot.

This approach was aimed at not only shedding light on changes in the expression of IgE-reactive proteins in ragweed pollen, but also gaining understanding of how and which allergenicity modulators from pollen released under conditions of elevated temperatures, elevated NO_2, and their combined treatments, could influence the allergenicity of ragweed allergens. Moreover, this study may contribute to predicting the potential changes in ragweed pollen allergenicity under scenarios where pollutants like nitrogen dioxide decrease, ideally reaching zero, while global warming persists unabated.

1.5.2 Are biotic factors such as plant associated fungi also to be considered in ragweed pollen allergenicity?

The existence of cross-reactions between ragweed pollen allergens and fungal allergens remains unknown. This study used a collection of field sampled ragweed seedling leaves to characterize the leaf associated fungal communities using Illumina sequencing. Additionally, the samples were used to isolate, culture and identify (morphology, Sanger sequencing) fungi associated with ragweed leaves. Finally, the fungal isolates were used to test the cross-reactions with ragweed pollen using enzyme-linked immune-sorbent assays (ELISA) with specific antibodies detecting Amb a 1 and Amb a 12, respectively.

1.5.3 Hypotheses

These analyses aim to validate the hypotheses that heightened metabolic activities in ragweed plants under elevated temperature and increased stress reactions under elevated NO_2 levels interact positively to increase allergenicity through different mechanisms [H1]. Specifically, elevated temperature do not directly lead to increased allergenicity of purified proteins but rather amplify stress reactions under elevated NO_2, resulting in the overexpression of stress-related proteins in pollen, including ragweed allergens, thereby potentially enhancing IgE binding activities in combined treatments [H1 a]. Another potential mechanism for increased allergenicity with elevated NO_2 treatments involves the nitration of allergens containing tyrosine residues in their protein sequence, potentially leading to enhanced IgE binding [H1 b]. Additionally, the activity of oxidative stress-related enzymes increases in response to elevated NO_2, moreover, being amplified with the addition of elevated temperature [H1 c]. Furthermore, a reduced flavonoid content in pollen under elevated temperature and an altered flavonoid composition under elevated NO_2 collectively reduce the potential to attenuate pollen allergenicity in combined treatments [H1 d]. Lastly, elevated temperature extends ragweed pollen exposure regardless of elevated NO_2, while also inducing a higher pollen load when combined with elevated NO_2 compared to instances of elevated temperature and elevated NO_2 alone [H1 e]. On the other hand, plant-associated fungi colonizing ragweed pollen during release potentially contribute to cross-sensitization among ragweed-allergic patients [H2].

These analyses offer insights into potential future changes in ragweed pollen allergenicity due to rising temperature and elevated nitrogen dioxide, posing threats to human health. Additionally, a preliminary investigation of the potential for cross-reactivity between ragweed-colonizing fungi and ragweed allergens has been explored.

2: Chapter – MATERIAL AND METHODS

2.1 Phytotron experiments simulating temperature increase and atmospheric NO_2 under actual and future (2070) conditions

2.1.1 Plant materials and growth conditions

2.1.1-1 Experimental design with temperature and NO_2 as factors

Two scenarios, simulating both current and future conditions, were independently conducted in two consecutive years: experiment 1 for the Munich area in 2018 and experiment 2 for the Würzburg area in 2019, as detailed below. Each experiment included two factors: temperature and NO_2. The investigation aimed to address whether a region with a currently moderate climate (e.g., Munich) might anticipate a higher risk of increased ragweed allergenicity compared to a region with an already warmer climate (e.g., Würzburg), the latter having a summer average 1.5 K higher than Munich.

As a simplified assumption, the same temperature course but at different levels based on an average seasonal course of climatic conditions between May and September in the Munich region (T_M) were used for both experiments. This foundational temperature profile was derived as an average from temperature data recorded at the Helmholtz Munich weather station between 2002 and 2005, a dataset previously utilized in phytotron experiments with ragweed (Kanter et al., 2013; Zhao et al., 2016). The climatological period of 1981 - 2010 served as the basis for calculating the temperature difference between Munich and Würzburg (Wetterdienst, 2016), resulting in the established 1.5 K temperature contrast between these two regions. Elevated temperature scenarios in both experiments were constructed by introducing a 4 °C increment to the temperature curves of the foundational profile in each experiment, thus approximately representing the anticipated increase under the RCP 8.5 scenario for 2070 (Hausfather, 2019; IPCC, 2013; Loucks, 2021; Riahi et al., 2011).

As an additional factor, NO_2 was maintained at a constant concentration of 80 ppb, following the protocol established in prior experiments (Zhao et al., 2016). This approach served the dual purpose of testing the reproducibility of experimental conditions and facilitating meaningful comparisons of results. In addition, the selected concentration aligns closely with current elevated levels of NO_2 observed near heavily trafficked roads (Agency, 2023), such as those places near motorways and busy urban roads in the Munich area, where concentrations can reach up to 106 ppb (Zhu et al., 2020). Moreover, it is worth noting that achieving an air quality status of 0 ppb NO_2 would be considered ideal, contingent upon a cessation of all fossil fuel burning.

In each experiment and each chamber, two sub-chambers served as technical repetitions (Table 2 - 1). The resulting treatments were coded by Temperature (T), region Munich (M), Würzburg (W), temperature increase by 4 °C (4), and addition of 80 ppb in the sub-chamber atmosphere (N). The corresponding abbreviations used in text, tables and figures are T_M, T_MN, T_M4, T_M4N for the experiment "Munich" (2018), and T_W, T_WN, T_W4, T_W4N for the experiment "Würzburg" (2019), respectively.

Table 2 - 1: Illustration of experimental design for the phytotron chamber experiments with temperature and NO_2 as factors. $T_W = T_M + 1.5$ °C.

Scenario	Phytotron Subchamber (EK)	Treatment Abbreviation	Temperature	NO_2 (ppb)
"Munich" (M)	EK 1.1	T_MN	current M	80
	EK 1.2	T_M	current M	0
	EK 1.3	T_MN	current M	80
	EK 1.4	T_M	current M	0
	EK 2.1	T_M4	current M + 4 °C	0
	EK 2.2	T_M4N	current M + 4 °C	80
	EK 2.3	T_M4	current M + 4 °C	0
	EK 2.4	T_M4N	current M + 4 °C	80
"Würzburg" (W)	EK 1.1	T_W	current W	80
	EK 1.2	T_WN	current W	0
	EK 1.3	T_W	current W	80
	EK 1.4	T_WN	current W	0
	EK 2.1	T_W4	current W + 4 °C	0
	EK 2.2	T_W4N	current W + 4 °C	80
	EK 2.3	T_W4	current W + 4 °C	0
	EK 2.4	T_W4N	current W + 4 °C	80

2.1.1-2 Phytotron chamber conditions and plants cultivation

For both aforementioned experiments, seeds of common ragweed (*A. artemisiifolia* L.) were provided by Julius Kühn Institut Braunschweig, Germany. These seeds, sourced from outdoors, and harvested in 2012, were used to eliminate any potential impact of parental life history conditions on the subsequent generation's growth and development (Elwell et al., 2011). Sowing took place on standard soil (Floradur®, Bayerische Gärtnerei Genossenschaft, München, Germany), and the seeds were then placed in a conventional growth chamber with a day/night temperature maintenance of 20/18 °C and a light/dark cycle of 14/10 hours. After four weeks, the seedlings were transplanted into 17 cm round pots using the same substrate as for initial sowing. Subsequently, they were transferred to phytotron chambers following the procedures outlined in previous experiments (Kanter et al., 2013).

However, logistical challenges encountered by the technical staff during the annual chamber maintenance resulted in unintended deviations from the planned protocol and resulted in different conditions for seedling growth in the two experiments during the early growth phases as detailed in Figure 2 - 1. Here, DAS is an abbreviation for days after sowing. Before placement in the growth chamber, seeds underwent vernalization in trays with plastic lids, kept in darkness at 4 °C for 48 hours in experiment 1 and 64 hours in experiment 2, respectively. Transplantation occurred for seedlings at DAS 25 in experiment 1 and DAS 38 in experiment 2. The inevitable delay in transplanting during the second experiment was a result of phytotron chamber maintenance. Figure 2 - 1 provides comprehensive details on the experimental timeline, highlighting key points such as seeding, transplanting, initiation of treatments, and measurement of average plant height in the initial nine weeks of both experiments.

In both experiments, following acclimatization to the controlled conditions in the phytotron chambers, the future temperature treatment (Figure 2 - 2) and the NO_2 treatment (Figure 2 - 3) commenced at DAS 59. The temperature elevation was implemented gradually, with a 2 °C increase over two consecutive days. Throughout plant growth, indoor conditions, including light intensity and relative humidity, were meticulously regulated, and also recorded by sensors, with specifics documented and presented in Figure 2 - 4. Each plant received daily individualized

irrigation to prevent both drought stress and overwatering (administered automatically via drip irrigation). The irrigation volume and frequency adjusted according to plant growth. Manual supplementation of water occurred when heightened evaporation from the plant leaves transpired due to the elevated temperature treatment.

However, in addition to the previously discussed inevitable annual maintenance (refer to Figure 2 - 1) and network malfunctions (refer to Figure 2 - 4), the temperatures recorded on some specific days during experiment 2 (2019) in the Würzburg scenario, did not align with the anticipated values (Figure 2 - 2). Firstly, the average daily temperatures recorded for T_W from DAS 38 to DAS 58, preceding the stress application, exhibited an increase $(0.86 \pm 0.49\,°C)$ compared to the mean daily temperature for T_W4. Following this, T_{W4} experienced intermittent delays in temperature increase on specific days: DAS 62 to DAS 65, DAS 75, DAS 77 to DAS 79, and DAS 100 to DAS 104, with mean daily temperature differences of $1.83 \pm 0.04\,°C$, $0\,°C$, $1.83 \pm 0.16\,°C$, and $0.33 \pm 0.03\,°C$, respectively, in comparison to those observed for T_W. In addition, the daily mean temperature of T_W exhibited an anomalous increase on DAS 76, resulting $1.47\,°C$ higher than that recorded of T_W4 on the same day. Excluding these 14 specific values, the daily temperature difference between T_W4 and T_W remained at $3.14 \pm 0.34\,°C$, spanning from DAS 60 to DAS 154 (indicated by blue and red triangles in Figure 2 - 2 B). While in experiment 1, the average daily temperature difference between T_M4 and T_M, recorded throughout experiment 1 (2018) of the Munich scenario from DAS 60 to DAS 128, consistently held at $3.54 \pm 0.11\,°C$ (indicated by blue and red circles in Figure 2 - 2 A). Although the recorded temperature differences fell slightly below the anticipated $4\,°C$, they aligned with the temperature increases projected by RCP 8.5 for the year 2100 (increase by $3.0 - 5.1\,°C$) (Hausfather, 2019). Consequently, the ensuing discussion remains applicable to the expected temperature rise (plus $4\,°C$) for a future simulation. To investigate the impact of temperature (a $4\,°C$ temperature increase), elevated NO_2, and their interaction on the differential plant traits and pollen properties in ragweed, the eight conditions derived from the two sets of experiments were categorized into 10 clusters. Experiment 1 yielded five clusters: T_MN vs T_M, T_M4N vs T_M4, T_M4 vs T_M, T_M4N vs T_MN, and T_M4N vs T_M. Experiment 2 contributed other five clusters: T_WN vs T_W, T_W4N vs T_W4, T_W4 vs T_W, T_W4N vs T_WN, and T_W4N vs T_W.
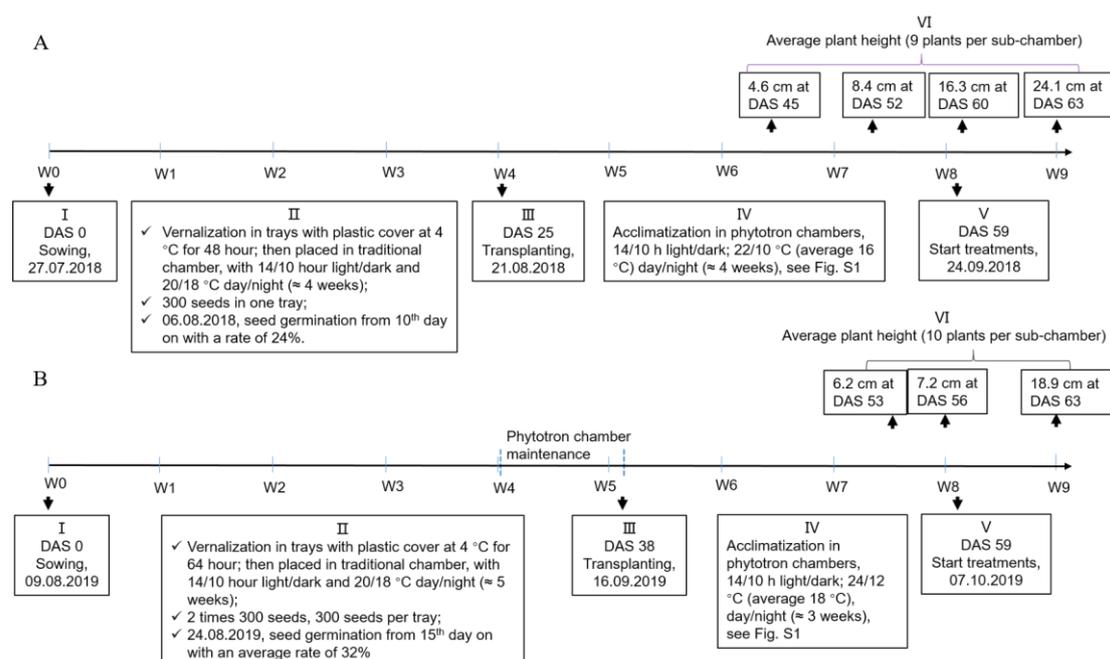
Figure 2 - 1: Schematic diagram showing raising and growth of ragweed plants in the first nine weeks under simulated temperatures in two regions of Bavaria: "Munich" (A) and "Würzburg" (B). DAS, days after sowing. I, sowing of seeds, at DAS 0 in both scenarios; II, vernalization and germination of seeds, from DAS 0 to DAS 25 in the Munich scenario and from DAS 0 to DAS 38 in the Würzburg scenario (the longer period in B was due to maintenance of the phytotron chambers); III, transplanting, DAS 25 in the Munich scenario and DAS 38 in the Würzburg scenario; IV, acclimatization of seedlings, from DAS 25 to DAS 59 in the Munich scenario, from DAS 38 to DAS 59 in the Würzburg scenario; V, start of experimental treatments (temperature and NO_2), at DAS 59 in both scenarios; VI, measured average plant height, at DAS 45, DAS 52, DAS 60 and DAS 63 in the Munich scenario and DAS 53, DAS 56 and DAS 63 in the Würzburg scenario, respectively. Scaled long and narrow arrows indicated the number of days after sowing (DAS) in weeks (W); other short fat arrows indicated special dates mentioned during two independent experiments.
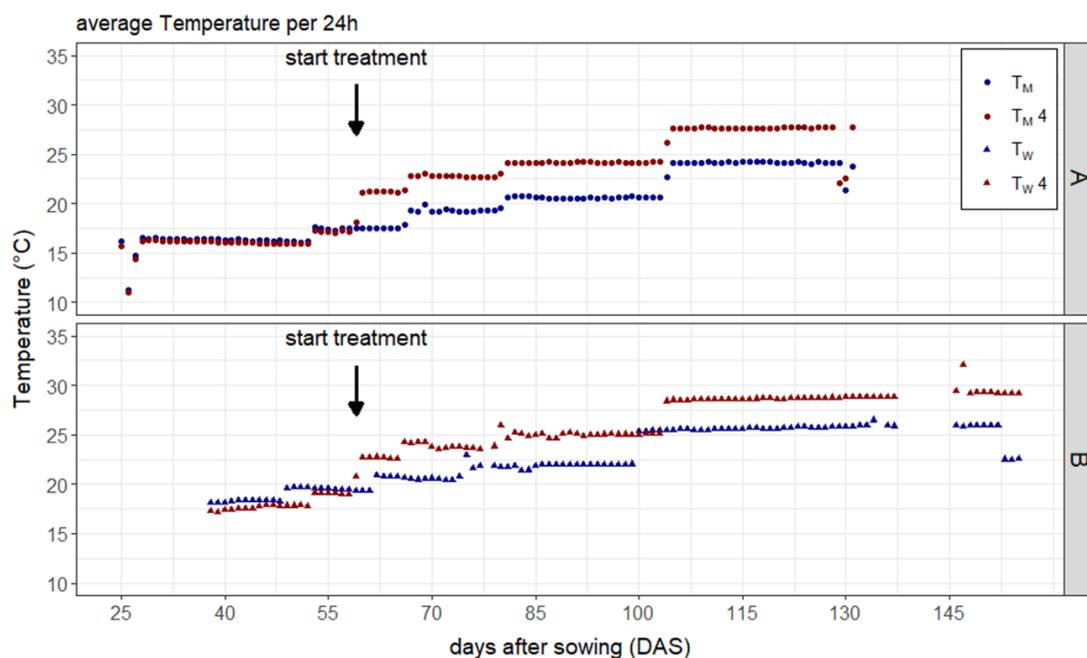
DAS	$\Delta T_{average}$ ($T_W 4$ - T_W) (mean ± sd)
DAS 62 - 65	1.83 ± 0.04 °C
DAS 75	0
DAS 76	- 1.47 °C
DAS 77 - 79	1.83 ± 0.16 °C
DAS 100 - 104	0.33 ± 0.03 °C

Figure 2 - 2: Daily average temperature based on real-time records in the four individual sub-chambers (1-4) of each phytotron chambers in two independent experiments simulating temperatures in two regions in Bavaria: "Munich" control (A, blue circles) and elevated temperature (A, red circles), "Würzburg" control (B, blue triangles) and elevated temperature (B, red triangles). The following table lists the daily average temperature differences (mean ± standard deviation) recorded for $T_W 4$ and T_W on specific dates (from DAS 62 to DAS 65, at DAS 75 and DAS 76, from DAS 77 to DAS 79, and from DAS 100 to DAS 104). Abbreviations: DAS, days after sowing; T_M, current temperature Munich, T_W, current temperature Würzburg; $T_M 4$, and $T_W 4$, temperature increase by 4 °C in Munich and Würzburg, respectively. Identical and vital phytotron chamber experiments started in "Munich" at day 25 and in "Würzburg" at day 38. Harvests was completed in "Munich" at day 130 and in "Würzburg" at days 154.
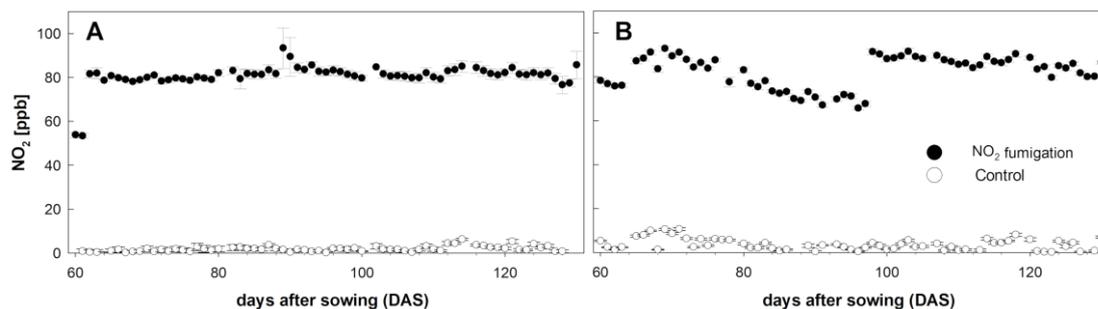
Figure 2 - 3: Daily average of NO$_2$ concentrations in the phytotron sub-chambers during two independent experiments simulating (A) Munich and (B) Würzburg temperature conditions. Sub-chambers were flushed with purified air (Ghirardo et al., 2020b) without NO$_2$ addition (white symbols) or fumigated to a final NO$_2$ concentrations of 80 ppb (black symbols); means ± sd (n=4 sub-chambers). DAS, days after sowing. NO$_2$ fumigation started at day 59 in the both scenarios.

2.1.2 Pollen harvest and characterization of plant traits

Owing to substantial growth variations among plant individuals, periodic trimming of plants became necessary during the experiments to prevent uneven shading among neighboring plants. Ultimately, the harvest included nine plants per sub-chamber in experiment 1 and ten plants per sub-chamber in experiment 2. Pollen harvesting utilized modified Aracon® systems, conducted when 80% of the plants released pollen as per the methodology outlined previously (Kanter et al., 2013). The pollen collection concluded on DAS 130 for experiment 1 and on DAS 154 for experiment 2 (refer to Figure 2 - 2), and the collected pollen was stored at -80 °C for subsequent analysis. Twelve plants from each treatment were randomly selected to determine the following traits: 1) Average plant height (cm): the upper part above the soil in all scenarios per plant was measured shortly before pollen harvesting. The average plant height was calculated; 2) The average length of male inflorescence size (cm): the average of all male racemes per plant was taken and analyzed; 3) Pollen season period from the beginning to the end (days): The onset of pollination was recorded from daily care recording in all scenarios since the sowing date of ragweed seeds. The start of the pollen release was set at the first time-point at which obvious yellow airborne pollen could be observed. The end of the pollen season was noted when no more pollen came out from the male flowers. Statistically, the average pollen season length has been assessed, i.e., Duration (days) = the end (days) - the start (days). The average values of the start and period of pollen season were calculated; 4) Average pollen yield per male inflorescence (mg):

pollen from each plant was pooled after pollen harvesting and weighed. The average weight was calculated per inflorescence.
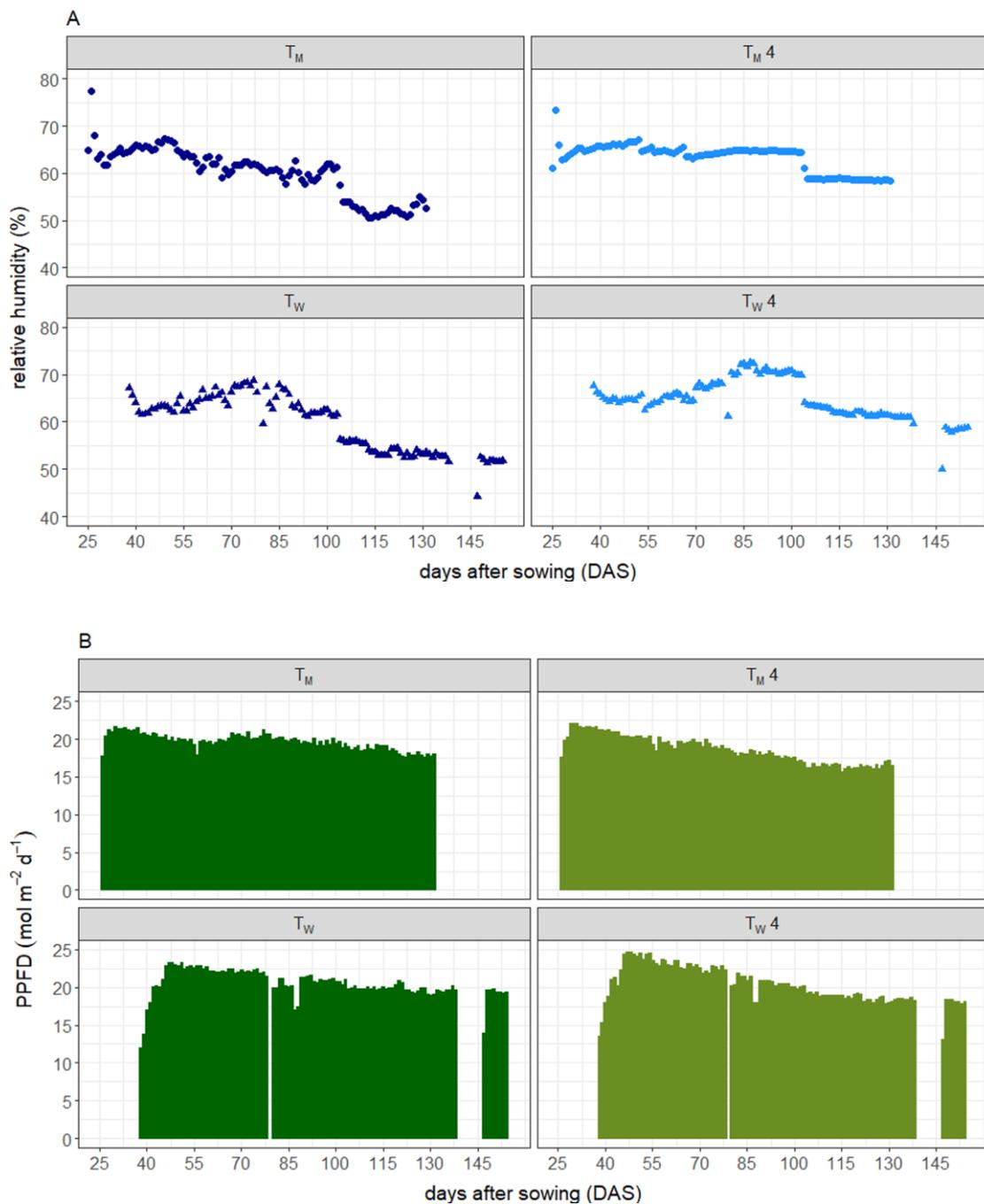
Figure 2 - 4: Daily average of relative humidity (rh) (A) and light intensity with daily sum of PPFD (B) in the phytotron chambers during two independent experiments simulating temperatures in two regions in Bavaria ("Munich" and "Würzburg") under control ("T_M", "T_W") and future ("T_M4", "T_W4") temperature conditions. DAS, days after sowing. PPFD = Photosynthetic Photon Flux Density (in the experiment "Würzburg" climatic conditions no data recording between day 138 and 147 due to network malfunction).

2.1.3 Sera pool and aqueous pollen extract preparation

2.1.3-1 Patient sera pools

The sera from 29 patients, individually sensitized to ragweed pollen and with Immuno CAP®
classes > 3 (refer to Table 2 - 2) (kindly provided by Prof. Dr.med.Dr.h.c Thomas Ruzicka,
Klinikum der Universität München, Munich, Germany), were pooled (designated as pool 1) for
the dot blot assays. The serum pool 1 was aliquoted into 300 µL and stored at – 20 °C until use.
Simultaneously, sera from 28 atopic individuals allergic to ragweed pollen, showcasing a diverse
range of Immuno CAP® classes (see Table 2 - 3), were pooled (designated as pool 2) for two-
dimensional immunoblot assays. Pool 2 was aliquoted into 400 µL and stored at – 20 °C until use.
Additionally, the sera from 16 patients in list 3 (refer to Table 2 - 4) were employed for cross-
reactivity assessments through ELISA and dot blot assays, maintained at – 20 °C until use. Sera
from nine healthy volunteers were used as controls, as previously mentioned (Zhao et al., 2016).

Table 2 - 2: Details of 29 patients' sera in pool 1 used in the immunoblot assays

Name	Concentration (kU/I)	CAP-Class *	Name	Concentration (kU/I)	CAP-Class *	Name	Concentration (kU/I)	CAP-Class *
1	0.46	1	11	1.06	2	21	1.17	2
2	0.58	1	12	1.49	2	22	0.75	2
3	1.96	2	13	2.27	2	23	1.61	2
4	1.13	2	14	7.05	3	24	2.76	2
5	2.65	2	15	9.78	3	25	10.6	3
6	2.07	2	16	30.6	4	26	1.86	2
7	1.62	2	17	5.45	3	27	31.7	4
8	5.02	3	18	4.46	3	28	1.14	2
9	1.86	2	19	1.73	2	29	0.72	2
10	2.59	2	20	0.92	2			

*Methods: CAP-FEIA (Fluorescent Enzyme Immunoassay); Spec. IgE: CAP-Class 0: < 0.35 kU/I; CAP-Class 1:
0.35 - < 0.7 kU/I; CAP-Class 2: 0.7 - < 3.5 kU/I; CAP-Class 3: 3.5 - < 17.5 kU/I; CAP-Class 4: 17.5 - < 50.0 kU/I;
CAP-Class 5: 50.0 - 100.0 kU/I; CAP-Class 6: > 100.0 kU/I.

2.1.3-2 Pollen sample pools

The specifics of the assays, the biological samples analyzed for each treatment, the counting
units constituting one biological replicate per treatment, the technical replicates utilized, and the

quantity of initial pollen/protein in the relevant solvent are detailed in Table 2 - 5. Varying numbers of individual plants were employed across different assays, as indicated in the table. Consistently, three technical replicates were executed for all assays; however, liquid chromatography with tandem mass spectrometry (LC-MS/MS) sequencing was conducted only once for each biological sample. The starting amounts of pollen in both experiments were consistent. The assays of NAD(P)H oxidase initiated with 20 milligrams (mg) of pollen in 600 µL of 0.1 M NH_4HCO_3 buffer, while H_2O_2 assays commenced with 10 mg of pollen in 100 µL of water. Both dot blot assays for total allergenicity and LC-MS/MS sequencing were initiated with 30 mg of pollen in 600 µL of NH_4HCO_3 buffer. The two-dimensional immunoblotting assays initiated with 60 mg of pollen in 1.5 mL of the same buffer. Moreover, both flavonoid content and composition measurements were initiated with 20 mg of pollen in 400 µL of methanol (Merck, Germany).

Table 2 - 3: Details of 28 patients' sera in pool 2 used in the immunoblot assays

Name	Concentration (kU/I)	CAP-Class *	Name	Concentration (kU/I)	CAP-Class *
1	5.02	3	15	1.86	2
2	7.05	3	16	2.59	2
3	9.78	3	17	1.06	2
4	30.6	4	18	1.49	2
5	8.02	3	19	2.27	2
6	9.54	3	20	1.73	2
7	5.45	3	21	0.92	2
8	4.46	3	22	1.17	2
9	89.8	5	23	0.75	2
10	4.18	3	24	1.61	2
11	4.47	3	25	2.76	2
12	10.6	3	26	1.86	2
13	31.7	4	27	1.14	2
14	1.96	2	28	0.72	2

*Methods: CAP-FEIA (Fluorescent Enzyme Immunoassay); Spec. IgE: CAP-Class 0: < 0.35 kU/I; CAP-Class 1: 0.35 - < 0.7 kU/I; CAP-Class 2: 0.7 - < 3.5 kU/I; CAP-Class 3: 3.5 - < 17.5 kU/I; CAP-Class 4: 17.5 - < 50.0 kU/I; CAP-Class 5: 50.0 - 100.0 kU/I; CAP-Class 6: > 100.0 kU/I.

Table 2 - 4: Sera list 3 of 16 patients' sera used in the cross-reactivity via ELISA

Name	Concentration (kU/I)	CAP-Class *	Sampling Date	Name	Concentration (kU/I)	CAP-Class *	Sampling Date
1	0.46	1	31.01.2013	9	5.02	3	08.10.2013
2	0.58	1	31.01.2013	10	1.86	2	12.11.2013
3	1.96	2	08.04.2013	11	2.59	2	12.11.2013
4	1.13	2	10.04.2013	12	1.06	2	08.03.2014
5	2.65	2	23.04.2013	13	1.49	2	22.07.2014
6	2.07	2	07.05.2013	14	2.27	2	14.08.2014
7	1.62	2	14.05.2013	15	7.05	3	14.07.2015
8	2	2	16.05.2013	16	31.7	4	2021

*Methods: CAP-FEIA (Fluorescent Enzyme Immunoassay); Spec. IgE: CAP-Class 0: < 0.35 kU/I; CAP-Class 1: 0.35 - < 0.7 kU/I; CAP-Class 2: 0.7 - < 3.5 kU/I; CAP-Class 3: 3.5 - < 17.5 kU/I; CAP-Class 4: 17.5 - < 50.0 kU/I; CAP-Class 5: 50.0 - 100.0 kU/I; CAP-Class 6: > 100.0 kU/I.

2.1.3-3 Aqueous pollen extract preparation

Aqueous protein extracts from the pollen used in this study were acquired through hydrating the pollen in 0.1 M ammonium bicarbonate (NH_4HCO_3, pH 8.1, Carl Roth GmbH, Karlsruhe, Germany), except for determining the H_2O_2 content where H_2O was used as solvent.

Protease inhibitors (1 tablet in 10 ml buffer, cOmplete™, Mini, EDTA-free, Sigma-Aldrich, Taufkirchen, Germany) were added to the aqueous solutions. The methodology of extracting pollen with an 0.1 M NH_4HCO_3 solution has been previously implemented on birch pollen (Buters et al., 2005).

Pollen was hydrated using the aforementioned buffer, and subsequently, the pollen-hydration buffer system underwent agitation at room temperature (RT) for one hour at 1,400 rpm (Janke & Kunkel Orbital Shaker Table, Germany). Afterwards, the pollen suspension was centrifuged (Thermo Scientific Fresco 21) at 21,100 g for 30 minutes at 4 °C. The supernatant was collected as crude protein extract, aliquoted, and preserved at - 20 °C until subsequent analysis.

Table 2 - 5: Pollen samples used for analyses

Method(s)_assays	Biological replicates per treatment	Unit for biological replicate per treatment	Technical replicates	Start pollen amount in solvent
NAD(P)H oxidase in both experiments	8	per plant	3	20 mg / 600 µL NH_4HCO_3
H_2O_2 in both experiments	10	per plant	3	10 mg / 100 µL ddH_2O
Dot blot assay in experiment 1	11	per plant	3	30 mg / 600 µL NH_4HCO_3
Dot blot assay in experiment 2	17	per plant	3	30 mg / 600 µL NH_4HCO_3
Flavonoids of concentration in both experiments	12	per plant	3	20 mg / 400 µL Methanol
Flavonoids immunoblot assays in both experiments	1	Pool of 12 samples	3	20 mg / 400 µL Methanol
2D-immunoblot in both experiments	3	Pool of 4 samples	3	60 mg / 1.5 ml NH_4HCO_3
Label-free LC-MS/MS in experiment 1	12 from T_M, 14 from T_MN, 16 from T_M4, 17 from T_M4N	per plant	1	30 mg / 600 µL NH_4HCO_3
Label-free LC-MS/MS in experiment 2	6	per plant	1	30 mg / 600 µL NH_4HCO_3

2.1.3-4 Protein purification by methanol and chloroform precipitation

The crude protein extract underwent additional purification for dot-blot analysis, 2D immunoblot assays, and LC-MS/MS sequencing, utilizing methanol and chloroform (Merck, Germany) precipitation (Wessel and Flügge, 1984).

In detail, 200 µL of the protein supernatant was blended with 800 µL of methanol and then centrifuged at 9,000 g for 30 seconds. Subsequently, the solution was mixed with 200 µL of chloroform and subjected to another centrifugation at 9,000 g for 30 seconds. This was followed

by the addition of 600 µL of ddH$_2$O, and the samples were centrifuged at 9,000 g for 30 seconds. The resultant solution exhibits three layers: a circular protein flake in the interphase layer which is needed, a substantial aqueous layer on the top, and a smaller chloroform layer at the bottom. Thus, the upper portion of the aqueous layer was discarded firstly, trying not to disturb the protein flake. Then, methanol (600 µL) was introduced, and the solution underwent centrifugation at 16,000 g for 2 minutes. As much of the methanol-chloroform solution was removed. Pellets were air-dried for 10 minutes and then re-suspended with 100 µL of ¼ Buffer B (25 mM NaH$_2$PO$_4$, 2.5 mM Tris-HCl, 2 M urea, adjusted to pH 8.0 with NaOH) or 1% SDS (Sigma, Germany) (exclusively for LC-MS/MS). Protein dissolution was achieved by shaking at 1400 rpm for 10 minutes. The supernatant was collected following a 5-minute centrifugation at 21,100 g at 4 °C and stored at -20 °C until use.

2.1.3-5 Quantification of proteins

Two methods were employed in this study for the quantification of protein concentrations. The first method utilized the Bradford assay (Protein Dye Reagent, BioRad, Germany) (Bradford, 1976), employing bovine serum albumin (BSA) as the internal standard. Quick Start Bovine Serum Albumin Standard (BioRad, Germany) was used for calibration. Protein samples (5 µL unknown protein sample/3 µL BSA standard + 20 µL/22 µL ddH$_2$O, respectively) were combined with 200 µL of reagent dye (1:4 dilution) and loaded into Nunclon 96-well flat-bottom and transparent plates (Thermo Fisher Scientific, Denmark). Following a 5-minute incubation at RT, absorbance was measured at 595 nm using a Tecan Reader Infinite M1000 Pro (Tecan, Germany).

The second method involved the use of the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Germany) following the manufacturer's instructions with some modifications. Quick Start Bovine Serum Albumin Standards were employed for calibration instead of the BSA stock solution provided in the kit. Protein samples (5 µL of unknown protein sample/3 µL of BSA standard + 20 µL/22 µL of ddH$_2$O, respectively) were combined with 200 µL of reagent dye (1 part of solution A diluted with 50 parts of solution B) and loaded into 96-well plates. After a 30-minute incubation at 37 °C, absorbance was measured at 562 nm using a Tecan Reader Infinite M1000 Pro.

2.1.4 Dot-blot assay

2.1.4-1 Applications of dot blot assay

Dot blot assay served multiple applications in this study, including: a) exploring the impact of flavonoids on the binding activity between allergens and immunoglobulin E (IgE) with sera from patients allergic to ragweed pollen; b) evaluating the total allergenicity of ragweed pollen; c) examining whether tetranitromethane (TNM) induction can modify the allergenicity of any rAmb a protein; and d) investigating the cross-reactions between fungal isolates and ragweed pollen (Table 2 - 6).

2.1.4-2 Procedure of dot blot assay

For dot blot analysis, 30 µL of each sample was evenly applied per well on a nitrocellulose (NC) membrane (0.2 µm) (GE Healthcare Life Sciences, Amersham, Germany) using the Bio-Dot® Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA). Subsequently, the NC membrane was stained with a ponceau S acidic solution (0.1% ponceau S in 5% acetic acid) for 5 minutes, following the method outlined previously (Bannur et al., 1999), and the staining was documented in image format (600 dots per inch resolution) using a scanner.

The stained NC membrane was then immersed in a blocking buffer [TBST (TBS: 10 mM Tris–HCl (pH 7.5), 0.9% NaCl, 1 mM $MgCl_2 \cdot 6H_2O$; T: 0.5% Tween 20) supplemented with 5% milk powder (blotting grade, low fat, Carl Roth GmbH, Karlsruhe, Germany)] for 1 hour at RT. Subsequently, the NC membrane was transferred to the first detecting antibody buffer and left overnight at 4 °C. Details of the applied detection antibodies and their dilutions are provided in Table 2 - 6. Following three washes with TBST for 10 minutes each, the NC membrane was incubated with detecting antibody buffer for 1 hour at RT. After three additional washes in TBST for 10 minutes, the NC membrane was transferred to the antibody buffer [TBST + 1:10,000 diluted monoclonal rat anti-mouse IgG2b antibody horseradish peroxidase (HRP) conjugated (provided by MAB Monoclonal Antibody Core Facility, HMGU, Munich, Germany)] for 1 hour at RT, if the detecting antibody was the monoclonal unconjugated mouse anti-human IgE antibody (Sigma-Aldrich, St. Louis, USA). Finally, after three washes in TBST buffer for 10 minutes each,

the NC membrane was incubated with ECL Select Western Blotting Detection Reagent (GE Healthcare, Amersham Place, UK) for 5 minutes at RT. Imaging and quantification were performed following Zhao et al. (2016) with a FUSION-FX7 Spectra System (Vilber, Eberhardzell, Germany) after 5 minutes of exposure, using the Image J 1.5.2 software (open source). For total allergenicity assays, the ratios of the integrated optical density (IOD) of immuno-reactive spots to the IOD of Ponceau S dye-stained spots were calculated.

Table 2 - 6: Applications of dot blot assay and the applied detecting antibodies in the assay

Purposes	Primary antibody	Secondary detecting antibody	Further detecting antibody
Flavonoids f immunoblot assays	Sera pool 2 (1:200 diluted)	Monoclonal unconjugated mouse anti-human IgE antibody (1: 50,000 diluted)	Monoclonal rat anti-mouse IgG2b antibody HRP conjugated (1:10,000 diluted)
Total allergenicity	Sera pool 1 (1:100 diluted)	Monoclonal unconjugated mouse anti-human IgE antibody (1: 50,000 diluted)	Monoclonal rat anti-mouse IgG2b antibody HRP conjugated (1:10,000 diluted)
Altered allergenicity after tyrosine nitration	Anti-nitrotyrosine antibody (monoclonal, mouse generated, clone 1A6; 1:2000 diluted)	Rat-anti-mouse IgG 2b conjugated with HRP (1:20,000 diluted)	No need
Cross-reactivity	Sera pool 3 and sera list 3	Monoclonal unconjugated mouse anti-human IgE antibody	Monoclonal rat anti-mouse IgG2b antibody HRP conjugated

2.1.5 The role of flavonoids interfering with IgE binding activities

2.1.5-1 Determination of total flavonoid content in pollen

The concentration of flavonoids in pollen extracts was determined using the AlCl$_3$ colorimetric method following published protocols (Gentili et al., 2019; Pękal and Pyrzynska, 2014) with slight modifications outlined below.

For each assay (refer to Table 2 - 5), 20 mg of ragweed pollen was mixed with 400 µL of methanol. The mixture was shaken for 1 h at RT with a speed of 1400 rpm. Subsequently, the soluble fraction was obtained through two rounds of centrifugation at 21,100 g for 10 min at 4 °C and stored at - 20 °C until use. Pollen extract samples (8 µL pollen extract + 72 µL ddH$_2$O) were loaded into 96-well plates, with each pollen sample prepared in six copies to ensure robustness. Following this, all samples were combined with 24 µL of NaNO$_2$ (5% w/v) (Sigma Aldrich, Germany) and allowed to incubate for 5 minutes. Subsequently, a triplicate of the sextuplicate pollen extract samples underwent a 6-minute incubation with 24 µL of AlCl$_3$ (10% w/v) (Sigma Aldrich, Germany), serving as a specific treatment, while the remaining triplicate, considered as a control, received an equal volume of ddH$_2$O. Neutralization of all samples with 160 µL of 1M NaOH followed, and the entire process was carried out at RT over a 10-minute period. Absorbance at 510 nm was quantified using the Tecan Reader Infinite M1000 Pro.

Each technical replicate (3 per biological replicate) underwent three measurements. To eliminate background absorbance, the average values from triplicates without AlCl$_3$ were subtracted from those with AlCl$_3$. A linear calibration curve was established using quercetin (Sigma Aldrich, USA) dissolved in methanol at concentrations of 50 µg/mL, 100 µg/mL, 200 µg/mL, 500 µg/mL, and 750 µg/mL. Results were expressed as milligrams of quercetin equivalents per gram of pollen (mg QE/g pollen).

2.1.5-2 Determining the influence of flavonoids in the allergen-IgE binding activity

A pool of 24 randomly selected samples covering all treatments was assembled for analysis. Protein content (µg/mg pollen) and total flavonoid content (µg/mg pollen) were assessed for each

sample (Figure 2 - 5). Utilizing the linear regression established between protein concentration and total flavonoid concentration per mg of pollen, varying amounts (0.1, 0.3, 1.6, and 3.2 µg) of flavonoids within a realistic range were chosen when 3 µg of protein was employed in the dot blot assays. Protein concentration in this context was determined using the Bradford assay (refer to section 2.1.3-5). Flavonoid content was quantified using the $AlCl_3$ colorimetric method (refer to section 2.1.5-1).

The BSA (Sigma Aldrich, USA) powder was dissolved to the desired concentration in 0.1 M NH_4CO_3 supplemented with protease inhibitor, EDTA-Free. CRP, serving as a comprehensive pool of all ragweed allergens (Gentili et al., 2019), was utilized in this study. A portion of pollen extract was prepared from 50 mg of CRP in 1.0 mL 0.1 M NH_4HCO_3 supplemented with protease inhibitor, following the procedure outlined in section 2.1.3-3. Subsequently, proteins from the pollen extract were purified and precipitated using the methanol-chloroform precipitation method (refer to section 2.1.3-4). Quantification of protein concentration was conducted through the Bradford assay (refer to section 2.1.3-5).
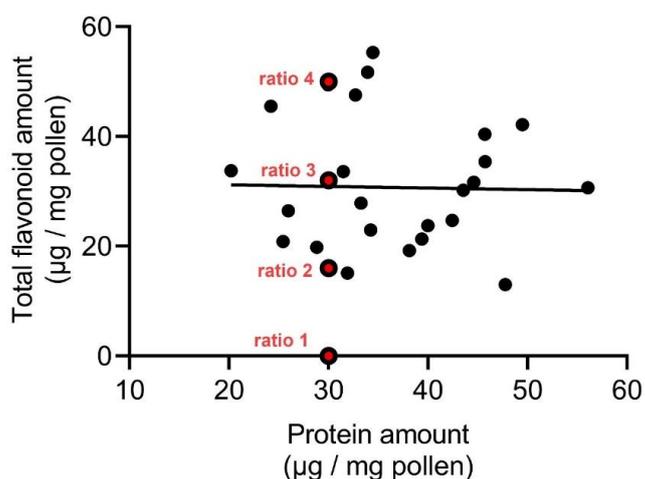
Figure 2 - 5: Linear regression between protein concentration and total flavonoid concentration per mg pollen. Red dots indicate the 4 ratios between protein concentration and total flavonoid concentration used at 1:10 dilution in the experiment. "ratio 1" = 30 µg / 0 µg, "ratio 2" = 30 µg / 16 µg, "ratio 3" = 30 µg / 32 µg, "ratio 4" = 30 µg / 50 µg. Black dots indicate randomly selected pollen samples from all treatments for regression, the black line represents the best fit for the given selected samples (Cheng et al., 2023).

rAmb a 1 was acquired following the procedure outlined in section 2.2.1 and quantified using the Bradford assay (refer to section 2.1.3-5).

Equivalent quantities of flavonoids from distinct biological replicates under the same treatment were extracted following the guidelines in section 2.1.5-1 and then combined. The mixtures of flavonoid extracts underwent vacuum drying at 4 °C using the Univapo 100H device (Uniequip, Germany) to remove methanol. Subsequently, the flavonoid pellets were reconstituted in ddH$_2$O to maintain a consistent flavonoid concentration. Additionally, commercial quercetin was diluted in ddH$_2$O to achieve the desired concentration.

Protein and flavonoid samples were mixed in a 96-well plate, reaching a total volume of 50 µL (with only 30 µL loaded) shortly before the initiation of the immunoblot. Controls were introduced for both flavonoids and proteins, incorporating samples without flavonoids and vice versa, to assess their background signals during the immunoblot.

In the dot blot assays, the procedure outlined in section 2.1.4-2 was adjusted as follows: the first detecting antibody buffer consisted of TBST-2% milk plus a 1:200 dilution of sera pool 2; the secondary detecting antibody buffer was TBST with a 1:50,000 dilution of monoclonal unconjugated mouse anti-human IgE antibody; and the subsequent detecting antibody buffer was TBST with a 1:10,000 dilution of monoclonal rat anti-mouse IgG2b antibody HRP conjugated.

2.1.5-3 UPLC-MSMS analysis of flavonoids profiles

For ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MSMS) analyses, the flavonoids extracted in section 2.1.5-1 and processed in section 2.1.5-2 were combined (600 µL methanol / 30 mg fresh pollen), followed by methanol removal using N$_2$ stream. The resulting extract was finally re-suspended in 400 µL of 50% (v/v) acetonitrile in water. After centrifugation for 20 minutes at 21,100 g and 4 °C, 350 µL of the supernatant was transferred into amber glass vials (350 µL each). The samples underwent processing by the Environmental Simulation Unit (EUS) at the Helmholtz Zentrum München, Munich, Germany. Details of non-targeted metabolomics analysis, including chromatography and MS parameters, were executed following the protocols outlined in their published work (Bertić et al., 2021;

Ghirardo et al., 2020a). All LC-MS hyper-grade chemicals (methanols/water) were purchased from Merck (Darmstadt, Germany), and acetonitrile was obtained from Honeywell (Puchheim, Germany). Data obtained from measurements were processed using Metaboscape 4.0 (Bruker), with parameter settings consistent with those detailed in previous work (Bertić et al., 2021).

2.1.6 Oxidative stress indicators in ragweed pollen

2.1.6-1 NAD(P)H oxidase levels

The detection of ROS-related compounds, such as NAD(P)H oxidase, indicative of oxidative stress, was conducted through the nitro blue tetrazolium (NBT) assay, following the procedures outlined previously (Bacsi et al., 2005; Boldogh et al., 2005).

Pollen grains (20 mg) were hydrated in 600 µL of 0.1 M NH_4HCO_3 containing proteinase inhibitor at RT for 1 h, with a stirring speed of 1,400 rpm. After a 30-minute centrifugation at 4 °C with a speed of 21,100 g, the supernatant was collected. A 25 µL solution (per assay) was combined with 2 mM NBT (Sigma), with or without 100 U superoxide dismutase (SOD, Sigma), along with 100 µM NADH (ROTH) or 100 µM NADPH (ROTH), and adjusted to a final volume of 240 µL using ddH_2O. The reaction assay underwent incubation at 37 °C for 30 min with a stirring speed of 1,000 rpm, followed by the removal of the supernatant through a 5-minute centrifugation with a speed of 21,100 g at 4 °C. Residual NBT was eliminated by washing with 1 mL phosphate-buffered saline (PBS) buffer (0.127 M NaCl, 7 mM $Na_2HPO_4•7H_2O$, 3 mM $NaH_2PO_4•H_2O$, pH adjusted to 7.4 using NaOH). The precipitate was completely dissolved in 250 µL methanol with gentle rotation for 20 min at RT. After a 5-minute centrifugation at a speed of 21,100 g at 4 °C, 200 µL of supernatant per assay was collected and measured in 96-well microplates with an absorbance of 530 nm. The results were expressed as A_{530} per mg of pollen. Eight randomly selected plants per treatment underwent the assay in triplicates.

2.1.6-2 H_2O_2 content

Ten mg of pollen were suspended in 100 µL of water and incubated at 4 °C for 30 minutes with agitation at 1000 rpm, followed by a 20-minute centrifugation at 4 °C with a speed of 21,100

g. The resulting supernatant was collected, and the H_2O_2 content was quantified precisely following the Peroxide Assay Kit (Catalog Number MAK311, Sigma) manual. Absorbance at 585 nm was taken, and the results were expressed as $\mu Mol\ \mu g^{-1}$. Each assay was conducted in triplicate.

2.1.7 Pollen allergen immune-reactivity detection

2.1.7-1 Dot-blot assays for total allergenicity

Eleven pollen samples from experiment 1 and 17 samples from experiment 2 were randomly chosen for each treatment (refer to Table 2 - 5). Each sample utilized a starting pollen amount of 30 mg in 600 μL of 0.1 M NH_4HCO_3. Pollen extracts were treated with the methanol-chloroform precipitation (refer to section 2.1.3-4), dissolved in 1/4 Buffer B, and quantified through the Bradford assay (refer to section 2.1.3-5).

An equivalent volume of protein extracts (3 μg in 30 μL TBS buffer) was loaded and conducted following the procedures outlined in sections 2.1.4-2. The only modification was the dilution of the first detecting antibody buffer (TBST-2% milk + 1:100 dilution of the sera pool 1). Images were captured and processed as described above (see 2.1.4-2).

2.1.7-2 Two-dimensional gel electrophoresis immunoblot

Two-dimensional immunoblot assays were conducted to delve deeper into each IgE-reactive protein's contribution to the overall pollen allergenicity, as indicated by dot blot analyses. Moreover, the use of LC-MS/MS for spot identification allows for the characterization of novel IgE-reactive proteins.

Equal amounts of pollen samples from four plants were randomly pooled, resulting in three biological samples for each treatment, and three technical replicates were independently performed (refer to Table 2 - 5). The pollen pools (60 mg / 1.5 mL) were re-suspended in 0.1 M NH_4HCO_3 with proteinase inhibitor and shaken at RT for 1 h. After a 30-minute centrifugation at 4 °C, the pollen extracts were aliquoted and stored at –20 °C until use.

The protein solution underwent purification using the methanol-chloroform precipitation method (refer to 2.1.3-4), was re-suspended in 1/4 Buffer B, and quantified using the BCA kit (refer to 2.1.3-5). Following the BCA kit assay, the volume of the protein extract, containing 15 µg of proteins for each biological sample, was calculated. A total volume of 100 µL for each sample was achieved by supplementing with 1/4 Buffer B. For further protein purification, the 2D Clean-Up Kit (Cytiva 80-6484-51, Sigma) was employed, adhering precisely to the provided protocol. Additionally, three internal standards, containing 15 µg of protein each, were prepared: one comprised the pool of 12 biological samples from experiment 1, another involved the pool of 12 biological samples from experiment 2, and the last one was the amalgamation of 24 biological samples from both Experiments.

After purification using the 2D Clean-Up Kit, protein pellets were re-suspended in 125 µL of rehydration buffer [7 M urea, 2 M Thiourea (PlosOne), 2% Chaps (PlosOne), 0.5% IPG buffer (GE Healthcare), 0.8% DL-Dithiothreitol (DDT, Sigma), 0.002% Bromophenol blue (BPB, Sigma)]. Subsequently, the re-suspended protein solution was loaded onto Immobiline® Drystrips (pH 3-11 NL, 7 cm, GE Healthcare) with the Drystrip Cover Fluid (GE Healthcare) in the Immobiline Drystrip Reswelling tray (GE Healthcare) at RT for 20 to 24 h. After rehydration, the strips were processed according to previously published method (Zhao et al., 2016), with adjustments made to isoelectric focusing. Specifically, the times and voltages (V) were modified as follows: 20 V for 12 h, a gradient from 100 V to 500 V for 1 h, a gradient from 500 V to 1000 V for 1 h, a gradient from 1000 V to 2000 V for 1 h, a gradient from 2000 V to 4000 V for 1 h, a gradient from 4000 V to 7000 V for 1 h, and 7000 V for 3 h, totaling 32350 Vh. Subsequently, the strips were transferred to 4 – 20% Mini-PROTEAN TGX Precast Protein gels (BIO-RAD) and run in a buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at RT, set at 100 V for 1.5 h. Upon completion, the separated proteins were transferred to a 0.2 µm NC membrane using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) with transfer buffer (running buffer + 20% methanol) set at 100 V for 1 h 15 min. The NC membrane was subsequently processed as described above in a dot blot assay (refer to sections 2.1.4-2). However, the first detecting antibody buffer was modified to TBST-2% milk plus a 1:50 dilution of the previously mentioned

sera pool 2. Image acquisition and visualization, and the subsequent spots intensity quantification followed the procedures described earlier (see section 2.1.4-2).

The values of spot intensity were expressed as a ratio (relative abundance) comparing to those corresponding in the internal standard. All spots were matched by gel-to-gel comparisons and the changes of the relative abundance of each spot were analyzed. Those spots, the abundance of which varied at least ± 2-fold between the treated and control pollen (fold change, FC), were considered as interesting spots and picked up for the following LC-MS/MS analysis.

2.1.7-3 Preparative gels and in-gel digestion and LC-MS/MS analysis

Fifteen µg of protein pooled from both experiments underwent two-dimensional separation, as previously described. Visualization of proteins in the gel was achieved using the silver stain method. Specifically, the resulting 2D gels were subjected to fixation in a solution of 50% (v/v) methanol and 12% (v/v) acetic acid for two 15-minute cycles, followed by three washes in 50% (v/v) methanol for 20 minutes each. Subsequent steps involved sensitizing the gels in 0.02% (w/v) $Na_2S_2O_3$ for 1 to 2 minutes, followed by a 5-minute wash with ddH_2O. The gels were then stained with a solution consisting of 2 g/L $AgNO_3$ and 0.075% (v/v) formaldehyde (37%) for 20 minutes. After a 1-minute wash with ddH_2O, the gels were developed in a solution containing 60 g/L Na_2CO_3, 5 mg/L $Na_2S_2O_3$, and 0.05% (v/v) formaldehyde (37%) until spots became visible (2 to 3 minutes in this study). To prevent excessive darkening of the gel background, a stop solution containing 0.5% (w/v) glycine was applied for 15 minutes. Finally, the gels were stored in a solution composed of 20% (v/v) ethanol and 2% (v/v) glycerin. It is essential to prepare the staining and developing solutions shortly before use.

In the storage solution, the visualized protein spots on the two-dimensional gels were picked up and rinsed in ddH_2O. Subsequently, the samples were dispatched to the Research Unit Protein Science at HMGU, Munich, Germany, where they assisted in the meticulous washing, digestion, and analysis of the spots through LC-MS/MS. Peptide identification was executed using Mascot version 2.6.2 (Matrix Science, Boston, USA), where the spectra were matched against the SwissProt_GreenPlants_spiked database (37335 proteins). Filtering criteria for peptides and spectra included the use of trypsin as the specific protease, allowance for one missed cleavage, a

parent ion tolerance of 10 ppm, a fragment ion tolerance of 0.02 Da, a fixed carbamidomethylation modification, and variable modifications such as deamination of glutamine or asparagine, or methionine oxidation. Subsequently, the acquired metadata underwent further analysis using Scaffold software version 5.0.1 (Proteome Software).

For protein identification, peptides were exclusively accepted and counted if the Mascot ion score exceeded 30 and met the criteria of a 95% minimum in peptide thresholds and a minimum of 2 unique peptides in the protein thresholds. Razor peptides were also incorporated into analysis. Among the identified proteins, only the top five proteins according to their respective protein scores are listed. This process was consistent with the established procedures outlined in several studies (Holzmeister et al., 2011; Zhao et al., 2016).

2.1.8 Comparative proteomic analysis

2.1.8-1 Sample preparation for LC-MS/MS

The protein samples underwent analysis at the Research Unit Protein Science over two consecutive years. All experiment 1 samples (pertaining to the Munich scenario), including 12 from T_M, 14 from $T_M N$, 16 from $T_M 4$, and 17 from $T_M 4N$, were subjected to LC-MS/MS sequencing (refer to Table 2 - 5). However, owing to a shift in financial support during this timeframe, the number of biological replicates per treatment for sequencing was reduced. Ultimately, six plants were randomly chosen per treatment in experiment 2.

For each sample, 30 mg of pollen was hydrated in 600 µL of 0.1 M NH_4HCO_3, following the procedure outlined in section 2.1.3-3. After employing the methanol-chloroform precipitation method, the resulting pellets were re-suspended in 1% SDS, dissolved in 1/4 Buffer B, and quantified using the Pierce™ BCA Protein Assay Kit. Subsequently, ten µg of protein in 10 µL of 1% SDS per sample were dispatched to the Research Unit Protein Science at HMGU, Munich, Germany.

2.1.8-2 Data processing for qualitative analysis based on peak intensities

Finally, an untargeted LC-MS/MS proteomic analysis was conducted on trypsin-digested pollen fractions. The data were processed according to Zhao et al. (2016). For quantification, the peak intensity of each peptide, based on normalized abundances, was utilized, and the median value for each peptide across biological replicates was extracted. Notably, Amb a 1, 8, 9, and 12 exhibit the presence of two or more isoforms. Due to high amino acid sequence similarities among these isoforms, this study considered the relevant isoforms as one protein group. Consequently, all peptides attributed to the same proteome were summed up and assigned to the respective identified protein, encompassing both unique and razor peptides in this investigation.

The differential expression patterns of proteins in ragweed pollen under different treatments were investigated. In addition, the expression profiles of ten well-established ragweed pollen allergens (indicated in Table 1 - 1) and other IgE-reactive proteins (indicated in Table 3 - 6) were accessible, and differential expression analysis of these allergenic proteins in the mentioned 9 clusters were conducted. Moreover, those proteins exhibiting an abundance change of at least $\pm$ 1.5-fold (Kanter et al., 2013; Zhao et al., 2016) with statistically differences were subjected to gene ontology (GO) classification via UniProtKB entries and the Panther Classification System database. As a result, significantly upregulated and downregulated proteins in the aforementioned 9 clusters were annotated with GO terms, such as biological processes (BP), molecular function (MF) and cellular component (CC), and Reactome pathways. The classification of these proteins into specific functional categories was facilitated by a set of plant-specific GO slims available at https://www.uniprot.org/uploadlists/ and https://pantherdb.org/.

2.2 Recombinant Amb a and TNM induction on rAmb a allergenicity

2.2.1 rAmb a expression in *E.coli*

2.2.1-1 Gene cloning of rAmb a and vector construction in *E.coli*

The preparation of vectors for rAmb a construction was conducted collaboratively with Dr. Feng Zhao, encompassing gene cloning and vector construction of rAmb a in *E. coli* (unpublished work). A comprehensive outline of the principal steps and results is presented below.

Genomic DNA from commercial ragweed pollen (CRP, Thermo Fisher Scientific, Sweden) served as the template for gene cloning. The nucleotide information of the 10 target genes underwent alignment with the National Center for Biotechnology Information (NCBI) database. Primers were strategically designed in the N-terminal to C-terminal order, incorporating restriction enzymes, as illustrated in Table 2 - 7.

The PCR products obtained from gene cloning were ligated into the pETM-14 vector (EMBL Protein Expression and Purification Core Facility) (Figure 2 - 6) after being digested with the specified restriction enzymes (Table 2 - 7), respectively. Following this, the reaction system underwent transformation into BL21 (DE3) pLysS *E. coli* (Promega, Germany). Finally, the confirmed bacterial targets resistant to Kanamycin (Kan$^+$) were preserved in sterile glycerol at a final concentration of 30% (v/v) and stored at - 80 °C until needed.
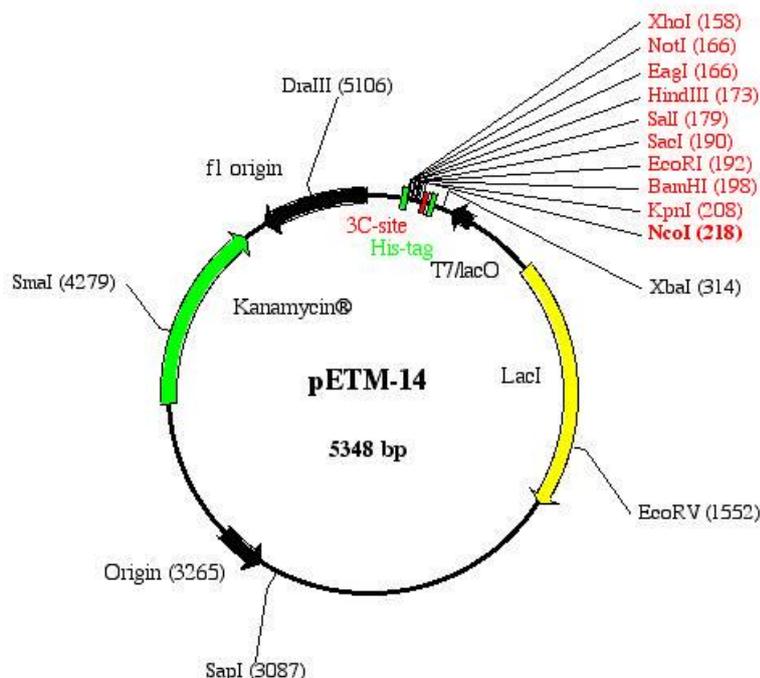
Figure 2 - 6: Map of the pETM-14 vector including tags and protease recognition sites.

2.2.1-2 Expression and purification of recombinant ragweed allergens

The expression and purification of rAmb a under denatured conditions, following the QIAexpress system, were carried out with the assistance of two master's students, Nikolaus Repgen and Julia Ruiz Capella. The 6 x His affinity tag was employed for the expression and purification of rAmb a (see Figure 2 - 6).

In general, the procedures for expressing and purifying rAmb a in *E. coli* adhered to the protocol outlined in the QIAexpress system manual with slight modifications. Notably, during the reactivation and amplification of positive colonies of rAmb a in (Luria-Bertani) liquid medium (LB) (Protocols, 2006) at 37 °C, all rAmb a were shaken at 200 rpm/min, except for rAmb a 1, which was shaken at 100 rpm/min. Additionally, when isopropyl β-D-1-thiogalactopyranoside (IPTG) was introduced to induce the expression of the target protein in *E. coli*, a concentration of 0.5 mM IPTG was added for all rAmb a variants, and the system was shaken at 37 °C for 4 hours at 200 rpm/min, except for rAmb a 1. For rAmb a 1, 0.1 mM IPTG was added, and the system was shaken overnight at 28 °C at 100 rpm/min.

2.2.1-3 Quality control and quantification of the purified rAmb a

Prior to utilizing the purified rAmb a, a thorough quality control assessment was conducted using 4 – 20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, USA), applying 150 V for 1 h. Protein visualization on the gel was achieved using Coomassie Brilliant Blue. The gel underwent staining for 30 minutes in a staining buffer (0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% ddH_2O) and was subsequently destained overnight in a destaining buffer (50% (v/v) methanol in water with 10% (v/v) acetic acid). For the quantification of rAmb a, the Bradford method (refer to section 2.1.3-5) was employed. Ultimately, the protein solution concentration was adjusted to 0.4 μg/μL.

Table 2 - 7: Primer sequences used for gene cloning

Allergens	GenBank Nucleotide	Forward and reverse primers (5' - 3')	Restriction enzymes	Additional Information
Amb a 1.0301	M62961	F CATGCCATGGCCAGCCGAAGGGGTCGGGG R CCGCTCGAGCGGGCAAGGTGCTCCAGGATGGC	Nco I Xho I	Signal Peptides were removed
Amb a 3.0101		F CATGCCATGGCCAGGAAAAGGTCTACCTTGTCGGTGG R CCGCTCGAGCGGGCTAGGGGGTAACATTGGCTACAAAC	Nco I Xho I	Signal Peptides were removed
Amb a 4.0101	FN687761	F CATGACATGTCAAAACTATGTGAAAAACCGAGTGTAACTT R CCGCTCGAGCGGGACCTCCTGAAGGAGGGGAAGG	Pci I Xho I	Signal Peptides were removed, repeat region at 3' end was removed
Amb a 5.0101		F CATGACATGTCA TTAG/CTGCCTTGTGCCTGGGC R CCGCTCGAGCGG ATTTTGCCGCCATTTTTT/GGCTG	Pci I Xho I	
Amb a 6.0101	U89793	F CATGTCATGACAGCATCGCCAACATGTGATACTGT R CGCGGATCCGCGTCATACTGGAAGTTTTGAACAGTCGA	Bsph I BamH I	Signal Peptides are removed
Amb a 8.0101	AY894660	F CATGACATGTCA ATGTCGTGGCAGACTTATGTGGAT R CCGCTCGAGCGG TTACATGCCTTGATCCACAAGATAAT	Pci I Xho I	
Amb a 9.0101	AY894657	F CATGACATGTCA ATGGCAGAGGAAGAAGACAAGGC R CCGCTCGAGCGG TTAGAATATCTTGCCAACGTCCTTCA	Pci I Xho I	
Amb a 10.0101	AY894659	F CATGACATGTCA ATGGCGCCAGAAAAATAACAAACA R CCGCTCGAGCGG TTACATTGCACCATCACCTCCCT	Pci I Xho I	
Amb a 11.0101	KF528831	F CATGACATGTCA TTCCATTACCCATGAGAGAGAGCTC R CCGCTCGAGCGG TCACAACTCCAACAATTTAGTCCTTAT	Pci I Xho I	Signal Peptides are removed
Amb a 12.0102	KU593513.1	F CAT GCC ATG GCA ATGGCAACCATCAAGGCAG R CCGCTCGAGCGGCTAGTAGGGTTCCACTGGCTTG	Nco I Xho I	

2.2.2 Tyrosine-nitration detection of rAmb a

2.2.2-1 Induction of rAmb a

Firstly, all ten rAmb a were expressed and purified following the procedures outlined in section 2.2.1-2. Qualitative confirmation was obtained through SDS-PAGE gel analysis, revealing the molecular weight of the rAmb a (refer to Figure 2 - 7).
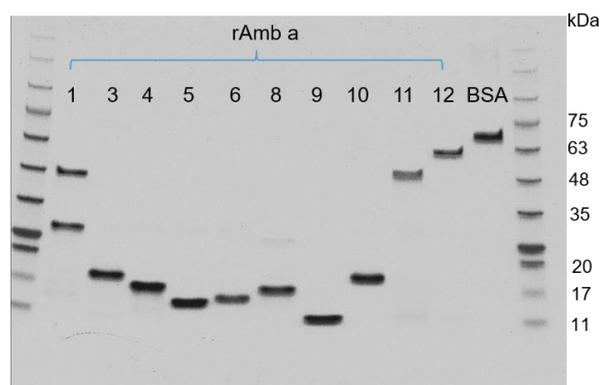
Figure 2 - 7: Visulization of rAmb a on 4 – 20 % Tris-Glycine SDS-PAGE gel stained with Coomassie Brilliant Blue. Lanes: 1 and 13, molecular weight markers; 2 - 11, rAmb a 1, 3, 4, 5, 6, 8, 9, 10, 11 and 12; 12, bovine serum albumin (BSA).

Subsequently, all ten rAmb a underwent *in vitro* treatment with TNM. A follow-up investigation to determine whether tyrosine nitration occurred in any of the TNM-treated rAmb a was conducted using dot blot analysis, as outlined below. By comparing signal intensities of the same protein with and without TNM treatment, it can be inferred that rAmb a 1, rAmb a 3, rAmb a 8, rAmb a 11, and rAmb a 12 may undergo nitration by TNM *in vitro* (refer to Figure 2 - 8). Furthermore, an evaluation of the altered allergenicity of rAmb a due to TNM treatment was conducted through dot blot analysis using a pool of patient sera (Figure 2 - 9).
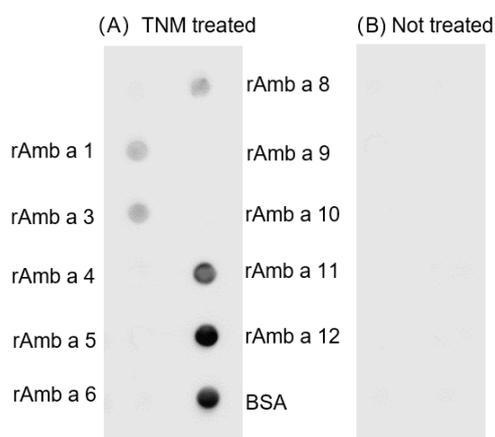
Figure 2 - 8: *In vitro* induction of rAmb a treated with TNM (A) and without TNM (B) determined by dot blot assays. The order of the protein samples was shown in A. The order of the samples in B was identical to that in A.
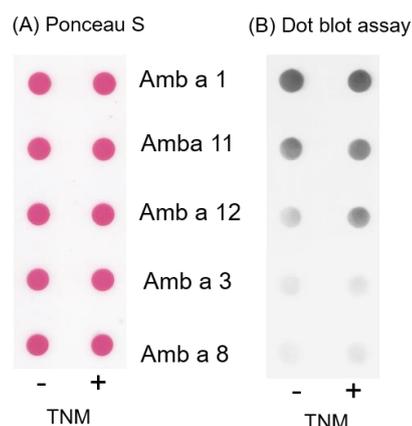
Figure 2 - 9: Altered allergenicity of rAmb a (Amb a 1, 11, 12, 3 and 8) with (+) and without (-) TNM treatment. Representative dot blot membranes stained with Ponceau S dye (A) and then probed with a pool of selected patient sera (B).

The results of the pre-experiment demonstrated a clear increase in the allergenicity of rAmb a 12 following TNM treatment, as observed with mixed sera from ragweed sensitized patients (refer to Figure 2 - 9). Subsequent investigations delved into the impact of TNM-nitrated variants of rAmb a 1, rAmb a 3, rAmb a 8, rAmb a 11, and rAmb a 12 on individuals allergic to ragweed pollen. Consequently, *in vitro* tyrosine nitration of rAmb a (1, 3, 8, 11, and 12) induced by TNM was performed with a TNM/Tyr ratio set at 5:1 (Yang et al., 2010). The TNM stock solution utilized in the study was in methanol at a final concentration of 0.5 M. The volumes of TNM added varied depending on the Tyr content of each allergen protein, as outlined in Table 2 - 8. The reaction between TNM and the protein occurred on ice for 30 minutes. BSA served as a negative control, demonstrating resistance to nitration by TNM.

Table 2 - 8: The volumes of diluted protein solution and stock solution of TNM used for *in vitro* tyrosine nitration

Allegens	Volume of diluted protein solution (0.4 µg/µL) (µL)	Volume of 0.5 M TNM (µL)
rAmb a 1	53	1
rAmb a 3	23	1
rAmb a 8	23	1
rAmb a 11	17	1
rAmb a 12	17	1

2.2.2-2 Detection of nitrated proteins by dot blot

The dot-blot assay aimed to ascertain potential nitration of rAmb a by TNM. Following the procedure outlined earlier (refer to 2.1.4-2), slight modifications were introduced for this dot-blot assay, involving the use of two detection antibodies. The initial detection antibody was anti-nitro tyrosine (monoclonal, mouse-derived, clone 1A6; EMD Millipore, USA) diluted at 1:2000 in TBST. Subsequently, the second detection antibody employed was rat-anti-mouse IgG 2b conjugated with HRP (provided by MAB Monoclonal Antibody Core Facility, HMGU, Munich, Germany) at a dilution of 1:20,000 in TBST (refer to Table 2 - 6). Membrane visualization was conducted using the aforementioned fusion system, with an exposure time of 5 minutes. Image analysis utilized Image J, following established protocols.

2.2.2-3 Detection of allergenicity by immunoassay

Generally, 3 µg of proteins underwent treatment with and without TNM nitration, and the resulting allergenicity of the recombinant proteins was assessed and compared through dot-blot immunoblotting analysis. The dot-blot assay procedure mirrored the previously outlined steps (refer to 2.1.4-2). Notably, serum from serum list 3 was used as the first detection antibody in the primary phase of the study, at a dilution of 1:100. The exposure time for membrane visualization was standardized at 5 minutes. All other procedural steps remained consistent with the previously described protocol. Ultimately, the ratio between IgE binding activity levels (post-TNM/pre-TNM nitration) was calculated and established.

2.3 Analysis of leave associated fungal communities via Illumina MiSeq Sequencing

2.3.1 Genomic DNA preparation

2.3.1-1 Field sampling of ragweed leaves

To explore fungi associated with *A. artemisiifolia* (for both pathogenic and endophytic), two fresh leaves per plant were gathered from 18 naturally growing plants of comparable size in August 2019. The selected plants were situated in an open waste grassland near Building 41b on the HMGU campus in Neuherberg, Munich, Germany. Leaf samples were individually stored at -80 °C until processing.

2.3.1-2 DNA extraction

Before DNA extraction, each leaf sample was placed in 2 mL tubes containing two stainless steel beads (Ø 5mm), swiftly frozen in liquid nitrogen for 10 seconds, and homogenized five times at $6.0 \, ms^{-1}$ for 1 minute using a FastPrep 24 machine (MP Biomedicals, USA). Following each FastPrep program, samples were frozen again in liquid nitrogen for 10 seconds. About 60 mg of the sample was utilized for DNA extraction with the DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany). Steps 1 and 2 in the protocol were omitted, as cell disruption was accomplished through the aforementioned procedure. The subsequent steps adhered strictly to the manufacturer's instructions. The experiment included two negative controls: one employing sterile water instead of leaf samples and the other utilizing sterile water indicating the aseptic operating environment. Genomic DNA quantification was conducted using the NanoDrop System (ND-1000 Spectrophotometer) at wavelengths of 230, 260, and 280 nm.

2.3.2 Preparation for Illumina sequencing

2.3.2-1 PCR amplification

Genomic DNA served as templates, and Next-Generation Sequencing (NGS) techniques, specifically Illumina MiSeq, were employed to assess the species richness of fungal associates naturally occurring on ragweed leaves. Barcode gene sequencing, utilizing the ITS2 region, was conducted with primer ITS3mixF/ITS4mixR (Tedersoo et al., 2015), including adaptor sequences as outlined in the MiSeq Amplicon guide (Table 2 - 9). The PCR amplification setup is detailed in Table 2 - 10, and the amplification procedure followed the program outlined in Table 2 - 11. Each sample underwent three independent PCRs, executed with 28, 30, and 32 cycles in the PCR program, respectively.

The PCR products obtained underwent processing through agarose gel electrophoresis on a 1.5% gel, composed of 2.25 g of agarose (USB Corporation, Cleveland, OH, USA), 150 mL of 1× Tris-Acetate-EDTA (TAE) buffer, and 0.1% EtBr (Ethidium Bromide solution, Sigma-Aldrich, Buchs, Switzerland). A running buffer of 1× TAE was utilized. The 50× TAE buffer stock solution (1 L) comprised 242 g of Tris base in ddH$_2$O, 57.1 mL of glacial acetic acid, and 100 mL of 0.5 M EDTA solution (pH 8.0). Electrophoresis was conducted using a Consort model instrument (peqlab, Erlangen, Germany). 10 µL of each sample was mixed with 2 µL of 6× DNA loading dye (Thermo Fisher Scientific). As a DNA standard, 3 µL of GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) was employed. Gels were run at 120 V for 1.5 hours. Gel documentation was executed using a Gel Documentation system (Quantum M1 1100/26 MX Gel Documentation, Vilber Lourmat, Germany) and MegaCapt software (Version 16.0.7.0, Vilber Lourmat, Germany).

2.3.2-2 PCR products purification

Three PCR products per sample were pooled and subjected to purification using the Agencourt AMPure XP PCR purification procedure (Beckman Coulter, Inc., USA). The purification was conducted in accordance with the manufacturer's instructions in a 96-well format. An AMPure XP volume of 50 µL was added to the 50 µL PCR reaction volume. The purified products were then analyzed on a 1.5% gel, running at 120 V for 1.5 hours, as previously described.

Table 2 - 9: Sequencing primers used for ITS2 PCR as described previouly (Nickel et al., 2018)

Primer name	Orientation	Primer Sequence	Target family
ITS3-Mix1	fw	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG* CATCGATGAAGAACGCAG	Fungi
ITS3-Mix2	fw	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG* CAACGATGAAGAACGCAG	Chytridiomycota
ITS3-Mix3	fw	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG* CACCGATGAAGAACGCAG	Sebacinales
ITS3-Mix4	fw	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG* CATCGATGAAGAACGTAG	Glomeromycota
ITS3-Mix5	fw	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG* CATCGATGAAGAACGTGG	Sordariales
ITS4-Mix1	rv	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* TCCTCCGCTTATTGATATGC	Fungi
ITS4-Mix2	rv	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* TCCTGCGCTTATTGATATGC	Chaetothyriales
ITS4-Mix3	rv	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* TCCTCGCCTTATTGATATGC	Archaeorhizomycota
ITS4-Mix4	rv	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* TCCTCCGCTGAWTAATATGC	Tulasnellaceae

fw = forward, rv = reverse. Primer sequences consist of adaptor sequences (MiSeq Amplicon guide, bold and italic) and ITS primer.

Table 2 - 10: Reaction setup for PCR amplification

Component	50 µL Reaction
Template DNA (10 ng/µL)	1 µL
10 µM Forward Primer	0.5 µL
10 µM Reverse Primer	0.5 µL
NEB Next High-Fidelity Master Mix	10 µL
H$_2$O molecular grade	to 20 µL

Table 2 - 11: Thermo-cycling program for the PCR amplification

Step	Temperature	Time
Initial polymerase activation	95 °C	5 minutes
28 cycles	95 °C	30 seconds
	55 °C	30 seconds
	72 °C	1 min
Final Extension	72 °C	10 minutes
Hold	4 °C	

2.3.2-3 DNA quantification

The resulting products were quantified using the Biotium AccuClear Ultra High Sensitivity dsDNA Quantification Kit, including 7 DNA standards (Biotium, USA), following the manufacturer's instructions. Specifically, 10 µL of DNA standard was pipetted into the respective wells and thoroughly mixed. The prepared 96-well plate (Nunclon 96 Flat Bottom Black Polystyrol, ThermoFisher Scientific, Denmark), containing both PCR products and DNA standards, was immediately shaken inside the Tecan Reader and read after a 10-minute incubation, employing the settings detailed in Table 2 - 12.

Table 2 - 12: Tecan reader setting for Biotium AccuClear Ultra High Sensitivity dsDNA quantification

Mode	Fluorescence top reading
Excitation Wavelength	468 nm
Emission wavelength	507 nm
Excitation bandwidth	5 nm
Emission bandwidth	5 nm
Gain	100 Manual
Number of flashes	50
Flash frequency	400 Hz
Integration time	20 µs
Lag time	0 µs
Settle time	10 ms
Z-Position (Manual)	20000 µm

2.3.2-4 Indexing and DNA sequence library preparation

Sequencing adapters from the Nextera Index Kit v2 Set A (Illumina, Hayward, USA) were employed for indexing (see Table 2 - 13). Considering the previously measured DNA concentrations, each sample was diluted to a concentration of 10 ng/µL for the Indexing PCRs, following the program outlined in Table 2 - 14.

Table 2 - 13: Reaction setup for indexing PCR

Component	50 µL Reaction
DNA (10 ng/µL)	1 µL
Primer 1 (i7 series)	2.5 µL
Primer 2 (i5 series)	2.5 µL
NEB Next High-Fidelity Master Mix	12.5 µL
H_2O molecular grade	to 25 µL

Table 2 - 14: Thermo-cycling program for the PCR

Step	Temperature	Time
Initial polymerase activation	95 °C	5 minutes
8 cycles	95 °C	30 seconds
	55 °C	30 seconds
	72 °C	1 min
Final Extension	72 °C	10 minutes
Hold	4 °C	

The obtained products from the indexing PCR underwent purification using the Agencourt AMPure XP PCR purification procedure, following the aforementioned steps. Subsequently, 20 µL of ddH$_2$O was utilized to elute the DNA pellet, and 16 µL was extracted for the subsequent phase. Reassurance of successful indexing was achieved by re-evaluating the fragment length on a gel, which indicated an augmented length post-indexing. Five PCR products, both pre- and post-indexing, were subjected to 1.5% gel electrophoresis at 120 V for 1.5 hours, and the fragment sizes were compared to affirm the indexing PCR quality. The Biotium AccuClear Ultra High Sensitivity dsDNA Quantification Kit, inclusive of 7 DNA standards, facilitated the quantification of the purified indexing PCR products, employing the same previously described procedure.

Following quantification, the purified indexing PCRs were diluted to a concentration of 10 nM per sample. A pooled solution was created by combining 3 µL from each sample and 1 µL from each negative control into a single 500 µL reaction tube.

The DNA sequence library, once constructed, underwent quantification using Qubit (Qubit 3, Invitrogen, Thermo Fisher Scientific) to determine nucleotide concentration. Subsequently, a 50 µL aliquot of the sequence library was prepared at a concentration of 4 nM. This prepared aliquot was dispatched to the sequencing laboratory (Core Facility Genomics at HMGU, Munich, Germany) equipped with the necessary kit (MiSeq v3 Kit, Illumina). The sequencing was carried out employing the Illumina MiSeq system.

2.3.2-5 Bioinformatics sequence analysis

Sequencing data were supplied in FASTQ format for subsequent analysis with the help of Dr. Fabian Weikl. The raw data underwent processing using the PIPITS v2.1 ITS analysis pipeline (Gweon et al., 2015), executed on Linux Ubuntu (version 16.04.02). The UNITE reference database version 7.2 (Kõljalg et al., 2013) was utilized during this process. PIPITS initially readied the MiSeq sequences for ITS extraction, eliminating potentially erroneous sequences. Subsequently, fungal ITS sub-regions, specifically the ITS2 regions, were extracted and matched against the reference database.

To maintain analytical integrity, samples with low sequence counts were excluded to prevent bias in the analyses. Sequences lacking affiliation with any organism in the public database were excluded from subsequent analyses. Furthermore, sequences attributed to protozoa, protista, plantae, and chromista, along with those associated with Tracheophyta (primarily assigned to *A. artemisiifolia*), were removed. The resulting sequences were categorized into OTUs (operational taxonomic units) and phylotypes. Following the definitions described previously (Gweon et al., 2015), OTUs encompass sequence groups exceeding 97% identity. Phylotypes, on the other hand, denote sequence groups grouped under the same taxonomic units, such as genus or species.

2.4 Fungal isolates and their identification and cross-reactivity with ragweed pollen

2.4.1 Isolation and cultivation of fungal isolates

2.4.1-1 Isolation from ragweed leaves

Fresh leaf samples (refer to section 2.3.1-1) were individually immersed in 15 mL of 0.5% Tween 20 buffer, and gently rotated in a 50 mL Falcon tube at 15 rpm for 10 minutes. The washing process involved two replacements of the 0.5% Tween 20 buffer, followed by surface drying of the leaves using sterile filter paper. Subsequently, the cleaned leaves were arranged on sterile, single-layer filter paper and placed in petri dishes containing 10 mL of distilled water supplemented with 0.1% ampicillin (Sigma-Aldrich, Taufkirchen, Germany) and 0.1% kanamycin. Incubation occurred at 25 °C under natural day/night conditions for 7 days.

After this period, the growing fungal hyphae on the incubated leaves were examined using a dissecting microscope. The solution was homogenized with gently shaking and mixing, and 100 µL of the solution was extracted. Following a 10-fold dilution with sterile water, the diluted solution was evenly inoculated onto 2% malt extract agar (MEA) consisting of 2% malt extract and 1.5% agar, with a final pH of 5.5 ± 0.3, supplemented with 0.1% ampicillin and 0.1% kanamycin. Two aliquots of each raw solution sample were stored in glycerol with a final concentration of 30% at -80 °C.

Inoculated plates were kept under natural diurnal conditions at 25 °C. Following a 5-day incubation, the plates underwent examination, and each newly emerging colony was meticulously picked and transferred to a fresh MEA plate. After another seven days of growth, all isolated colonies underwent an initial screening for morphological similarity on the MEA medium. Among the screened colonies sharing similar traits, only one colony was chosen for the subsequent isolation step. By incorporating synthetic nutrient agar (SNA), a low-nutrient medium conducive to the sporulation of various fungi, some colonies could be characterized based on microscopic

features. The SNA medium composition included 1 g/L KH_2PO_4, 1 g/L KNO_3, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 0.2 g/L glucose, 0.2 g/L sucrose, and 20 g/L agar.

2.4.1-2 Purification of fungal isolates by single spore technique

After morphological and microscopic characterization of fungal isolates (Table 3 - 11), 15 cultures underwent further purification, employing single-spore isolation techniques (Choi et al., 1999; Noman et al., 2018), for fungal species identification and subsequent examination of cross-reactivity between fungal extracts and ragweed pollen allergens.

In detail, fungal isolates cultured on MEA were transferred to new SNA plates and incubated for 7 days. Using a dissecting microscope, 100-300 fungal spores in 0.1 mL of 0.5% Tween 20 were inoculated onto 2% water agar (WA, 20 g/L agar in ddH_2O with a pH of 5.6 ± 0.3). The spore-solution was spread using stainless steel spreaders, then air-dried for 10 minutes. Specifically, the WA was prepared two days in advance and air-dried at room temperature for 2 hours to remove excess water film. The air-dried WA plates were subsequently incubated at 28 °C for 2 to 4 hours. Following this, a small piece (0.5 x 0.5 cm) of agar medium containing one germinated spore was excised and placed on 2% potato dextrose agar (PDA, 4 g/L potato extract, 20 g/L dextrose, 15 g/L agar with a final pH of 5.6 ± 0.3). Fungal growth was monitored after 7 days of incubation at RT, and pure colonies of fungal isolates were further cultured on MEA. Additionally, for fast-growing colonies, pure white, largely appressed hyphal tips were transferred to obtain a pure culture.

2.4.2 Identification of fungal isolates by Sanger Sequencing

2.4.2-1 DNA extraction

After further single-spore purification on water agar, 26 morphologically distinct colonies (with two isolates being morphologically similar) underwent additional characterization based on their DNA sequences, amplified by Sanger sequencing.

Mycelia were aseptically harvested from 10-day cultures for DNA extraction using the DNeasy® PowerSoil® Kit. The procedure precisely followed the steps outlined in section 2.3.1-2,

which included homogenization via the FastPrep 24 machine. Genomic DNA quantification was conducted with the NanoDrop System at 230, 260, and 280 nm. Subsequently, DNA concentration measurements guided the dilution of DNA to 10 ng/µL.

2.4.2-2 ITS PCR for Sanger sequencing

The genomic DNA obtained served as the template for PCR amplification, employing Q5 Hot Start High-Fidelity DNA Polymerase (NEB, UK) in 50 µL volumes. The PCR setup is detailed in Table 2 - 15, with the modification of incorporating 100 ng DNA. Primers ITS1-F (TCCGTAGGTGAACCTGCGG) and ITS4-R (TCCTCCGCTTATTGATATGC) (White et al., 1990) were utilized for amplifying the ITS region (ITS1 and ITS2) along with the 5.8S ribosomal RNA gene. The amplification process adhered to the program outlined in Table 2 - 16. Subsequently, the PCR products obtained underwent processing in a 2% gel, running at 125 V for 1.5 hours, as detailed in section 2.3.2-1. Hereafter, the PCR products were purified using the Agencourt AMPure XP PCR purification procedure, precisely following the instructions outlined in section 2.3.2-2, and subsequently controlled using a 1.5% gel, running at 120 V for 1.5 hours, as detailed earlier in section 2.3.2-1.

PCR products were successfully generated from 27 fungal isolates, targeting the fungal ITS1 + ITS2 + 5.8S regions. Due to morphological similarities and nearly identical PCR fragment lengths, 15 fungal isolates were selected for Sanger sequencing. Eurofins MWG GmbH facilitated the sequencing process, and the obtained sequences were subjected to a BLAST search against the UNITE database (https://unite.ut.ee/analysis.php#). The results with the highest similarity score were considered for further analysis.

Table 2 - 15: Reaction setup for PCR

Component	50 μL Reaction	Final Concentration
5 X Q5 reaction buffer	10 μL	1X
5 X Q5 High GC Enhancer (optional)	10 μL	1X
10 mM dNTPs	1 μL	200 μM
Template DNA	5 μL	< 1,000 ng
10 μM Forward Primer	2.5 μL	0.5 μM
10 μM Reverse Primer	2.5 μL	0.5 μM
Q5 Hot Start High-Fidelity DNA Polymerase	0.5 μL	0.02 U/μL
Nuclease-Free Water	to 50 μL	

Table 2 - 16: Thermo-cycling program for the PCR

Step	Temperature	Time
Initial polymerase activation	98 °C	30 seconds
32 cycles	98 °C	10 seconds
	63 °C	30 seconds
	72 °C	30 seconds
Final Extension	72 °C	2 minutes
Hold	8 °C	

2.4.3 Cross-reactions between fungal isolates and ragweed pollen

Dot blot assay was used to screen the IgE-reactive patterns of fungal isolates, ragweed pollen and their combination, probed with patients' sera allergic to ragweed pollen, respectively. Aqueous extracts of fungal isolates and ragweed pollen were prepared following the procedure outlined in section 2.1.3-3. Notably, preceding the hydration of fungal mycelia in NH_4HCO_3, an additional homogenization step was executed using the FastPrep 24 machine, with program details precisely specified in section 2.3.1-2. Protein concentrations for both extracts were quantified using the Bradford method, as described in section 2.1.3-5. The procedures for dot blot assay was exactly following the steps as described in section 2.1.4.

2.5 Statistics

The data underwent initial processing for normal distribution using the Shapiro-Wilk test through IBM SPSS Statistics 21. Two-way ANOVA, complemented by Fisher's LSD tests, was employed when the data satisfied the criteria of normal distribution and homogeneity of variance ($p > 0.05$), considering temperature and NO_2 as factors, along with their interaction. GraphPad Prism software for Windows (version 9.0.2) facilitated these analyses. In cases where the data did not meet the assumptions, the Kruskal-Wallis test and Dunn's test were utilized for statistical testing. Additionally, the unpaired two-tailed Student's t-test was applied. Principal component analysis score plots and loading plots were generated using RStudio (version 2022–02–16 for Windows) (Cheng et al., 2023). Tables were created in Excel 2016, and figures were plotted using GraphPad Prism. Venn diagrams were generated online (Tang et al., 2023), and Multivolcano plots were produced online using BioLadder (bioladder.cn). Detailed procedures for each analysis are presented in the respective results sections.

3: Chapter – RESULTS

3.1 Plant growth characteristics in response to elevated temperatures and elevated NO_2

Alterations in plant sizes, such as plant height and male inflorescence size, and observations on pollen release are presented and compared within treatments, for both experiments separately. A comparison between the two experiments was impeded by a technical failure of temperature control in the second experiment which prevented comparable temperature curves between the two experimental runs. However, distinct responses in plant growth parameters and pollen release are reported and compared with the necessary care in the following paragraphs.

3.1.1 Plant height indicating plant growth and development

In both experiments, ragweed plants in all conditions showed a classic sigmoid increase in height throughout the period spanning from DAS 45 to DAS 126 for experiment 1 (the Munich scenario, Figure 3 - 1 A), and from DAS 53 to DAS 122 for experiment 2 (the Würzburg scenario, Figure 3 - 1 B). In experiment 1, ragweed plants subjected to elevated temperature-related conditions (T_M4 and T_M4N) experienced a delayed onset of the stationary phase, occurring approximately 17 days later (initiating around DAS 104), in comparison to those grown under ambient temperature-related conditions (T_M and T_MN), which entered the stationary phase around DAS 87. In contrast, in experiment 2, ragweed plants exposed to the T_W4 condition displayed a delay in the stationary phase by about 14 days (commencing at approximately DAS 115) compared to ragweed plants subjected to T_W, T_WN, and T_W4N conditions (commencing at approximately DAS 101).
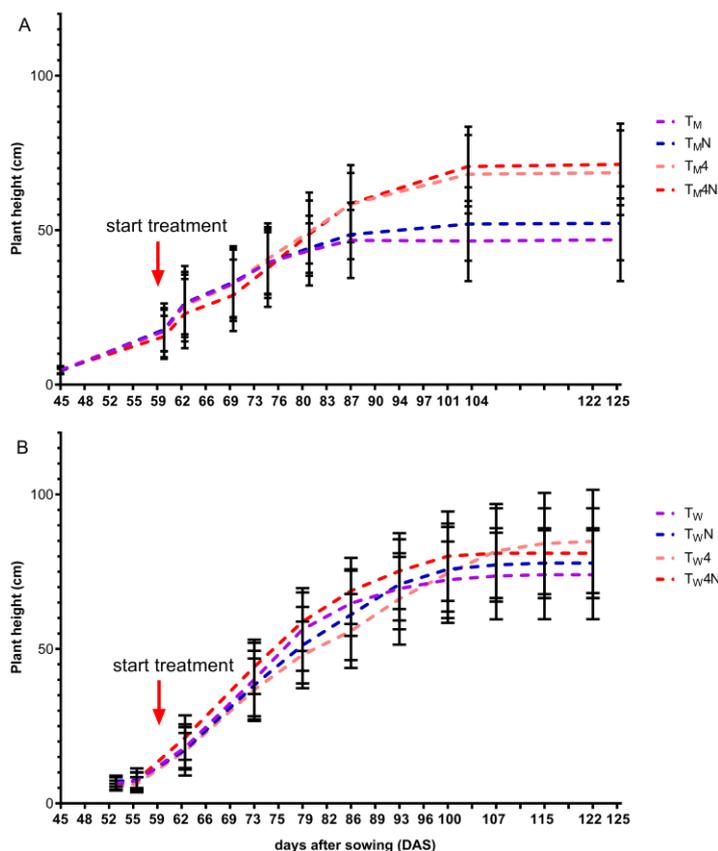
Figure 3 - 1: Time courses of the average plant height (12 plants per condition, mean ± standard deviation) of *A. artemisiifolia* plants grown in two experiments under current (T) and future temperature (T4) simulations with 80 ppb (N) and no (0 ppb) NO_2 in the ambient air: A), the "Munich" scenario, measured between DAS 45 and DAS 126; and B), the "Würzburg" scenario, measured between DAS 53 and DAS 122. Red arrows in the figure indicate the initiation of experimental treatments on DAS 59. Abbreviations: DAS, days after sowing; T_M, current temperature Munich; T_W, current temperature Würzburg; T_M4, and T_W4, temperature increase by 4 °C in Munich and Würzburg, respectively.

Moreover, in both experiments, there were no significant differences in ragweed plant heights among experimental conditions within the same year when stress treatments were applied at DAS 59 and DAS 60 (Table 3 - 1 and Figure 3 - 2). However, in experiment 1, the heights of ragweed plants subjected to elevated temperature-related conditions (T_M4 and T_M4N) were consistently and significantly higher ($p < 0.05$) than those of plants grown under ambient temperature-related conditions (T_M and T_MN) at DAS 87, DAS 104 and DAS 126 (Table 3 - 1 A). Specifically, a temperature increase by 4 °C significantly augmented plant height at DAS 126, manifesting

notable increases of 46.3% and 36.6% in the final height of ragweed plants subjected to treatments represented by clusters T_M4 vs T_M, and T_M4N vs T_MN, respectively. Conversely, in experiment 2, ragweed plants exposed to the T_M4N condition exhibited significantly greater heights at DAS 80 and DAS 87 compared to those subjected to the T_M4 condition, demonstrating substantial increases of 22.2% and 23.3%, respectively (Table 3 - 1 B). Furthermore, the significant differences in plant heights between conditions subsequently diminished, with only a slight increase in final plant height of 14.8% (p = 0.17) observed in the T_W4 vs T_W cluster as the temperature increased by 4 °C.

Table 3 - 1: Plant heights (12 plants per condition, mean ± standard deviation) of *A. artemisiifolia* plants measured on specific days in the "Munich" scenario (A) and "Würzburg" scenario (B), in agreement with the data plotted in Figure 3 - 1. Statistically significant results (ANOVA) followed by Fisher's LSD test are indicated by different letters (n = 12, *p* < 0.05). The ANOVA results revealed a statistically significant source of variation (*p* < 0.05) for plant height at DAS 87, DAS 104, and DAS 126 in experiment 1 (A), and at DAS 80 and DAS 87 in experiment 2 (B), respectively. *F* test; DFn = degrees of freedom in the numerator; DFd = degrees of freedom in the denominator; *p* < 0.05. Abbreviations for experimental settings are as in Figure 3 - 1.

A	DAS	Plant height (cm) in experimnt 1 (the Munich scenario)				Two-way ANOVA		
		T_M	T_MN	T_M4	T_M4N	Source of variation	*F* (DFn, DFd)	*p*
	45	4.7 ± 1.3	4.4 ± 0.9	4.7 ± 1.1	4.8 ± 1.2			
	60	17.3 ± 9.0	17.8 ± 7.0	17.5 ± 6.8	15.6 ± 6.7			
	63	26.2 ± 12.2	26.4 ± 10.1	25.5 ± 10.1	23.0 ± 11.2			
	70	32.7 ± 12.1	33.1 ± 11.2	32.6 ± 11.1	28.9 ± 11.6			
	75	39.5 ± 11.5	39.4 ± 10.0	40.7 ± 11.5	37.7 ± 12.5			
	81	43.4 ± 11.3	44.2 ± 8.0	49.4 ± 10.2	48.7 ± 13.5			
	87	46.7 ± 12.2 a	48.6 ± 8.0 a	58.4 ± 10.1 b	58.6 ± 12.5 b	Temperature increase	*F* (1, 44) = 10.9	< 0.05
	104	46.5 ± 13.0 a	52.0 ± 11.9 a	68.1 ± 12.7 b	70.6 ± 12.9 b	Temperature increase	*F* (1, 44) = 27.7	< 0.05
	126	46.9 ± 13.4 a	52.2 ± 12.2 a	68.6 ± 13.7 b	71.3 ± 13.2 b	Temperature increase	*F* (1, 44) = 26.6	< 0.05

B	DAS	Plant height (cm) in experimnt 2 (the Würzburg scenario)				Two-way ANOVA		
		T_W	T_WN	T_W4	T_W4N	Source of variation	*F* (DFn, DFd)	*p*
	53	6.5 ± 2.0	7.2 ± 1.8	5.3 ± 1.1	5.8 ± 1.6			
	56	7.3 ± 2.8	7.5 ± 3.9	6.4 ± 2.1	7.5 ± 2.5			
	63	18.1 ± 6.6	17.3 ± 8.3	16.9 ± 5.9	21.3 ± 7.2			
	73	40.1 ± 11.9	38.3 ± 11.1	36.8 ± 10.1	44.2 ± 8.8			
	80	56.3 ± 13.4 ab	51.2 ± 12.4 ab	48.1 ± 10.8 a	58.8 ± 9.5 b	Interaction	*F* (1, 44) = 5.06	0.03
	87	64.8 ± 10.6 ab	61.1 ± 14.7 ab	55.8 ± 12.0 a	68.8 ± 10.7 b	Interaction	*F* (1, 44) = 5.23	0.03
	94	69.5 ± 10.3	70.9 ± 14.6	66.2 ± 14.8	75.2 ± 12.3			
	101	72.4 ± 12.4	75.8 ± 13.7	74.5 ± 16.1	80.0 ± 14.5			
	108	73.6 ± 14.0	77.2 ± 11.9	81.7 ± 15.2	81.0 ± 14.6			
	115	74.0 ± 14.4	77.8 ± 11.3	84.1 ± 16.4	81.0 ± 14.6			
	122	74.0 ± 14.4	77.8 ± 11.3	84.8 ± 16.7	81.0 ± 14.6			
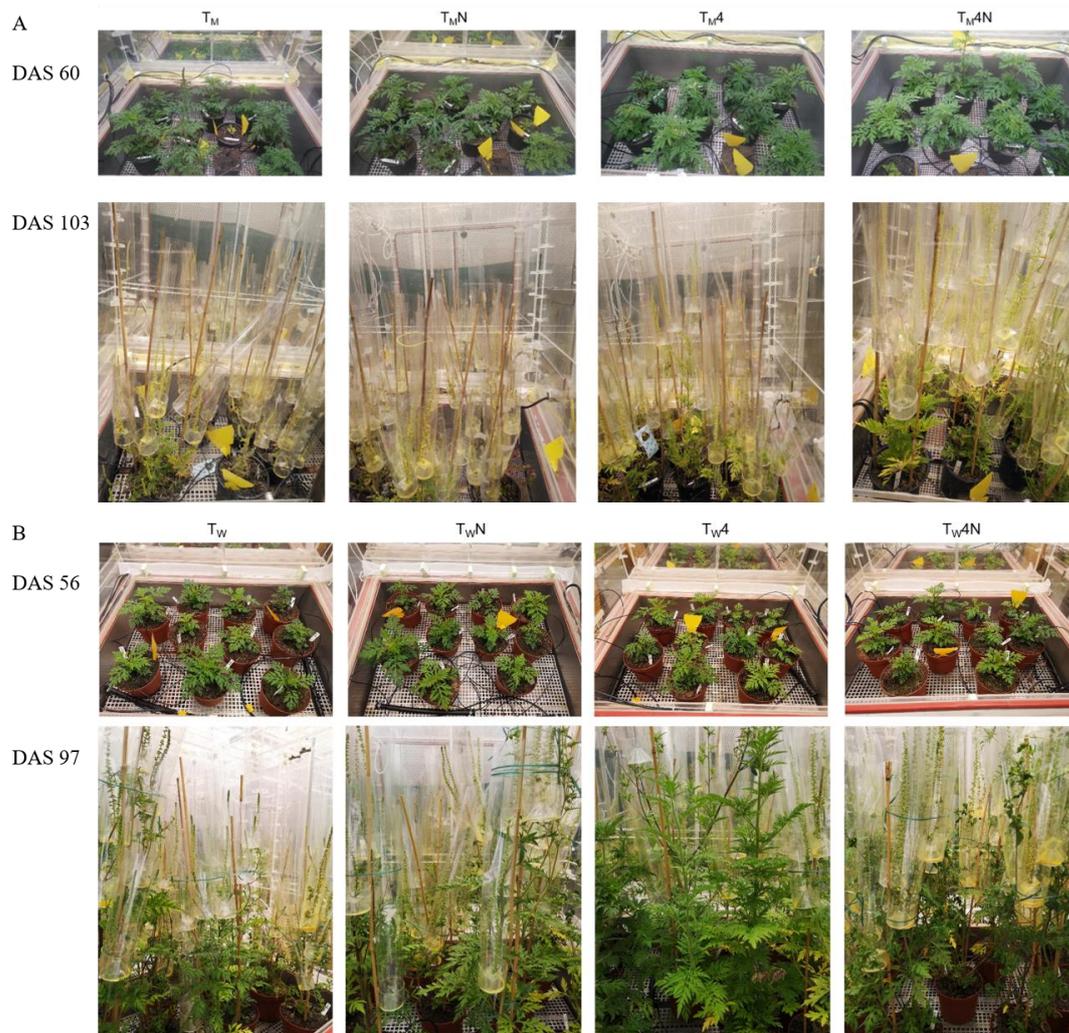
Figure 3 - 2: Representative pictures of *A. artemisiifolia* plant grown under the corresponding conditions for the Munich scenario at DAS 60 and DAS 103 (A), and Würzburg scenario at DAS 56 and DAS 97 (B), respectively. Experimental settings and abbreviations in the figures remain the same as in Figure 3 - 1.

3.1.2 Male inflorescences size at harvest

In experiment 1, there were significant enhancements in the average length of male inflorescences per plant, showing increases of 90.9% and 81.8% in clusters T_M4 vs T_M and T_M4N vs T_MN, respectively, in response to the 4 °C temperature increase (Figure 3 - 3 A). Similarly, in the second experiment, elevated temperature significantly stimulated the average length of male inflorescences in plants in clusters T_W4 vs T_W and T_W4N vs T_WN, with increases of 26.1% and 18.1%, respectively (Figure 3 - 3 B).

3.1.3 Pollen release

In experiment 1, both the initiation and duration of the ragweed pollen season exhibited significant responses to elevated temperature (Table 3 - 2). The commencement of pollen release was notably delayed by 6.5 and 5.4 days, respectively, in clusters T_M4 vs T_M and T_M4N vs T_MN, due to elevated temperature (Figure 3 - 3 C). Conversely, elevated temperature resulted in evident extensions of pollen durations by 13.0 and 11.4 days in clusters T_M4 vs T_M and T_M4N vs T_MN, respectively (Figure 3 - 3 E). However, in experiment 2, the onset of pollen release was significantly influenced by the interaction between elevated NO_2 and elevated temperature (Table 3 - 2). A noticeable advancement of 10.5 days in pollen release was observed due to elevated NO_2 under conditions related to elevated temperature (refer to the T_W4N vs T_W4 cluster) (Figure 3 - 3 D). Moreover, the duration of pollen release was substantially affected by both elevated NO_2 and elevated temperature (Table 3 - 2), resulting in notable extensions of 11.0 days ($p < 0.05$), 7.4 days ($p = 0.09$), and 8.1 days ($p = 0.06$) in pollen durations in clusters T_W4 vs T_W, T_W4N vs T_WN, T_WN vs T_W, respectively (Figure 3 - 3 F).

Furthermore, pollen release per male inflorescence was predominantly influenced by elevated NO_2 in experiment 1 (Table 3 - 3), resulting in notable inductions of 56.5% and 25.3% in pollen release in clusters T_MN vs T_M, T_M4N vs T_M4, respectively. A similar but not significant NO_2 induction of 32.7% in pollen release in experiment 2 was observed in the T_W4N vs T_W4. In contrast, a significant reduction of 45.8% in pollen release was observed in the cluster T_W4 vs T_W in response to elevated temperature (Figure 3 - 3 G and H).
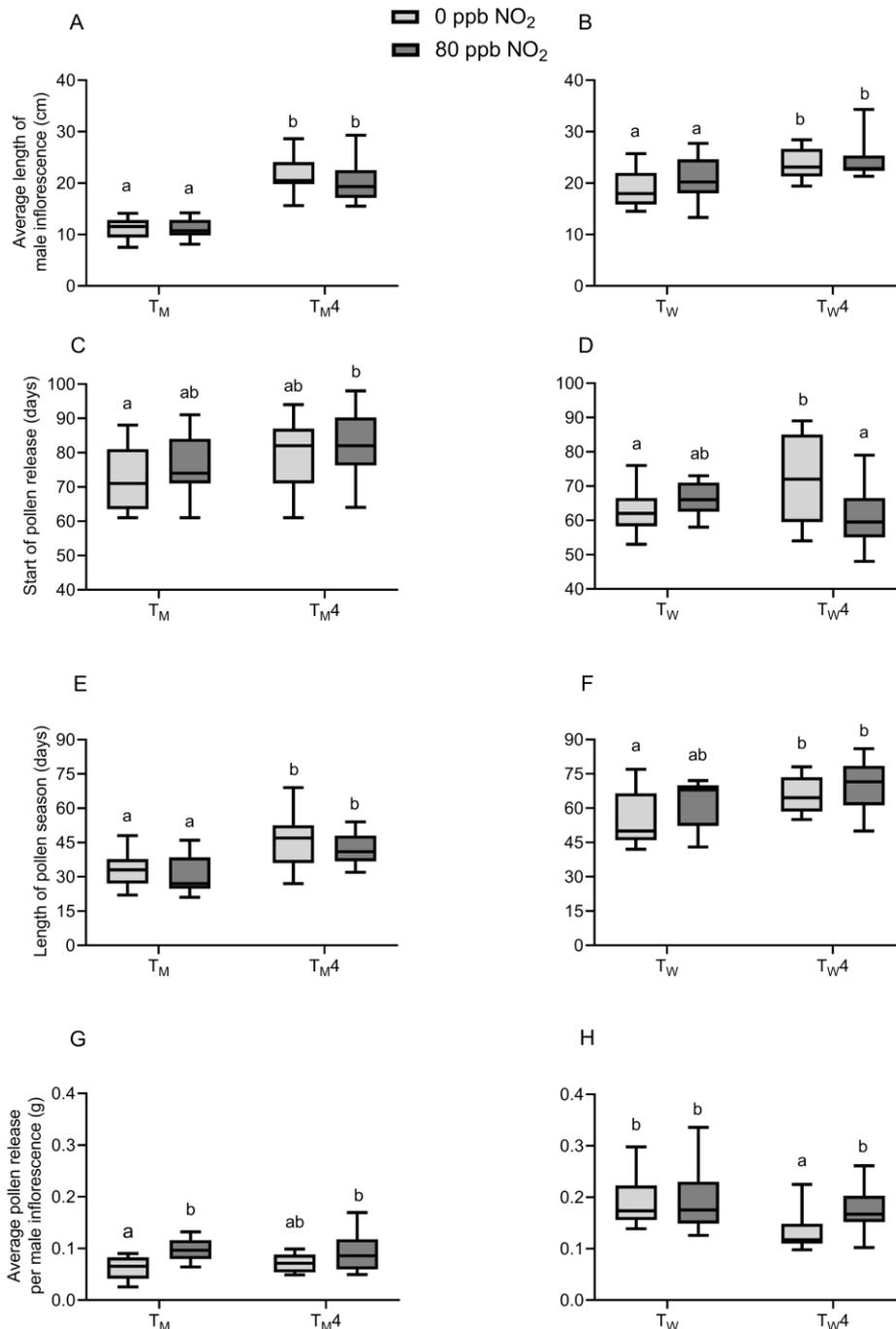
Figure 3 - 3: Growth characteristics of *A. artemisiifolia* plants in two experiments ("Munich", "Würzburg") under current (T) and future temperature (T4) simulations with 80 ppb and 0 ppb NO_2 in the ambient air. A and B), average length of male inflorescence; C and D), start of pollen release; E and F), length of pollen season; G and H), average pollen release per male inflorescence. Experimental settings and abbreviations in the figures remain the same as in Figure 3 - 1. Boxplots with median from 12 plants in each condition, min/max whiskers were applied for plotting. Statistically significant results (ANOVA) followed by Fisher's LSD test are indicated by different letters for all parameters, with the exception that average pollen amount per male inflorescence, which was tested using Kruskal-Wallis test and Dunn's test ($p < 0.05$) (Cheng et al., 2023).

Table 3 - 2: Results of Two-way ANOVA with temperature increase (T+4°C) and addition of 80 ppb NO_2 (NO_2) as factors in two experiments simulating current and future scenarios in the region of Munich and Würzburg, respectively. Measured parameters (quantitative variables, n = 12 plants per condition) were "average length of male racemes per plant", "start of pollen release", and "length of pollen season"; significant factors are indicated in bold; df = degree of freedom, F test, $p < 0.05$.

Parameter	Factor	Munich			Würzburg		
		df	*F*	*p*	*df*	*F*	*p*
Average length of	T+ 4.0 °C	1	119	**<0.0001**	1	17	**0.0002**
male inflorescence	NO_2	1	0.6	0.4367	1	1	0.2760
per plant [cm]	T+ 4.0 °C xNO_2	1	0.4	0.5302	1	0.3	0.5706
	Residual	44			44		
Start of pollen	T+ 4.0 °C	1	4	**0.0400**	1	0.4	0.5206
release [days]	NO_2	1	1	0.2487	1	2	0.1652
	T+ 4.0 °C xNO_2	1	0.04	0.8483	1	8	**0.0081**
	Residual	44			44		
Length of pollen	T+ 4.0 °C	1	21	**<0.0001**	1	9	**0.0040**
season [days]	NO_2	1	1	0.2545	1	4	**0.0439**
	T+ 4.0 °C xNO_2	1	0.09	0.7652	1	0.3	0.5577
	Residual	44			44		

Table 3 - 3: Results of One-way ANOVA (Kruskal-Wallis test) with temperature increase (T + 4 °C) and addition of 80 ppb NO_2 (NO_2) as factors impacting average pollen release per male inflorescence. Experimental settings and abbreviations in the figures remain the same as in Figure 3 - 1. Significant factors are indicated in bold; N = number; *df* = degree of freedom, p < 0.05.

Variable	Groups	N	Median	*df*	Test statistics (Chi-square)	*p* (adjusted for ties)
NO_2	T_M + T_M4	24	0.0673	1	10.48	**0.001**
	T_MN + T_M4N	24	0.0911	1		
	T_W + T_W4	24	0.1546	1	2.266	0.132
	T_WN + T_W4N	24	0.1673	1		
T	T_M + T_MN	24	0.0798	1	0.061	0.805
	T_M4 + T_M4N	24	0.0791	1		
	T_W + T_WN	24	0.1734	1	7.075	**0.008**
	T_W4 + T_W4N	24	0.1523	1		

3.2 The role of flavonoids in the IgE binding activity

3.2.1 Total flavonoids concentration (TFC) in pollen

Both elevated NO_2 and temperature increase significantly influenced the TFC in ragweed pollen, as illustrated in Table 3 - 4 and Figure 3 - 4. Notably, in experiment 1, there were distinct reductions of 15.4% and 39.2% in TFC in pollen extracts due to elevated NO_2 treatment in clusters $T_M N$ vs T_M, $T_M 4N$ vs $T_M 4$, respectively. Conversely, in experiment 2, considerable increases of 29.0% and 22.4% in TFC were observed in elevated temperature treatment in clusters $T_W 4$ vs T_W and $T_W 4N$ vs $T_W N$, respectively.
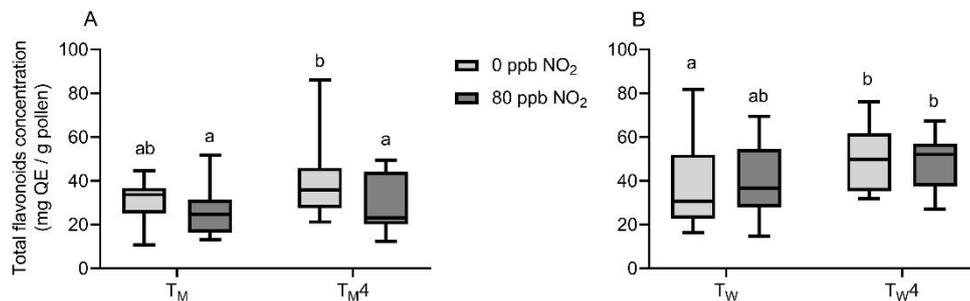
Figure 3 - 4: Total flavonoids concentration of *A. artemisiifolia* pollen in the Munich scenario (A), and Würzburg scenario (B). Description for pollen samples and abbreviations in the figures remain the same as in Figure 3 - 1. Kruskal-Wallis test, and the Dunn's test were indicated by different letters (boxplots with min/max whiskers, 12 plants per condition) ($p < 0.05$).

Table 3 - 4: Results of One-way ANOVA (Kruskal-Wallis test) with temperature increase (T + 4 °C) and addition of 80 ppb NO_2 (NO_2) as factors impacting total flavonoids concentration. Experimental settings and abbreviations in the figures remain the same as in Figure 3 - 1. Significant factors are indicated in bold; N = number; *df* = degree of freedom, $p < 0.05$.

Variable	Groups	N	Median	*df*	Test statistics (Chi-square)	*p* (adjusted for ties)
NO_2	$T_M + T_M 4$	24	33.7099	1	4.423	**0.035**
	$T_M N + T_M 4N$	24	23.3840	1		
	$T_W + T_W 4$	24	45.5778	1	0.043	0.837
	$T_W N + T_W 4N$	24	43.8827	1		
T	$T_M + T_M N$	24	29.5568	1	0.715	0.398
	$T_M 4 + T_M 4N$	24	30.0815	1		
	$T_W + T_W N$	24	35.4568	1	4.868	**0.027**
	$T_W 4 + T_W 4N$	24	51.3210	1		

3.2.2 Flavonoid composition

3.2.2-1 Overview of flavonoid composition in ragweed pollen

A total of 62 flavonoid metabolites were identified through LC-MS/MS in both experiments. These profiles were categorized into five groups exhibiting similar patterns, with groups contributing less than 0.5% to the total flavonoid composition assigned to the category of "others" (Figure 3 - 5). Clearly, flavonoids belonging to the quercetins group were predominant in ragweed pollen samples, constituting an average of 77.5% of the total flavonoid composition. The second-largest contribution to the total flavonoid composition came from the coumarins and hydroxycoumarins group (averaging 12.3%), followed by the kaempferols group (averaging 5.5%), the naringenins group (averaging 1.1%), and the genisteins group (averaging 0.6%). The "others" group comprised 2.9% on average of the total flavonoid composition.
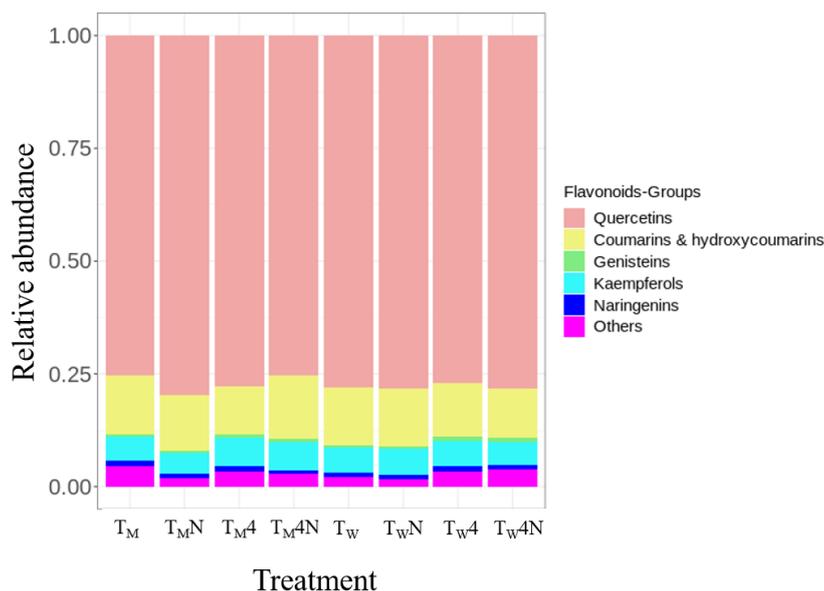
Figure 3 - 5: Relative abundance of each flavonoid group contributing to the flavonoid composition in ragweed pollen for eight conditions across two experiments. Three independent measurements per condition were averaged, and each flavonoid group is distinguished by a unique color. Experimental settings and abbreviations in the figures remain the same as in Figure 3 - 1.

3.2.2-2 Effects of elevated NO_2 and temperatures on the flavonoid composition in ragweed pollen

Principal Component Analysis (PCA) unveiled distinct differences in the distribution of the 62 flavonoid components in ragweed pollen under simulated conditions (refer to Figure 3 - 6). These differences are depicted in the score plots, where three independent samples corresponding to each condition are represented by closely clustered scatters of the same color. The resulting eight clusters across two experiments signify a good repeatability and a high degree of similarity among samples within the conditions, while also revealing marked differentiation between the conditions.

PCA for experiment 1 revealed that a 4 °C temperature increase accounted for most of the variance (PC1, 43.7%). Additionally, a notable NO_2 effect was observed, with the flavonoid composition undergoing substantial changes, more pronounced in NO_2-free treatments than in elevated NO_2 treatments. Elevated NO_2 significantly altered the flavonoid composition in ambient temperatures, explaining 32% of the variance, while a similar but milder effect was observed in elevated temperatures (Figure 3 - 6 A). Likewise, PCA for experiment 2 indicated that elevated temperature explained 53.4% of the variance in flavonoid composition. Moreover, elevated NO_2 contributed to 19.5% of the variance in flavonoid composition in conditions involving T_W4-temperature. However, a noteworthy temperature effect emerged in PCA, with the impact on alterations in flavonoid composition remarkably minimized in conditions involving T_W-temperature compared to those involving T_W4-temperature (Figure 3 - 6 B).

A more intricate categorization facilitated the classification of 62 flavonoid compounds into 13 subgroups. These subgroups, along with the respective compound counts, were as follows: flavonoid glycosides (14), flavones and flavonols (12), hydroxycoumarins (8), coumarins (7), isoflavonoids (6), O-methylated flavonoids (5), flavanonols (3), flavanones (comprising 2 flavonoid species), chalcones and dihydrochalcones (1), epigallocatechins (1), flavonoid-3-O-glycosides (1), isoflavonoid O-glycosides (1), and pterocarpans (1). Simultaneously, in light of the high degree of similarity observed in the PCA score plots among the three samples within the same condition, the abundance of each flavonoid component from each condition was averaged.

The discerned variations in the top 30 flavonoid composition in ragweed pollen under different conditions in PCA were visualized through heat maps, supplemented with subgroup information. Evidently, the clustering of treatments in both experiments in the heat maps aligns with the observations in PCA, indicating that temperature was a key factor explaining a higher variance in the flavonoid composition of ragweed pollen (Figure 3 - 7).

In experiment 1, the clustering of flavonoid composition revealed that among the 14 flavonoid glycosides, four were the most abundant in ragweed pollen in all four conditions. Notably, a specific compound (with the reference ID rpn 128) belonging to the coumarins subgroup exhibited higher enrichment in the elevated NO_2-treatment compared to NO_2-free treatment, contributing greatly to the altered flavonoid composition due to NO_2. Conversely, one compound from the flavones and flavonols subgroup (with the reference ID rpn 136), two from the hydroxycoumarins subgroup (with the reference IDs rpn 163 and rpn 164), and one from the O-methylated flavonoids subgroup (with the reference ID rpn 133), showed substantial variations between ambient and elevated temperatures, playing crucial roles in the temperature-induced alterations in flavonoid composition. Moreover, two compounds (with the reference IDs rpn 138 and rpn 155) from the flavones and flavonols subgroup, along with one (with the reference ID rpn 149) from the flavonoid glycosides subgroup, displayed alterations under both elevated NO_2 and elevated temperature treatments (Figure 3 - 7 A).

In experiment 2, one among the 12 flavones and flavonols, and one among the 8 hydroxycoumarins ranked first and second, respectively, in relative abundance in ragweed pollen under all four conditions. Additionally, the only flavonoid-3-O-glycoside and one among the 14 flavonoid glycosides followed. Furthermore, one hydroxycoumarin (with the reference ID rpn 159) was enriched in NO_2-free treatments, remarkably contributing to the altered flavonoid composition due to NO_2. Conversely, two compounds from the flavones and flavonols subgroup (with the reference IDs rpn 135 and rpn 182) exhibited significantly increased relative abundance due to elevated temperature. Their enrichment in the respective elevated temperature treated conditions notably contributed to the alteration in flavonoid composition induced by temperature increases. Additionally, two compounds from the hydroxycoumarins group (with the reference IDs rpn 161 and rpn 165), one from the flavonoid glycosides group (with the reference ID rpn

116), and one from the isoflavonoid O-glycosides group (with the reference ID rpn 171)

responded to both temperature increase and elevated NO_2 with changes in their respective relative

abundance (Figure 3 - 7 B).
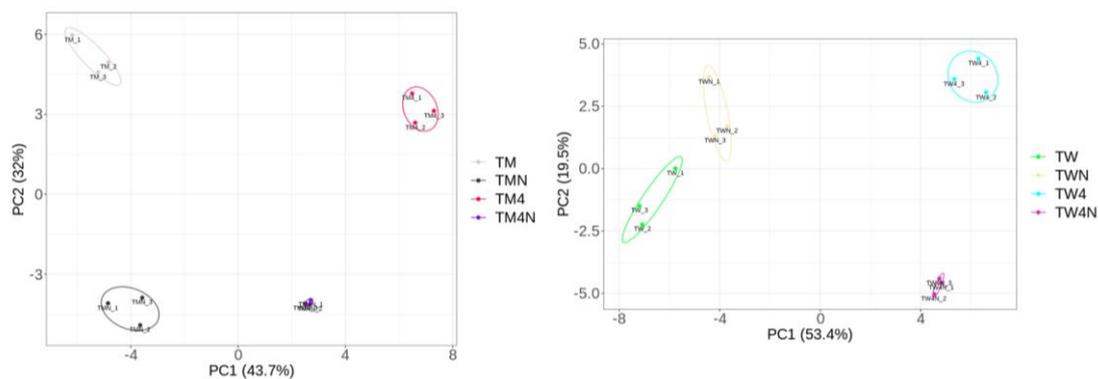
Figure 3 - 6: Principal Component Analysis (PCA) revealing a larger impact of elevated temperature (A, experiment 1; B, experiment 2) than elevated NO_2 on flavonoid composition in ragweed pollen. The abbreviations for experimental settings are as described previously. Each condition was repeated in triplicate. Eight conditions in this study were distinguished by unique colors, while ellipses indicate a 95% confidence for each condition.
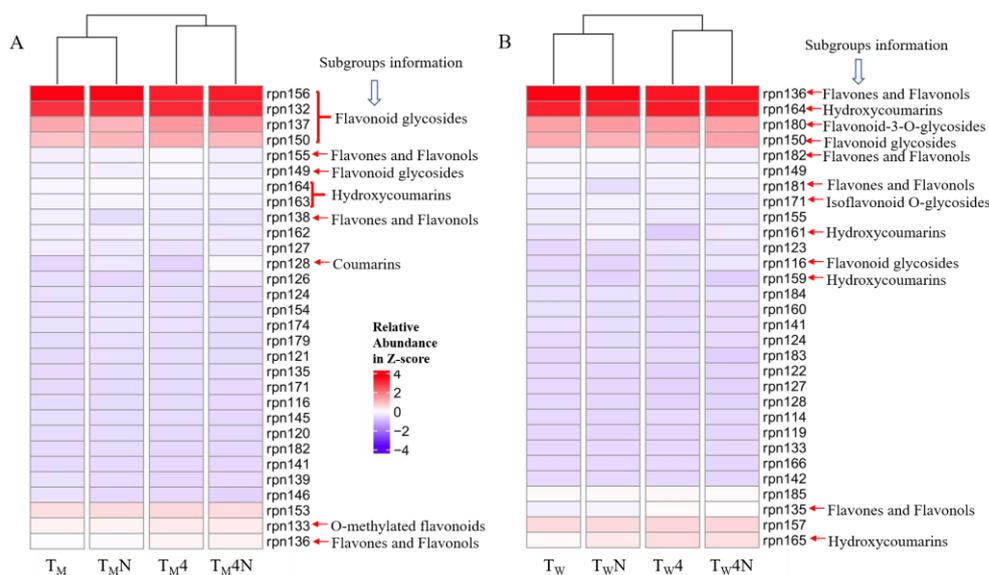
Figure 3 - 7: Heat maps of the top 30 differential flavonoids at the subgroup level for ragweed pollen samples from conditions with treatments of elevated temperature and elevated NO_2 in experiment 1 (panel A), and experiment 2 (panel B). The conditions are indicated at the bottom of the figures. The color gradient (red – white – blue) indicates the relative abundance in Z-score (high – median – low) of flavonoid compounds, respectively. The color scheme was applied consistently in both heat maps. Clustering of conditions was performed using the average linkage algorithm with the Euclidean distance. Flavonoids exhibiting noticeable differences in color intensities are pointed out and accompanied by corresponding subgroup information at the right side of each panel. The abbreviations for conditions are consistent with those previously described in Figure 3 - 1.

3.2.3 IgE-binding activities modulated by the addition of flavonoids

Due to their prominent representation among flavonoids (Figure 3 - 5), quercetins were selected as a model to investigate the potential interference of flavonoids with antigen-IgE binding activity. The addition of 0.3, 1.6, and 3.2 µg of quercetin, respectively, led to a significant inhibition of IgE binding when using ragweed-sensitized patients' sera against the major ragweed allergen, Amb a 1 (5 µg) (Figure 3 - 8 A). Similarly, the IgE-binding activities against 3 µg protein extracted from commercial ragweed pollen (CRP) were obviously inhibited by an addition of quercetin at 1.6 and 3.2 µg, respectively (Figure 3 - 8 B). Notably, the respective control groups containing BSA or no protein did not exhibit any signal (Figure 3 - 8 A).

Primarily, to delve deeper into the impact of added flavonoids on allergen-IgE binding, specific quantities of flavonoids, namely 1.6 and 3.2 µg, were employed in subsequent experiments (Figure 3 - 8 B). Flavonoids obtained from pollen samples in the two phytotron chamber experiments exhibited varying interfering effects on allergen-IgE binding activity under distinct treatments (Figure 3 - 8 C and D). In experiment 1, flavonoids extracted from the ambient temperature conditions (T_M and $T_M N$) displayed the most potent inhibition of allergen-IgE binding at both 1.6 and 3.2 µg, while interference by $T_M 4N$ was evident only at 3.2 µg (Figure 3 - 8 C). In comparison, in experiment 2, only flavonoids derived from ambient temperature T_W (T_W and $T_W N$) exhibited significant inhibition of allergen-IgE binding at the highest tested amount of 3.2 µg (Figure 3 - 8 D).
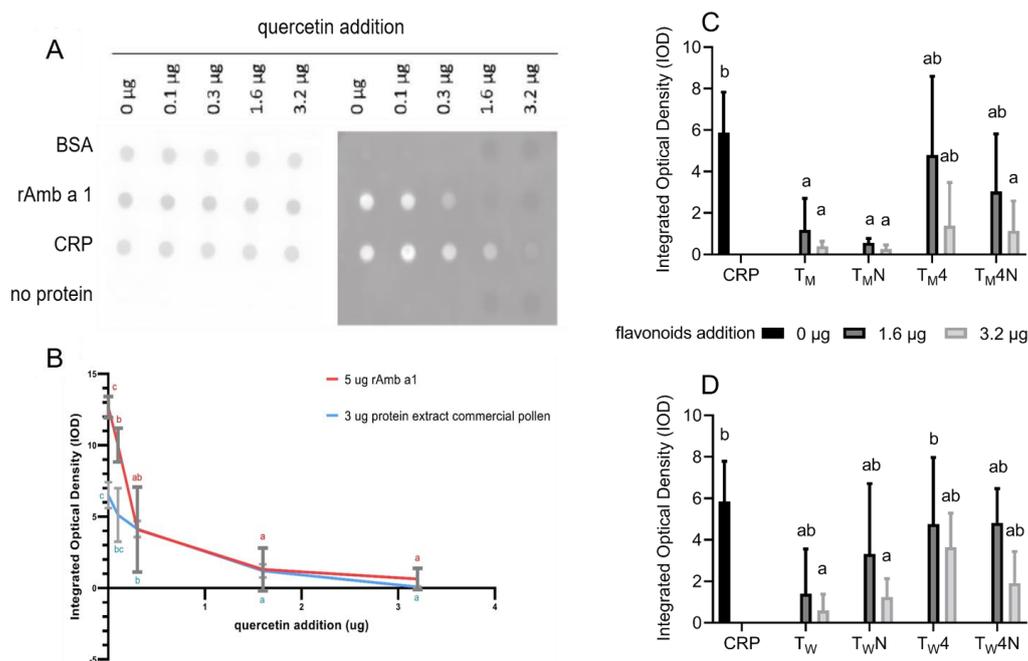
Figure 3 - 8: Interference of quercetin with the binding between patient sera IgE and protein extract from commercial ragweed pollen (CRP) or recombinant ragweed major allergen Amb a 1 (rAmb a 1). (A) Representative images of dot blot membranes showing protein spots stained with Ponceau S (left) and the corresponding immunoblots (right). (B) Integrated optical density of IgE-reactivity of immunoblotted spots upon quercetin addition to rAmb a 1 (red curve) or protein extract from commercial ragweed pollen (CRP) (blue curve). Interference of flavonoid extracts from different conditions with IgE-binding activity of 3 µg purified commercial ragweed pollen protein (CRP), (C) in experiment 1 and (D) in experiment 2. Description for pollen samples and abbreviations in the figures remain the same as in Figure 3 - 1. Columns represent the means of three independent dot blot assays. Statistical analysis by unpaired two-tailed Student's t-test was used. Differences are indicated by letters ($p < 0.05$) (mean ± standard deviation, n = 3) (Cheng et al., 2023).

3.3 Quantification of the allergenicity associated compounds in ragweed pollen

3.3.1 NAD(P)H oxidases activities

In the NBT assay results, the introduction of exogenous NADPH or NADH significantly elevated the NBT reduction activity across all conditions when compared to the baseline results of the initial column lacking SOD, NADPH or NADH. Moreover, this reduction of NBT by ragweed pollen extracts was significantly prevented by the addition of SOD, suggesting that NAD(P)H

oxidase is the main source of these ROS-related compounds in ragweed pollen (Figure 3 - 9 A and D).
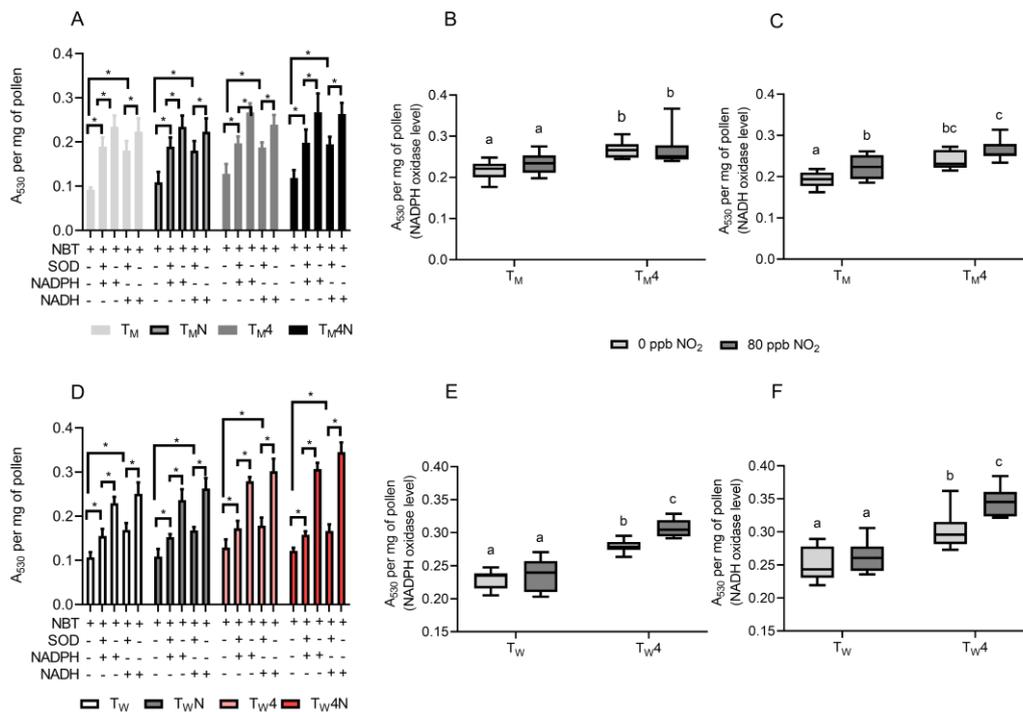
Figure 3 - 9: Assays for NAD(P)H oxidase activities (A, experiment 1; D, experiment 2), NADPH oxidase levels (B and E) and NADH oxidase levels (C and F) in ragweed pollen. A and D): Description for pollen samples and abbreviations in the figures remain the same as in Figure 3 - 1. A and D): NAD(P)H oxidase activity was assayed in the absence and presence of SOD, NADH and NADPH (indicated with "+" and "-", which is equal to "in presence" and "in absence", respectively). The data are means of three independent experiments and each experiment was performed with 8 samples per condition. Only the significant differences regarding the assay were shown in the graphs by stars ($p < 0.05$). B, C, E and F): NAD(P)H oxidase activity was assayed by adding extra NADH and NADPH. Statistically significant results (ANOVA) followed by Fisher's LSD test are indicated by different letters ($p < 0.05$); boxplots with min/max whiskers (Cheng et al., 2023).

ANOVA analysis revealed that both elevated temperature and elevated NO_2 had significant induction effects on NADPH oxidase and NADH oxidase in ragweed plant pollen in both experiments. However, no significant effect of NO_2 on the induction of NADPH oxidase was found in ragweed pollen from experiment 1 (the Munich scenario). In addition, the interaction between elevated temperature and NO_2 tended to be significant in the induction of NADPH oxidase ($p = 0.0866$) and NADH oxidase ($p = 0.0858$) in ragweed pollen from experiment 2 (the Würzburg scenario) (Table 3 - 5). In experiment 1, an overall increase of 18.4% in NADPH

oxidase (Figure 3 - 9 B) and 20.8% in NADH oxidase (Figure 3 - 9 C) was observed in ragweed pollen from elevated temperature treated conditions compared to ambient temperature treated conditions. In addition, elevated NO_2 led to an overall increase of 12.6% in NADH oxidase when comparing ragweed pollen from the elevated NO_2 treated conditions and the NO_2-free treated conditions (Figure 3 - 9 C). Similarly, the comparison of ragweed pollen from the T_W4 condition and T_W condition in experiment 2, revealed significant alterations in the levels of NADPH oxidase (Figure 3 - 9 E) and NADH oxidase (Figure 3 - 9 F) due to elevated temperature, with overall increases of 25.8% and 26.0%, respectively. A less but also significant overall increase of 6.8% in NADPH oxidase (Figure 3 - 9 E) and 10.0% in NADH oxidase (Figure 3 - 9 F) was observed in response to elevated NO_2 in experiment 2.

Table 3 - 5: Results for allergenic indicators of ragweed pollen using Two-way ANOVA with temperature increase (T + 4 °C) and addition of 80 ppb NO_2 (NO_2) as factors in two experiments. Measured parameters (quantitative variables) were indicators for pollen allergenicity in ragweed pollen extracts: "total allergenicity", "NADPH oxidase", "NADH oxidase", and "H_2O_2 content". Significant factors are indicated in bold; df = degree of freedom, F test, $p < 0.05$.

Parameters	Source of variation	Munich			Würzburg		
		df	*F (dfn, dfd)*	*p*	*df*	*F (dfn, dfd)*	*p*
NADPH oxidase	T+ 4.0 °C	1	$F(1, 28) = 16$	**0.0004**	1	$F(1, 28) = 105$	**<0.0001**
	NO_2	1	$F(1, 28) = 0.8$	0.376	1	$F(1, 28) = 9$	**0.0065**
	T+ 4.0 °C x NO_2	1	$F(1, 28) = 0.7$	0.413	1	$F(1, 28) = 3$	0.0866
	Residuals	28			28		
NADH oxidase	T+ 4.0 °C	1	$F(1, 28) = 25$	**<0.0001**	1	$F(1, 28) = 57$	**<0.0001**
	NO_2	1	$F(1, 28) = 10$	**0.004**	1	$F(1, 28) = 10$	**0.0042**
	T+ 4.0 °C x NO_2	1	$F(1, 28) = 0.1$	0.7246	1	$F(1, 28) = 3$	0.0858
	Residuals	28			28		
H_2O_2 content	T+ 4.0 °C	1	$F(1, 36) = 0.03$	0.8593	1	$F(1, 36) = 0.4$	0.5456
	NO_2	1	$F(1, 36) = 0.5$	0.4731	1	$F(1, 36) = 0.006$	0.9403
	T+ 4.0 °C x NO_2	1	$F(1, 36) = 12$	**0.0013**	1	$F(1, 36) = 0.7$	0.4067
	Residuals	36			36		
Total allergenicity	T+ 4.0 °C	1	$F(1, 40) = 5$	**0.0356**	1	$F(1, 64) = 6.097$	**0.0162**
	NO_2	1	$F(1, 40) = 33$	**<0.0001**	1	$F(1, 64) = 3.352$	0.0718
	T+ 4.0 °C x NO_2	1	$F(1, 40) = 5$	**0.0274**	1	$F(1, 64) = 1.055$	0.3081
	Residuals	40			64		

3.3.2 H_2O_2 level

In experiment 1, the H_2O_2 contents were 66.1% higher in pollen due to the elevated NO_2 treatment only under elevated temperature treated conditions (T_M4N vs T_M4), and were 52.0% lower under 4 °C higher temperature compared to ambient temperature (T_M4 vs T_M) (Figure 3 - 10 A). Therefore, the lack of a significant difference in the H_2O_2 content in the combined treatment indicated a significant interaction between temperature and NO_2 (Table 3 - 5). In experiment 2, neither NO_2 nor temperature increase exhibited significant effects on the H_2O_2 content in the pollen extracts (Figure 3 - 10 B).

3.3.3 Total allergenicity of ragweed pollen protein extracts

In experiment 1, elevated temperature, elevated NO_2, and their interaction significantly triggered pollen allergenicity (Table 3 - 5). Elevated NO_2 led to significantly higher allergenicity levels in pollen extracts from conditions of T_MN and T_M4N compared to those from conditions of T_M and T_M4 (NO_2-free treatment), with T_MN intensity being 1.5 folds that of T_M, and T_M4N intensity being 2.1 folds that of T_M4. Additionally, elevated temperature, when combined with elevated NO_2, significantly heightened pollen allergenicity, as indicated by T_M4N intensity being 1.4 times higher than that of T_MN. Notably, the dot-blot assay revealed the highest total allergenicity of the allergenic proteins under the combined condition T_M4N (Figure 3 - 10 C).
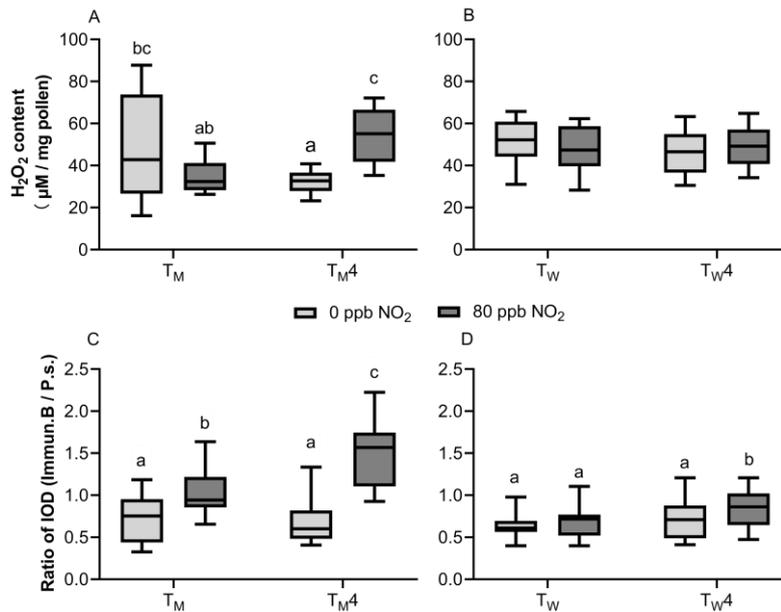
Figure 3 - 10: H_2O_2 content (A and B) and total allergenicity of *A. artemisiifolia* pollen determined by dot blot assays (C and D) in ragweed pollen samples: A and C for experiment 1; B and D for experiment 2. Description for pollen samples and abbreviations in the figures remain the same as in Figure 3 - 1. The data are means of three independent assays with 10 pollen samples each for H_2O_2 content in both experiments (A and B), 11 pollen samples for total allergenicity in experiment 1 (C), and 17 pollen samples for total allergenicity per condition in experiment 2 (D). Significant differences based on results of two-way ANOVA followed by Fisher's LSD test are indicated by different letters (boxplots with min/max whiskers, $p < 0.05$) (Cheng et al., 2023).

Similarly, in experiment 2, elevated temperature played a significant role in inducing pollen allergenicity. Pollen allergenicity was notably influenced by the higher temperature at T_W4N vs T_WN, with an increase of 24.2%. The same effect of NO_2 was observed but was not significant ($p = 0.0718$). A significant increase in NO_2-induced allergenic potential of ragweed pollen was observed in T_W4-treated plants, as evidenced by a 19.0% increase in the intensity of T_W4N compared to T_W4 (Table 3 - 5 and Figure 3 - 10 D).

3.4 IgE-reactive proteins in pollen and their contributions to total allergenicity

3.4.1 The contribution of each IgE-reactive protein to total allergenicity

3.4.1-1 Overview of the IgE-reactive spots

The allergenic potential of aqueous proteins extracted from ragweed pollen was assessed by summarizing the IgE binding intensity of each protein spot to the patient's sera following their separation based on isoelectric point and molecular mass, as described in section 2.1.7-2. In both experiments, 25 spots with molecular weights (Mw) of 11 - 17 kDa (spots 1 - 5), 25 - 35 kDa (spots 6 - 9), 35 - 40 kDa (spots 9, 10 - 20, 25), and 48 - 52 kDa (spots 21 - 24) were identified (Figure 3 - 11). In experiment 1, the cumulative pollen allergenicity, especially affected by elevated NO_2 and the combination of elevated temperature and elevated NO_2, as determined by 2D immunoblotting (Figure 3 - 12 A), aligned with the findings from total allergenicity assessed through dot blotting (Figure 3 - 10 C). For experiment 2, the pollen allergenicity, as evaluated by 2D immunoblotting, exhibited a similar trend in response to temperature increase compared to total allergenicity determined by dot blotting (Figure 3 - 12 B and Figure 3 - 10 D), although the difference was not statistically significant.

In experiment 1, the allergenic potential of each spot and its contribution to total allergenicity is indicated in Table 3 - 6. Notably, five spots (10, 13, 14, 15, and 19) consistently contributed more than 5% to total allergenicity across all conditions. Additionally, spot 8 in T_M4, spot 11 in T_MN, spot 12 in T_M and T_M4, and spot 18 in T_M4 and T_M4N exceeded the 5% threshold. Cumulatively, these spots accounted for over 50% of the total allergenicity in all conditions. Among them, spot 14, with a molecular weight (Mw) range of 35 - 40 kDa, exhibited the highest contribution to total allergenicity across all conditions, and its intensity was most pronounced under the combined T_W4N condition (Table 3 - 6).

In experiment 2, five spots (9, 10, 13, 14, and 19) contributed more than 5% to the total allergenicity under T_W– treated conditions, with the additional inclusion of spot 15 in T_WN

surpassing the 5% threshold. For the two T_W4- treated conditions, four spots (13, 14, 15, and 19) contributed more than 5% to the total allergenicity, and spots (8, 9, and 10) in T_W4, as well as spots (12 and 18) in T_W4N, exceeded the 5% threshold. Cumulatively, these spots accounted for over 50% of the total allergenicity in their respective conditions (Table 3 - 6).

3.4.1-2 Effects of elevated NO_2 and elevated temperatures on IgE reactivity of ragweed pollen allergens

In experiment 1, NO_2 significantly increased the allergenic potential in spots 6, 7, 14, and 19 under elevated temperature-treated conditions (T_M4N vs T_M4 cluster), while in spots 8, 10, 11, and 16 under ambient temperature-treated conditions (T_MN vs T_M cluster). Additionally, elevated temperature significantly heightened the pollen allergenic potential in spot 22 under elevated NO_2 treated conditions (T_M4N vs T_MN cluster). Furthermore, a noteworthy positive interaction between elevated temperature and NO_2, inducing the allergenic potential of ragweed pollen, was observed in spots 1, 2, 4, 8, 10, 15, 18, 19, 20, 21, 22, 23, 24, and 25 in the T_M4N vs T_M cluster (Figure 3 - 13 A).

In experiment 2, elevated NO_2 notably heightened the allergenic potential of ragweed pollen in spot 22; conversely, it considerably diminished the allergenic potential in spot 16 under both temperature-treated conditions. Additionally, significant changes in allergenic potential resulting from temperature increase were identified in spots 13 and 14, exhibiting increases in the T_W4N vs T_WN cluster. Conversely, a reduction was observed in spot 10 within the T_W4N vs T_WN cluster, and reductions were noted in spots 16, 22 and 23 within the T_W4 vs T_W cluster. Additionally, a significant interaction between elevated temperature and NO_2 was observed in the T_W4N vs T_W cluster. This interaction resulted in an augmentation of ragweed pollen allergenicity in spots 14, 15, 17, and 18, while mitigating ragweed pollen allergenicity in spots 10 and 16 (Figure 3 - 13 B).

Figure 3 - 11: Representative 2-DE and Immunoblotting using pollen proteins of *A. artemisiifolia* from all conditions. A-H) Immunoreactive proteins spots detected with pooled sera from allergic patients using 2-DE immunoblotting; I) 2-DE of total soluble proteins stained with Coomassie blue. Description for pollen samples and abbreviations in the figures remain the same as in Figure 3 - 1.
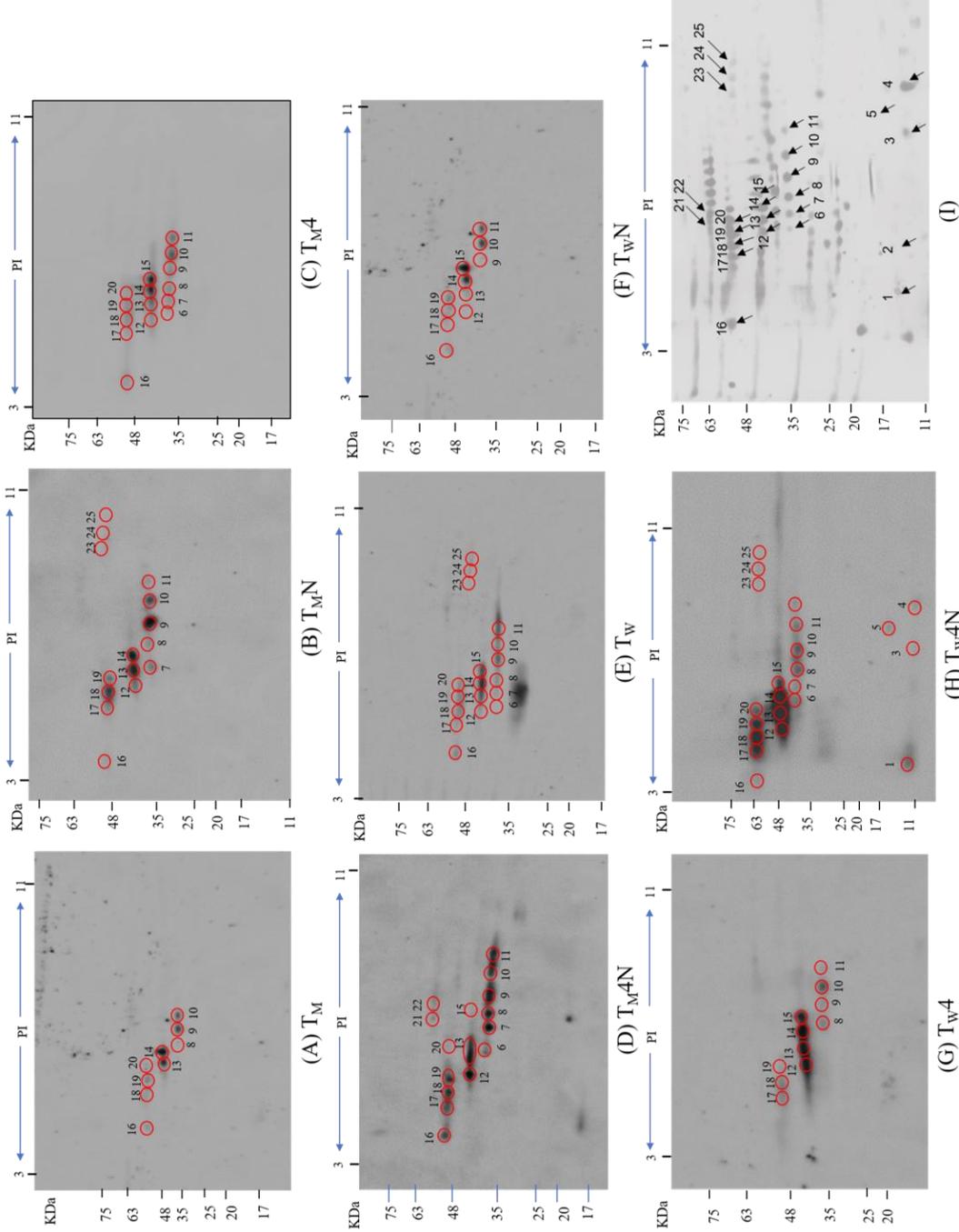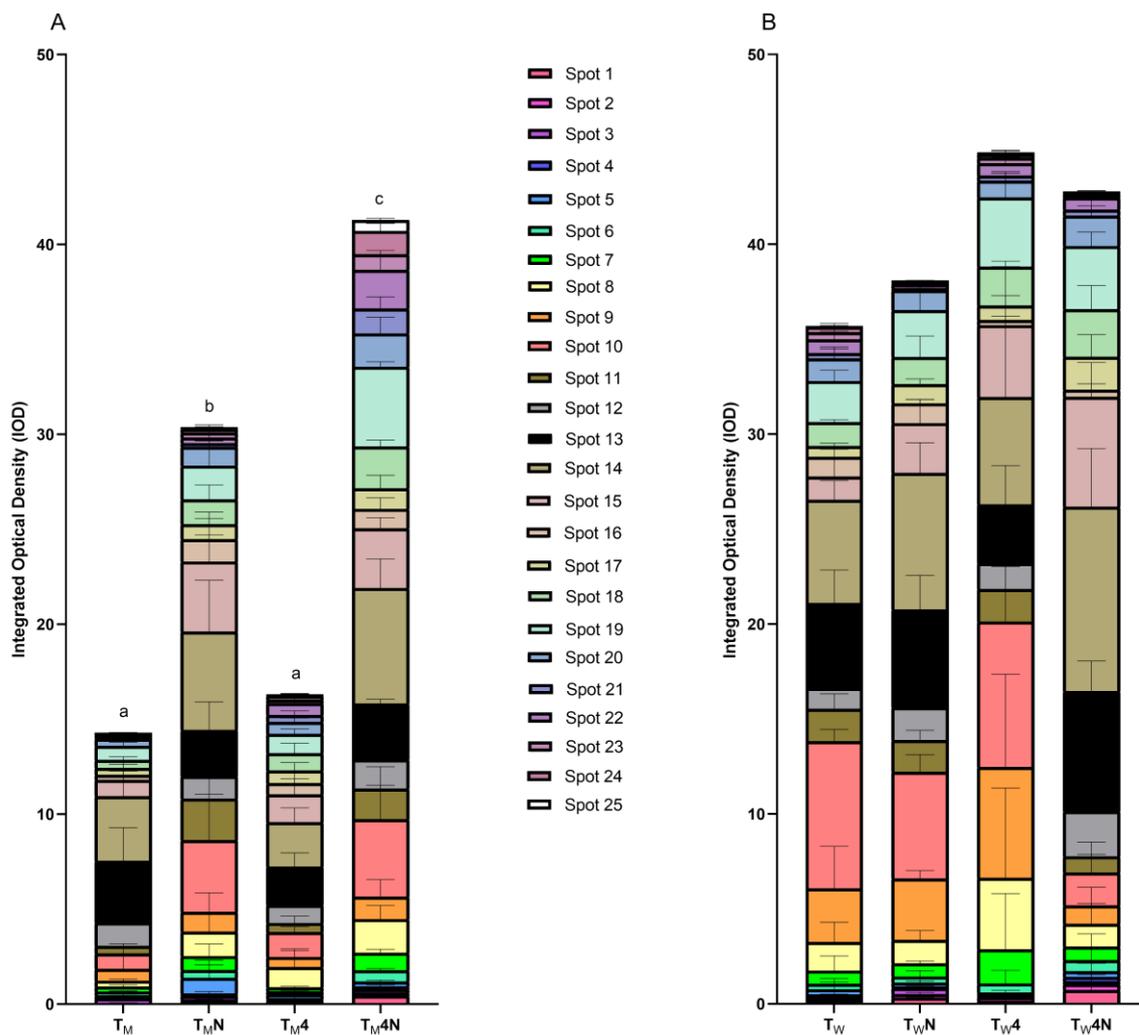
Figure 3 - 12: Contribution of the single spots visualized by 2D immunoblot to the total allergenicity of ragweed pollen in experiment 1 (A) and experiment 2 (B). Three pollen sample pools per condition and each pool from 4 plants were used in each assay. Bar plots with mean ± standard deviation. Description for pollen samples and abbreviations in the figures remain the same as in Figure 3 - 1.

Table 3 - 6: The mean percentage of each individual spot (25 spots in total) contributing to the total allergenicity in ragweed pollen across conditions in two experiments obtained by two-dimension immunoblot. Description for pollen samples and abbreviations in the figures remain the same as in Figure 3 - 1. The contribution of each spot to the total allergenicity is distinguished by a color gradient, where the lowest contribution is given in light gray, the middle contribution in light blue, and the highest contribution in red. The top five spots in each condition that contributed most to the total allergenicity are shown in bold.

Spots	Experiment 1					Experiment 2			
	T_M	T_MN	T_M4	T_M4N		T_W	T_WN	T_W4	T_W4N
Spot 1	0.22%	0.44%	0.63%	1.07%		0.57%	0.87%	0.39%	1.70%
Spot 2	0.17%	0.36%	0.38%	0.41%		0.20%	0.49%	0.41%	0.74%
Spot 3	1.67%	0.64%	0.39%	0.42%		0.46%	0.84%	0.17%	0.34%
Spot 4	0.18%	0.37%	0.43%	0.37%		0.15%	0.18%	0.12%	0.53%
Spot 5	1.00%	2.73%	1.26%	0.63%		0.77%	0.53%	0.23%	0.72%
Spot 6	1.39%	1.35%	1.11%	1.41%		0.79%	0.87%	1.07%	1.35%
Spot 7	1.79%	2.42%	1.22%	2.22%		1.97%	1.81%	4.00%	1.68%
Spot 8	2.16%	4.25%	**6.51%**	4.30%		4.23%	3.27%	**8.40%**	2.77%
Spot 9	4.45%	3.44%	3.26%	2.86%		**7.89%**	**8.48%**	**13.02%**	2.27%
Spot 10	**5.58%**	**12.43%**	8.03%	9.87%		**21.69%**	**14.75%**	**17.09%**	4.06%
Spot 11	2.84%	**7.18%**	2.97%	3.93%		4.77%	4.37%	3.83%	1.99%
Spot 12	**8.66%**	3.90%	5.81%	3.71%		3.08%	4.57%	3.02%	5.59%
Spot 13	**22.72%**	8.02%	**12.41%**	7.14%		**12.55%**	**13.50%**	6.91%	**14.75%**
Spot 14	**23.78%**	**17.01%**	**14.28%**	**14.72%**		**15.22%**	**18.89%**	**12.61%**	**22.71%**
Spot 15	**6.04%**	**12.13%**	**9.05%**	**7.64%**		3.43%	**6.88%**	**8.43%**	**13.52%**
Spot 16	1.82%	3.91%	3.58%	2.47%		2.90%	2.75%	0.61%	0.90%
Spot 17	2.55%	2.59%	4.06%	2.61%		1.59%	2.63%	1.70%	4.03%
Spot 18	2.96%	4.29%	5.59%	5.34%		3.52%	3.76%	4.54%	**5.86%**
Spot 19	5.14%	5.84%	6.27%	**10.15%**		**6.06%**	6.49%	8.14%	**7.78%**
Spot 20	2.73%	3.30%	3.87%	4.26%		3.35%	2.76%	1.97%	3.75%
Spot 21	0.41%	0.61%	2.21%	3.18%		0.78%	0.26%	0.61%	0.73%
Spot 22	1.16%	0.97%	3.84%	4.94%		2.02%	0.52%	1.46%	1.50%
Spot 23	0.25%	0.85%	1.14%	2.00%		1.06%	0.19%	0.65%	0.35%
Spot 24	0.28%	0.61%	1.22%	2.96%		0.82%	0.22%	0.41%	0.28%
Spot 25	0.07%	0.35%	0.49%	1.41%		0.11%	0.13%	0.22%	0.10%

3.4.2 Identification of new IgE-reactive proteins

Further identification of 17 spots (indicated in Figure 3 - 11) was performed using LC-MS/MS. The IgE-reactive proteins identified within these spots mostly matched the expected size of the protein ladder, except for the major allergen Amb a 1 isoforms with molecular weights ranging from 42.3 to 44.1 kDa, which were predominant in all spots except spots 1 and 2. Another known ragweed pollen allergen with a similar molecular weight (43.2 kDa) as Amb a 11 was detected in spots 9, 10, and 11. Several known minor allergens of *A. artemisiifolia* pollen were also identified: Amb a 12 with a molecular weight of 48.0 kDa was present in spots 18, 19, 21, and 22; Amb a 3 with a molecular weight of 11.4 kDa was found in spots 4 and 5; Amb a 8 with a molecular weight of 14.2 kDa was detected in spots 1 and 2, and Amb a 10 with a molecular weight of 17.8 kDa was identified in spot 1 (refer to Table 3 - 7).

Additionally, homology database searches revealed six other allergenic proteins, namely calmodulin in spot 1, peptidyl-prolyl cis-trans isomerase FKBP15-2 in spot 3, nucleoside diphosphate kinase IV in spot 4, phosphoglycerate kinase (cytosolic) in spot 15, calreticulin in spot 16, and triosephosphate isomerase (cytosolic) in spots 18, 19, and 21 (Table 3 - 7).
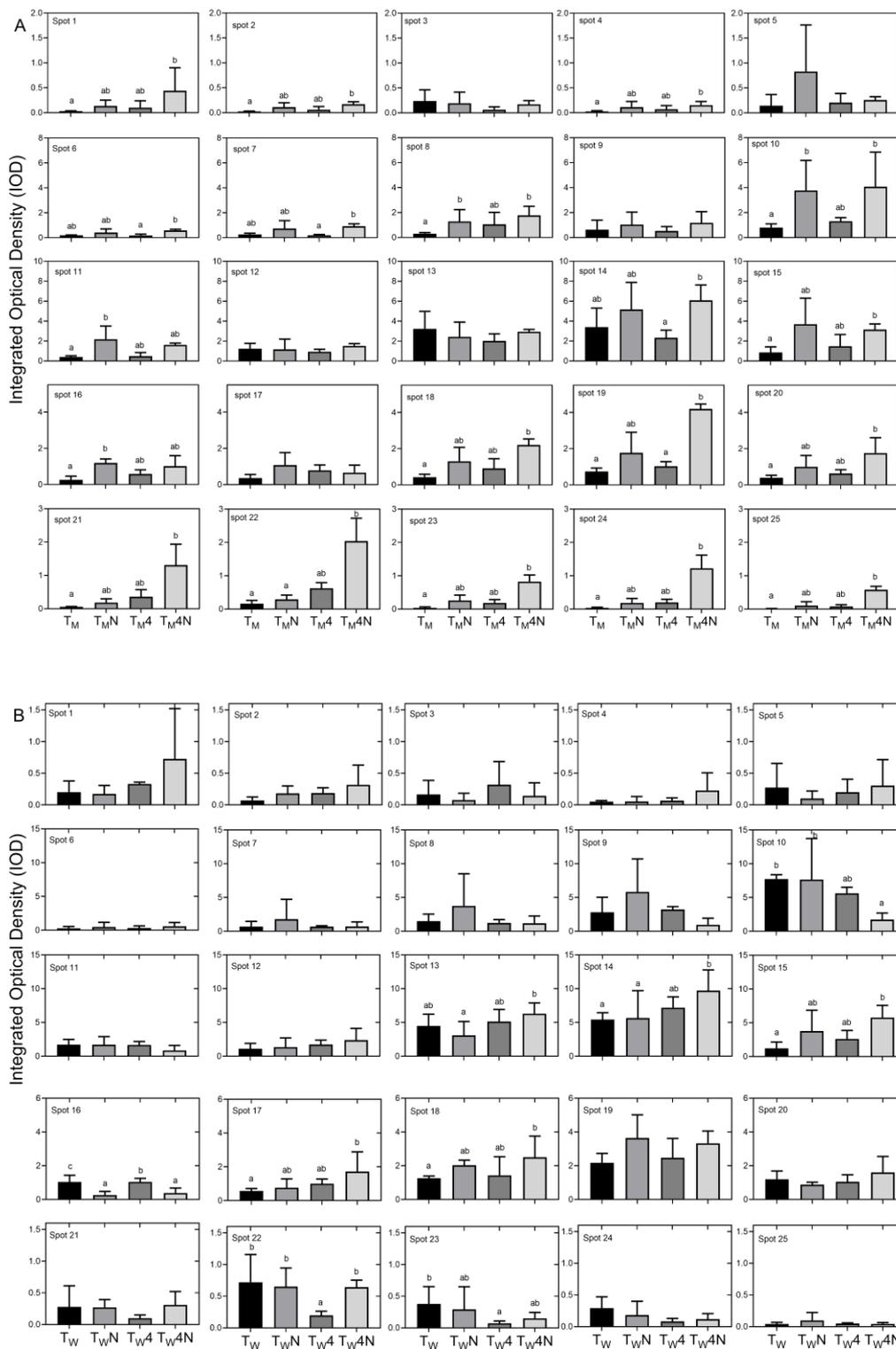
Figure 3 - 13: Individual spot intensity based on IgE binding after 2-D gel electrophoresis of pollen extracts representing pollen allergenicity under four conditions in experiment 1 (A) and in experiment 2 (B), respectively. Description for pollen samples and abbreviations in the figures remain the same as in Figure 3 - 1. Significant differences based on results of two-way ANOVA followed by Fisher's LSD test are indicated by letters (p < 0.1).

Table 3 - 7: Top five proteins ranked by protein score among identified allergenic proteins per spot in ragweed pollen. Spot number is as indicated in Figure 3 - 11.

Spot Nr.	Accession Number (UniProtKB)	Protein name	Allergen Name	Sequence coverage (%)	Nr. of unique peptide	Mw (kDa)	Best Mascot Ion Score
Spot 1	Q2KN25	Calcium-binding protein	Amb a 10.0101	63.70%	8	17.8	777.1
	P93171	Calmodulin		52.30%	3	16.9	707.1
	Q2KN24	Profilin	Amb a 8.0101	55.60%	3	14.2	298.9
Spot 2	Q2KN24	Profilin	Amb a 8.0101	42.90%	2	14.2	287.8
Spot 3	P27759	Pectate lyase 5	Amb a 1.0101	14.10%	4	42.7	466.8
	P27762	Pectate lyase 4	Amb a 1.0501	10.60%	2	44.0	253.4
	E1XUM1	Pectate lyase	Amb a 1.0502	11.80%	2	44.0	245.7
	Q38936, Q41649	Peptidyl-prolyl cis-trans isomerase FKBP15-2		9.20%	2	16.2	209.9
Spot 4	P27759	Pectate lyase 5	Amb a 1.0101	14.10%	4	42.7	470.6
	P00304	Pollen allergen Amb a 3	Amb a 3.0101	28.70%	3	11.4	351
	E1XUL9	Pectate lyase (Fragment)	Amb a 1.0402	11.40%	2	42.3	250
	P27762	Pectate lyase 4	Amb a 1.0501	10.60%	2	44.1	223
	Q8LAH8, Q8RXA8	Nucleoside diphosphate kinase IV, chloroplastic/mitochondrial		8.02%	2	25.8	209.8
Spot 5	P27759	Pectate lyase 5	Amb a 1.0101	13.90%	3	42.7	476.3
	P00304	Pollen allergen Amb a 3	Amb a 3.0101	26.70%	2	11.4	213.5
Spot 9	E1XUL5	Pectate lyase	Amb a 1.0305	40.10%	8	43.0	1321.9
	E1XUL9	Pectate lyase (Fragment)	Amb a 1.0402	28.90%	8	42.3	1041.5
	P27759	Pectate lyase 5	Amb a 1.0101	27.00%	7	42.7	866.2
	V5LU01	Cysteine protease	Amb a 11.0101	18.40%	5	43.2	641.1
Spot 10	E1XUL5	Pectate lyase	Amb a 1.0305	63.50%	11	43.0	1442.6

Spot Nr.	Accession Number (UniProtKB)	Protein name	Allergen Name	Sequence coverage (%)	Nr. of unique peptide	Mw (kDa)	Best Mascot Ion Score
Spot 10	V5LU01	Cysteine protease	Amb a 11.0101	32.60%	8	43.2	948
	E1XUL9	Pectate lyase (Fragment)	Amb a 1.0402	26.10%	6	42.3	947.4
	P27759	Pectate lyase 5	Amb a 1.0101	29.50%	6	42.7	726.4
Spot 11	E1XUL5	Pectate lyase	Amb a 1.0305	63.50%	11	43.0	1311
	V5LU01	Cysteine protease	Amb a 11.0101	24.60%	6	43.2	837.8
	E1XUL9	Pectate lyase (Fragment)	Amb a 1.0402	16.50%	3	42.3	584.9
	P27759	Pectate lyase 5	Amb a 1.0101	14.90%	3	42.7	543
	P27762	Pectate lyase 4	Amb a 1.0501	15.10%	2	44.1	446.9
Spot 15	P27759	Pectate lyase 5	Amb a 1.0101	55.60%	15	42.7	2001.5
	E1XUL5	Pectate lyase	Amb a 1.0305	66.80%	12	43.0	1411.3
	E1XUM1	Pectate lyase	Amb a 1.0502	38.30%	2	44.0	1095.3
	P27762	Pectate lyase 4	Amb a 1.0501	37.00%	2	44.1	1023.5
	Q42962	Phosphoglycerate kinase, cytosolic		26.40%	4	42.4	745.4
Spot 16	P27759	Pectate lyase 5	Amb a 1.0101	29.80%	7	42.7	878.2
	Q9ZPP1	Calreticulin		11.50%	2	47.9	609.2
	Q9SLY8	Calreticulin		8.25%	2	48.3	398.5
Spot 18	A0A1B2H9Q5	Enolase 2	Amb a 12.0102	42.20%	5	48.0	1652.6
	P27759	Pectate lyase 5	Amb a 1.0101	23.50%	6	42.7	855.2
	E1XUL5	Pectate lyase	Amb a 1.0305	30.00%	6	43.0	673.2
	E1XUL9	Pectate lyase (Fragment)	Amb a 1.0402	16.50%	3	42.3	608.5
	P48493	Triosephosphate isomerase, cytosolic		14.40%	2	20.5	184.6
Spot 19	A0A1B2H9Q5	Enolase 2	Amb a 12.0102	55.30%	6	48.0	2029.7
	P27759	Pectate lyase 5	Amb a 1.0101	30.30%	9	42.7	1216

Spot Nr.	Accession Number (UniProtKB)	Protein name	Allergen Name	Sequence coverage (%)	Nr. of unique peptide	Mw (kDa)	Best Mascot Ion Score
Spot 19	E1XUL5	Pectate lyase	Amb a 1.0305	34.30%	7	43.0	936.5
	E1XUL9	Pectate lyase (Fragment)	Amb a 1.0402	16.50%	3	42.3	760.7
	P48493	Triosephosphate isomerase, cytosolic (Fragment)		25.60%	3	20.5	265.3
Spot 21	A0A1B2H9Q5	Enolase 2	Amb a 12.0102	37.50%	4	48.0	1151.1
	P27759	Pectate lyase 5	Amb a 1.0101	23.20%	5	42.7	691.3
	E1XUL9	Pectate lyase (Fragment)	Amb a 1.0402	16.50%	3	42.3	575.7
	E1XUL5	Pectate lyase	Amb a 1.0305	21.40%	4	43.0	497.4
	P48493	Triosephosphate isomerase, cytosolic (Fragment)		20.50%	2	20.5	253.6
Spot 22	A0A1B2H9Q5	Enolase 2	Amb a 12.0102	27.90%	4	48.0	1019.5
	E1XUL9	Pectate lyase (Fragment)	Amb a 1.0402	16.50%	3	42.3	603.2
	P27759	Pectate lyase 5	Amb a 1.0101	19.90%	4	42.7	600.5
	E1XUL5	Pectate lyase	Amb a 1.0305	26.20%	5	43.0	587.7
Spot 23	P27759	Pectate lyase 5	Amb a 1.0101	14.90%	3	42.7	455.4
	P27762	Pectate lyase 4	Amb a 1.0501	18.60%	2	44.1	429.8
Spot 24	P27759	Pectate lyase 5	Amb a 1.0101	14.90%	3	42.7	379.7
Spot 25	P27759	Pectate lyase 5	Amb a 1.0101	9.34%	2	42.7	340.7

3.5 Comparative proteomic analysis of ragweed pollen aqueous extracts from temperature and NO₂ treatments

3.5.1 Differential expression patterns of proteins in ragweed pollen in response to elevated NO₂ and elevated temperature

3.5.1-1 Abundance of established ragweed pollen allergens

In both experiments, the identified ragweed allergens consisted of isoforms of Amb a 1 [a 1.0101, a 1.0201, a 1.0202, a 1.0304, a 1.0305, a 1.0401 (only in experiment 1), a 1.0402, a 1.0501, a 1.0502], as well as Amb a 3. 0101, Amb a 4.0101, Amb a 5.0101, Amb a 6.0101, Amb a 8 (a 8.0101 and a 8.0102), Amb a 9.0102, Amb a 10.0101, Amb a 11.0101, and Amb a 12.0102 (Table 3 - 8).

In experiment 1, with fold change limits of 1.5 (upregulation) and 0.667 (downregulation), a significant upregulation in the abundance in response to elevated NO₂ were observed in Amb a 1.0305 in T_M4N vs T_M4 cluster. Conversely, downregulations in response to elevated NO₂ were observed in Amb a 1.0201 and Amb a 1.0401 in T_MN vs T_M cluster, without statistically significant differences. Moreover, a 4 °C temperature increase markedly amplified the abundance of Amb a 1.0201 by 47-fold in the T_M4N vs T_MN cluster. Conversely, there was a downregulation in the abundance of Amb a 1.0305 (p = 0.085) in response to the same temperature increase in the T_M4 vs T_M cluster (Table 3 - 8).

In experiment 2, with the same 1.5 fold change limits as before, the abundance of Amb a 5.0101 was significantly upregulated due to elevated NO₂ in T_W4N vs T_W4 cluster. Similarly, abundance upregulations in response to elevated NO₂ were observed in Amb a 1.0201 and Amb a 5.0101 in T_WN vs T_W cluster, however, without statistically significant differences. In contrast, in the T_WN vs T_W cluster, elevated NO₂ downregulated the abundance of Amb a 4.0101, Amb a 8.0102, Amb a 9.0102 and Amb a 10.0101, with statistical differences in Amb a 10.0101 (p < 0.05) and Amb a 8.0102 (P = 0.068) (Table 3 - 8). In addition, elevated temperature resulted in noticeable increases in the abundance of Amb a 1.0305 (p = 0.056, p > 0.1) and Amb a 1.0402

(both p values less than 0.05) in both the T_W4 vs T_W and T_W4N vs T_WN clusters, respectively. Similar trends of increased abundance were observed for Amb a 1.0201 (p = 0.098) in T_W4 vs T_W cluster and Amb a 1.0101 (p < 0.05) in T_W4N vs T_WN cluster in response to the same temperature increase. Conversely, substantial reductions in the abundance of Amb a 4.0101 (p < 0.05, p > 0.1) and Amb a 10.0101 (both p values less than 0.05) due to elevated temperature were observed in both T_W4 vs T_W and T_W4N vs T_WN clusters, respectively. Moreover, significant downregulations in the abundance of Amb a 5.0101, Amb a 8.0101, and Amb a 8.0102 were observed in the T_W4 vs T_W cluster, as well as Amb a 9.0102, however, without statistically significant difference. Furthermore, the abundance of Amb a 1.0201, Amb a 1.0305, and Amb a 1.0402 was significantly higher in the T_W4N condition than in the T_W condition, with Amb a 1.0201 exhibiting a notable 12-fold increase in abundance in the T_W4N vs T_W cluster. In comparison, the abundances of Amb a 4.0101 (p = 0.05), Amb a 8.0102 (p < 0.05) and Amb a 10.0101 (p < 0.05), respectively, were obviously lower in the T_W4N condition than in the T_W condition (Table 3 - 8).

3.5.1-2 Abundance of the identified IgE-reactive proteins

In addition to the established ragweed pollen allergens (Amb a) and their isoforms, six other allergenic proteins (referred to Table 3 - 7), were identified in the ragweed pollen extract (Table 3 - 9). Notably, there were no significant changes in the abundance of the listed allergenic proteins within the elevated NO_2-treatment clusters (T_MN vs T_M, T_M4N vs T_M4, T_WN vs T_W, and T_W4N vs T_W4).

In experiment 1, significant downregulations in abundance were observed in response to a 4 °C temperature increase for cytosolic phosphoglycerate kinase and two calreticulins in the T_M4 vs T_M cluster, as well as one calreticulin (accession number Q9SLY8) in the T_M4N vs T_MN cluster. Identical significant downregulations for these three proteins were observed under T_M4N compared to ambient temperature T_M (Table 3 - 9).

In experiment 2, temperature increase caused significant downregulations in the abundance of calmodulin, chloroplastic/mitochondrial nucleoside diphosphate kinase IV (with the exception

of $p > 0.1$ in T_W4 vs T_W cluster), and cytosolic phosphoglycerate kinase in both T_W4 vs T_W and T_W4N vs T_WN clusters. Additionally, one calreticulin (accession number Q9SLY8) in the T_W4 vs T_W cluster and the other calreticulin (accession number Q9ZPP1) in the T_W4N vs T_WN cluster were significantly downregulated in response to elevated temperature (Table 3 - 9). Furthermore, significant downregulations in the abundance of the listed IgE-reactive proteins (excluding the cytosolic triosephosphate isomerase) were observed under T_W4N compared to temperature T_W (Table 3 - 9).

Table 3 - 8: Expression profiles of allergens in nine clusters in ragweed pollen by LC-MS/MS, including isoform name, the abundance ratio (FC), and the corresponding p value. Description for abbreviations in the tables remain the same as in Figure 3 - 1. FC = 1.5 (0.667 for down regulation limit) was set in both experiments; Statistics was performed by unpaired two-tailed t-test (p<0.05). Values meeting the criteria of $|log_{1.5}$ FC$| > 1.0$ and p-values below 0.05 were highlighted in both bold and yellow, underscoring their significance. Additionally, p-values falling between 0.05 and 0.1 were also emphasized, using bold and green for clarity. NaN: not present.

Isoforms	T_MN vs T_M		T_M4N vs T_M4		T_M4 vs T_M		T_M4N vs T_MN		T_M4N vs T_M		T_wN vs T_w		T_w4N vs T_w4		T_w4 vs T_w		T_w4N vs T_wN		T_w4N vs T_w	
	FC	p	FC	p	FC	p	FC	p	FC	p	FC	p	FC	p	FC	p	FC	p	FC	p
Amb a 1.0101	0.92	0.99	1.30	0.20	0.80	0.97	1.14	0.32	1.04	0.66	0.93	0.33	1.24	0.12	1.18	0.51	**1.60**	**0.01**	1.44	0.02
Amb a 1.0201	**0.02**	0.35	1.02	1.00	0.73	1.00	**47.03**	0.65	0.74	0.96	**1.60**	0.14	0.84	0.82	**2.87**	*0.10*	1.20	0.61	**12.87**	**0.03**
Amb a 1.0202	0.96	0.98	1.22	0.16	0.78	0.19	1.00	0.99	0.96	1.00	1.08	0.28	1.12	0.05	1.31	0.02	1.40	0.01	1.49	0.00
Amb a 1.0304	1.00	1.00	1.08	0.75	0.86	0.03	0.93	0.27	0.93	0.30	1.06	0.50	1.03	0.49	1.15	0.31	1.13	0.29	1.18	0.07
Amb a 1.0305	0.79	0.11	**1.50**	0.96	**0.51**	*0.09*	0.97	0.97	0.77	0.28	1.46	0.22	1.02	0.63	**2.94**	*0.06*	**1.86**	0.45	**2.67**	**0.02**
Amb a 1.0401	**0.62**	0.61	0.77	0.92	1.03	1.00	1.27	0.91	0.79	0.95	NaN		NaN		NaN		NaN		NaN	
Amb a 1.0402	1.03	1.00	1.15	0.53	1.05	1.00	1.18	0.41	1.21	0.40	0.98	0.29	1.07	0.96	**2.00**	**0.00**	**2.14**	**0.00**	**2.20**	**0.01**
Amb a 1.0501	1.10	0.94	1.20	0.50	0.89	1.00	0.97	1.00	1.07	0.81	1.02	0.83	1.03	0.83	1.43	0.03	1.39	0.04	1.46	0.04
Amb a 1.0502	0.96	1.00	1.22	1.00	0.92	0.98	1.17	1.00	1.12	0.99	1.05	0.74	1.38	0.14	0.76	0.10	1.14	0.98	1.05	0.74
Amb a 3.0101	0.82	0.04	1.03	0.96	0.68	0.00	0.86	0.81	0.71	0.00	1.01	0.81	0.95	0.38	0.86	0.64	0.82	0.03	0.82	0.23
Amb a 4.0101	0.85	0.97	1.09	0.98	0.83	0.79	1.07	1.00	0.91	0.97	**0.63**	0.32	1.01	0.65	**0.38**	**0.03**	**0.65**	0.18	**0.38**	*0.05*
Amb a 5.0101	1.05	0.99	1.04	0.96	1.29	0.99	1.27	0.48	1.34	0.79	**1.60**	0.37	**2.65**	**0.03**	**0.63**	**0.03**	1.18	0.86	1.46	0.34
Amb a 6.0101	0.90	1.00	0.91	0.99	0.90	0.94	0.91	0.77	0.82	0.76	1.01	0.57	0.92	0.79	1.01	0.68	0.96	0.21	0.94	0.39
Amb a 8.0101	0.94	1.00	0.93	0.99	0.84	0.16	0.83	0.04	0.79	0.07	0.85	0.12	1.07	0.98	**0.64**	**0.00**	0.81	0.00	0.69	0.00
Amb a 8.0102	0.96	0.87	0.92	0.83	0.90	0.47	0.86	0.45	0.83	0.08	**0.66**	*0.07*	1.07	0.62	**0.42**	**0.00**	0.71	0.00	**0.47**	**0.00**
Amb a 9.0201	0.74	0.99	1.04	1.00	0.84	1.00	1.18	1.00	0.87	1.00	**0.45**	0.15	0.85	0.33	**0.50**	0.22	0.91	0.37	0.42	0.10
Amb a 10.0101	1.12	0.83	1.14	0.61	0.82	0.47	0.84	0.56	0.93	1.00	**0.62**	**0.01**	1.08	0.78	**0.33**	**0.00**	**0.54**	**0.02**	**0.34**	**0.00**
Amb a 11.0101	0.83	0.47	1.09	0.70	0.69	0.00	0.90	0.37	0.75	0.01	1.05	0.93	1.05	0.55	0.85	0.00	0.95	0.01	0.89	0.01
Amb a 12.0102	1.04	1.00	1.12	0.61	0.73	0.00	0.78	0.00	0.81	0.00	0.93	0.20	0.98	0.32	0.83	0.00	0.86	0.00	0.80	0.00

Table 3 - 9: Expression profiles of seven IgE-reactive proteins (refer to Table 3 - 7) in ragweed pollen accessed by LC-MS/MS. Description for clusters, abbreviations, column names and statistics in the table remain the same as in Table 3 - 8..

Accession Nr.	Protein description	T_MN vs T_M		T_M4N vs T_M4		T_M4 vs T_M		T_M4N vs T_MN		T_M4N vs T_M		T_w4 vs T_w		T_w4N vs T_wN		T_wN vs T_w		T_w4N vs T_w4		T_w4N vs T_w	
		FC	p	FC	p	FC	p	FC	p	FC	p	FC	p	FC	p	FC	p	FC	p	FC	p
P93171	Calmodulin	1.099	0.959	1.073	0.869	1.150	0.573	1.122	0.424	1.233	0.138	0.338	0.001	0.579	0.010	0.822	0.021	1.156	0.792	0.414	0.001
Q38936	Peptidyl-prolyl cis-trans isomerase FKBP15-2	0.874	0.614	1.000	0.950	0.676	0.026	0.774	0.145	0.676	0.004	0.718	0.017	0.750	0.045	0.869	0.487	0.899	0.237	0.644	0.006
Q8LAH8	Nucleoside diphosphate kinase IV, chloroplastic/mitochondrial	0.992	0.983	0.936	0.966	0.798	0.126	0.753	0.092	0.747	0.031	0.648	0.459	0.628	0.030	1.010	0.772	0.946	0.453	0.645	0.017
Q42962	Phosphoglycerate kinase, cytosolic	0.913	0.998	1.098	0.973	0.588	0.000	0.707	0.001	0.646	0.000	0.615	0.001	0.601	0.011	0.934	0.917	0.949	0.321	0.579	0.000
Q9SLY8	Calreticulin	0.860	0.696	0.953	1.000	0.517	0.000	0.573	0.000	0.493	0.000	0.642	0.001	0.749	0.001	0.870	0.197	1.023	0.545	0.667	0.000
Q9ZPP1	Calreticulin	0.830	0.728	0.921	0.999	0.615	0.000	0.682	0.001	0.566	0.000	0.687	0.010	0.613	0.001	1.061	0.569	0.922	0.404	0.643	0.003
P48493	Triosephosphate isomerase,	0.867	0.982	1.091	0.946	0.715	0.000	0.900	0.010	0.780	0.003	0.907	0.022	1.052	0.044	1.023	0.351	1.146	0.411	1.067	0.105
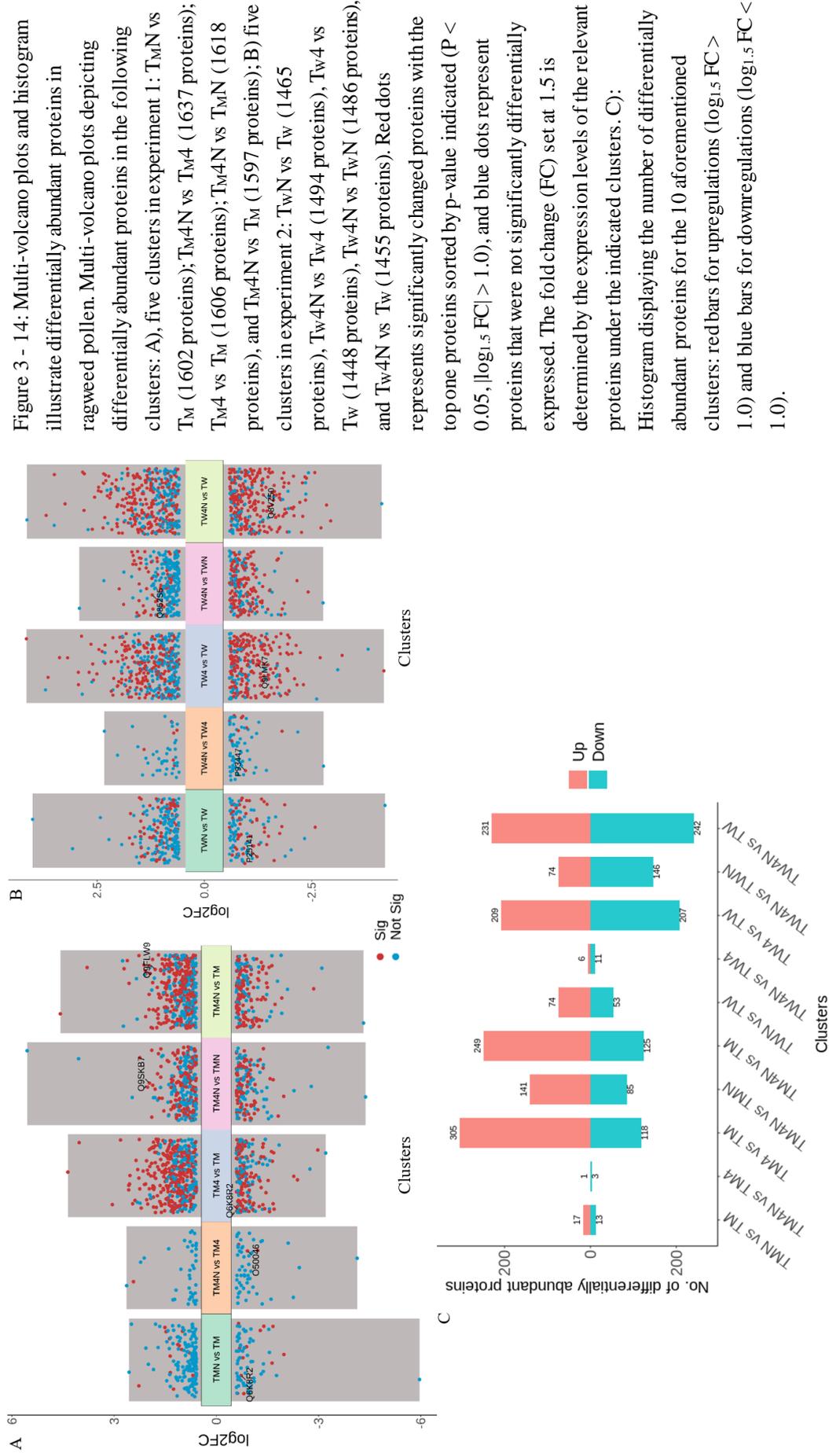
Figure 3 - 14: Multi-volcano plots and histogram illustrate differentially abundant proteins in ragweed pollen. Multi-volcano plots depicting differentially abundant proteins in the following clusters: A), five clusters in experiment 1: T_MN vs T_M (1602 proteins); T_M4N vs T_M4 (1637 proteins); T_M4 vs T_M (1606 proteins); T_M4N vs T_MN (1618 proteins), and T_M4N vs T_M (1597 proteins); B) five clusters in experiment 2: T_WN vs T_W (1465 proteins), T_W4N vs T_W4 (1494 proteins), T_W4 vs T_W (1448 proteins), T_W4N vs T_WN (1486 proteins), and T_W4N vs T_W (1455 proteins). Red dots represents significantly changed proteins with the top one proteins sorted by p-value indicated (P < 0.05, |$\log_{1.5}$ FC| > 1.0), and blue dots represent proteins that were not significantly differentially expressed. The fold change (FC) set at 1.5 is determined by the expression levels of the relevant proteins under the indicated clusters. C): Histogram displaying the number of differentially abundant proteins for the 10 aforementioned clusters: red bars for upregulations ($\log_{1.5}$ FC > 1.0) and blue bars for downregulations ($\log_{1.5}$ FC < 1.0).

Experiment 1 (A)
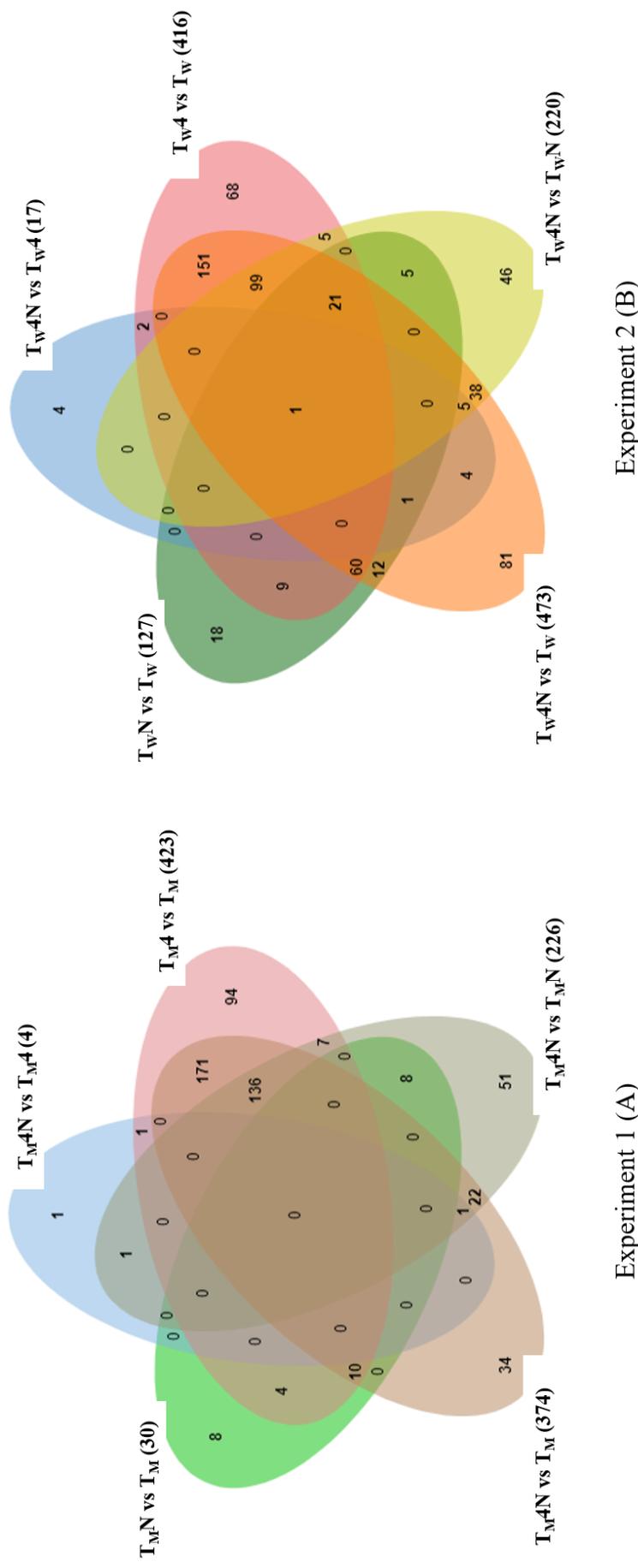
Experiment 2 (B)

Figure 3 - 15: The Venn diagrams showing the number of differentially expressed proteins due to treatments, including elevated temperature, elevated NO_2 and their interaction, in experiment 1 (A) and experiment 2 (B), respectively. Description for abbreviations in the tables remain the same as in Figure 3 - 1.

3.5.1-3 Expression levels of proteins in ragweed pollen

As a result, experiment 1 yielded a total of 6268 identified peptides, corresponding to 1602 proteins in T_MN vs T_M, 1637 proteins in T_M4N vs T_M4, 1606 proteins in T_M4 vs T_M, 1618 proteins in T_M4N vs T_MN, and 1597 proteins in T_M4N vs T_M (Figure 3 - 14 A). In experiment 2, 5555 peptides were identified, representing 1465 proteins in T_WN vs T_W, 1494 proteins in T_W4N vs T_W4, 1448 proteins in T_W4 vs T_W, 1486 proteins in T_W4N vs T_WN, and 1455 proteins in T_W4N vs T_W (Figure 3 - 14 B). To visually depict significant changes in protein abundance within specific clusters, volcano plots [log10 (P-value) vs log2 (fold change of condition/another condition)] and Venn diagrams were generated for a comprehensive representation of quantitative data (Figure 3 - 14 and Figure 3 - 15, A and B, respectively).

Specifically, in response to elevated NO_2, clusters T_MN vs T_M, T_M4N vs T_M4, T_WN vs T_W, and T_W4N vs T_W4 exhibited significant differential expression in 30 (17 upregulations and 13 downregulations), 4 (one upregulation and three downregulations), 127 (74 upregulations and 53 downregulations), and 17 (6 upregulations and 11 downregulations) proteins, respectively. In comparison, a 4 °C temperature increase led to significant changes in 423 (305 upregulations and 118 downregulations), 226 (141 upregulations and 85 downregulations), 416 (209 upregulations and 207 downregulations) and 220 (74 upregulations and 146 downregulations) proteins in clusters T_M4 vs T_M, T_M4N vs T_MN, T_W4 vs T_W and T_W4N vs T_WN, respectively. Furthermore, in the combination treatments of clusters T_M4N vs T_M and T_W4N vs T_W, 374 (249 upregulations and 125 downregulations) and 473 (231 upregulations and 242 downregulations) proteins displayed significant alterations, respectively (Figure 3 - 14 C). Among the 374 proteins significantly differentially expressed in response to the combined treatments in experiment 1, 329 overlapped with those affected by elevated temperature alone. Additionally, 11 proteins were shared as influenced by both elevated NO_2 and elevated temperature. Notably, no proteins were identical only to those altered by elevated NO_2 (Figure 3 - 15 A). However, in experiment 2, out of the 473 proteins significantly differentially expressed under the combined treatments, 288 proteins were commonly shared with those affected by elevated temperature alone, while 17 proteins showed common alterations in response to elevated NO_2 alone. Moreover, 86 proteins were shared

between those influenced by both elevated NO_2 and elevated temperature. Remarkably, one protein was significantly expressed under elevated NO_2 (TwN), elevated temperature (Tw4) and the combined treatment (Tw4N), with respect to the control treatment (Tw), respectively (Figure 3 - 15 B).

In summary, in experiment 1, elevated temperature exerted a more pronounced impact on alterations in the pollen proteome than elevated NO_2, evident in the increased number of red points ($P < 0.05$, $|\log_{1.5} FC| > 1.0$) in clusters T_M4 vs T_M (423) and T_M4N vs T_MN (226) compared to clusters T_MN vs T_M (30) and T_M4N vs T_M4 (4), respectively. Through the comparisons between clusters T_M4N vs T_M (374), T_M4 vs T_M (423), and T_MN vs T_M (30), it is evident that the impact of elevated temperature on alterations in the pollen proteome was mitigated with the addition of elevated NO_2. Similarly, in experiment 2, the introduction of elevated NO_2 mitigated the impact of elevated temperature on altering the pollen proteome, as evidenced by fewer red points in the Tw4N vs TwN cluster compared to the Tw4 vs Tw cluster. Furthermore, elevated NO_2 under Tw conditions played a more pivotal role in shaping the pollen proteome than under Tw4 conditions, as indicated by a higher number of red points in the TwN vs Tw cluster compared to the Tw4N vs Tw4 cluster. These findings collectively underscore the greater influence of temperature compared to elevated NO_2 in shaping the pollen proteome.

3.5.2 Classification of differentially expressed proteins in ragweed pollen

3.5.2-1 Overview of Gene ontology (GO) annotation for proteins significantly differentially expressed in ragweed pollen

Eventually, among the proteins exhibiting significant up- or down-regulation (a total of 1837 proteins, as illustrated in Figure 3 - 14 C) within 10 specific clusters, GO term assignments were made for 30 proteins in T_MN vs T_M, 4 proteins in T_M4N vs T_M4, 423 proteins in T_M4 vs T_M, 226 proteins in T_M4N vs T_MN, 374 proteins in T_M4N vs T_M, 127 proteins in TwN vs Tw, 17 proteins in Tw4N vs Tw4, 416 proteins in Tw4 vs Tw, 220 proteins in Tw4N vs TwN, and 473 proteins in Tw4N vs Tw. Notably, these significantly altered proteins were distributed across three GO

categories, with 2016 (87.3%) assigned to cellular component (CC), 2234 (96.7%) to molecular function (MF), and 2121 (91.8%) to biological process (BP) (Figure 3 - 16).

Clearly, the GO classification of the terms related to CC, MF, and BP exhibited differences between the clusters subjected to elevated NO_2 and elevated temperature. The temperature-treatment-related clusters showed a higher degree of enrichment and a greater number of annotated GO terms in each of the three GO categories compared to elevated NO_2 treatment-related clusters (Figure 3 - 16).

In experiment 1, in the elevated NO_2 treatments, the $T_M N$ vs T_M cluster exhibited enrichment in 11 CC terms (49 counts), 8 MF terms (53 counts), and 9 BP terms (53 counts), while the $T_M 4N$ vs $T_M 4$ cluster showed enrichment in 3 CC terms (3 counts), 4 MF terms (6 counts), and 3 BP terms (4 counts). In contrast, elevated temperature resulted in a notable increase in annotated terms. Evidently, a 4 °C temperature increase enriched the $T_M 4$ vs T_M cluster with 16 CC terms (820 counts), 14 MF terms (825 counts), and 15 BP terms (719 counts), and the $T_M 4N$ vs $T_M N$ cluster with 15 CC terms (422 counts), 13 MF terms (449 counts), and 14 BP terms (373 counts). Additionally, the combined effect of elevated temperature and elevated NO_2 showed comparable enrichment in the $T_M 4N$ vs T_M cluster with 15 CC terms (732 counts), 14 MF terms (724 counts), and 15 BP terms (633 counts) when compared to the clusters subjected to elevated temperature treatments (Figure 3 - 16).

In comparison, in experiment 2, the elevated NO_2 treatments ($T_W N$ vs T_W and $T_W 4N$ vs $T_W 4$ clusters) resulted in 14 CC terms (227 counts), 13 MF terms (242 counts), and 12 BP terms (210 counts), and 10 CC terms (21 counts), 6 MF terms (29 counts), and 7 BP terms (25 counts), respectively. Elevated temperature enriched the $T_W 4$ vs T_W cluster with 16 CC terms (731 counts), 13 MF terms (789 counts), and 15 BP terms (673 counts), and the $T_W 4N$ vs $T_W N$ cluster with 16 CC terms (373 counts), 13 MF terms (406 counts), and 12 BP terms (357 counts). Furthermore, the concurrent rise in temperature and NO_2 led to greater GO enrichment in the $T_W 4N$ vs T_W cluster. Specifically, there were 16 CC terms (842 counts), 13 MF terms (911 counts), and 14 BP terms (768 counts), surpassing the enrichment observed with elevated temperature or elevated NO_2 alone (Figure 3 - 16).

Figure 3 - 16: GO analysis illustrating classes of proteins in ragweed pollen differing in 10 clusters. Proteins with significant differences were subjected to GO classification in terms of cellular component (CC) (A), molecular function (MF) (B), and biological process (BP) (C). A color gradient from dark orange to white indicates GO enrichment counts from high to low, while values are indicated in bars (blue for CC, pink for MF, and orange for BP). Description for abbreviations in the tables remain the same as in Figure 3 - 1.

In summary, in both experiments, the temperature-treatment-related clusters showed a higher degree of enrichment and a greater number of annotated GO terms in each of the three GO categories in contrast to elevated NO_2 treatment-related clusters. Consequently, the comparisons within treatments indicate that temperature exerts a greater influence than NO_2 on the proteome of ragweed pollen (Figure 3 - 16).

3.5.2-2 Association between applied treatments and the GO functional classifications of differentially expressed proteins

In order to enhance the focus of the analysis and provide clearer insights into the association between applied treatments and the GO functional classifications of differentially expressed proteins, only those classifications with a minimum of 5 proteins in each treatment-related cluster were taken into consideration (based on the results shown in Figure 3 - 16). As a result, the T_M4N vs T_M4 cluster was excluded from the analysis and clusters treated with elevated NO_2 included T_MN vs T_M, T_WN vs T_W, and T_W4N vs T_W4. Moreover, the elevated temperature treatments comprised elevated temperature alone and elevated temperature combined with elevated NO_2. These treatments were represented by the clusters T_M4 vs T_M, T_M4N vs T_MN, T_W4 vs T_W, T_W4N vs T_WN, T_W4N vs T_W, and T_W4N vs T_W (Figure 3 - 17).

For the CC category, the five most abundantly regulated groups, both up- and down-regulated, consistently observed in elevated NO_2- and/or elevated temperature- treated clusters were "cytoplasm", "membrane", "cytosol", "nucleus", and "mitochondrion". In addition, exclusively in the elevated temperature-treated clusters, upregulated proteins were associated with "ribosome", "proteasome", "endosome" and "chromosome", while downregulated proteins were linked to the "organelle lumen" (Figure 3 - 17 A and B).

In the MF category, the GO term "catalytic activity" emerged as the most abundant among both up- and down-regulated proteins, exhibiting enrichment across all clusters. Binding-related GO terms, including "nucleotide binding", "metal ion binding", and "protein binding", followed closely, being enriched in both treatments related clusters. Conversely, GO terms such as "signal transducer activity", "DNA binding", and "motor activity" were enriched exclusively in the elevated temperature-treated clusters. Notably, proteins within the "structural molecular activity"

GO term exhibited significant upregulation in response to elevated temperature. Similar patterns were evident in proteins annotated with "RNA binding" and "transporter activity", with a majority of these proteins upregulated in the elevated temperature-treated clusters (Figure 3 - 17 C and D).
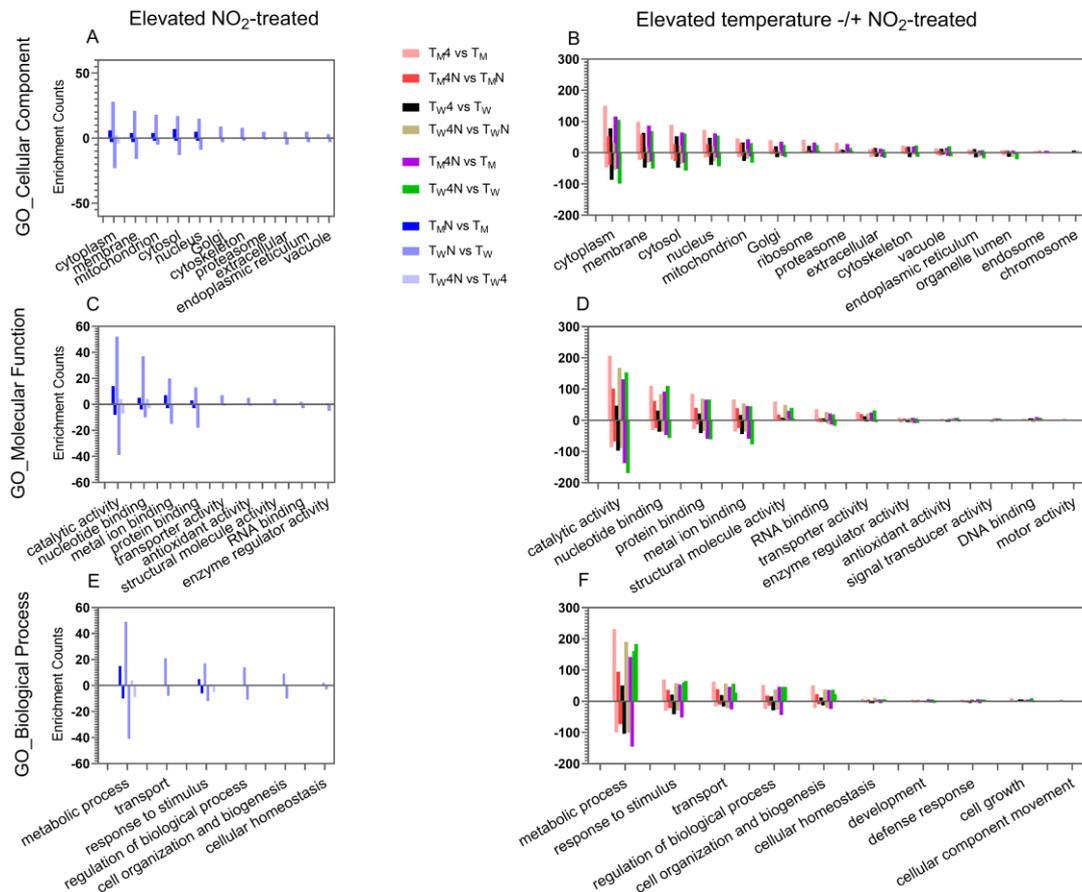
Figure 3 - 17: Proteins with significant differences (upregulation in the upper part and downregulations in the lower part) in each treatment related cluster, subjected to GO classification in terms of cellular component (A) and (B), molecular function (C) and (D) and biological function (E) and (F) with a threshold of 5 proteins (sum of up- and down-regulations) per classification. Blues represent elevated NO$_2$-treated clusters; reds, black and brown signify clusters subjected to elevated temperature treatment; purple and green shading indicate clusters subjected to a combination treatment. Description for abbreviations in the figures remain the same as in Figure 3 - 1.

In the BP category, the GO term "metabolic process" ranked as predominant among both up- and down-regulated proteins, demonstrating consistent enrichment across all clusters. Furthermore, a substantial number of proteins were associated with GO terms such as "response to stimulus", "transport", "regulation of biological process", and "cell organization and biogenesis", particularly enriched in the elevated temperature-treated clusters. Meanwhile, a limited number of proteins were linked to the GO terms "cellular homeostasis,", "development", "defense response",

"cell growth", and "cellular component movement", with the latter four terms exclusively enriched in response to elevated temperature treatments (Figure 3 - 17 D and E).

3.5.2-3 Annotation of differentially expressed proteins based on specific functional category

A more detailed classification of these significantly altered proteins into functional groups based on their predicted functions is presented, with the protein count assigned to each respective function detailed in Table 3 - 10 for experiment 1 and Table 3 - 11 for experiment 2. In both experiments, the identified functional categories encompassed: (1) allergens, with other allergen in both experiments and *Ambrosia* allergens exclusively in experiment 2; (2) cell wall biogenesis/degradation; (3) cytoskeleton dynamics; (4) glucose metabolism, including glycolysis; (5) metabolic process, including one-carbon metabolic process, nitrogen metabolism, sulfur metabolism, carbon utilization and NAD(P)H dehydrogenase activity; (6) protein biosynthesis, folding, and degradation processes; (7) translation; (8) transport-related; (9) protein modification, such as methylation, phosphorylation, ubiquitination, and protein glycosylation (only in experiment 1); (10) secondary metabolite; (11) signaling; (12) stress and defense-related; (13) reproductive structure development; (14) photosynthesis, and (15) tricarboxylic acid cycle.

Notably, in experiment 1, significantly differentially expressed proteins homologous to other allergens, and involved in sulfur metabolism, as well as in the biosynthesis process of flavonoid, isoprene, riboflavin, lignin and steroid, were consistently down-regulated in three clusters (T_M4 vs T_M, T_M4N vs T_MN, T_M4N vs T_M), each of which were related to treatments with a 4 °C increase in temperature. Conversely, a consistent up-regulation was observed in proteins associated with NAD(P)H dehydrogenase activities, protein glycosylation, and fatty acid biosynthesis within the same clusters from elevated temperature treated conditions. In addition, functional categorization of both significantly up- and down-regulated proteins, more pronouncedly observed in the same three clusters under elevated temperature treatment compared to elevated NO_2, encompassed cytoskeletal dynamics and cell wall biogenesis/degradation, influencing cellular structure. Moreover, alterations were noted in glycolysis, the tricarboxylic acid cycle and photosynthesis,

pivotal for cellular energy and nutrient provision, alongside the modulation of signaling pathways, especially those activated by plant growth regulators (Table 3 - 10).

In experiment 2, the expression of pollen allergens varied significantly under both, elevated temperature and elevated NO_2 treatments. Amb a 1 isoforms in ragweed pollen were consistently upregulated in response to elevated temperature. Conversely, Amb a 4, Amb a 5 and Amb a 8 isoforms (*A. artemisiifolia*), Bet v 6 (*Betula pendula*), Cat r 1 (*Catharanthus roseus*), Sal k 4 (*Kali turgidum*), Sal k 4 (*Kali turgidum*), and Gly m 3 (*Glycine max*) were downregulated in response to elevated temperature. Notably, Amb a 5 showed an exceptional upregulation in response to elevated NO_2 in the T_W4N vs T_W4 cluster (Table 3 - 12).

Table 3 - 10: Functional categories of the differentially expressed proteins with corresponding clusters under different treatments (elevated temperature, elevated NO_2 and the combination) in experiment 1. Description for abbreviations in the tables remain the same as in Figure 3 - 1. The number of specified proteins in the up-regulated group is shown in bold and highlighted in red.

| | Number of assigned proteins | | | | | | | | | |
Functional category	T_{M4} vs T_M Up (305)	Down (118)	T_{M4N} vs T_{MN} Up (141)	Down (85)	T_{M4N} vs T_M Up (249)	Down (125)	T_{MN} vs T_M Up (17)	Down (13)	T_{M4N} vs T_{M4} Up (1)	Down (3)
(1) Other allergen		3		3		2				
Ole e 12 (*Olea europaea*)		1		1		1				
Cat r 1 (*Catharanthus roseus*)		1		1		1				
Mal d 4 (*Malus domestica*)				1						
Gly m 3 (*Glycine max*)		1								
(2) Cell wall biogenesis/degradation	**6**	9	**3**	4	**5**	8				
(3) Cytoskeleton dynamics	**23**	5	**16**	5	**19**	6	**1**			
(4) Glucose metabolism	**26**	11	**8**	4	**19**	5	**2**			
Glycolysis	13	3	5	2	11	2				
(5) Metabolic process	**31**	19	**18**	16	**24**	20	**2**	3		
One-carbon metabolic process	1	5	1	4	1	4				
Nitrogen metabolism	3		2	2	1			1		
Carbon utilization	1									
NAD(P)H dehydrogenase activity	9		6		8		1			
(6) Protein biosynthesis, folding and degradation process	**41**	12	**17**	9	**37**	13				1
(7) Translation	**43**	6	**8**	3	**34**	8	**1**	1		

Functional category	T_{M4} vs T_M		T_{M4N} vs T_{MN}		T_{M4N} vs T_M		T_{MN} vs T_M		T_{M4N} vs T_{M4}	
	Up (305)	Down (118)	Up (141)	Down (85)	Up (249)	Down (125)	Up (17)	Down (13)	Up (1)	Down (3)
(8) Transport related	**39**	8	**22**	6	**35**	12	**2**	2		1
(9) Protein modification	**15**	7	**11**	2	**11**	8	**1**	1	**1**	1
Methylation	4		3		4	2				
Phosphorylation	7	3	5		5	3				
Ubl conjugation pathway	3	4	3	2	1	3	1	1		
Glycosylation	1				1					
(10) Secondary metabolite	**14**	7	**7**	10	**12**	8	**4**	1		
Fatty acid biosynthesis	3		3		3		1			
Lipid biosynthesis	7	2	1	2	4		1			
Oxylipin biosynthesis	2	1	3		2			1		
Flavonoid biosynthesis.		1		1	1	1				
Isoprene biosynthesis		2		3		3				
Lignin biosynthesis				1						
Riboflavin biosynthesis		1		1		1				
Steroid biosynthesis				1		1				
(11) Signaling	**9**	4	**2**	2	**5**	6				
Plant growth regulator	8	4	2	1	4	3				
(12) Stress and defense related	**19**	11	**13**	11	**14**	13	**2**	3		1
(13) Reproductive structure development	**17**	5	**7**	1	**13**	6	**2**	1		
(14) Photosynthesis	**3**	6		6	**3**	7				
(15) Tricarboxylic acid cycle	**13**	1	**7**	1	**11**	1				
(16) Others	**6**	4	**2**	2	**7**	2	**2**	1		

Table 3 - 11: Functional categories of the differentially expressed proteins with corresponding clusters under different treatments (elevated temperature, elevated NO_2 and the combination) in experiment 2. Description for abbreviations in the tables remain the same as in Figure 3 - 1. The number of specified proteins in the up-regulated group is shown in bold and highlighted in red.

Functional category		Number of assigned proteins								
	Tw4 vs Tw		Tw4N vs TwN		Tw4N vs Tw		TwN vs Tw		Tw4N vs Tw4	
	Up (209)	Down (207)	Up (74)	Down (146)	Up (231)	Down (242)	Up (74)	Down (53)	Up (6)	Down (11)
(1) A. *artemisiifolia*										
Amb a 1	1	4	2		2	2			1	1
Amb a 4		1		2	1	1				
Amb a 5		1				1				1
Amb a 8		2				1				
(2) Other allergen		4				4				
Bet v 6 (*Betula pendula*)		1		2		1				
Cat r 1 (*Catharanthus roseus*)		1		1		1				
Sal k 4 (*Kali turgidum*)		1		1		1				
Gly m 3 (*Glycine max*)		1				1				
(3) Cell wall										
biogenesis/degradation	3	4	1	3	3	3	1	3		
(4) Cytoskeleton dynamics	20	7	3	4	22	7	8	3	1	
(5) Glucose metabolism	13	21	6	12	18	25	7	4	3	2
Glycolysis	8	8	3	5	12	9	5	1		2
(6) Metabolic process	29	44	3	33	25	55	7	13	1	2
One-carbon metabolic process	1	5		2	2	7	2			
Nitrogen metabolism	2	1			1					
Carbon utilization						1				

Functional category	Tw4 vs Tw Up (209)	Down (207)	Tw4N vs TwN Up (74)	Down (146)	Tw4N vs Tw Up (231)	Down (242)	TwN vs Tw Up (74)	Down (53)	Tw4N vs Tw4 Up (6)	Down (11)
NAD(P)H dehydrogenase activity	8	4	2	5	6	4	3	3		
(7) Protein biosynthesis, folding and degradation process	20	17	6	13	21	24	7	5		1
(8) Translation	37	21	18	8	44	28	4	3		3
(9) Transport related	39	20	19	15	45	19	13	7		1
(10) Protein modification	3	10		9	5	10	1	2		
Methylation					1					
Phosphorylation	1	2		2	3	3		2		
Ubl conjugation pathway	1	8		5	1	7	1			
(11) Secondary metabolite	3	6	2	2	2	7	2	3		
Fatty acid biosynthesis	2				1		1			
Lipid biosynthesis		1	2			3				
Flavonoid biosynthesis.		1		1		1		2		
Lignin biosynthesis	1	1				1				
Riboflavin biosynthesis		1				1	1			
(12) Signaling	8	9	1	5	6	6	4	1		
Plant growth regulator	8	2		2	5	1	4			
(13) Stress and defense related	19	18	9	17	18	20	10	3		1
(14) Reproductive structure development	4	2	3	2	6	2	3	1		
(15) Photosynthesis	3	4	1	4	5	6	1	2		
(16) Tricarboxylic acid cycle	3	6	1	4	5	9	4	1		
(17) Others	4	10		16	4	15	2	2		1

3.6 Tyrosine nitration in recombinant ragweed allergens

The allergenicity of proteins, including rAmb a 1, 3, 8, and 11, as well as commercial ragweed pollen (CRP), exhibited distinct alterations upon TNM nitration when tested against 16 patient sera (Table 3 - 12). These variations aligned with patient-specific responses in allergic reactions. Notably, rAmb a 1 and rAmb a 8 displayed the highest likelihood (31.25%) of increased allergenicity after TNM nitration. Similarly, TNM nitration of rAmb a 3, 11, and CRP indicated potentially induced allergenicity, with percentages of 18.75%, 25.00%, and 20.00%, respectively. Conversely, there were higher probabilities of reduced allergenicity after TNM nitration for rAmb a 1, 3, and CRP, with percentages of 43.75%, 37.50%, and 33.33%, respectively. rAmb a 8 and 11 exhibited an equal likelihood of increased and decreased allergenicity after TNM nitration, at 31.25% and 25%, respectively. Furthermore, in the case of rAmb a 12, consistent down-regulation of allergenicity was observed across all 16 patient sera tested.

Table 3 - 12: The altered allergenicity of rAmb a proteins (Amb a 1, 11, 12, 3 and 8) accessed with and without TNM treatment, followed by probing with 16 individual patient (P) serum. The allergenicity index was computed by subtracting the integrated optical density (IOD) of the immunoblotting after TNM treatment from the IOD of the immunoblotting without TNM. Criteria for determining up- or down-regulation in allergenicity were established as follows: up, ↑, index > 0.1; no change, →, -0.1 < index < 0.1; down, ↓, index < -0.1. NA means no data.

Patient	rAmb a 1	rAmb a 3	rAmb a 8	rAmb a 11	rAmb a 12	CRP
P1	↑	→	↑	↑	↓	→
P2	↓	→	→	↑	↓	↓
P3	↓	→	→	→	↓	↓
P4	↓	↑	↑	↑	↓	↓
P5	→	→	↑	→	↓	→
P6	↑	↑	↑	↑	↓	NA
P7	→	→	↓	→	↓	→
P8	↑	↑	↑	→	↓	→
P9	↑	↓	↓	→	↓	↓
P10	→	→	→	→	↓	↑
P11	→	↓	↓	↓	↓	↑
P12	↓	↓	→	↓	↓	↓
P13	↓	↓	↓	↓	↓	→
P14	↓	↓	→	→	↓	↑
P15	↓	↓	↓	↓	↓	→
P16	↑	→	→	→	↓	→
Up-regulation (%)	31.25%	18.75%	31.25%	25.00%		20.00%
No change (%)	25.00%	43.75%	37.50%	50.00%		46.67%
Down-regulation (%)	43.75%	37.50%	31.25%	25.00%	100.00%	33.33%

3.7 Characterization of ragweed plant-associated fungi

3.7.1 Community analysis of leave associated fungi of common ragweed by Illumina sequencing

3.7.1-1 Evaluation of the primary steps for creating the sequence library

PCR amplifications of genomic DNA extracted from leaves of 18 plants, distinguishing between the leaves at the upper and lower positions, yielded bands within the appropriate range of ITS2 sequence sizes, approximately 500 bp per sample. Two negative controls, one for DNA extraction and the other for PCR amplification, confirmed the absence of contamination during DNA extraction (see Figure 3 - 18). Analysis of fragment lengths demonstrated an average increase of 70 bp in PCR products following indexing, providing evidence of successful indexing for Illumina sequencing library preparation (refer to Figure 3 - 19).
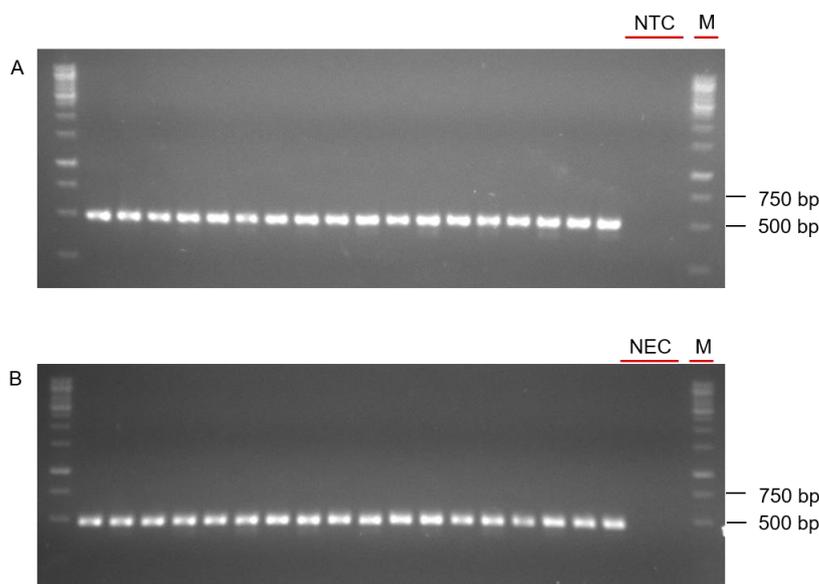
Figure 3 - 18: Representative fungal ITS2 PCR products of the 18 samples from upper (A) and lower (B) leaves. NTC, negative technical control for PCR amplification; NEC, negative environmental control for DNA extraction; M, Marker (GeneRuler 1 kb DNA Ladder, Thermo Fisher Scientific).
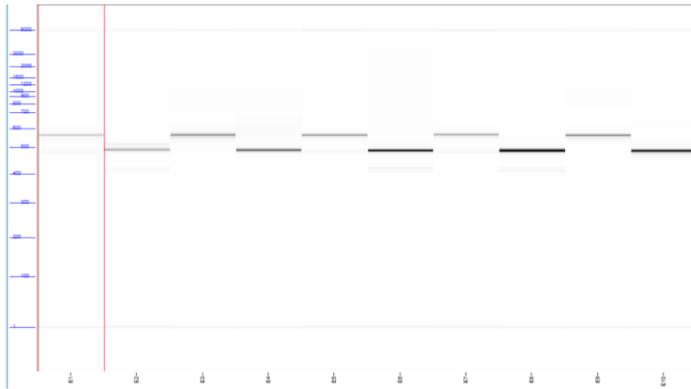
Figure 3 - 19: Five PCR products were randomly selected for fragment length analysis using the Fragment Analyzer. Upon comparing the paired gel bands after (higher band) and before (lower band) indexing, a consistent pattern emerged, indicating that the majority of the samples exhibited a size difference of 70 bp.

3.7.1-2 Overview of the fungal community

Following processing and quality filtering conducted by PIPITS, DNA sequencing of the ITS2 region produced a total of 80,070 high-quality reads, ranging from 61 to 8,045 reads per sample (Table 3 - 13). These sequences were classified into 415 OTUs (operational taxonomic units), with the majority assigned to three fungal phyla. Ascomycota emerged as the predominant phylum, constituting 66.5%, followed by Basidiomycota (23.9%). A minor portion of OTUs belonged to the Chytridiomycota phylum, accounting for 0.2%. Approximately 7.5% of OTUs could not be assigned to a specific phylum due to either an unclear ITS sequence or an unclarified taxonomic position of the reference sequence. Additionally, 2.2% of the sequences lacked an entry in the database (Figure 3 - 20 A). Further classification at lower taxonomic levels grouped these OTUs into 17 classes and 24 orders. At the class level, Dothideomycetes dominated (36.9%), followed by four other classes each exceeding 5%: Leotiomycetes (8.9%), Agaricomycetes (8.7%), Tremellomycetes (8.7%), and Sordariomycetes (8.2%) (Figure 3 - 20 B). Furthermore, at the order level, a substantial number of OTUs were assigned to Pleosporales, constituting 27.3% (Figure 3 - 20 C).

A filter was implemented, setting a minimum sequence count threshold of 150 per sample (refer to Table 3 - 13). Subsequent analysis of alpha diversity indices revealed significant differences in the fungal composition at the two leaf positions within sampled ragweed plants. These distinctions encompassed changes in both the abundance extent within fungal communities (richness index, $p < 0.01$) and the distribution of abundance among species (evenness index, $p < 0.01$) on the two leaf positions. The variations in fungal patterns were predominantly influenced by low-abundance species (Shannon index, $p = 0.01$) rather than high-abundance species (Simpson index, $p = 0.82$) (Figure 3 - 21 A - D). In contrast, there were no significant differences observed either in the abundance of fungal species (richness index, $p = 1$) or in the distribution of abundance among species (evenness index, $p = 0.98$) within the respective leaf position across plants (Figure 3 - 21 E, F).

Table 3 - 13: The sequence counts of fungal ITS2 sequences per sample determined by Illumina sequencing, considering both the lower and upper positions of leaves for each plant. When the numbers in front of two sample IDs match, it signifies that the samples originated from the same plant. The concluding letter in the ID specifies the specific section from which the sample was collected. In this context, the label "M" designates samples sourced from the lower leaves, while "T" designates samples from the upper leaves. The color gradient, ranging from dark to light, visually denotes the decreasing height of the harvested samples. Similarly, the length of the bar graph corresponds to the sequence counts, illustrating a gradient from the highest to the lowest counts.

Plant ID (Lower layer)	Harvest height (cm)	Sequence Counts (sum per sample)	Plant ID (Upper layer)	Harvest height (cm)	Sequence Counts (sum per sample)
15OM	20	8045	6FT	45	1162
20TM	23.5	5771	17QT	57	812
11KM	24	5664	9IT	55	679
3CM	29.5	5051	21UT	40	632
17QM	28.5	4878	20TT	47	533
22SM	35.5	4509	11KT	48	484
21UM	20	4461	13MT	50	463
8HM	21.5	4278	1AT	57	453
9IM	27.5	4010	22ST	71	387
16PM	21	3424	14NT	51	338
7GM	20	3323	8HT	43	312
18RM	18	3166	3CT	59	283
13MM	25	3085	18RT	36	255
6FM	22.5	2923	2BT	59	251
5EM	25	2639	7GT	40	249
1AM	28.5	2506	5ET	50	217
14NM	25.5	2352	15OT	40	150
2BM	29.5	2264	16PT	42	61
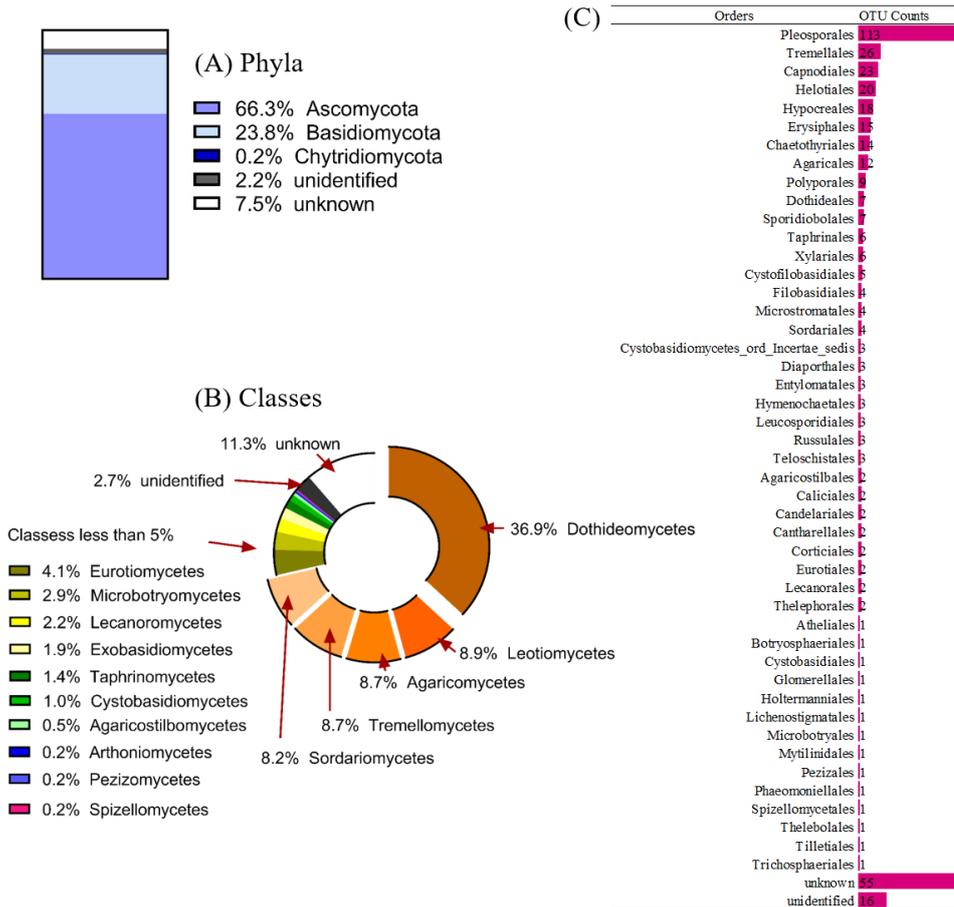
Figure 3 - 20: Taxonomic classification of obtained OTUs at the phylum (A), class (B) and order (C) levels.

Furthermore, Principal Coordinates Analysis (PCoA) illustrated a distinct fungal pattern distribution between the two leaf positions in ragweed plants as the 95% confidence intervals generated therein did not overlap. Conversely, symbols representing sampled plants (each identified by distinct colors) exhibit an almost uniform distribution. The nearly overlapping confidence intervals suggest that the disparities in fungal communities among the sampled plants were not statistically significant. Additionally, sampled height demonstrated a greater impact on the community compared to plant height and plant cube volume, aligning with the correlation between leaf positions and sampled height in the sampling methodology (Figure 3 - 21 G).
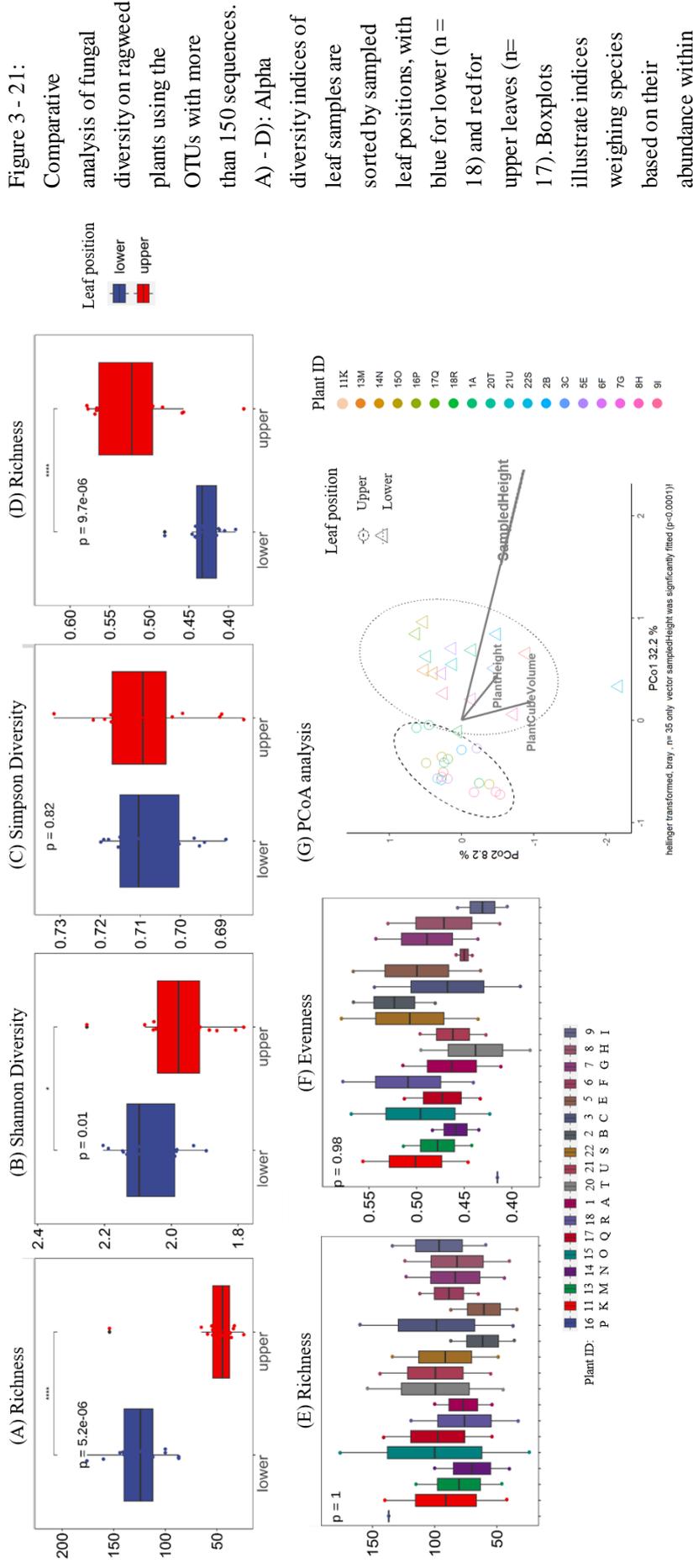
Figure 3 - 21: Comparative analysis of fungal diversity on ragweed plants using the OTUs with more than 150 sequences. A) - D): Alpha diversity indices of leaf samples are sorted by sampled leaf positions, with blue for lower (n = 18) and red for upper leaves (n= 17). Boxplots illustrate indices weighing species based on their abundance within fungal community, including the richness index (p < 0.01) and the evenness index (p < 0.01). E), F): Boxplots of richness indices reflect that neither the abundance of fungal species (richness index, p = 1) nor the distribution of abundance among those species (evenness index, p = 0.98) significantly differed between plants (n = 18 and respective color indicated in figures). Kruskal-Wallis test (indicated by asterisks, p < 0.05) and post-hoc comparisons using Dunn's test were performed in the analysis of diversity indices. G): PCoA (Bray-Curtis-Index) of fungal community composition patterns on two ragweed leaves (upper: n = 17, circles; lower: n = 18, triangles). The 95%-confidence intervals showed minimal overlap, indicating distinct fungal patterns between the two leaf positions rather than sampled plants. Sampled height showed a clear influence on the fungal community.

Furthermore, the top 30 OTUs collectively accounted for over 85% of the total fungal community within the samples. A comparison between a-diversity indices showed a consistent similarity of richness (Figure 3 - 21 E), and evenness (Figure 3 - 21 F) in individual plants at the corresponding leaf position. Notably, lower leaves exhibited a higher fungal species richness compared to upper leaves, aligning with the previously mentioned richness index ($p < 0.01$). Moreover, the relative abundance of each OTU in every sample, denoted by the respective colored bar length, exhibited a similar pattern within positions but differed between them, corroborating the findings of the evenness index ($p < 0.01$) mentioned earlier (Figure 3 - 22 A).

Moreover, sample-to-sample differentials in the heat map, coupled with the sorting of samples based on sampled height, were examined to elucidate variations in the relative abundance of fungal species at the genus taxonomic level across samples (Figure 3 - 22 B). Notably, species affiliated with the genus taxon "unknown_Capnodiales (Order)" were present in all leaf samples, displaying the highest relative abundance. Subsequently, fungal species associated with the genus taxon "unknown_Didymellaceae (Family)" and *Podospora* were enriched in leaf samples at different leaf positions, predominantly situated at the lower leaves (indicated by lighter green colors in Figure 3 - 22 B), indicating a potential spatial distribution of these fungi. Additionally, the enrichment of fungal species from the genera *Pseudopithomyces*, *Bipolaris*, *Neoascochyta*, *Sporobolomyces*, and *Aureobasidium* exhibited variations based on leaf positions. The first three genera were more abundant in the upper leaves, while the last two were more prevalent in the lower leaves.
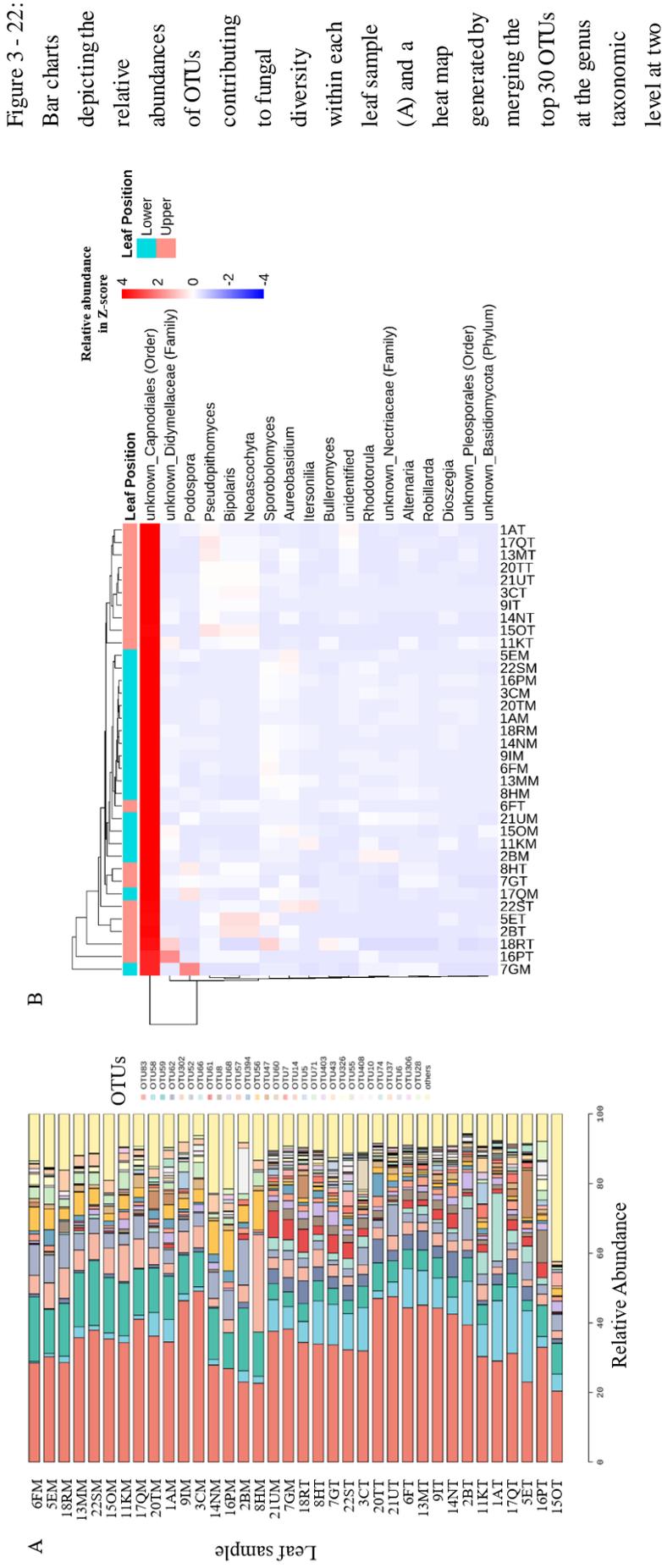
Figure 3 - 22: Bar charts depicting the relative abundances of OTUs contributing to fungal diversity within each leaf sample (A) and a heat map generated by merging the top 30 OTUs at the genus taxonomic level at two leaf positions (B). The abbreviations used to represent different leaf samples are detailed in Table 3 - 13, in which number = Plant ID, M and T are short for lower and upper leave, respectively. In figure A, there were 36 leaf samples, comprising 2 positions of leaf samples from each of the 18 plants. The top 30 OTUs were individually color-coded, while the remaining OTUs were collectively designated as "others". In figure B, the scale and color intensity reflect alterations in OTU relative abundance at the genus taxon across samples with varying sampled height. The green gradient from dark to light represents the sampling height from high to low, respectively. The color gradient (dark – light- blue) indicates the relative abundance in Z-Score (high – median – low) of fungal genus, respectively. The term "Unknown" is employed to signify that the genus of OTUs at this taxonomic level remains unknown, implemented with the lowest determinable taxonomic affiliation available for the OTUs in the database. Genus taxon clustering was performed using the average linkage algorithm with the Euclidean distance.

Table 3 - 14: Cultural and morphological characteristics of fungal isolates.

| Isolate list | Characteristics of cultures | | | | |
	Colony color	Shape/margin	Colony surface	Elevation	Additional description
Isolate 1	pink	circular/erose	downy	crateriform	
Isolate 2	pink	circular/undulate	downy	crateriform	
Isolate 3	pink	circular/entire	cottony	raised	
Isolate 4	brown black	circular/entire	downy	raised	
Isolate 5	pink with gey in the middle	circular/entire	cottony	raised	
Isolate 6	pink	circular/undulate	downy	raised	rosette
Isolate 7	pink	circular/erose	cottony	raised	
Isolate 8	pink	circular/entire	cottony	umbonate	
Isolate 9	pink	circular/undulate	downy	crateriform	smooth
Isolate 10	pink with gey in the middle	circular/undulate	cottony	convex	rough
Isolate 11	brown black	irregular/lobate	cottony	convex	
Isolate 12	black	irregular/undulate	cottony	umbonate	
Isolate 13	white with gey in the middle	circular/undulate	cottony	raised	rosette
Isolate 14	grey, white	irregular/filamentous	cottony	convex	moldy
Isolate 15	grey	circular/entire	downy	flat; filamentous	
Isolate 16	grey	circular/undulate	downy	raised	rosette
Isolate 17	white margin and brown in the middle	circular/erose	downy	raised	rosette
Isolate 18	pink	irregular/lobate	cottony	raised; filamentous	
Isolate 19	white	circular/erose	downy	umbonate	rosette
Isolate 20	white	circular/filamentous	cottony	umbonate	rosette
Isolate 21	pink with gey in the middle	circular/filamentous	cottony	convex	rosette

Isolate list	Characteristics of cultures				
	Colony color	Shape/margin	Colony surface	Elevation	Additional description
Isolate 22	brown black	circular/filamentous	cottony	convex	
Isolate 23	black with grey in the middle	filamentous/filamentous	cottony	flat	
Isolate 24	black with white in the middle	filamentous/filamentous	cottony	flat	
Isolate 25	white with gey in the middle	circular/entire	downy	umbonate	
Isolate 26	color deepening from margin to the middle	circular/entire	downy	flat	
Isolate 27	grey	circular/undulate	cottony	convex	rough
Isolate 28	pink with gey in the middle	circular/entire	cottony	flat	
Isolate 29	white with gey in the middle	circular/undulate	downy	flat	
Isolate 30	grey	irregular/undulate	carpet-like	convex	
Isolate 31	grey	circular/undulate	cottony	raised	rapid growth
Isolate 32	grey	irregular/filamentous	cottony	convex; filamentous	rapid growth
Isolate 33	grey	circular/filamentous	cottony	raised	rapid growth
Isolate 34	grey with pink margin	circular/entire	cottony	raised	rapid growth
Isolate 35	black bottom; off-white	circular/entire	carpet-like	flat	rapid growth
Isolate 36	off-white	filamentous/filamentous	cottony	convex	rapid growth
Isolate 37	skin-color	filamentous/filamentous	cottony	convex	rapid growth
Isolate 38	pink with gey in the middle	circular/undulate	downy	flat	rapid growth
Isolate 39	off-white	irregular	cottony	umbonate	rapid growth
Isolate 40	pink with gey in the middle	circular/entire	cottony	convex	rapid growth
Isolate 41	white	filamentous/filamentous	cottony	convex	rapid growth
Isolate 42	white margin with skin color in the middle	circular/entire	cottony/filamentous	convex	rapid growth
Isolate 43	brown black	irregular/filamentous	cottony/filamentous	convex	rapid growth
Isolate 44	brown black	irregular/filamentous	downy/filamentous	convex	rapid growth

Characteristics of cultures

Isolate list	Colony color	Shape/margin	Colony surface	Elevation	Additional description
Isolate 45	brown black	circular/entire	downy	raised	rapid growth
Isolate 46	brown black	curled/filamentous	carpet-like/filamentous	flat	rapid growth
Isolate 47	brown black	irregular	filamentous	flat	rough; rapid growth
Isolate 48	milk white	circular/entire	short aerial hyphae	flat	
Isolate 49	gey in the middle; pink; transparent margin	circular/entire	dry, powdery	flat	rotate; rapid growth
Isolate 50	gey	circular/undulate	short aerial hyphae	flat	rosette margin
Isolate 51	milk white	circular/entire	dry, powdery	convex	rotate in the middle
Isolate 52	grey in the middle; white margin	circular/undulate	dry, powdery	raised	
Isolate 53	color deepening from margin to the middle	circular/entire	short aerial hyphae; powdery margin	raised	rotate; rapid growth
Isolate 54	grey	circular/entire	powdery	convex	rapid growth
Isolate 55	grey margin; dark in the middle	circular/entire	dry, powdery	flat	rotate
Isolate 56	color deepening from margin to the middle	circular/undulate	short aerial hyphae	flat	rosette
Isolate 57	pink	circular/entire	dry, powdery	flat	rapid growth
Isolate 58	pink margin; pink-orange in the middle	circular/entire	dry, powdery	raised	rapid growth
Isolate 59	white	circular/entire	wrinkled; downy	raised	rotate; slow growth
Isolate 60	white	circular/entire	spiderweb	raised	slow growth
Isolate 61	white	circular/undulate	wrinkled/filamentous	raised	slow growth
Isolate 62	white	irregular/filamentous	cottony	raised	rapid growth
Isolate 63	milk white	irregular/filamentous	cottony	raised	rapid growth
Isolate 64	white	circular/undulate	spiderweb	umbonate	rotate; slow growth

Table 3 - 15: The blast results of 15 isolates obtained via UNITE using Sanger sequencing. Sequential to the sequencing sample number (Sample 1 - 15) for each fungal isolate, characters provide details about the corresponding leaf sample, serving as the source of the fungal isolate. Specific information about the leaf samples can be found in Table 3 - 13. The species identified per sample with the highest identity score were emphasized in bold. Owing to their high sequence similarities, additional potential identifications for certain candidates were explored, accompanied by their respective identity scores. The table exclusively provides relevant information for the primary species when multiple species were identified in blast results from the same sample and shared identical taxonomic levels of genus, family, and order.

Fungal isolate	Species (blast via UNITE)	Identity score (%)	E Value	Genus	Family	Order
Sample1-19VT	**B. bassiana**	99.63	0	*Beauveria*	Cordycipitaceae	Hypocreales
Sample2-9IT	**A. brassicae**	98.87	0	*Alternaria*	Pleosporaceae	Pleosporales
	A. sp.	98.87	0			
	A. alternata	98.87	0			
Sample3-16PT	**P. corylophilum**	99.82	0	*Penicillium*	Aspergillaceae	Eurotiales
Sample4-7GT	**A. sp.**	99.63	0	*Alternaria*	Pleosporaceae	Pleosporales
	A. arborescens	99.62	0			
	A. alternata	99.62	0			
Sample5-19VT	**A. tenuissima**	96.24	0	*Alternaria*	Pleosporaceae	Pleosporales
	G. indica	94.97	0	*Gibberella*	Nectriaceae	Hypocreales
Sample6-9IT	**P. chartarum**	99.3	0	*Pseudopithomyces*	Didymellaceae	Pleosporales
Sample7-7GT	**Clonostachys**	99.81	0	*Clonostachys*	Bionectriaceae	Hypocreales
	C. epichloe	98.31	0			
Sample8-8HM	**G. tricincta**	100	0	*Gibberella*	Nectriaceae	Hypocreales
	G. acuminata	99.81	0			
Sample9-8HM	**A. fumigatus**	99.63	0	*Aspergillus*	Aspergillaceae	Eurotiales
	P.	99.26	0	*Penicillium*	Aspergillaceae	Eurotiales
	P. solitum	99.26	0			
	P. commune	99.08	0			
Sample10-8HM	**A. alternata**	99.62	0	*Alternaria*	Pleosporaceae	Pleosporales
	A.	99.81	0			
	A. pomicola	100	0			
Sample11-8HM	**M. bolleyi**	100	0	*Microdochium*	Pleosporaceae	Xylariales
Sample12-3CT	**B. sorokiniana**	99.45	0	*Bipolaris*	Pleosporaceae	Pleosporales
Sample13-3CT	**C. cladosporioides**	99.61	0	*Cladosporium*	Cladosporiaceae	Capnodiales
Sample14-9IT	**C. protuberata**	99.64	0	*Curvularia*	Pleosporaceae	Pleosporales
Sample15-8HT	**C. lunata**	99.11	0	*Curvularia*	Pleosporaceae	Pleosporales

3.7.2 Identification of ragweed plant-associated fungi by Sanger sequencing

3.7.2-1 Characterization of fungal isolates based on cultivation

Among the fungal isolates cultured on MEA plates, a total of 64 distinct early-emerging colonies were identified, distinguished by their cultural and morphological traits (Table 3 - 14).

3.7.2-2 Identification of fungal isolates by Sanger sequencing

The complete ITS sequences were subjected to blasting against the UNITE database (https://unite.ut.ee/analysis.php#), resulting in the identified candidates with their respective identity scores (Table 3 - 15). By selecting the highest identity score, seven genera encompassing fifteen distinct fungal species were identified. These include *Beauveria* (with one species, *B. bassiana*), *Alternaria* (comprising four species - *A. brassicae*, *Alternaria sp.*, *A. tenuissima*, and *A. alternata*), *Penicillium* (with one species, *P. corylophilum*), *Pseudopithomyces* (with one species, *P. chartarum*), *Clonostachys* (with one species, *Clonostachys*), *Gibberella* (with one species, *G. tricincta*), *Aspergillus* (with one species, *A. fumigatus*), *Microdochium* (with one species, *M. bolleyi*), *Bipolaris* (with one species, *B. sorokiniana*), *Cladosporium* (with one species, *C. cladosporioides*), and *Curvularia* (comprising two species, *C. protuberate* and *C. lunata*).

When comparing the ITS-based identification of isolates with the ITS2 sequences obtained from Illumina sequencing, at the lowest taxonomic level, six fungal isolates were successfully matched at the genus level. These include *Alternaria* (4 isolates), *Penicillium* (1 isolate), *Pseudopithomyces* (1 isolate), *Bipolaris* (1 isolate), *Cladosporium* (1 isolate), and *Curvularia* (2 isolates). Additionally, four fungal isolates were linked to the family taxonomic level, corresponding to *Bionectriaceae*, *Nectriaceae*, *Aspergillaceae*, and *Pleosporaceae*, with each family associated with a single fungal isolate. Furthermore, *B. bassiana* was associated in the ITS2-derived fungal community at the order level (Table 3 - 15 and Figure 3 - 22).

3.8 Immuno-reactivity analysis of fungal proteins and ragweed pollen extract

Dot blotting of ragweed pollen extracts, along with the addition of 3 µg CRP, when probed with patient sera, revealed distinct signals, indicating successful IgE-reaction detections in the assay. Adding CRP to sample 1 (referencing *B. bassiana*) resulted in significantly higher IgE-binding compared to sample 1 alone. No significant IgE-binding was observed upon adding CRP to samples 8, 11, and 15, corresponding to *G. tricincta*, *M. bolleyi*, and *C. lunata*, respectively. Neither the remaining 11 fungal isolates nor their combinations with CRP showed any detectable IgE-binding signals (Figure 3 - 23).
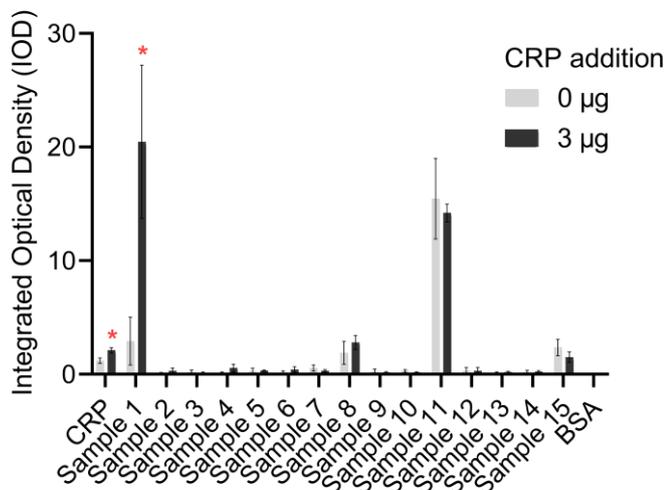
Figure 3 - 23: The integrated optical density of IgE reactivity measured between patient sera allergic to ragweed pollen and 3 µg of protein extracting from commercial ragweed pollen (CRP, represented by the first grey column), 15 fungal isolates (displayed in the remaining grey columns), and the addition of 3 µg of CRP to each sample (shown in the black columns). BSA (bovine serum albumin) serves as the negative control for measuring background signal. The identification numbers of the 15 fungal isolates correspond to the blast results outlined in Table 3 - 15. Columns represent the means of three independent dot blot assays. Statistical analysis by unpaired two-tailed Student's t-test was used for the comparisons between the addition of 0 µg and 3 µg of CRP to each sample. Differences are indicated by red stars ($p < 0.05$) (mean ± standard deviation).

4: Chapter – DISCUSSION

4.1 How do elevated temperature, elevated NO₂, and their interaction affect ragweed plant growth, traits and pollen release?

4.1.1 Experimental constraints

4.1.1-1 Conditions during early plant development

Two independent experiments were conducted simulating current ambient and future elevated temperatures in combination with and without NO_2 fumigation. The scenarios were chosen to represent a moderate (Munich) and warmer (Würzburg) urban area in Bavaria. The environmental parameters within the highly controlled phytotron chambers, encompassing light intensity, relative humidity (refer to Figure 2 – 4), UV radiation, atmospheric gas composition, and water supply, were meticulously standardized and have previously successfully been applied in published studies with ragweed (El-Kelish et al., 2014; Kanter et al., 2013; Zhao et al., 2016). However, seedling cultivation until the application of temperature regimes was different in the two experiments because of unforeseen needs for chamber maintenance in 2019 at the start of experiment 2 (refer to Figure 2 – 1 and Figure 2 – 2). Consequently, experiment 1 involved cultivating seedlings in a phytotron chamber, whereas experiment 2 necessitated using an alternative climate chamber with different light spectra and temperatures. These modifications might have induced unforeseen adaptive changes in the plants' size and growth rate, particularly during the initial stages of seedling growth.

Thus the observed differences in seedling growth may lead to part of the variances in plant and pollen traits at harvest, aligning with studies indicating that early seedling development influenced later-life features and stress tolerance through impacting resource capture in competitions for light and soil nutrients (Krishnan et al., 2011; Lowry and Smith, 2018; Tang et al., 2023). Consequently, the examination of plant traits and pollen properties focused on within-experiment factor effects rather than inter-experiment comparisons.

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4.1.1-2 Temperature simulations in experiment 2

Additionally, during DAS $62 - 65$, DAS 75, DAS $77 - 79$, and DAS $100 - 104$, the temperature differences between the elevated-temperature treatment chamber (T_W4) and the ambient-temperature treatment chamber (T_W) in experiment 2 were found to be 1.83 ± 0.04 °C, 0 °C, 1.83 ± 0.16 °C, and 0.33 ± 0.03 °C, respectively (Figure $2 - 2$). These values represent a deviation of 4 °C from the anticipated temperature difference. Specifically, DAS 38 - 48 corresponds to the lag phase of the growth curve, characterized by initial and slow plant development (Figure $3 - 2$). A mild temperature increase within the optimal range during this phase could facilitate robust seedling establishment, leading to increased cell elongation and expansion (Hartley et al., 2012), which lays the foundation for vigorous growth in subsequent stages. This may ultimately lead to an early flowering season (Burghardt et al., 2016; Capovilla et al., 2015b), as well as larger plant size (plant height and male inflorescence size) and higher yields at harvest (Gray and Brady, 2016; Schlenker and Roberts, 2009). Hence, the cumulative effects on plant size observed during the lag phase (Table $3 - 1$ B) might mitigate the impact of elevated temperature treatments in experiment 2 on plant height increase. This observation may partially elucidate the significant increase in plant height attributed to elevated temperature in experiment 1, whereas a similar effect was not observed in experiment 2. Moreover, during the lag phase, elevated temperatures can stimulate pollen production and release by boosting metabolic activity and enhancing resource availability (Borghi et al., 2019; Hartley et al., 2012). Thus, the higher temperatures recorded in the T_W-treated chamber during this period compared to T_W4-treated chamber may partially explain the greater pollen release observed in the T_W-treated plants as compared to the T_W4-treated plants in experiment 2.

4.1.2 Single and interactive effects of elevated temperature and NO_2 on plant growth

4.1.2-1 Elevated temperature plays a vital role in governing ragweed plant size

Generally warmer climates favor the growth of *A. artemisiifolia* (ragweed) as reflected by plant size (Deen et al., 1998). In the present experiments, this effect was manifested through the

promotion of both plant height and the average length of male inflorescences at harvest under elevated temperature treatments, as observed consistently in two independent experiments. These results align with greenhouse experiments showing that rising temperatures lead to proportional increases in plant size across various thermal regimes, including 15/9 h light-dark cycles of 18 - 14 °C, 24 - 20 °C, and 30 - 26 °C, respectively (Gentili et al., 2019). Furthermore, Skálová (2019) observed positive correlations between ragweed growth and temperature across five different temperature ranges ranging from 10 - 26 °C, supporting the notion that warmer temperatures enhance plant development.

However, it's crucial to note that in experiment 2, the height of ragweed plants did not significantly increase under elevated temperature treatment. Besides the above discussed influence in growth conditions during early seedling development, an important factor is likely the elevated temperature (T_W4) in the relatively warmer climate of the Würzburg experiment surpassing the optimal growth temperature for ragweed. This finding is consistent with previous research by Bazzaz (1974), who found that while ragweed growth responded positively to temperatures between 8 and 31.7 °C, it declined when temperatures exceeded 31.7 °C up to 40 °C.

Taken together, the overall argument supports the idea that warmer temperatures generally promote ragweed growth, but it also highlights the importance of considering optimal temperature ranges for different experimental conditions.

4.1.2-2 Interactive effects of elevated temperature and NO_2 treatments on ragweed plant size

In the current study, contrasting the effects of 0 ppb and 80 ppb of NO_2 treatments under ambient temperature conditions revealed no significant alterations in plant heights or male inflorescence size. This finding corroborates prior research indicating that ragweed plants exposed to 80 ppb NO_2 exhibited no significant change in male inflorescence size compared to those under 0 ppb or 40 ppb NO_2 (Zhao et al., 2017b).

Surprisingly, the positive effect of elevated temperature on promoting ragweed plant height was attenuated by elevated NO_2 under the elevated temperature treated conditions of experiment

2. This attenuation was observed in plant heights measured at DAS 80 and DAS 87. Furthermore, when the plants reached their final heights, no significant differences were observed across treatments. These findings suggest that there is an interaction between elevated temperature and elevated NO_2 in the relatively warmer climate and prompt consideration of potential mechanisms underlying this phenomenon. It is likely that when elevated temperature in the relatively warmer climate exceeds the optimum growth temperature for ragweed, as outlined in section 4.1.2-1, elevated NO_2 may prompt adjustments in plant nitrogen metabolism. These adjustments impact vital biological processes such as photosynthesis, protein synthesis, signaling transduction and transport (González-Pérez et al., 2008; Vranová et al., 2002; Wang et al., 2019; Zeevaart, 1976), thereby offsetting the heat stress-induced damage to the plant. This argument is supported by previous studies, which suggest that nitrogen dioxide can serve as an additional nitrogen source for plants (Takahashi and Morikawa, 2019a; Takahashi et al., 2011). Furthermore, nitrogen fertilization has been reported to alleviate the negative impacts of abiotic stresses, such as heat stress, on plant growth (Amarasekare and Savage, 2012; Waraich et al., 2011; Waraich et al., 2012). Overall, the argument presents a comprehensive analysis of the complex interplay between NO_2 and plant growth, shedding light on the multifaceted factors influencing plant responses to environmental stimuli.

4.1.3 Single and interactive effects of elevated temperature and NO_2 on pollen release of ragweed plant

4.1.3-1 Start of pollen release

The phenological development of common ragweed, specifically the timing of the pollen season, plays a critical role in determining the duration of the pollen season. Most pollen-monitoring studies do not clearly distinguish between the onset of pollen release and flower opening, as these events typically occur simultaneously (Kolek et al., 2021; Zhang et al., 2019).

In both experiments, plants were exposed to elevated temperature and NO_2 stress starting from DAS 59 and 60. Prior to this period, experimental setups such as humidity (Dahl et al., 2013) and light intensity (Deen et al., 1998; Deen et al., 2001), which may impact the phenological

development of ragweed plants, were consistent across all four conditions in both experiments. Regarding temperature, an additional factor influencing the phenological development (Deen et al., 1998; Prank et al., 2013), both temperature treatments were consistently controlled in experiment 1 but varied slightly in experiment 2, as previously outlined in section 4.1.1-2. Therefore, the disparity in the initial timing of pollen release among ragweed plants across treatments could be ascribed to the effects of elevated temperature and NO_2 post-DAS 60 but before pollen release.

4.1.3-1.1 Effects of elevated temperatures on pollen phenology

In experiment 1, an elevated temperature increase of 4 °C occurring several days before pollen release notably delayed the release. Similarly, in experiment 2, the impact of a 4 °C temperature rise on postponing pollen release was also observed under NO_2-free conditions. A similar observation has been documented by Barnes et al. (2001), indicating that lower temperatures during ragweed vegetation periods led to earlier airborne pollen release, but the effect depended upon interactions with wind disturbances and rainfall. These findings suggest that environmental temperatures shortly preceding pollen release are crucial in determining the initiation of pollen release. It is likely due to the increased sensitivity of plants to heat stress 7 to 15 days prior to anthesis, coinciding with pollen development (Sato, 2008). However, this finding contradicts the study by Gentili et al. (2019), which demonstrated that ragweed plants began flowering significantly earlier at intermediate (24 – 20 °C) and high (30 – 26 °C) thermal regimes compared to a low thermal regime (18 – 14 °C), while with no significant differences between the intermediate and high thermal regimes. One possible explanation for this discrepancy might be the even higher thermal regime resulting from the elevated temperature treatment in both climates in this study compared to the high thermal regime conducted by Gentili et al. (2019). Specifically, the temperatures in the Tw4-condition of this study, which included a 4 °C elevation, potentially surpassed the upper threshold for optimal flowering induction, thereby leading to a different phenological response. Moreover, Burghardt et al. (2016) demonstrated that fluctuating warm temperatures and constant warm temperatures resulted in opposite flowering phenology in several Arabidopsis accessions. Consequently, the simulated naturally occurring and fluctuating

temperatures in this study, compared to the constant warm thermal ranges used by Gentili et al. (2019), may also contribute to the observed inconsistencies in phenology.

Similar to the results in the present experiments on ragweed, advancements in pollen release have also been reported in other plant species, such as olive (Bonofiglio et al., 2013), birch (Méndez et al., 2005), and oak (Fernández-Martínez et al., 2012), in response to approximate increases of 1 °C, 0.4 – 0.7 °C, and 1.6 ± 0.6 °C in annual mean temperature, respectively.

Overall, these findings consistently indicate that temperature is a major environmental factor influencing the flowering phenology of certain plant species (Ziska et al., 2019). Moreover, considering all the findings, it suggests that the severity (Sato et al., 2000), timing (Lippmann et al., 2019; Springate and Kover, 2014), and fluctuations (Burghardt et al., 2016) of heat stress experienced during various plant developmental stages (Bernacchi et al., 2023) may influence phenological timings differently. Therefore, it is critical to consider the magnitude, the exposure period of temperature changes, moreover, the plant developmental stage when evaluating their effects on flowering phenology. Future research should aim to disentangle these complex interactions by conducting controlled experiments that systematically vary temperature increases, exposure durations, and seedling stages, providing a more comprehensive understanding of how climate change may impact plant reproductive timing.

4.1.3-1.2 Interactive effects of elevated temperatures and elevated NO_2 on pollen phenology

Surprisingly, a notable interaction between elevated NO_2 and elevated temperature on the onset of pollen release was observed in experiment 2. However, it should be noted that the onset of ragweed pollen release under the T_W4N condition in experiment 2 occurred shortly after the commencement of elevated NO_2 treatment, resulting in a relatively limited period of NO_2 exposure before pollen release began. This short treatment duration may not have been sufficient to accumulate signals that significantly affect pollen release. This is supported by previous studies, which have shown that the effects of environmental factors on plant reproductive development do not become apparent until a certain level of signaling molecules is accumulated or in shortage (Cho et al., 2017; Riboni et al., 2013; Shim and Jang, 2020). Therefore, the current experimental results may not fully reflect the potential effects of NO_2 on pollen release. To address this

limitation, future studies should consider extending the duration of NO_2 treatment and making observations at multiple stages of pollen development to assess the effects of NO_2 on pollen release fully. Additionally, a series of experiments could be designed to gradually increase the NO_2 treatment time and record its dynamic changes on pollen release to gain a deeper understanding of the effects of NO_2 on plant reproductive development.

4.1.3-2 Length of pollen reason

The duration of the ragweed pollen season is notably postponed by elevated temperature, in agreement with hypothesis H1 e. This finding is consistent with pollen release susceptible to termination by low temperatures and the occurrence of the first frosts, suggesting a potential delay in pollen season under warm temperatures at the end of the vegetation period (Deen et al., 1998). This study confirms such delays, revealing obviously extended durations of the ragweed pollen season under elevated temperatures in both experiments and irrespective of NO_2 concentrations. These findings align with a study that reported a lengthening of the ragweed pollen season over decades due to delayed fall frosts and an extended frost-free period along a latitudinal gradient in North America (Ziska et al., 2011). Similarly, other plant species, such as *Parietaria*, olive, and cypress in western Liguria, have exhibited substantial increases in the duration of pollen seasons over 27 years, associated with a rise in the number of days with temperatures exceeding 30 °C (Ariano et al., 2010). Collectively, the findings above indicating delayed senescence in ragweed plants under elevated temperature suggest that elevated temperature extended the durations of pollen release primarily by slowing down the plants' senescence rather than promoting earlier pollen release.

Remarkably, elevated NO_2 significantly lengthened the pollen season in the second experiment. Taken together, these findings suggest that under elevated temperature conditions in the relatively warmer climate, elevated NO_2 prolongs the pollen season by advancing the onset of pollen release rather than delaying the plants' senescence. The earlier onset and extended duration of pollen release can increase the exposure period to allergenic pollen, thereby potentially exacerbating symptoms and increasing healthcare burdens, in agreement with hypothesis H1 e.

4.1.3-3 Pollen release per male inflorescence

4.1.3-3.1 Elevated temperature in the relatively warmer climate reduces pollen release per male inflorescence

Elevated temperature showed no significant impact on pollen release per male inflorescence in a moderate climate while significantly reduced pollen release in the relatively warmer climate. Similarly, Schlenker and Roberts (2009) demonstrated that yield growth for annual crops, such as corn, soybean, and cotton, increased gradually with temperatures up to 29 °C to 32 °C but decreased sharply with temperatures exceeding this threshold.

These contrasting responses highlight the complexity of plant reproductive responses to temperature variations and underscore the importance of considering local climatic conditions when predicting the impacts of climate change on plant phenology. In moderate climates, the potential for increased total pollen release due to enhanced vegetative growth could contribute to higher pollen loads in the atmosphere, thereby exacerbating allergic responses in susceptible individuals. Conversely, in relatively warmer climates where temperatures may surpass the optimal range for reproductive development, reduced pollen release per inflorescence could offset the expected increase in pollen load, although this does not necessarily mitigate the overall public health impact due to prolonged pollen seasons. Taken together, these findings are in agreement with hypothesis H1 e.

4.1.3-3.2 Elevated NO_2 induces pollen release per male inflorescence

80 ppb NO_2 in the atmosphere significantly increased the average pollen release per male inflorescence. Due to pruning measures implemented during plant growth to mitigate excessive inter-plant competition within the spatially limited phytotron chambers, data on pollen yield per plant were not collected. Utilizing pollen release per male inflorescence as a comparable unit across treatments, the results reaffirmed the regulatory impact of elevated NO_2 on pollen release per inflorescence in ragweed plants, as observed in a previous study by Zhao et al. (2017b).

Overall, the effects of NO_2 on pollen release depend on the concentration of NO_2 and the specific plant species. In contrast to ragweed plants, elevated NO_2 (up to 36 ppb) resulted in a

shorter pollen season in birch (Kolek et al., 2021). Tomato plants exposed to 50 ppb of NO_2 exhibited an earlier and prolonged pollen season, along with increased pollen production, in comparison to those with no NO_2 exposure (Takahashi et al., 2011). Furthermore, *Nicotiana plumbaginifolia* seedlings subjected to 200 ppb of NO_2 for 10 weeks displayed a doubled shoot size and enhanced cell constituent content compared to those exposed to 100 ppb of NO_2 (Takahashi et al., 2005). A previous study involved horticultural plants exposed to 50, 100 and 200×10^3 ppb of NO_2 for 5-6 weeks, resulting in a twofold increase in biomass, both above and below ground, for each species compared to growth in the absence of NO_2 (below 5×10^3 ppb) (Adam et al., 2008). Collectively, in conjunction with the studies mentioned above (Takahashi et al., 2005), mass spectrometry analysis of carbon (C), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) content suggests that exogenous NO_2 functions more as a signaling molecule than a vital nutrient source in stimulating plant growth, nutrient uptake, and metabolism (Takahashi and Morikawa, 2019b).

Hence, considering the collective impact on ragweed plant growth and pollen release, it can be deduced that airborne NO_2 acts as a survival stressor, prompting plants to adapt to their surroundings by reallocating resources when confronted with this stress. Under such circumstances, plants might opt to channel more resources towards reproductive structures, such as pollen, without noteworthy alterations in other growth parameters. This shift in metabolic allocation serves as a survival strategy, enabling plants to uphold their reproductive capacity in challenging environments (Lundgren and Des-Marais, 2020; Shaar-Moshe et al., 2019).

4.1.4 Compounds in pollen affecting pollen allergenicity under elevated temperature and NO_2 treatments

4.1.4-1 Total flavonoid concentration was modulated by elevated temperature and NO_2

The total flavonoid concentration (TFC) in ragweed pollen exhibits contrasting responses to elevated temperature and elevated NO_2, as observed in the conducted experiments. The TFC in ragweed pollen increases in response to elevated temperature, contradicting hypothesis H1 d and the findings of Gentili et al. (2019), who reported an increase in the TFC of ragweed pollen with

decreasing temperatures. This divergence may stem from differences in experimental conditions, with day and night real-time temperature fluctuations while Gentili et al. (2019) used three constant temperature ranges, each differing by 6 °C, starting from seed germination. Burghardt et al. (2016) support this speculation with observations on *Arabidopsis thaliana*, indicating reversed reproductive development and physiological growth under fluctuating temperatures compared to constant ones.

Moreover, variations in seed origins and genetic backgrounds may also contribute to the disparate effects of elevated temperature on TFC in ragweed pollen. Seeds in this study originated from Braunschweig, Germany, while Gentili et al. (2019) used seeds from a rural site near Milan, Italy. Differences in genetic backgrounds among legume seeds have been observed to result in inconsistent relationships between treatments and flavonoid content (Davis et al., 2008), and this may also apply to ragweed. Furthermore, Gentili et al. (2019) used aqueous pollen extracts to simulate the hydration process of ragweed pollen inhaled in the airway, while the present study used methanolic extracts. Previous studies demonstrate that these two different solvents yield varying fractions and amounts of pollen TFC (El-Kelish et al., 2014; Zhao et al., 2017b), indicating that direct comparisons between the results of the two methods may not be appropriate.

Elevated NO_2 resulted in a significant decrease in the TFC of ragweed pollen samples, as evidenced in experiment 1, which is consistent with a previous study (Zhao et al., 2017b).

However, the significant induction of TFC by elevated temperature was only evident in experiment 2, while the impact of elevated NO_2 on TFC reduction diminished. A potential explanation for this discrepancy could be the prolonged exposure to elevated temperatures due to the delayed senescence of ragweed plants and the 1.5 °C higher environmental temperature applied in experiment 2. Several studies have demonstrated that flavonoid biosynthesis and accumulation are dynamic processes influenced by temporal factors such as the duration of stress exposure (Ahmed et al., 2021; Hakan et al., 2017). These findings suggest that extended exposure to elevated temperature may lead to greater TFC accumulation in ragweed pollen, potentially resulting in a more pronounced effect of elevated temperature in experiment 2.

4.1.4-2 Flavonoid composition

4.1.4-2.1 Elevated temperature has a greater effect on flavonoid composition than elevated NO$_2$

In both experiments, elevated temperature had a more substantial impact on the flavonoid composition of ragweed pollen compared to elevated NO$_2$, suggesting temperature as a primary driver of flavonoid changes under climate change conditions. This finding aligns with previous studies indicating that elevated temperature can disrupt metabolic pathways involved in flavonoid biosynthesis, resulting in changes in flavonoid profiles (Gouot et al., 2019; Mohammadrezakhani et al., 2018; Torres et al., 2017).

Moreover, the interaction between elevated temperature and elevated NO$_2$ yielded complex and varied responses in ragweed pollen flavonoid composition. Experiment 1 revealed that the combined presence of elevated temperature and NO$_2$ had less pronounced effects on flavonoid composition compared to individual treatments. This may indicate the prioritization of resources under combined stress conditions in a moderate climate (Nieves-Cordones et al., 2019; Zheng et al., 2016). However, experiment 2 revealed a contrasting outcome, with elevated NO$_2$ exhibiting a more pronounced effect in combination with elevated temperature on flavonoid composition in ragweed pollen. Under the above discussed condition of temperature in experiment 2 eventually surpassing the optimum, resources may be allocated towards defense mechanisms against oxidative stress induced by NO$_2$, thereby amplifying the effects of NO$_2$ on flavonoid metabolism (Pooja and Munjal, 2019; Rehman et al., 2023).

4.1.4-2.2 Expression of specific flavonoids regardless of treatments

Regardless of experimental treatments, methanolic extracts of ragweed pollen consistently revealed quercetins as the predominant flavonoid group. This observation aligns with the flavonoid composition of ragweed pollen cultivated under natural conditions (Mihajlovic et al., 2015) and under experimental conditions involving elevated ozone (Kanter et al., 2013) and elevated CO$_2$ (El-Kelish et al., 2014), respectively. Quercetin plays pivotal roles in various plant physiological processes, including seed germination, pollen growth, and photosynthesis (Singh et al., 2021). Notably, when cultivated on quercetin-supplemented media, model plants such as

Arabidopsis thaliana, *Nicotiana tabacum*, and *Lemna gibba* exhibited enhanced growth compared to control groups. Additionally, these plants demonstrated reduced levels of oxidized proteins, suggesting that quercetin acts as a broad-spectrum antioxidant stress protectant for plants (Kurepa et al., 2016).

4.1.4-2.3 Expression of specific flavonoids in response to elevated temperatures with or without elevated NO$_2$

Specifically, elevated temperature treatment notably affected derivatives within the flavones and flavonols, hydroxycoumarins, and O-methylated flavonoids subgroups. Previous studies have shown that elevated temperatures can influence the expression and accumulation of these specific flavonoid compounds in various plant species, such as *Betula pendula* (Lavola et al., 2013), *Salix myrsinifolia* (Lavola et al., 2013), *Populus tremula* (Randriamanana et al., 2015) and *Brassica Oleracea* (Mølmann et al., 2015). These findings indicate a temperature-sensitive regulation of biosynthetic pathways leading to the production of these flavonoid subgroups and their derivatives (Jamloki et al., 2021). Particularly, the flavonol subgroup has been recognized for its role as a crucial antioxidants in plants, contributing to the maintenance of cellular reactive oxygen species homeostasis under heat stress, promoting pollen viability, pollen tube growth, and thereby enhancing plant reproduction (Muhlemann et al., 2018).

In comparison, elevated NO$_2$ treatment-induced changes in the coumarins subgroup and its hydroxycoumarins derivatives. This specific response may indicate the activation of defense mechanisms against oxidative stress triggered by elevated NO$_2$. It has been reported that coumarins possess antioxidant properties and are implicated in plant responses to oxidative stress and pathogen defense (Lei et al., 2015). Therefore, the enrichment of coumarin derivatives under elevated NO$_2$ conditions suggests a targeted metabolic response aimed at mitigating the adverse effects of reactive nitrogen species on cellular homeostasis (Farnese et al., 2016).

Furthermore, exposure to the combined elevated NO$_2$ and elevated temperature led to alterations in specific derivatives belonging to flavonoid glycosides and isoflavonoid O-glycosides subgroups. It has been reported that glycosylation of flavonoids enhances plant stress defense by improving their stability, accessibility, antioxidant activity, detoxification capacity, and signaling

properties (Behr et al., 2020; Yang et al., 2018; Zhang et al., 2022). Thus, the observed changes in glycosylated flavonoid derivatives may represent adaptive strategies aimed at enhancing stress tolerance and maintaining cellular homeostasis in ragweed pollen exposed to concurrent elevated temperature and elevated NO_2. Additionally, the exclusive enrichment of flavonoid-3-O-glycoside in experiment 2 may suggest a heightened stress level for ragweed plant growth. Taken together, these responses reflect targeted metabolic adjustments aimed at mitigating oxidative stress induced by each stressor.

4.1.4-3 Interference of flavonoids in IgE-binding activities

Remarkably, flavonoids extracted from pollen samples of ragweed plants cultivated at lower temperatures exhibited a more pronounced inhibition of allergen-IgE-binding activities compared to those from plants grown at higher temperatures in both experiments. This observation suggests that certain flavonoid compounds expressed in ragweed pollen under ambient temperatures may possess the ability to inhibit allergen-IgE-binding activities effectively.

In particular, rutin, a distinct type of flavonoid glycoside, has been shown to respond to the combined treatment of elevated temperature and elevated NO_2. Previous research has documented rutin's impact on IgE-allergen binding activities in ragweed pollen (Gentili et al., 2019). The concentration of rutin displayed a negative correlation with the intensity of ragweed allergen-IgE binding, highlighting its potential role in reducing pollen allergenicity. Additionally, other temperature-responsive flavonols present in ragweed pollen, such as kaempferol-type flavonoids and naringenins, were identified to interfere with IgE-allergen binding. Studies have demonstrated that the flavonoid-Bet v 1 complex can cover allergen epitopes (Grutsch et al., 2014; Kofler et al., 2012; Seutter-Von Loetzen et al., 2014). However, future investigations should focus on isolating differentially expressed flavonoid compounds in ragweed pollen under different treatments, and independent studies should be conducted to elucidate their specific functions in IgE-allergen binding.

Moreover, specific flavonoid compounds with distinct functions in anti-allergy therapy have been identified (Bansode et al., 2018; Castell et al., 2014; Vo, 2020). Augmenting the quantity of added quercetins to the allergen significantly impeded the binding of IgE to the ragweed allergen.

Beyond ragweed allergen, the protein-quercetin complex emerged as the primary phytochemical covalently bound to peanut proteins, leading to a significant reduction in IgE binding to peanut proteins (Plundrich et al., 2017). Subsequent *in vivo* and *in vitro* studies demonstrated that conjugating quercetin with ovalbumin, a well-known food allergen, not only diminished IgE-protein binding but also reduced the overall allergenicity of ovalbumin (Zhang et al., 2020b). Moreover, quercetin-type flavonols have been associated with temperature responses, with their concentrations increasing at lower temperatures (Albert et al., 2009; Schmidt et al., 2010). Hence, the study's findings suggest that ragweed pollen grown under ambient temperature conditions exhibits a lower allergenic potential due to potentially higher quercetin amounts compared to pollen grown under elevated temperatures and its robust inhibition of IgE binding to antigens. Unfortunately, pollen samples from the control conditions were running out; thus, in future work, there is abundant room for further progress in determining individual flavonoid components in pollen using LC-MS/MS.

4.1.5 Effect of temperature and elevated NO_2 on oxidative indicators regulating pollen allergenic potential

4.1.5-1 NAD(P)H oxidase

Both elevated NO_2 and elevated temperature could enhance NADPH and NADH oxidase in ragweed pollen. The finding that elevated NO_2 increased reactive oxygen species (ROS)-related enzyme activities [NAD(P)H oxidase] aligns with a study indicating significantly higher NADPH oxidase activity in pollen samples from ryegrass plants exposed to high levels of NO_2 and SO_2 compared to samples from less air-polluted areas (Lucas et al., 2019). Similar effects on inducing these oxidative stress marker enzymes in ragweed pollen have been reported for ozone exposure (Pasqualini et al., 2011) and drought (El-Kelish et al., 2014). Furthermore, when exposed to half, equal, and twice the legal limit for European vegetation protection for 6 hours separately, most forest pollen species (*Acer negundo*, *Betula pendula*, *Quercus robur*) exhibited a significant increase in NADPH oxidase activity at the highest concentration of NO_2. However, in the same study, exposure to the same concentration gradient of ozone for 6 hours resulted in varied NADPH oxidase activity among pollen species (Pereira et al., 2021). The differences in NADPH oxidase

activity among pollen from different plant species influenced by environmental factors can be attributed to their varying intensity and localization, thereby indicating different functions based on the plant families (Smiljanic et al., 2017; Wang et al., 2009).

The NAD(P)H oxidase complex, known as a respiratory burst response to environmental stress in plants (Lamb and Dixon, 1997; Speranza and Scoccianti, 2012), suggests that ragweed plants in this study, indicated by elevated NAD(P)H activities under increased temperature and NO_2, experienced and reacted to stressful conditions. This strengthens the hypothesis of H1 c that both elevated NO_2 and increased temperature contribute to oxidative stress in ragweed pollen, potentially exacerbating its allergenicity.

4.1.5-2 H_2O_2 content

Both elevated NO_2 under elevated temperature conditions and elevated temperature under elevated NO_2 conditions significantly increase the H_2O_2 content in ragweed pollen in experiment 1. This observation suggests synergistic effects between these treatments on oxidative stress pathways in ragweed pollen. Moreover, the interaction between elevated NO_2 and elevated temperature in inducing H_2O_2 content in ragweed pollen is emphasized, contrary to hypothesis H1 c. This interaction reflects a cumulative impact on oxidative stress pathways, potentially affecting pollen viability and reproductive fitness (Wahid et al., 2007). Notably, under NO_2-free conditions, elevated temperature significantly reduced the H_2O_2 content. This finding highlights the contrasting effects of elevated temperature alone, indicating a temperature-dependent modulation of ROS homeostasis in ragweed pollen (De-Pinto et al., 2015; Locato et al., 2008).

However, contrasting results are observed in experiment 2, where neither elevated NO_2 alone, elevated temperature alone, nor their combination significantly affected the H_2O_2 content of ragweed pollen extracts. As for other factors discussed above, it is plausible that the elevated temperature conditions in experiment 2 already induced maximal H_2O_2 production, thereby masking any additional effects of NO_2 or their combination.

4.1.5-3 Other oxidative indicators

Additionally, it is crucial to consider the potential limitations of these responses, as ROS responses in plants are dynamic processes involving transformations between different reactive forms, such as superoxide anion, H_2O_2, and hydroxyl radical (Krumova and Cosa, 2016; Madkour, 2019). In agreement, published work suggests that other enzyme systems influence ROS dynamics under various environmental conditions and pollution levels. For example, superoxide dismutase (SOD) converts the superoxide anion to the more stable hydrogen peroxide (Hayyan et al., 2016; Wang et al., 2018). In *in vitro* exposure experiments with *Dactylis glomerata* pollen, O_3 fumigation at 61 ppb for 6 hours did not affect SOD activity but doubled H_2O_2 content in pollen, whereas NO_2 fumigation at 106 ppb for 6 hours doubled SOD activity but did not alter H_2O_2 content (Galveias et al., 2021). Similarly, peroxidase (POD) and catalase (CAT) are involved in the breakdown of hydrogen peroxide (Almagro et al., 2009; Nokthai et al., 2010).

Moreover, glutathione peroxidase (GPX) and glutathione reductase (GR) are intracellular antioxidant enzymes that enzymatically reduce hydrogen peroxide to water to limit its harmful effects (Iskusnykh et al., 2013). These enzymes are the chief oxidants in plant cells, participating in cell signal transduction and redox balance (Harohalli Masthigowda et al., 2022; MeŞE and Turfan, 2019; Xie et al., 2022). The involvement of these enzyme systems in ROS metabolism highlights the complexity of antioxidant defense mechanisms in plants. Therefore, the observed changes in H_2O_2 content may represent only a snapshot of the overall ROS dynamics in ragweed pollen. The interconversion between ROS species is influenced by various factors, including enzymatic activities and environmental conditions, which may not be fully captured by H_2O_2 measurements alone.

In conclusion, the argument contributes to the broader field of ragweed plant physiology and stress ecology by elucidating the complex interactions between elevated NO_2, elevated temperature, and oxidative stress pathways. Further research involving NAD(P)H oxidase activities and H_2O_2 content in ragweed pollen can offer valuable insights into these mechanisms, advancing our understanding of plant responses to environmental stressors.

4.1.6 Immunoblotting analysis of ragweed pollen

4.1.6-1 Changes in the allergenicity of aqueous proteins of ragweed pollen

In both scenarios, elevated temperature and elevated NO_2 enhanced the total allergenicity of ragweed pollen, based on the results obtained both by dot bot and 2D-immunoblot (Table 3 – 5 and Figure 3 – 10). The effect of elevated temperature on inducing pollen allergenicity is remarkably consistent with findings of increased allergenicity of ragweed pollen in the study of Gentili et al. (2019), despite different experimental settings.

In addition, the finding that 80 ppb NO_2 increased allergenicity under both temperature regimes reproduced the results of Zhao et al. (2016) based on the same T_M scenario and the same level of 80 ppb NO_2 in their experiment. Moreover, a consistent induction of NO_2 resulting in heightened IgE recognition in pollen was noted in *Betula pendula*, *Ostrya carpinifolia*, and *Carpinus betulus* (Cuinica et al., 2014), and in *Acer negundo* pollen (Sousa et al., 2012), when subjected to *in vitro* NO_2 exposure, in comparison to samples without NO_2 exposure. Furthermore, a field study indicated that ragweed pollen sampled along high-traffic roads exhibited greater allergenicity compared to pollen collected from low-traffic roads and vegetated areas, as reported by Ghiani et al. (2012). Thus the consistent results in both experiments in the present work suggest that the effect of NO_2 on pollen allergenicity has been intensified at higher temperatures.

4.1.6-2 Alterations in the abundance of major IgE-reactive proteins in ragweed pollen

4.1.6-2.1 Dominance of Amb a 1 isoforms in ragweed pollen allergenicity

The predominance of Amb a 1 isoforms in the total allergenicity of ragweed pollen was evident in 2D immunoblots, consistent with prior studies (Gadermaier et al., 2008; Gentili et al., 2019; Zhao et al., 2016). Moreover, these results align with earlier studies that showed the presence of Amb a 1 isoforms, Amb a 11, and Amb a 12 in spots where their allergenic potential was affected by elevated NO_2 (Zhao et al., 2016) and responsive to increased temperatures (Gentili et al., 2019).

Similar increments in Amb a 1 isoforms correlated with heightened pollen allergenicity under conditions like elevated CO_2 (Choi et al., 2018; El-Kelish et al., 2014; Singer et al., 2005), drought (El-Kelish et al., 2014), and high-traffic roads (Ghiani et al., 2012), suggesting a potential exacerbation of ragweed pollen allergy in urban and climate change-affected areas. Similar inductions were noted for homologous pectate lyase allergens in *Arizona* cypress pollen exposed to traffic-related air pollution (Shahali et al., 2009), and in *Humulus Scandens* pollen collected from areas with air pollutants, including PM 10, PM 2.5 and O_3 (Lu et al., 2020). Therefore, these findings underscore the significant roles of pectate lyase proteins in facilitating plants' adaptation to environmental stimuli. They also highlight the intricate interplay between environmental factors and allergen expression, with potential implications for public health.

4.1.6-2.2 Contribution of Amb a 11 and Amb a 12 in ragweed pollen allergenicity

The high sensitization rate observed for Amb a 11, up to 66%, underscores its substantial contribution to the augmentation of IgE responses. This finding aligns with previous research indicating the pivotal role of Amb a 11 in allergic reactions triggered by ragweed pollen exposure (Bouley et al., 2015; Groeme et al., 2016). However, the detection of Amb a 11 presents a challenge due to its structural resemblance to Amb a 1, necessitating sophisticated techniques such as 2D blotting analysis for accurate visualization and differentiation (Bordas-Le Floch et al., 2015; Bouley et al., 2015; Chen et al., 2018a; Gentili et al., 2019; Zhao et al., 2016). Consequently, limited research has demonstrated the specific expressions of CP allergens in pollen under different environmental conditions, aside from investigations conducted with ragweed pollen subjected to elevated levels of NO_2 (Zhao et al., 2016) and increased temperatures (Gentili et al., 2019). Nevertheless, the alteration of Amb a 11 under simulated conditions could be mainly attributed to two aspects.

On one hand, proteins belonging to the cysteine protease family, which degrade certain proteins to enable nutrient remobilization, particularly nitrogen (N) (Díaz-Mendoza et al., 2014; Roberts et al., 2012), induce a broad spectrum of defense responses, including leaf senescence and plant cell death (Cai et al., 2018; Ge et al., 2016; Misas-Villamil et al., 2016). This suggests that plants can recycle proteins in response to abiotic stress through the hydrolysis of plant proteins as a first line of defense for plant survival (Liu et al., 2018a). On the other hand, various studies have

revealed that under stressful conditions such as drought, salt stress, cold and endoplasmic reticulum stress, plants often regulate the gene expression or protein activity of cysteine proteases through regulatory signals, such as phytocystatins (Kunert et al., 2015; Mangena, 2020; Subburaj et al., 2017), to impact plant growth and enhance plant stress tolerance. These phytocystatins participate in abscisic acid-mediated stress signal transduction (Rabbani et al., 2003; Subburaj et al., 2017; Tan et al., 2017) or engaging in the salicylic acid signaling pathway (Van-Der Linde et al., 2012a; Van-Der Linde et al., 2012b).

Therefore, these prior studies propose that under stress-inducing conditions, such as elevated NO_2 and elevated temperature, there is a likelihood of heightened degradation of various substances and activation of signal transduction, potentially leading to the induction of Amb a 11 in ragweed pollen. Moreover, the induction of Amb a 11 in ragweed pollen may result in higher pollen allergenicity. However, the direct association between Amb a 11 induction in pollen and its allergenicity in patients sensitized to ragweed pollen remains inadequately explored. Further research elucidating the molecular mechanisms underlying Amb a 11-mediated immune responses is warranted, offering insights into potential therapeutic strategies for mitigating allergic reactions associated with ragweed pollen exposure.

In addition, the induction of Amb a 12 may also contribute to increased IgE-reactive intensity, given its significant prevalence of IgE reactivity (Bordas-Le Floch et al., 2015; Zhao et al., 2016). Amb a 12, an enolase, plays a crucial role in providing additional ATP by regulating the glycolytic pathway, aiding plants in coping with environmental stresses (Giegé et al., 2003; Plaxton, 1996). Its consistent induction has been observed in ragweed plants under various stressors (Zhao et al., 2016), *Populus euphratica* facing heat stress (Ferreira et al., 2006), and *Mesembryanthemum crystallinum* under salt stress (Barkla et al., 2009), demonstrating its role in environmental adaptation.

Hence, an explanation for the heightened allergenicity lies in the augmented abundance of allergens in response to treatments, particularly the induction of Amb a 1 and its isoforms, and additionally, the overexpression of minor allergens, such as Amb a 11 and Amb a 12.

4.1.6-3 Identification of potential IgE-reactive proteins in ragweed pollen

In this context, the discovery of additional potential allergenic proteins beyond the known allergens presents an opportunity to deepen our understanding of allergic responses. For instance, the peptidyl-prolyl cis-trans isomerase protein detected in spot 3, which has also been observed in elevated NO_2-treated ragweed pollen (Zhao et al., 2016), has garnered attention due to its identification in pollen and fungal allergens (Cadot et al., 2000; Ghosh et al., 2014; Pemberton, 2006; Sircar et al., 2020).

Moreover, the identification of calmodulin, a calcium-modulated protein, as a putative allergen highlights the phenomenon of cross-reactivity among pollen allergens. Studies implicating calmodulin homologs in various pollen species, including Amb a 9 and Amb a 10 in common ragweed pollen (Wopfner et al., 2008), an allergenic calmodulin in *Amaranthus palmeri* pollen (Bonura et al., 2008; Gómez-Esquivel et al., 2021), Bet v 4 in birch pollen (Engel et al., 1997), Ole e 3 and Ole e 8 in olive pollen (Ledesma et al., 1998; Ledesma et al., 2000), and Phl p 7 in timothy grass (Niederberger et al., 1999). These findings underscore the potential for enhanced IgE-allergen binding activity, suggesting a broader immunological significance beyond individual allergen specificity. Expanding upon these insights, it's crucial to consider the implications of allergen cross-reactivity in clinical settings. High cross-reactivity among allergens from the calmodulin family, as evidenced by sensitization prick tests with pollen-allergic patients (Garmatiuk et al., 2013; Hayek et al., 1998; Moya et al., 2021; Vakili-Moghaddam et al., 2019; Valenta et al., 1998; Verdino et al., 2002; Wopfner et al., 2007), underscores the need for comprehensive diagnostic approaches to assess allergic sensitization profiles accurately. Moreover, elucidating the structural and functional characteristics of allergenic proteins can provide valuable insights into the molecular basis of allergic responses, informing the development of targeted therapeutic interventions.

Additionally, the discovery of calreticulin, a high-capacity calcium-binding protein, sharing homology with *Penicillium chrysogenum* allergen Pen ch 31 (Gelebart et al., 2005), suggests its potential as an IgE-reactive allergen in ragweed pollen susceptible individuals. Studies by Wang et al. (2017) and Gong et al. (2022) have further indicated that calreticulin may function as both an

allergen and an adjuvant, thereby promoting immune responses and contributing to the development of allergic diseases. Moreover, calreticulin's ability to interact with immune cells and influence the overall immune response to allergen exposure (Raghavan et al., 2013; Williams et al., 2016) underscores its immunological significance in allergic reactions. Further research is warranted to elucidate the molecular mechanisms underlying its involvement in allergic responses and explore its potential as a therapeutic target for allergic diseases.

The nucleoside diphosphate kinase identified in spot 4 and the cytosolic triosephosphate isomerase identified in spots 18, 19, and 21, with no homologs identified and in allergens such as food, fungi, and pollen, indicate that these two proteins may not be associated with pollen allergenicity. The highly conserved nature of these two proteins and their widespread presence in various tissues suggest that they are likely to have been detected by chance.

4.1.7 Comparative analysis of protein expression profiles in ragweed pollen

4.1.7-1 Amb a 1 and its isoforms in ragweed pollen

4.1.7-1.1 Environmental effects on the abundance of Amb a 1 isoforms

Amb a 1.0201 and Amb a 1.0402 displayed consistent significant increases in abundance due to elevated temperature. This suggests that these isoforms may be upregulated as part of a stress response mechanism, potentially enhancing the allergenic properties of ragweed pollen in warmer climates. Additionally, the differential regulation of Amb a 1 isoforms in the two experiments indicates complex regulatory mechanisms, which highlights tthe sensitivity of allergen expression to environmental changes (Ciappetta, 2016). Collectively, the consistent upregulation across experiments underscores the robustness of this response, highlighting the importance of temperature as a key factor influencing ragweed allergen expression (Ciappetta, 2016). The temperature-dependent response aligns with research indicating that environmental temperature significantly influences alternative splicing events, leading to the generation of distinct isoforms (Capovilla et al., 2015a; Del-Mondo et al., 2022). Taken together, these findings emphasize the importance of considering multiple environmental factors in understanding and predicting allergen responses.

Similar selective modulation of elevated NO_2 on the expression of specific Amb a 1 isoforms was demonstrated by the significant increase in the abundance of Amb a 1.0305 and the significant decrease in the abundance of Amb a 1.0401. These findings align with the previous studies that NO_2 exposure potentially alters the allergenic profile of ragweed pollen in polluted environments (Ghiani et al., 2012; Zhao et al., 2016). Alterations in isoform abundance in response to elevated NO_2 imply potential protein modifications during metabolism, particularly through NO_2-induced S-nitrosylation (Kovacs and Lindermayr, 2013; Lindermayr et al., 2006; Zhao et al., 2016) and protein nitrosylation (Holtgrefe et al., 2008), well-documented processes affecting protein function and signaling pathways.

In addition to individual effects, an interactive effect is evidenced by the significantly enhanced abundance of Amb a 1.0101, which was observed exclusively under elevated NO_2 conditions in experiment 2. Additionally, elevated NO_2 under ambient temperature conditions produced opposite results in the abundance change of Amb a 1.0201, with a significant decrease in experiment 1 and a substantial increase in experiment 2. The contrasting changes in Amb a 1.0201 abundance between the two experiments highlight the nuanced nature of the interaction between a mild temperature increase of 1.5 °C and elevated NO_2.

The specific responses of different Amb a 1 isoforms to these environmental factors suggest that the allergenic profile of ragweed pollen could vary significantly under future climate change scenarios, contrary to hypothesis H1 a, but consistent with the study of Verhage et al. (2017). Hence, future research should focus on elucidating the pathways through which NO_2 and temperature jointly influence allergen expression. Controlled experiments that systematically vary temperature and NO_2 conditions will be essential for understanding the thresholds and limits of these effects. Additionally, studying a wider range of plant species and environmental conditions can help determine if similar patterns are observed across different ecosystems.

4.1.7-1.2 Varying allergenicity within Amb a 1 isoforms

Remarkably, despite a dynamic balance between expression levels, it is plausible that Amb a 1 isoforms with similar abundance may possess varying allergenicity, potentially due to differences in secondary structure. This finding supports the research that variations in amino acid

sequences or secondary structures among different Amb a 1 isoforms resulted in distinct patterns of IgE binding and immunogenicity (Buzan et al., 2022). For instance, Amb a 1β (amino acid residues located at 26–180) displayed higher IgE-binding activity compared to Amb a 1α (amino acid residues located at 181–396) (Wopfner et al., 2009), and Amb a 1.01 exhibited higher IgE-binding activity compared to Amb a 1.02 or 03 isoforms (Wolf et al., 2017). These observations suggest that even minor structural differences among isoforms can significantly impact their allergenic potential.

As mentioned above, alternative splicing events due to elevated temperature and post-translational modifications by elevated NO_2 could lead to changes in protein stability and function (Zhang and Mount, 2009), emphasizing the complexity of environmental factors on allergen expression. Understanding these molecular mechanisms is crucial for deciphering the underlying biology driving allergen responses to environmental cues and informing targeted strategies for allergy management and prevention (Zahid et al., 2022).

4.1.7-2 Established minor allergens in ragweed pollen

The finding that significant decreases observed in Amb a 8.0101 and Amb a 8.0102 in response to elevated temperature aligns with the finding by El-Kelish et al. (2014), who demonstrated downregulation of the Amb a 8.1 transcript under elevated CO_2 conditions in a greenhouse experiment. Moreover, the overall effect of elevated temperature on decreasing these allergen proteins may be attributed to their involvement in essential metabolic pathways sensitive to temperature changes. Notably, this profilin, a protein reported in numerous plants (Vidali et al., 2009) and yeast species (Lu and Pollard, 2001), may be particularly susceptible to such disruptions.

Furthermore, the significant induction of Amb a 5.0101, a cysteine-rich protein, in response to elevated NO_2 may be attributed to its involvement in nitrogen metabolism (Pan and Wang, 2017) and the antioxidant response triggered in pollen by increased NO_2 concentrations (Gow et al., 2004; Holtgrefe et al., 2008). In addition, treatment with elevated NO_2 could influence the regulation of metabolic pathways in plants, potentially leading to a redistribution of resources within the plant (Savchenko and Tikhonov, 2021). Consequently, plants may adjust the rate of

synthesis of proteins such as defensins-like proteins (including Amb a 4.0101) and polcalcin-like proteins (including Amb a 10.0101) to adapt to changes in metabolic pathways, resulting in decreased expression of these proteins (Liu et al., 2018a).

Together, these novel findings, presented for the first time, unveil alterations in the levels of minor allergens in ragweed pollen, mainly involved in responding to environmental changes rather than contributing to variations in pollen protein allergenicity.

4.1.7-3 Protein sequence homologies to known allergens in other plant species

Consistent down-regulation of four allergen homologs was observed under elevated temperature conditions in both experiments. This finding of the downregulated profilin proteins (Mal d 4 homologue in apple) due to elevated temperature aligns with the decreased abundance of Amb a 8 isoforms discussed in section 4.1.7-2, suggesting that elevated temperature may reduce the allergenicity of certain pollen types. Additionally, a similar reduction was observed in an isoflavone reductase (Ole e 12 homologue in olive pollen)) in experiment 1 and a phenylcoumaran benzylic ether reductase (Bet v 6 homologue in birch pollen) in experiment 2. This finding is consistent with a recent study showing that heat stress in sweet corn seedlings led to the down-regulation of genes encoding phenylcoumaran benzylic ether and isoflavonoid reductases (Wang et al., 2023). These enzymes share up to 81% sequence similarity (Karamloo et al., 2001) and are crucial for plant metabolism, particularly in the biosynthesis of lignans and isoflavonoids, which are essential for plant defense and adaptation to environmental challenges (Del-Mondo et al., 2022; Niculaes et al., 2014; Wei et al., 2022). Consequently, it can be inferred that heat stress decreases lignan and isoflavonoid biosynthesis, leading to the loosening of the cell wall and promoting plasma membrane fluidity to facilitate the influx of substances, biomass, and information (Niculaes et al., 2014).

Furthermore, the observed down-regulation of cyclophilins (Cat r 1 homologue in *Catharanthus roseus*) under elevated temperature conditions in both experiments is notable. Cyclophilins are involved in protein folding and function as chaperones in stress responses (Andreeva et al., 1999). Their reduction, contrary to the expected increase in alternative splicing events under heat stress (Jo et al., 2022), as discussed in section 4.1.7-1, suggests a complex

regulatory mechanism governing gene expression and protein synthesis in response to elevated temperatures. This finding warrants further investigation to understand the precise role of cyclophilins in heat stress adaptation.

Collectively, these results highlight the adaptive strategies in different metabolic pathways employed by plants to cope with elevated temperatures. The reduction in reductases regulating lignan and isoflavonoid biosynthesis, coupled with changes in profilin and cyclophilin levels, indicates a shift in metabolic and defensive priorities. This shift could enhance the plant's ability to maintain cellular integrity and function under stress but may also alter its interaction with the environment, including its allergenic properties.

4.1.7-4 IgE-reactive proteins with sequences homologies to known allergens in ragweed pollen

Among the potential IgE-reactive proteins discussed in section 4.1.6-3, the peptidyl-prolyl cis-trans isomerase protein and its isoforms with their overall expression exhibited a significant decrease in response to combined elevated temperature and elevated NO_2 in experiment 2. The reduction of this protein is in accordance with the significantly decreased expression of cyclophilin discussed in section 4.1.7-3, which belongs to the immunophilin superfamily and regulates the peptidyl-prolyl cis-trans isomerase activity (Kaur et al., 2015; Noir et al., 2005). However, this finding contrasts with the results of Zhao et al. (2016), who observed a significant increase of this protein in ragweed pollen treated with elevated NO_2 compared to pollen treated without NO_2. The discrepancy may be primarily attributed to the introduction of elevated temperature treatment, potentially reducing the synthesis or stability of this protein, as discussed in section 4.1.7-2.

Moreover, LC-MS/MS quantification of calmodulin in this study revealed a significant decrease in samples treated with elevated temperature. This finding correlates with the reduced expression of Amb a 9 and Amb a 10 due to elevated temperature treatment in experiment 2 and aligns with the observed reduction of cyclophilin discussed above (Kaur et al., 2015).

Furthermore, calreticulin, cytosolic phosphoglycerate kinase and chloroplastic/mitochondrial nucleoside diphosphate kinase IV exhibited a similar elevated temperature-dependent reduction. This protein, crucial for various cellular processes including protein folding, glycolysis, and nucleotide metabolism, respectively (Jia et al., 2009), also displayed sensitivity to heat stress (Conway et al., 1995) and induction in rice under cold treatment (Guo et al., 2023).

Collectively, these findings suggest that elevated temperature, especially when combined with elevated NO_2, exerts a multifaceted impact on the expression and stability of key proteins involved in plant defense and metabolism. Although these proteins may be induced by environmental stress at the transcriptional level, they may not remain stable at higher temperatures eventually. The reduction in the expression of these proteins under stress conditions points to a potential weakening of the plant's defensive capabilities and metabolic efficiency, which could have broader implications for plant health and productivity under future climate scenarios.

4.1.7-5 Protein *in vitro* tyrosine nitration in ragweed pollen

In this study, *in vitro* nitration experiments were conducted on recombinant allergens, revealing potential modifications by nitration in Amb a 1, Amb a 3, Amb a 8, Amb a 11, and Amb a 12. With the exception of Amb a 12, the other four proteins exhibited a 20-30% possibility of enhancing the IgE response based on the tested sera following nitration, in line with hypothesis H1 b. Despite the relatively low probability, the potential for structural conformational changes in allergens, resulting in altered allergenicity through post-translational modifications, remains significant, as discussed above in sections 4.1.6-3 and 4.1.6-4. In agreement, NO_2 was found to induce S-nitrosylation of five Amb a 1 isoforms and Amb a 3 in ragweed pollen, potentially exacerbating the allergenicity of ragweed pollen under elevated NO_2 treatments, as documented by Zhao et al. (2016). A similar augmentation effect of NO_2 on the allergenicity of birch pollen allergen Bet v 1, attributed to the formation of nitro-tyrosine residues, was also observed (Gruijthuijsen et al., 2006; Karle et al., 2012). In this regard, conducting additional immunoprecipitation analyses using anti-biotin and anti-nitrotyrosine antibodies in future studies could provide further evidence and insights into these mechanisms.

4.1.8 GO Annotation of differentially expressed proteins in ragweed pollen

4.1.8-1 Molecular function with catalytic activity

In the category of molecular function, the most abundant group across all treatments was the catalytic activity, which involves the catalysis of biochemical reactions at physiological temperatures, known as enzymes (Cooper, 2000), and highlights their importance in pollen for all processes during pollen tube growth and interactions during fertilization (Krichevsky et al., 2007; Wang et al., 2010). These enzymatic activities also act as secondary messengers, integrating environmental and intracellular signals to optimize pollen tube performance under varying physiological conditions (Grudkowska and Zagdańska, 2004; Zheng et al., 2019).

Simultaneously, some allergenic proteins in ragweed pollen have specific molecular functions and some of them are related to catalytic processes. For instance, Amb a 1, the major allergen in ragweed pollen, exhibits pectate lyase activity, which is crucial for pectin degradation (Marín-Rodríguez et al., 2002). Amb a 12, an enolase, plays a key role in specific peptide-bond cleavages and energy production by regulating the glycolytic pathway (Grijincu et al., 2023; Zhao et al., 2016). Amb a 11, a cysteine protease, along with its homologous proteins, play crucial roles in regulating anther development and pollen formation in various plants (Bouley et al., 2015; Groeme et al., 2016; Li et al., 2021). Thus, the critical role and high demand of catalytic reactions in pollen growth and development suggest the relatively high prevalence of Amb a 1, Amb a 11, and Amb a 12. This observation aligns with the results from 2D-immunoblots, as discussed in section 4.1.6-2.

4.1.8-2 Molecular function with binding-related terms

The second most abundant group across all treatments consisted of binding-related terms, including nucleotide binding, metal ion binding, and protein binding. Several allergenic proteins in ragweed pollen exhibited these binding activities, implying their crucial roles in the physiological processes of ragweed pollen and their contribution to enhanced allergenic potential (Abdullah et al., 2016; Chen et al., 2016).

Particularly, certain molecular functions, including DNA binding and RNA binding, were significantly upregulated exclusively under elevated temperature treatments. This finding indicates a robust transcriptional and post-transcriptional regulatory response in ragweed pollen. This response may facilitate rapid adaptation to thermal stress by promoting the expression of stress-responsive genes and alternative splicing events, which generate protein isoforms better suited to the altered environmental conditions (Jo et al., 2022; Liu et al., 2022; Shiina and Shimizu, 2020). This is in accordance with the findings discussed in section 4.1.7-1. Similarly, the increased activities of molecular functions, including signal transducer activity, motor activity, structural molecular activity, and transporter activity, associated with cytoskeleton dynamics under elevated temperature conditions highlight their crucial role in the directionality and speed of pollen tube elongation (Chen et al., 2023; Yuan et al., 2023). These findings align with previous studies, which indicated that these activities were highly sensitive to temperature fluctuation (Prasad et al., 2015; Prasad et al., 2008). The actin-binding protein Amb a 8, a profilin, and its homologies are particularly important in this context, as they regulate actin polymerization, which is vital for cytoskeletal reorganization during pollen tube growth (Sunyer et al., 2009; Vidali et al., 2001). This is in accordance with the findings discussed in sections 4.1.7-2 and 4.1.7-3. These findings suggest that elevated temperatures not only impact the expression of allergenic proteins but also modulate the molecular functions that are critical for pollen viability and allergenicity.

4.1.8-3 Annotations in biological processes

Among the biological process categories, the annotation with metabolic process ranked the highest in number across all treatments. These metabolic processes are fundamental to maintaining cellular energy balance and biosynthesis, essential for pollen viability and fertilization capacity (Liu et al., 2023; Zhu and Thompson, 2019). The consistent enrichment of this term suggests that both up- and down-regulated proteins contribute to the dynamic regulation of metabolic pathways, facilitating pollen adaptation to fluctuating environmental conditions.

Proteins associated with the response to stimulus and defense response were particularly enriched due to elevated temperature, highlighting ragweed pollen's ability to perceive and react to environmental changes.

Similarly, the enrichment of the GO terms "transport", "regulation of biological process", "cellular homeostasis", and "cellular component movement" indicates a robust network of transport proteins and regulatory molecules. These elements ensure the efficient movement of ions, metabolites, and signaling molecules, which is essential for maintaining cellular homeostasis under stress conditions, such as elevated temperature (Chowdhary et al., 2011; Slieker, 2024).

Moreover, the specific enrichment of cell organization and biogenesis proteins in elevated temperature-treated clusters emphasizes the need for structural and functional reorganization of cellular components to cope with thermal stress. This reorganization is crucial for sustaining pollen tube growth and ensuring successful fertilization, as discussed earlier in relation to cytoskeleton dynamics (Chen et al., 2009; Yuan et al., 2023). Collectively, these insights provide a comprehensive understanding of the molecular basis of ragweed pollen's response to elevated temperatures, with implications for managing allergenicity in the context of climate change.

4.1.8-4 Annotations in cellular component

The consistent regulation of proteins associated with the cytoplasm, membrane, cytosol, nucleus, and mitochondrion across all treatments underscores the central role of these cellular components in pollen. In accordance with previous research, the cytoplasm and cytosol are the primary sites for metabolic activities and signal transduction pathways, potentially facilitating immediate responses to elevated NO_2 and elevated temperature (Hoppert and Mayer, 1999; Verkman, 2002). Additionally, the involvement of the membrane highlights its role in maintaining cellular integrity and mediating transport processes essential for cellular homeostasis (Conde et al., 2011). Moreover, the regulation of the nucleus is crucial for the transcriptional reprogramming necessary to adapt to stress (Mammoto et al., 2012), while the involvement of the mitochondrion emphasizes the importance of energy metabolism in supporting stress responses (Picard et al., 2018). Collectively, the enriched differentially expressed proteins align with the annotations for cellular components, as well as those for molecular functions and biological processes discussed above.

The exclusive upregulation of proteins associated with the ribosome, proteasome, endosome, and chromosome in elevated temperature-treated clusters further emphasizes the complex adaptive

mechanisms activated in response to thermal stress. The regulation of the chromosome may be attributed to its role in stress perception and signal transduction when plants encounter oxidative stress caused by elevated temperature (Balestrazzi et al., 2013). This leads to the modulation of gene expression, ultimately resulting in a plant response. The upregulation of ribosomal proteins indicates an increased demand for protein synthesis to support cellular functions and repair mechanisms under stress (Petibon et al., 2021). The involvement of the proteasome, especially the ubiquitin-proteasome system, suggests enhanced protein degradation processes to remove damaged or misfolded proteins, maintaining cellular proteostasis (Sun et al., 2021; Wang and Schippers, 2019). The upregulation of the endosome highlights its role in trafficking and sorting proteins and lipids, crucial for maintaining cellular organization and function under stress conditions (Geldner, 2004; Muller et al., 2007). Conversely, the downregulation of proteins linked to the organelle lumen in elevated temperature-treated clusters suggests a reduction in activities associated with specific organelles, possibly to reallocate resources towards more critical stress response functions (Sasaki and Yoshida, 2015). This downregulation could indicate a strategic shift in cellular priorities, focusing on immediate survival and adaptation rather than routine organelle maintenance.

4.1.9 Functional categories of significantly regulated proteins in ragweed pollen

4.1.9-1 Enhanced ragweed plant growth is mainly associated with increased metabolic activities

Pollen is essential for plant reproduction, and changes in protein composition and expression levels in plants due to environmental conditions can also affect the protein profiles of mature pollen (Hinojosa et al., 2019). Thus, in terms of functional category, the induced plant height in ragweed plants due to the individual and combined effects of elevated temperature and elevated NO_2 may be reflected in the enhanced abundant proteins related to metabolic activities and plant growth regulators, observed in the protein profiles of their pollen (Khan et al., 2019).

Primarily, proteins related to metabolic processes in response to elevated temperature were notably more abundant among up-regulated proteins in experiment 1 than that in experiment 2,

indicating a positive plant growth in experiment 1. These findings align with the previously described opposite effects of elevated-temperature treatments on plant height in the two climates, outlined in section 4.1.2-1.

Additionally, in both climates, several proteins involved in plant growth regulator-activated signaling pathway were more significantly up-regulated by elevated temperatures compared to elevated NO_2. This association might be attributed to the conducive temperatures that stimulate the metabolic process within plants, especially the synthesis of growth regulators, activating cell division and elongation, subsequently impacting plant height, as previously reported in crop species (Fahad et al., 2016; Patel and Franklin, 2009).

It is noteworthy that the intense differential changes in proteins associated with the photosynthetic process were caused by elevated temperature rather than by elevated NO_2. The photosynthesis process, including the Calvin cycle (Bertsch et al., 1993; Meissner et al., 1999), C4 acid pathway (Niessen et al., 2012), and nitrate assimilation (Kawahara et al., 2013), was mostly downregulated in response to elevated temperature. This finding is contrast to a prior study highlighting the notable adaptability of ragweed plants across a broad temperature range (5 to 35 °C) and their heightened photosynthetic activity under elevated temperatures, as observed in mature ragweed plants (Bazzaz, 1974). However, proteins involved in carbon fixation and energy production, which are highly regulated by photosynthetic process, showed significant upregulations in response to elevated temperature. This enhancement promotes greater biomass accumulation and inflorescence development, as evidenced by the increased plant size and male inflorescence length observed in *Arabidopsis thaliana* (Meyer et al., 2007). Thus, it can be speculated that ragweed plants are more likely to achieve positive growth despite a deficient photosynthesis, suggesting a hermetic dose response in ragweed plants under elevated temperature stress in a moderate climate (Agathokleous, 2021).

4.1.9-2 Effects of elevated temperature and elevated NO_2 on ragweed reproductive response

The comprehensive characterization of functional categories of significantly regulated proteins in ragweed pollen provides a molecular framework for understanding its physiological

responses and adaptive strategies. By elucidating the roles of reproductive structure development factors, stress-responsive proteins, and secondary metabolite biosynthesis enzymes, this study contributes to the broader understanding of ragweed pollen biology, shedding light on ragweed pollen's resilience and reproductive strategies (Khan et al., 2018; Zenda et al., 2022).

4.1.9-2.1 Reproductive structure development

Reproductive structure development factors include processes during reproductive structure development, such as pollen tube elongation, growth, and overall development, along with flower development, embryo and seed development, which are tightly regulated and essential for successful pollination and fertilization (Shi and Yang, 2010).

Elevated temperature significantly upregulated proteins associated with reproductive structure development in experiment 1, but exhibited the opposite trend in experiment 2. This finding aligns with previous studies indicating that increases in temperature within an optimal range can stimulate reproductive processes and enhance pollen output in various plant species (Gray and Brady, 2016; Hatfield and Prueger, 2015). However, while surpassing the temperature threshold, the resulting heat stress can lead to the denaturation of proteins and impair enzymatic functions critical for reproductive development (Bita and Gerats, 2013; Resentini et al., 2023). This observation is consistent with the hypothesis in the section 4.1.3-3.1. Overall, the impact of elevated temperature on reproductive structure development aligns with previous research by Hatfield and Prueger (2015) and Poidevin et al. (2021), underscoring the vulnerability of pollination to heat stress across diverse species. Given the anticipated rise in temperatures associated with climate change, particularly during crucial developmental stages, the potential impact on production is significant.

Particularly, in experiment 2, elevated temperature resulted in the up-regulation of proteins associated with pollen development and pollen tube growth, which are linked to reproductive structure development. Conversely, proteins related to flower development were predominantly down-regulated. The up-regulation of proteins involved in pollen tube growth under elevated temperature conditions highlights the plant's attempt to ensure that the limited pollen produced is more efficient in achieving fertilization. Pollen tube growth is a critical phase in the reproductive

process, as it determines the successful delivery of sperm cells to the ovule (Hepler et al., 2013). In addition, the down-regulation of flower development proteins may explain the observed decrease in pollen release per male inflorescence under elevated temperature conditions, as outlined in the section 4.1.3-3.1. Reduced investment in flower development likely results in fewer and less robust flowers, thereby decreasing the overall pollen output. This phenomenon aligns with previous research indicating that elevated temperatures can negatively impact flower formation and development, leading to reduced reproductive success (Bita and Gerats, 2013). Collectively, the findings from experiment 2 underscore the complex interplay between elevated temperature and plant reproductive strategies. The up-regulation of proteins associated with pollen development and tube growth, coupled with the down-regulation of flower development proteins, suggests an adaptive mechanism to ensure reproductive success despite reduced pollen quantity under stress conditions.

Similarly, elevated NO_2 induced differential expression of multiple proteins associated with the development of reproductive structures under ambient temperature conditions. This finding suggests induced pollen production due to elevated NO_2, consistent with the observation discussed in section 4.1.3-3.2 and previous research by Zhao et al. (2016). Notably, under elevated temperature conditions, the NO_2-induced differential expression of these proteins was no longer observed. These findings imply a complex interplay between elevated NO_2 and elevated temperature in regulating the development of reproductive structures in ragweed, suggesting that the combined stressors of elevated NO_2 and elevated temperature may overwhelm the plant's adaptive mechanisms. Elevated temperatures alone already impose significant stress on plants, leading to substantial changes in protein expression associated with various physiological processes, including pollen development and tube growth (Bita and Gerats, 2013). The additional stress from elevated NO_2 may disrupt these processes, preventing the plant from effectively up-regulating proteins involved in reproductive structure development.

4.1.9-2.2 Cytoskeleton dynamics

Polarization through rearrangement of the cytoplasm and cytoskeleton is essential for pollen germination. After pollen tube growth reaches its maximal tip region, there must be a continual deposition of new cell walls and plasma membranes (Mascarenhas, 1993). Thus, cytoskeleton

dynamics, including cytoskeletal organization and cell wall remodeling, is essential to pollen's resilience and reproductive strategies under stress conditions (Mollet et al., 2013; Selinski and Scheibe, 2014).

Obviously, proteins associated with cytoskeleton dynamics were significantly differentially expressed under elevated temperature conditions in both experiments. This finding aligns with previous research indicating that proteins, such as actin and tubulin isoforms, involved in cytoskeletal functions like pollen tube elongation and growth, are sensitive to temperature changes (Cai et al., 2005). Elevated temperatures can disrupt the organization of cytoskeletal elements, affecting pollen tube growth and directional guidance toward the ovule, as shown in previous studies (Bita and Gerats, 2013; Resentini et al., 2023).

Similarly, the differential expression of cytoskeleton-related proteins due to elevated NO_2 was observed particularly under ambient temperature conditions in experiment 2, suggesting that elevated NO_2 in a relatively warmer climate facilitates reorganization in mature pollen. This facilitation could enhance pollen germination and rapid tube elongation and orientation, promoting successful fertilization (Palevitz et al., 1994; Vidali et al., 2001). However, while ach environmental factor individually promoted the reorganization of cytoskeletal elements, their concurrent presence appears to diminish this effect. This negative interaction could result from the plants' need to balance the competing demands of managing oxidative stress induced by NO_2 and thermal stress, leading to a compromise in cytoskeletal protein expression.

4.1.9-2.3 Energy generation

Energy generation processes, including glycolysis, tricarboxylic acid cycle, and Calvin cycle, along with metabolic processes, are crucial for pollen tube elongation and growth (Chen et al., 2017; Choi et al., 2014; Noir et al., 2005). Obviously, proteins associated with metabolic processes, glucose metabolism (including glycolysis), and tricarboxylic acid cycle exhibited more significant up-regulation under elevated temperature treatment in experiment 1, but demonstrated a considerable down-regulation in experiment 2. These findings again support an optimal temperature range can positively impact ragweed growth by optimizing metabolic processes (Fahad et al., 2016; Khan et al., 2019; Patel and Franklin, 2009). While temperatures beyond the

optimal range can lead to thermal stress, disrupting enzymatic activities and metabolic pathways (Liu et al., 2023; Zenda et al., 2022). The down-regulation of proteins involved in glucose metabolism, the tricarboxylic acid cycle and the Calvin cycle reflects a shift towards a stress-induced metabolic state, where energy-consuming processes are down-regulated to prioritize cellular maintenance and survival (Liu et al., 2023; Zhu and Thompson, 2019).

Similarly, a greater number of proteins associated with glycolysis and tricarboxylic acid cycle was upregulated in response to elevated NO_2 under ambient temperature conditions than under elevated temperature conditions alone. The findings suggests that elevated NO_2 under the ambient temperature conditions enhances metabolic activity, potentially increasing energy production and promoting pollen production. This aligns with previous findings that NO_2 can act as a signaling molecule and nitrogen fertilization, stimulating similar induction of Amb a 12 and enhancing pollen development and production (Grijincu et al., 2023; Zhao et al., 2016; Zhao et al., 2017b). In contrast, these findings also suggest that the combined stress of elevated temperature and elevated NO_2 may exceed the plant's metabolic capacity, and the overall metabolic burden may lead to a down-regulation of key metabolic proteins as a protective response to conserve energy and reduce damage.

4.1.9-2.4 Cellular transport and signaling

Each stage of pollen tube development and fertilization involves cell-to-cell signaling and transport-related molecules between the male gametophyte and female tissues. The up-regulation of V-type proton ATP synthase, ATP synthase and clathrin, as well as proteins involved in GTP-mediated signaling and calcium ion-dependent signaling, suggests that elevated temperature enhances the cellular machinery necessary for pollen tube elongation and growth (Chen et al., 2017; Choi et al., 2014; Li et al., 2010). This is consistent with previous findings that underscore the role of these proteins in facilitating the dynamic cellular changes required for effective pollen tube navigation (Song et al., 2009; Zhao et al., 2016). Particularly, the observed up-regulation of calcium ion-dependent signaling under elevated temperature conditions highlights the pivotal role of calcium as a universal signaling molecule in pollen tube growth (Shi and Yang, 2010; Zheng et al., 2019). Calcium ions play a critical role in maintaining the oscillatory nature of pollen tube growth, affecting various downstream targets that regulate actin dynamics and vesicle trafficking

(Geldner, 2004; Muller et al., 2007). This finding aligns with prior studies indicating that elevated temperatures can enhance calcium-dependent processes, thereby supporting the rapid elongation and directional growth of pollen tubes towards the ovule (Wopfner et al., 2008).

On the other hand, the up-regulation of ATP synthase in response to elevated NO_2, as observed in this study and corroborated by Zhao et al. (2016), points to an increased energy demand under elevated NO_2 treatment. ATP synthase is vital for ATP production in mitochondria, providing the necessary energy for various cellular processes, including those involved in stress responses and pollen tube growth (Li et al., 2010). This indicates that elevated NO_2 may enhance the metabolic activity in pollen, facilitating the energy-intensive process of tube elongation under stress conditions.

4.1.9-3 Stress and defense-related proteins

It is known that the stress and defense responses reflected in the mature pollen proteome include not only extracellular stresses after release from the anther but also intracellular stresses caused by the active metabolism of germinating pollen and its interaction with the cells of the stigma and style (Dai et al., 2006). In this study, a notable portion of stress-related proteins, such as heat shock proteins (HSPs), chitinase, catalases, peroxiredoxins, chaperonin, and several other antioxidant enzymes, along with several 26S proteasome non-ATPase regulatory subunits were identified in ragweed pollen. This finding aligns with previous studies on NO_2-treated (Zhao et al., 2016) and elevated O_3 fumigated (Kanter et al., 2013) ragweed pollen, as well as environmental stressor-treated or non-treated pollen from various plant species (Agrawal and Rakwal, 2008). Hence, it can be inferred that these proteins in ragweed pollen play crucial roles in maintaining cellular homeostasis, detoxification, and defense against environmental stressors and reactive oxygen species (ROS), which may be affected by the individual and combined treatments of elevated temperature and elevated NO_2. These findings align with previous studies on environmental stress responses in plants, indicating a conserved mechanism of stress tolerance across different species (Foyer and Noctor, 2005; Gray and Brady, 2016; Vaidyanathan et al., 2003).

A larger proportion of proteins potentially associated with stress and defense was elevated temperature-dependent differential expressed than elevated NO_2 did. HSPs, particularly HSP 70 and HSP 90, and molecular chaperones are crucial for mitigating the adverse effects of temperature stress on reproductive processes (Wang et al., 2004). For example, HSP70 and HSP90 are known to chaperone misfolded proteins and stabilize cellular structures under heat stress conditions (Wahid et al., 2007; Wang et al., 2004). The chaperone proteins assist in the proper folding of newly synthesized proteins, prevent aggregation of misfolded proteins, and stabilize cellular structures under heat-stress conditions (Pant et al., 2020). Collectively, the upregulations of HSPs and chaperonin in response to elevated temperature may help maintain the structural integrity of pollen grains and support proper pollen tube elongation, ensuring successful fertilization even under stressful conditions.

Moreover, the identification of proteins such as chitinase, catalases, peroxiredoxins, and various antioxidant enzymes emphasizes their role in protecting pollen cells from oxidative damage, indicating an overall higher oxidative stress level under the elevated temperature treatment. The expression of chitinase was exclusively observed as a downregulation due to individual elevated temperature and elevated NO_2. This is consistent with a converse upregulation of this protein due to cold stress in crops (Iwuala et al., 2020; Vaghela et al., 2022). Additionally, the significant upregulations of catalases and peroxiredoxins, consistent with their critical roles in detoxifying hydrogen peroxide, a reactive oxygen species (ROS) that can accumulate under heat stress (Locato et al., 2008) and other abiotic stresses like UV (Rao et al., 1996), ozone (Rao et al., 1996), drought (Basu et al., 2010; Sharma and Dubey, 2005), and cause cellular damage. Collectively, the presence of these enzymes suggests an intricate defense mechanism that allows ragweed pollen to cope with abiotic stresses.

Additionally, the detection of 26S proteasome non-ATPase regulatory subunits indicates the involvement of the ubiquitin-proteasome pathway in protein degradation and quality control. This pathway is essential for removing damaged or misfolded proteins and regulating various cellular processes, including stress responses (Kosová et al., 2021). The upregulation of these proteasome subunits in response to elevated temperature and NO_2 treatments suggests an enhanced capacity for protein quality control and turnover, contributing to cellular homeostasis.

Furthermore, the increased expression of stress-related proteins in response to elevated temperature and NO₂ treatments has significant implications for both plant reproductive success and human health. On the one hand, the enhanced stress tolerance of ragweed pollen suggests a higher likelihood of successful fertilization under adverse environmental conditions, contributing to the spread and persistence of this allergenic plant. On the other hand, the increased allergenicity of the pollen under these conditions poses a greater risk to individuals with pollen allergies, potentially leading to more severe allergic reactions during periods of elevated temperatures, which is in accordance with the findings outlined in section 4.1.6-1.

4.1.9-4 Secondary metabolite

The identified enzymes in the biosynthesis pathways of secondary metabolites, such as those involved in fatty acid, lipid, oxylipin, flavonoid, isoprene, lignin, riboflavin, and steroid biosynthesis, were observed to significantly differential expression due to elevated temperature and elevated NO₂. The presence of enzymes involved in the biosynthesis of a wide range of secondary metabolites indicates a sophisticated metabolic network in ragweed pollen that supports its adaptability and reproductive success under varying environmental conditions (Paupière et al., 2017; Rehman et al., 2023). For instance, fatty acids and lipids are essential for the formation of pollen cell membranes and energy storage, contributing to pollen viability and germination (Traidl-Hoffmann et al., 2002; Zhu and Thompson, 2019). Oxylipins, derived from fatty acids, are involved in plant defense responses and signaling pathways that mediate interactions with pathogens and herbivores (Adigun et al., 2023; Ponce de León et al., 2015). Flavonoids have antioxidant properties and play a role in protecting pollen from UV radiation and oxidative stress (Chutipaijit et al., 2009; Woo et al., 2002).

The detection of biosynthetic enzymes for secondary metabolites such as flavonoids and lignin suggests their involvement in structural and protective functions within the pollen. Flavonoids, apart from their antioxidant roles, are crucial in regulating pollen tube growth and fertilization processes, especially during high-temperature stress (Muhlemann et al., 2018). Lignin a structural component, reinforces pollen walls, enhancing their resilience to environmental stresses (Liu et al., 2018b). This protein was observed with an accumulation in the process of cold

acclimation in woody evergreens (Wei et al., 2006) and an induced deposition due to heat treatment in citrus fruit (Yun et al., 2013). Moreover, both heat and low temperatures were found to reduce the lignification in loquat fruit (Zeng et al., 2016). Steroids were found to influence pollen tube growth and fertilization by modulating cellular processes and signaling pathways in rice during heat stress (Fahad et al., 2016). Additionally, riboflavin biosynthesis is integral for cellular metabolism and energy production, ensuring the energetic demands of pollen germination and tube elongation are met (Powers et al., 2012).

4.1.9-5 Protein modifications

The differential expression of proteins with PTMs, particularly under elevated temperature and elevated NO_2 treatments, highlights the dynamic nature of the pollen proteome in response to environmental stressors. The upregulation of protein methylation and phosphorylation in response to elevated temperature in experiment 1 indicates an active modulation of signaling pathways and transcriptional programs to cope with heat stress. This is consistent with the findings of Han et al. (2022), who reported similar PTM patterns in crops under warm-temperature stress. In contrast, the mixed patterns of up- and down-regulation observed in experiment 2, which simulated a relatively warmer climate, suggest a more complex regulatory response to elevated temperature stress, potentially due to the higher baseline temperatures exceeding the optimal range for ragweed growth.

The observed PTMs play crucial roles in maintaining cellular homeostasis and ensuring the functionality of stress response mechanisms. For example, the upregulation of protein phosphorylation under elevated temperature conditions can enhance the activation of heat shock factors (HSFs) and other transcription factors that drive the expression of heat shock proteins (HSPs) and other protective proteins (Jo et al., 2022). This aligns with the upregulation of HSPs observed in the study, which helps mitigate the adverse effects of temperature stress by stabilizing cellular structures and facilitating proper protein folding.

Similarly, protein methylation can regulate gene expression by modifying histones and influencing chromatin structure, thereby altering the accessibility of DNA to transcriptional machinery (Pacchierotti and Spanò, 2015; Shiina and Shimizu, 2020). The induced expression of

methylated proteins under elevated temperature conditions in experiment 1 suggests that epigenetic modifications are a key component of the plant's adaptive response to heat stress. This is further supported by studies showing that methylation patterns can change in response to environmental stressors, leading to long-term changes in gene expression and stress tolerance (Breton-Larrivée et al., 2019).

Glycosylation, another critical PTM, significantly upregulated in response to elevated temperature independent of elevated NO_2, affecting the stability and function of many proteins involved in stress responses in ragweed pollen. For instance, glycosylated proteins are often involved in cell wall remodeling and signal transduction, processes that are essential for maintaining cell integrity and communication under stress conditions (Behr et al., 2020). The identification of glycosylated proteins in response to elevated temperature suggests that this modification is crucial for pollen viability and stress resilience.

Taken together, these modifications play critical roles in regulating protein function, stability, and interactions, thereby enabling plants to quickly adjust their transcriptional programs and signaling pathways in response to environmental changes.

4.2 Diversity of leaf-colonizing fungal species of ragweed and their potential interaction with pollen allergens

4.2.1 Characterization of ragweed plant-associated fungi

4.2.1-1 Diversity and niche preference

The findings from Illumina sequencing analysis clearly demonstrate a disparity in fungal colonization between the upper and lower levels of the ragweed plant, with the former exhibiting a more diverse fungal community. This observation suggests a potential compartmentalization of fungal colonization within the plant, underscoring the significance of spatial dynamics in plant-fungal interactions. The microbial composition of the upper leaf regions may be closely associated with the microbial community found in pollen which could not be studied in the present work.

The consistent presence and high relative abundance of species affiliated with the genus taxon "unknown_Capnodiales (Order)" across all leaf samples is a noteworthy finding of this study. This taxon's prevalence suggests a strong association with ragweed leaves, indicating leaves as a preferred habitat for this particular fungal group. The persistence of this taxon throughout different leaf layers implies its adaptability to varying micro-environmental conditions within the leaf structure. Moreover, the identification of a specific fungal isolate belonging to this genus taxon (Table 3 – 15) opens avenues for experimental studies to characterize its physiology, host specificity, and pathogenicity.

In addition, the differential enrichment of fungal species affiliated with the genus taxon "unknown_Didymellaceae (Family)" and *Podospora* across leaf samples at different positions further highlights the spatial dynamics of fungal colonization within ragweed leaves. This result indicates a potential preference for lower leaf positions by these fungal taxa, suggesting spatial niche differentiation within the plant host. The family Didymellaceae encompasses a diverse group of fungi known for their plant pathogenicity and saprophytic lifestyles (Aveskamp et al., 2010; Aveskamp et al., 2008; Marin-Felix et al., 2017). The enrichment of "unknown_Didymellaceae (Family)" taxa in lower leaf samples may reflect specific ecological preferences or physiological adaptations to conditions prevailing in the lower leaf layers. Lower leaf positions often experience reduced light exposure, higher humidity, and altered nutrient availability, which may favor the growth and proliferation of certain fungal taxa. The identification of a fungal isolate (Table 3 – 15) affiliated with the family Didymellaceae underscores the importance of characterizing individual taxa to understand their ecological significance and potential impacts on host health.

Similarly, the enrichment of *Podospora* in lower leaf samples suggests a spatial distribution pattern within the ragweed phyllosphere. *Podospora spp.* are commonly found in soil and decaying plant material, indicating a potential role in leaf litter decomposition or nutrient recycling processes (Kazerooni et al., 2022). The preferential colonization of lower leaf layers by *Podospora* may be linked to increased organic matter accumulation and microbial activity in these regions; moreover, it indicates the interactions between the plant-soil-system.

Pseudopithomyces, *Neoascochyta*, and *Aureobasidium*, all belonging to the family Didymellaceae, exhibited higher abundance in the upper leaves, whereas *Bipolaris*, a member of the family Pleosporaceae, was more prevalent in the lower leaves. These findings suggest differential adaptation to micro-environmental conditions and resource availability within the ragweed phyllosphere. In contrast, the enrichment of *Bipolaris* and *Sporobolomyces* in lower leaves implies a distinct ecological niche within the lower leaf layers. *Bipolaris spp.* are known for their saprophytic and pathogenic lifestyles, often associated with leaf spots and blights in various plant hosts (Gupta et al., 2018; Kazerooni et al., 2022). The prevalence of *Bipolaris* in lower leaves may reflect the accumulation of organic matter and higher microbial activity in these regions, providing favorable conditions for its growth and colonization. Similarly, *Sporobolomyces*, a ubiquitous yeast-like fungus commonly found in soil and plant surfaces, may thrive in the more sheltered and humid microenvironments of lower leaf layers (Fatemi et al., 2022).

Most of the identified fungal taxa with differential enrichment based on leaf position belonged to a broad spectrum of fungal taxa with no apparent host specificity. This finding underscores the generalist nature of fungal colonization within the ragweed phyllosphere, reflecting the diverse array of environmental sources contributing to fungal inoculum.

4.2.1-2 Potential allergenic fungi associated with ragweed

Several fungal genera identified in this study, including *Bipolaris*, *Aureobasidium*, *Pseudopithomyces*, *Neoascochyta*, and *Sporobolomyces*, have been implicated in allergic respiratory diseases and hypersensitivity reactions in susceptible individuals. These fungi produce airborne spores that can act as allergens, triggering allergic responses such as rhinitis, asthma, and allergic fungal sinusitis. *Bipolaris spp.*, for example, is known to produce potent allergenic proteins and enzymes that can elicit allergic reactions upon inhalation or skin contact (Chowdhary et al., 2011; Lim et al., 1995). Similarly, *Aureobasidium spp.* has been identified as a common indoor and outdoor allergen, with airborne spores contributing to allergic rhinitis and asthma in sensitized individuals (Taylor et al., 2006). The presence of *Aureobasidium* in ragweed leaves raises concerns about potential allergen exposure during outdoor activities or agricultural practices

involving ragweed-infested areas. *Sporobolomyces*, while less studied in the context of allergenicity, is also known to produce allergenic proteins and enzymes that can trigger allergic reactions in susceptible individuals (Damji et al., 2019; Fatemi et al., 2022). Their presence in ragweed leaves suggests a potential source of fungal allergens in the environment, particularly during ragweed flowering and spore release periods. It is important to note that the allergenic potential of fungal species can vary depending on factors such as spore morphology, allergen composition, and individual susceptibility. Therefore, further research is needed to characterize the allergenicity of specific fungal isolates identified in our study and assess their contribution to allergic disease burden in ragweed-infested regions.

4.2.2 Cross-reactivity between fungal proteins and aqueous ragweed pollen extract

The significantly higher IgE-binding observed upon adding CRP (commercial ragweed pollen) to sample 1, referencing *B. bassiana*, suggests a potential synergistic effect between *B. bassiana* antigens and CRP in eliciting allergic responses. This finding indicates that *B. bassiana* may contain allergenic proteins or epitopes that interact with CRP, enhancing their allergenic potential in sensitized individuals. However, further characterization of specific allergenic components in *B. bassiana* will be essential for understanding the mechanisms underlying this interaction and its relevance to allergic disease. Moreover, characterizing the epitopes involved could facilitate the development of targeted therapies or diagnostic tools for individuals sensitized to these allergens (Raghavan et al., 2013).

In contrast, no significant IgE-binding was observed upon adding CRP to samples 8 (*G. tricincta*), 11 (*M. bolleyi*), and 15 (*C. lunata*), indicating a lack of synergistic effects between CRP and these fungal isolates in eliciting IgE-mediated responses. This suggests that the allergenic potential of these isolates might be inherently lower, or their allergenic proteins do not synergize with CRP in the tested conditions. This finding is important as it highlights the variability in allergenic potential among different fungal species and the complexity of allergen interactions.

Moreover, none of the remaining 11 fungal isolates nor their combinations with CRP showed detectable IgE-binding signals. This finding suggests that these fungal isolates may exhibit low

allergenic potential or may lack allergenic proteins or epitopes recognized by IgE antibodies under the tested conditions. However, it is essential to consider that allergenicity is a multifaceted phenomenon influenced by various factors such as protein concentration, conformational stability, and individual immune variability (Stadler and Stadler, 2003; Traidl-Hoffmann et al., 2009). Therefore, while these findings provide a preliminary indication of the allergenic potential of these fungal isolates, comprehensive studies incorporating higher protein concentrations, different conformational states, and diverse individual sera are necessary to draw definitive conclusions.

The observed synergistic IgE-binding between *B. bassiana* and CRP underscores the potential for enhanced allergenic responses when certain fungal proteins interact with common environmental allergens. This highlights the importance of identifying specific allergenic components within *B. bassiana* and understanding their interaction mechanisms. Conversely, the lack of significant IgE-binding in other fungal isolates, such as *G. tricincta*, *M. bolleyi*, and *C. lunata*, suggests variability in allergenic potential and emphasizes the need for further research to explore these interactions comprehensively. Future studies should focus on detailed proteomic analyses of these fungal isolates to identify specific allergenic proteins and their epitopes. This knowledge is crucial for developing targeted interventions and mitigating allergic responses in sensitized individuals, ultimately improving public health outcomes related to fungal and pollen allergies.

5: Chapter – CONCLUSIONS AND OUTLOOK

Overall, this study highlights that the effects of the interaction between elevated temperature and elevated NO_2 on the environment and human health were underestimated because pollen from ragweed plants grown under combined conditions exhibited the highest allergenic potential compared to pollen from plants grown under single factor conditions (Figure 5 - 1). Elevated temperature, with or without elevated NO_2, promoted ragweed plant growth, delayed pollen release season and increased the allergenicity-related compounds, such as NADP(H) oxidase activity in pollen. Moreover, elevated temperatures resulted in more allergenic pollen and higher levels of flavonoids but with a reduced ability to bind IgE, while elevated NO_2 resulted in more allergenic pollen but a less total flavonoid content. Although a relatively warmer climate was expected to advance the onset of pollen release, the effect of combined elevated temperature and NO_2 on this onset warrants further investigation. In addition, the interference of phenolic metabolites and particularly flavonoids with allergen-antibody binding as modulators in immune reactions in atopics deserves further attention.

Proteomic analysis revealed significant up-regulation of proteins involved in metabolic processes, cytoskeleton dynamics, stress responses, and secondary metabolite biosynthesis under elevated temperature conditions. These proteins enhance pollen viability, reproductive success, and stress resilience, suggesting that elevated temperatures bolster adaptive and defensive mechanisms in ragweed pollen. Moreover, elevated temperature conditions in a warmer climate may surpass the optimal range for reproductive development. The interaction between elevated temperature and NO_2 showed complex, synergistic effects on the expression of allergenic and stress-related proteins, indicating that combined stressors, particularly in the warmer climate, can significantly influence pollen protein profiles and increase allergenic potential.

The study also observed synergistic IgE-binding between *B. bassiana* and commercial ragweed pollen (CRP), indicating enhanced allergic responses when fungal proteins interact with ragweed pollen allergens. Conversely, other fungal isolates showed no significant IgE-binding, suggesting variability in allergenic potential. This underscores the need for comprehensive studies on these interactions under diverse environmental conditions and individual immune profiles.

Furthermore, future research will explore the *in vivo* interactions of ragweed plants with associated fungi to assess cross-reactivity and their allergenic risks.
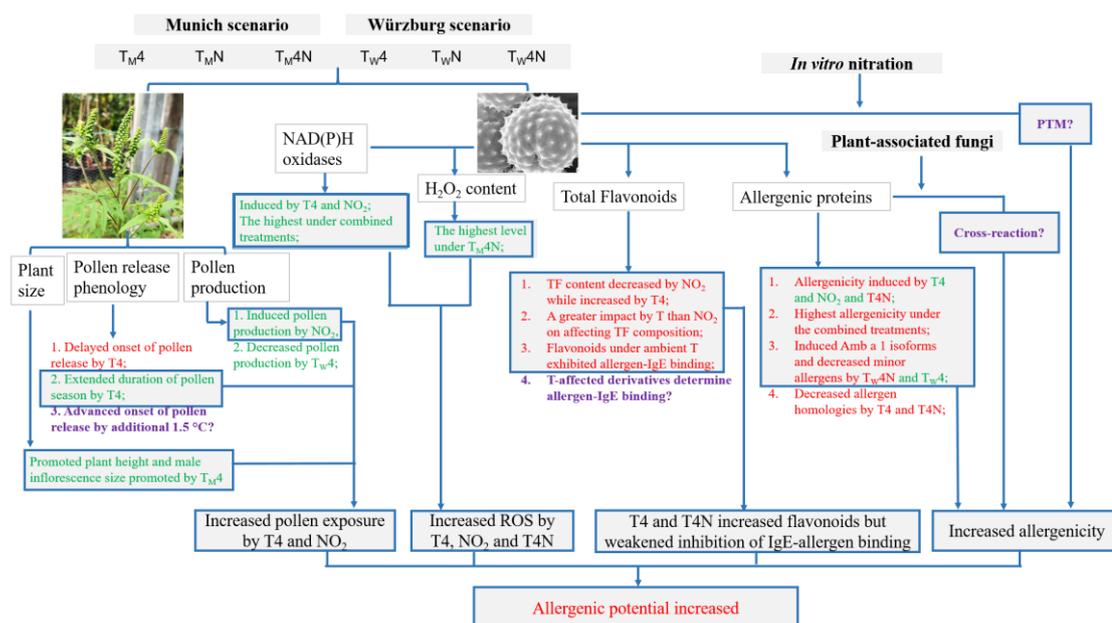
Figure 5 - 1: Proposed mechanisms for the induced allergenic potential of ragweed pollen under elevated temperature (T4), elevated NO_2 (TN) and their combination (T4N), plant-associated fungi and *in vitro* nitration. Two independent experiments ("Munich", "Würzburg") under current (T) and future temperature (T4) simulations with 80 ppb and 0 ppb NO_2 in the ambient air were simulated. Abbreviations: T_M4, T_MN, T_M4N refer to elevated temperature, elevated NO_2 and combined treatments in the Munich scenario; T_W4, T_WN, T_W4N refer to the same treatments in the Würzburg scenario; ROS = reactive oxygen species; PTM = post-translational modification. Findings highlighted in green indicate agreement with published studies; red denotes novel findings or results contrary to hypotheses in this study; and purple marks novel findings or findings need further investigation. Only the results text within the boxes lead to the next outcome, as directed by the arrows.

Overall, this study provides valuable insights into the proteomic responses of ragweed pollen to elevated temperature and NO_2, highlighting the intricate interplay between environmental stressors, pollen allergenicity and plant adaptation. Moreover, it underscores the importance of local solutions for NO_2 emission control and strategies to mitigate temperature increases to reduce ragweed-pollen-linked health risks and fungal allergies.

REFERENCES

Aalberse, R.C. (2007). Assessment of allergen cross-reactivity. Clin. Mol. Allergy 5, 2. https://doi.org/10.1186/1476-7961-5-2.

Abdullah, S.U., Alexeev, Y., Johnson, P.E.*, et al.* (2016). Ligand binding to an allergenic lipid transfer protein enhances conformational flexibility resulting in an increase in susceptibility to gastroduodenal proteolysis. Sci. Rep. 6, 30279. https://doi.org/10.1038/srep30279.

Ackaert, C., Kofler, S., Horejs-Hoeck, J.*, et al.* (2014). The impact of nitration on the structure and immunogenicity of the major birch pollen allergen Bet v 1.0101. PLOS ONE 9, e104520. https://doi.org/10.1371/journal.pone.0104520.

Adam, S.E.H., Shigeto, J., Sakamoto, A.*, et al.* (2008). Atmospheric nitrogen dioxide at ambient levels stimulates growth and development of horticultural plants. Botany 86, 213-217. https://doi.org/10.1139/b07-129.

Adigun, O.A., Pham, T.H., Grapov, D.*, et al.* (2023). Phyto-oxylipin mediated plant immune response to colonization and infection in the soybean-*Phytophthora sojae* pathosystem. Front. Plant Sci. 14, 1141823. https://doi.org/10.3389/fpls.2023.1141823.

Adolphson, C., Goodfriend, L., Gleich, G.J. (1978). Reactivity of ragweed allergens with IgE antibodies. Analyses by leukocyte histamine release and the radioallergosorbent test and determination of cross-reactivity. J. Allergy Clin. Immunol. 62, 197-210. https://doi.org/10.1016/0091-6749(78)90208-7.

Agathokleous, E. (2021). The rise and fall of photosynthesis: Hormetic dose response in plants. J. For. Res. 32, 889-898. https://doi.org/10.1007/s11676-020-01252-1.

Agency, G.E. (2001). Air quality qssessment in Germany. https://www.umweltbundesamt.de/en/data/air/air-data.

Agency, G.E. (2023). Air data in Germany: Annual tabulation of nitrogen dioxide in 2022. https://www.umweltbundesamt.de/en/data/air/air-data/annual-tabulation/eJxrWpScv9BwUWXqEiMDIyMAMK8Fsw==.

Aggarwal, R., Purwar, S., Kharbikar, L.*, et al.* (2011). Induction of a wheat β-1,3-glucanase gene during the defense response to *Bipolaris sorokiniana*. Acta Phytopathol. Entomol. Hung. 46, 39-47. https://doi.org/10.1556/aphyt.46.2011.1.5.

Agrawal, G.K., Rakwal, R. (2008). Plant Proteomics: Technologies, Strategies, and Applications. Wiley. https://books.google.com/books?id=CfDqXTCbWL4C

Ahlholm, J.U., Helander, M.L., Savolainen, J. (1998). Genetic and environmental factors affecting the allergenicity of birch (*Betula pubescens* ssp. *czerepanovii* [Orl.] Hämet-ahti) pollen. Clin. Exp. Allergy 28, 1384-1388. https://doi.org/10.1046/j.1365-2222.1998.00404.x.

Ahmed, U., Rao, M.J., Qi, C.*, et al.* (2021). Expression profiling of flavonoid biosynthesis genes and secondary metabolites accumulation in *Populus* under drought stress. Molecules 26, 5546. https://doi.org/10.3390/molecules26185546.

Alan, Ş., Şahin, A.A., Sarışahin, T.*, et al.* (2018). The effect of geographical and climatic properties on grass pollen and Phl p 5 allergen release. Int. J. Biometeorol. 62, 1325-1337. https://doi.org/10.1007/s00484-018-1536-0.

Albert, A., Sareedenchai, V., Heller, W.*, et al.* (2009). Temperature is the key to altitudinal variation of phenolics in *Arnica montana* L. cv. ARBO. Oecologia 160, 1-8. https://doi.org/10.1007/s00442-009-1277-1.

Alberternst, B., Nawrath, S. (2008). Spreading and distribution of common ragweed (*Ambrosia artemisiifolia*) in Germany. Proceedings of the 4th European Symposium on Aerobiology, Turku. 65-74.

Alberternst, B., Nawrath, S., Starfinger, U. (2016). Biodiversity impacts of common ragweed. Julius-Kühn-Archiv 455, 188-226. https://doi.org/10.5073/jka.2016.455.45.

Almagro, L., Gómez-Ros, L.V., Belchi-Navarro, S.*, et al.* (2009). Class III peroxidases in plant defence reactions. J. Exp. Bot. 60, 377-390. https://doi.org/10.1093/jxb/ern277.

Almonacid, L., Fuentes, A., Ortiz, J.*, et al.* (2015). Effect of mixing soil saprophytic fungi with organic residues on the response of *Solanum lycopersicum* to arbuscular mycorrhizal fungi. Soil Use Manage. 31, 155-164. https://doi.org/10.1111/sum.12160.

Amarasekare, P., Savage, V. (2012). A framework for elucidating the temperature dependence of fitness. Am. Nat. 179, 178-191. https://doi.org/10.1086/663677.

Anderegg, W.R.L., Abatzoglou, J.T., Anderegg, L.D.L.*, et al.* (2021). Anthropogenic climate change is worsening North American pollen seasons. Proc. Natl. Acad. Sci. 118, e2013284118. https://doi.org/10.1073/pnas.2013284118.

Andreeva, L., Heads, R., Green, C.J. (1999). Cyclophilins and their possible role in the stress response. Int. J. Exp. Pathol. 80, 305-315. https://doi.org/10.1046/j.1365-2613.1999.00128.x.

Arbes-Jr, S.J., Gergen, P.J., Elliott, L.*, et al.* (2005). Prevalences of positive skin test responses to 10 common allergens in the US population: Results from the third National Health and Nutrition Examination Survey. J. Allergy Clin. Immunol. 116, 377-383. https://doi.org/10.1016/j.jaci.2005.05.017.

Ariano, R., Berra, D., Chiodini, E.*, et al.* (2015). Ragweed allergy: Pollen count and sensitization and allergy prevalence in two Italian allergy centers. Allergy Rhinol. 6, e177–e183. https://doi.org/10.2500/ar.2015.6.0141.

Ariano, R., Canonica, G.W., Passalacqua, G. (2010). Possible role of climate changes in variations in pollen seasons and allergic sensitizations during 27 years. Ann. Allergy, Asthma, Immunol. 104, 215-222. https://doi.org/10.1016/j.anai.2009.12.005.

Artursson, V., Finlay, R.D., Jansson, J.K. (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ. Microbiol. 8, 1-10. https://doi.org/10.1111/j.1462-2920.2005.00942.x.

Asturias, J.A., Arilla, M.C., Gómez-Bayón, N.*, et al.* (1997). Cloning and expression of the panallergen profilin and the major allergen (Ole e 1) from olive tree pollen. J. Allergy Clin. Immunol. 100, 365-372. https://doi.org/10.1016/s0091-6749(97)70250-1.

Aveskamp, M.M., De-Gruyter, J., Woudenberg, J.H.*, et al.* (2010). Highlights of the *Didymellaceae*: A polyphasic approach to characterise *Phoma* and related pleosporalean genera. Stud. Mycol. 65, 1-60. https://doi.org/10.3114/sim.2010.65.01.

Aveskamp, M.M., Gruyter, J., Crous, P. (2008). Biology and recent developments in the systematics of *Phoma*, a complex genus of major quarantine significance. Fungal Divers. 31, 1-18. https://api.semanticscholar.org/CorpusID:40211158

Avilán, L., Gualdrón-López, M., Quiñones, W.*, et al.* (2011). Enolase: A key player in the metabolism and a probable virulence factor of trypanosomatid parasites-perspectives for its use as a therapeutic target. Enzyme Res. 2011, 932549. https://doi.org/10.4061/2011/932549.

Bacsi, A., Dharajiya, N., Choudhury, B.K.*, et al.* (2005). Effect of pollen-mediated oxidative stress on immediate hypersensitivity reactions and late-phase inflammation in allergic conjunctivitis. J. Allergy Clin. Immunol. 116, 836-843. https://doi.org/10.1016/j.jaci.2005.06.002.

Bae, J., Benoit, D.L., Watson, A.K. (2016). Effect of heavy metals on seed germination and seedling growth of common ragweed and roadside ground cover legumes. Environ. Pollut. 213, 112-118. https://doi.org/10.1016/j.envpol.2015.11.041.

Balestrazzi, A., Macovei, A., Donà, M.*, et al.* (2013). Genotoxic Stress, DNA Repair, and Crop Productivity, in: Tuteja, N., Gill, S.S. (Eds.), Crop Improvement Under Adverse Conditions. Springer New York, New York, NY, pp 153-169. https://doi.org/10.1007/978-1-4614-4633-0_7.

Ballizany, W.L., Hofmann, R.W., Jahufer, M.Z.Z.*, et al.* (2012). Multivariate associations of flavonoid and biomass accumulation in white clover (*Trifolium repens*) under drought. Funct. Plant Biol. 39, 167-177. https://doi.org/10.1071/FP11193.

Bannur, S.V., Kulgod, S.V., Metkar, S.S.*, et al.* (1999). Protein determination by ponceau S using digital color image analysis of protein spots on nitrocellulose membranes. Anal. Biochem. 267, 382-389. https://doi.org/10.1006/abio.1998.3020.

Bansode, R.R., Plundrich, N.J., Randolph, P.D.*, et al.* (2018). Peanut flour aggregation with polyphenolic extracts derived from peanut skin inhibits IgE binding capacity and attenuates RBL-2H3 cells degranulation via MAPK signaling pathway. Food Chem. 263, 307-314. https://doi.org/10.1016/j.foodchem.2018.05.007.

Barck, C., Lundahl, J., Halldén, G.*, et al.* (2005). Brief exposures to NO_2 augment the allergic inflammation in asthmatics. Environ. Res. 97, 58-66. https://doi.org/10.1016/j.envres.2004.02.009.

Barkla, B.J., Vera-Estrella, R., Hernandez-Coronado, M.*, et al.* (2009). Quantitative proteomics of the tonoplast reveals a role for glycolytic enzymes in salt tolerance. Plant Cell 21, 4044-4058. https://doi.org/10.1105/tpc.109.069211.

Barnes, C., Pacheco, F., Landuyt, J.*, et al.* (2001). The effect of temperature, relative humidity and rainfall on airborne ragweed pollen concentrations. Aerobiologia 17, 61-68. https://doi.org/10.1023/A:1007693032090.

Bashir, M.H., Mohamed, A.A., Muninnobpamasa, T.*, et al.* (2012). Ragweed pollen lipid is an immunoadjuvant of the humoral and cellular immune response in BALB/c mice. J. Allergy Clin. Immunol. 129, AB365. https://doi.org/10.1016/j.jaci.2012.01.006.

Baskin, J., Baskin, C. (1980). Ecophysiology of secondary dormancy in seeds of *Ambrosia Artemisiifolia*. Ecology 61, 475. https://doi.org/10.2307/1937410.

Bastia, T., Carotenuto, N., Basile, B.*, et al.* (2000). Induction of novel organelle DNA variation and transfer of resistance to frost and Verticillium wilt in *Solanum tuberosum* through somatic hybridization with 1EBN *S. commersonii*. Euphytica 116, 1-10. https://doi.org/10.1023/A:1003943704037.

Basu, S., Roychoudhury, A., Saha, P.P.*, et al.* (2010). Differential antioxidative responses of indica rice cultivars to drought stress. Plant Growth Regul. 60, 51-59. https://doi.org/10.1007/s10725-009-9418-4.

Baxter, A., Mittler, R., Suzuki, N. (2014). ROS as key players in plant stress signalling. J. Exp. Bot. 65, 1229-1240. https://doi.org/10.1093/jxb/ert375.

Bayden, A.S., Yakovlev, V.A., Graves, P.R.*, et al.* (2011). Factors influencing protein tyrosine nitration--structure-based predictive models. Free Radic. Biol. Med. 50, 749-762. https://doi.org/10.1016/j.freeradbiomed.2010.12.016.

Bazzaz, F.A. (1974). Ecophysiology of *Ambrosia artemisiifolia*: A successional dominant. Ecology 55, 112-119. https://doi.org/10.2307/1934623.

Beck, I., Jochner, S., Gilles, S.*, et al.* (2013). High environmental ozone levels lead to enhanced allergenicity of birch pollen. PLOS ONE 8, e80147. https://doi.org/10.1371/journal.pone.0080147.

Beggs, P.J. (2015). Environmental allergens: From asthma to hay fever and beyond. Curr. Clim. Change Rep. 1, 176-184. https://doi.org/10.1007/s40641-015-0018-2.

Behr, M., Neutelings, G., El-Jaziri, M.*, et al.* (2020). You want it sweeter: How glycosylation affects plant response to oxidative stress. Front. Plant Sci. 11, 571399. https://doi.org/10.3389/fpls.2020.571399.

Berg, G. (2009). Plant–microbe interactions promoting plant growth and health: Perspectives for controlled use of microorganisms in agriculture. Appl. Microbiol. Biotechnol. 84, 11-18. https://doi.org/10.1007/s00253-009-2092-7.

Bernacchi, C.J., Ruiz-Vera, U.M., Siebers, M.H.*, et al.* (2023). Short- and long-term warming events on photosynthetic physiology, growth, and yields of field grown crops. Biochem. J. 480, 999-1014. https://doi.org/10.1042/bcj20220433.

Bertić, M., Schroeder, H., Kersten, B.*, et al.* (2021). European oak chemical diversity – from ecotypes to herbivore resistance. New Phytol. 232, 818-834. https://doi.org/10.1111/nph.17608.

Bertsch, U., Schlicher, T.B., Schröder, I.*, et al.* (1993). Sequence of mature phosphoglycerate kinase from spinach chloroplasts. Plant Physiol. 103, 1449-1450. https://doi.org/https://doi.org/10.1104/pp.103.4.1449.

Bita, C., Gerats, T. (2013). Plant tolerance to high temperature in a changing environment: Scientific fundamentals and production of heat stress-tolerant crops. Front. Plant Sci. 4, 273. https://doi.org/10.3389/fpls.2013.00273.

Blanca, M., Victorio-Puche, L., Garrido-Arandia, M.*, et al.* (2020). Pru p 9, a new allergen eliciting respiratory symptoms in subjects sensitized to peach tree pollen. PLOS ONE 15, e0230010. https://doi.org/10.1371/journal.pone.0232301.

Bocsan, I.C., Muntean, I.A., Ureche, C.*, et al.* (2019). Characterization of patients with allergic rhinitis to ragweed pollen in two distinct regions of Romania. Medicina 55, 712. https://doi.org/10.3390/medicina55110712.

Boldogh, I., Bacsi, A., Choudhury, B.K.*, et al.* (2005). ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. J. Clin. Invest. 115, 2169-2179. https://doi.org/10.1172/jci24422.

Bonofiglio, T., Orlandi, F., Ruga, L.*, et al.* (2013). Climate change impact on the olive pollen season in Mediterranean areas of Italy: Air quality in late spring from an allergenic point of view. Environ. Monit. Assess. 185, 877-890. https://doi.org/10.1007/s10661-012-2598-9.

Bonura, A., Gulino, L., Trapani, A.*, et al.* (2008). Isolation, expression and immunological characterization of a calcium-binding protein from *Parietaria* pollen. Mol. Immunol. 45, 2465-2473. https://doi.org/10.1016/j.molimm.2008.01.009.

Bordas-Le Floch, V., Le-Mignon, M., Bouley, J.*, et al.* (2015). Identification of novel short ragweed pollen allergens using combined transcriptomic and immunoproteomic approaches. PLOS ONE 10, e0136258. https://doi.org/10.1371/journal.pone.0136258.

Borghi, M., Perez de-Souza, L., Yoshida, T.*, et al.* (2019). Flowers and climate change: A metabolic perspective. New Phytol. 224, 1425-1441. https://doi.org/10.1111/nph.16031.

Boulet, L.P., Turcotte, H., Laprise, C.*, et al.* (1997). Comparative degree and type of sensitization to common indoor and outdoor allergens in subjects with allergic rhinitis and/or asthma. Clin. Exp. Allergy 27, 52-59. https://doi.org/10.1111/j.1365-2222.1997.tb00672.x.

Bouley, J., Groeme, R., Le-Mignon, M.*, et al.* (2015). Identification of the cysteine protease Amb a 11 as a novel major allergen from short ragweed. J. Allergy Clin. Immunol. 136, 1055-1064. https://doi.org/10.1016/j.jaci.2015.03.001.

Bousquet, J., Khaltaev, N., Cruz, A.A.*, et al.* (2008). Allergic rhinitis and its impact on asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). Allergy 63 Suppl 86, 8-160. https://doi.org/10.1111/j.1398-9995.2007.01620.x.

Bousquet, P.J., Castelli, C., Daures, J.P.*, et al.* (2010). Assessment of allergen sensitization in a general population-based survey (European Community Respiratory Health Survey I). Ann. Epidemiol. 20, 797-803. https://doi.org/10.1016/j.annepidem.2010.05.012.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254. https://doi.org/10.1006/abio.1976.9999.

Brandes, D., Nitzsche, J. (2006). Biology, introduction, dispersal, and distribution of common ragweed (*Ambrosia artemisiifolia* L.) with special regard to Germany. Nachrichtenblatt-Deutschen Pflanzenschutzdienstes Braunschweig 58, 286-291. http://www.digibib.tu-bs.de/?docid=00016647

Breen, S., Williams, S.J., Outram, M., *et al.* (2017). Emerging insights into the functions of pathogenesis-related protein 1. Trends Food Sci. 22, 871-879. https://doi.org/10.1016/j.tplants.2017.06.013.

Breitenbach, M., Simon-Nobbe, B. (2002). The allergens of *Cladosporium herbarum* and *Alternaria alternata*, in: Breitenbach, M., Crameri, R., *et al.* (Eds.), Chem. Immunol., pp 48-72. https://doi.org/10.1159/000058862.

Breiteneder, H., Ferreira, F., Reikerstorfer, A., *et al.* (1992). Complementary DNA cloning and expression in *Escherichia coli* of *Aln g* I, the major allergen in pollen of alder (*Alnus glutinosa*). J. Allergy Clin. Immunol. 90, 909-917. https://doi.org/10.1016/0091-6749(92)90463-c.

Breton-Larrivée, M., Elder, E., McGraw, S. (2019). DNA methylation, environmental exposures and early embryo development. Anim. Reprod. 16, 465-474. https://doi.org/10.21451/1984-3143-ar2019-0062.

Breton, M.C., Garneau, M., Fortier, I., *et al.* (2006). Relationship between climate, pollen concentrations of *Ambrosia* and medical consultations for allergic rhinitis in Montreal, 1994–2002. Sci. Total Environ. 370, 39-50. https://doi.org/10.1016/j.scitotenv.2006.05.022.

Buhner, S., Schäuffele, S., Giesbertz, P., *et al.* (2023). Allergen-free extracts from birch, ragweed, and hazel pollen activate human and guinea-pig submucous and spinal sensory neurons. Neurogastroenterol. Motil. 35, e14559. https://doi.org/10.1111/nmo.14559.

Bullock, J.M., Chapman, D., Schäfer, S., *et al.* (2012). Assessing and controlling the spread and the effects of common ragweed in Europe.

Bundesamt, U. (2021). GE-I-3: Contamination with pollen of ragweed. 06.02.2024. https://www.umweltbundesamt.de/en/topics/climate-energy/climate-impacts-adaptation/impacts-of-climate-change/monitoring-report-2019/indicators-of-climate-change-impacts-adaptation/cluster-human-health/ge-i-3-contamination-pollen-of-ragweed#allergenic-plants-gaining-ground.

Burbach, G.J., Heinzerling, L.M., Röhnelt, C., *et al.* (2009). Ragweed sensitization in Europe - GA²LEN study suggests increasing prevalence. Allergy 64, 664-665. https://doi.org/10.1111/j.1398-9995.2009.01975.x.

Burge, H.A., Rogers, C.A. (2000). Outdoor allergens. Environ. Health Perspect. 108 Suppl 4, 653-659. https://doi.org/10.1289/ehp.00108s465.

Burghardt, L.T., Runcie, D.E., Wilczek, A.M., *et al.* (2016). Fluctuating, warm temperatures decrease the effect of a key floral repressor on flowering time in *Arabidopsis thaliana*. New Phytol. 210, 564-576. https://doi.org/10.1111/nph.13799.

Buters, J., Alberternst, B., Nawrath, S., *et al.* (2015). *Ambrosia artemisiifolia* (ragweed) in Germany – current presence, allergological relevance and containment procedures. Allergo J. Int. 24, 108-120. https://doi.org/10.1007/s40629-015-0060-6.

Buters, J.T., Kreyling, W.G., Behrendt, H. (2005). Bet v 1 monitoring in outdoor air. J. Allergy Clin. Immunol. 115, S98. https://doi.org/10.1016/j.jaci.2004.12.407.

Buters, J.T.M., Kasche, A., Weichenmeier, I., *et al.* (2008). Year-to-year variation in release of Bet v 1 allergen from birch pollen: Evidence for geographical differences

between West and South Germany. Int. Arch. Allergy Immunol. 145, 122-130. https://doi.org/10.1159/000108137.

Buzan, M.R., Zbîrcea, L.E., Gattinger, P., *et al.* (2022). Complex IgE sensitization patterns in ragweed allergic patients: Implications for diagnosis and specific immunotherapy. Clin. Transl. Allergy 12, e12179. https://doi.org/10.1002/clt2.12179.

Cadot, P., Díaz, J.F., Proost, P., *et al.* (2000). Purification and characterization of an 18-kd allergen of birch (*Betula verrucosa*) pollen: Identification as a cyclophilin. J. Allergy Clin. Immunol. 105, 286-291. https://doi.org/10.1016/S0091-6749(00)90078-2.

Cai, G., Ovidi, E., Romagnoli, S., *et al.* (2005). Identification and characterization of plasma membrane proteins that bind to microtubules in pollen tubes and generative cells of tobacco. Plant Cell Physiol. 46, 563-578. https://doi.org/10.1093/pcp/pci060.

Cai, Y.M., Yu, J., Ge, Y., *et al.* (2018). Two proteases with caspase-3-like activity, cathepsin B and proteasome, antagonistically control ER-stress-induced programmed cell death in *Arabidopsis*. New Phytol. 218, 1143-1155. https://doi.org/10.1111/nph.14676.

Campos, M., Markham, K.R., Mitchell, K.A., *et al.* (1997). An approach to the characterization of bee pollens via their flavonoid/phenolic profiles. Phytochem. Anal. 8, 181-185. https://doi.org/10.1002/(SICI)1099-1565(199707)8:4<181::AID-PCA359>3.0.CO;2-A.

Capovilla, G., Pajoro, A., Immink, R.G.H., *et al.* (2015a). Role of alternative pre-mRNA splicing in temperature signaling. Curr. Opin. Plant Biol. 27, 97-103. https://doi.org/10.1016/j.pbi.2015.06.016.

Capovilla, G., Schmid, M., Posé, D. (2015b). Control of flowering by ambient temperature. J. Exp. Bot. 66, 59-69. https://doi.org/10.1093/jxb/eru416.

Caraballo, L., Valenta, R., Puerta, L., *et al.* (2020). The allergenic activity and clinical impact of individual IgE-antibody binding molecules from indoor allergen sources. World Allergy Organ J. 13, 100118. https://doi.org/10.1016/j.waojou.2020.100118.

Carosso, A., Gallesio, M.T. (2000). Allergy to ragweed: Clinical relevance in Turin. Aerobiologia 16, 155-158. https://doi.org/10.1023/A:1007663506535.

Castell, M., Perez-Cano, F.J., Abril-Gil, M., *et al.* (2014). Flavonoids on allergy. Curr. Pharm. Des. 20, 973-987. https://doi.org/10.2174/13816128113199990041.

Cavaiuolo, M., Cocetta, G., Ferrante, A. (2013). The antioxidants changes in ornamental flowers during development and senescence. Antioxidants 2, 132-155. https://doi.org/10.3390/antiox2030132.

Cecchi, L., Malaspina, T.T., Albertini, R., *et al.* (2007). The contribution of long-distance transport to the presence of *Ambrosia* pollen in central northern Italy. Aerobiologia 23, 145-151. https://doi.org/10.1007/s10453-007-9060-4.

Celenk, S. (2019). Detection of reactive allergens in long-distance transported pollen grains: Evidence from *Ambrosia*. Atmos. Environ. 209, 212-219. https://doi.org/10.1016/j.atmosenv.2019.04.040.

Celenk, S., Malyer, H. (2017). The occurrence of *Ambrosia* pollen in the atmosphere of Northwest Turkey: Investigation of possible source regions. Int. J. Biometeorol. 61, 1499-1510. https://doi.org/10.1007/s00484-017-1328-y.

Chae, K., Lord, E.M. (2011). Pollen tube growth and guidance: Roles of small, secreted proteins. Ann. Bot. 108, 627-636. https://doi.org/10.1093/aob/mcr015.

Chaki, M., Valderrama, R., Fernández-Ocaña, A.M., *et al.* (2011). High temperature triggers the metabolism of S-nitrosothiols in sunflower mediating a process of nitrosative stress which provokes the inhibition of ferredoxin-NADP reductase by tyrosine nitration. Plant, Cell Environ. 34, 1803-1818. https://doi.org/10.1111/j.1365-3040.2011.02376.x.

Chakraborty, U., Pradhan, D. (2011). High temperature-induced oxidative stress in *Lens culinaris*, role of antioxidants and amelioration of stress by chemical pre-treatments. J. Plant Interact. 6, 43-52. https://doi.org/10.1080/17429145.2010.513484.

Chan-Yeung, M., Anthonisen, N.R., Becklake, M.R., *et al.* (2010). Geographical variations in the prevalence of atopic sensitization in six study sites across Canada. Allergy 65, 1404-1413. https://doi.org/10.1111/j.1398-9995.2010.02399.x.

Chang, C.Y., Chou, H., Tam, M., *et al.* (2002). Characterization of enolase allergen from *Rhodotorula mucilaginosa*. J. Biomed. Sci. 9, 645-655. https://doi.org/10.1159/000067279.

Chaudhary, P., Agri, U., Chaudhary, A., *et al.* (2022). Endophytes and their potential in biotic stress management and crop production. Front Microbiol 13, 933017. https://doi.org/10.3389/fmicb.2022.933017.

Chaudhary, S., Devi, P., Bhardwaj, A., *et al.* (2020). Identification and characterization of contrasting genotypes/cultivars for developing heat tolerance in agricultural crops: Current status and prospects. Front. Plant Sci. 11, 587264. https://doi.org/10.3389/fpls.2020.587264.

Chen, J.C., Chiu, L.L., Lee, K.L., *et al.* (2012). Identification of critical amino acids in an Immunodominant IgE epitope of Pen c 13, a major allergen from *Penicillium citrinum*. PLOS ONE 7, e34627. https://doi.org/10.1371/journal.pone.0034627.

Chen, K.W., Marusciac, L., Tamas, P.T., *et al.* (2018a). Ragweed pollen allergy: Burden, characteristics, and management of an imported allergen source in Europe. Int. Arch. Allergy Immunol. 176, 163-180. https://doi.org/10.1159/000487997.

Chen, M., Xu, J., Devis, D., *et al.* (2016). Origin and functional prediction of pollen allergens in plants Plant Physiol. 172, 341-357. https://doi.org/10.1104/pp.16.00625.

Chen, N., Qu, X., Wu, Y., *et al.* (2009). Regulation of actin dynamics in pollen tubes: Control of actin polymer level. J. Integr. Plant Biol. 51, 740-750. https://doi.org/10.1111/j.1744-7909.2009.00850.x.

Chen, N., Shao, Q., Xiong, Z. (2023). Isolation and characterization of a pathogenesis-related protein 1 (*SlPR1*) gene with induced expression in tomato (*Solanum lycopersicum*) during *Ralstonia solanacearum* infection. Gene 855, 147105. https://doi.org/10.1016/j.gene.2022.147105.

Chen, P., Li, Y., Zhao, L., *et al.* (2017). Genome-wide identification and expression profiling of ATP-binding cassette (ABC) transporter gene family in pineapple (*Ananas comosus* (L.) Merr.) reveal the role of *AcABCG38* in pollen development. Front. Plant Sci. 8, 2150. https://doi.org/10.3389/fpls.2017.02150.

Chen, W., Gong, P., Guo, J.*, et al.* (2018b). Glycolysis regulates pollen tube polarity via Rho GTPase signaling. PLOS Genet. 14, e1007373. https://doi.org/10.1371/journal.pgen.1007373.

Cheng, X., Frank, U., Zhao, F.*, et al.* (2023). Plant growth traits and allergenic potential of *Ambrosia artemisiifolia* pollen as modified by temperature and NO_2. Environ. Exp. Bot. 206, 105193. https://doi.org/10.1016/j.envexpbot.2022.105193.

Chiu, L.L., Lee, K.L., Lin, Y.F.*, et al.* (2008). Secretome analysis of novel IgE-binding proteins from *Penicillium citrinum*. Proteomics Clin. Appl. 2, 33-45. https://doi.org/10.1002/prca.200780050.

Cho, L.H., Yoon, J., An, G. (2017). The control of flowering time by environmental factors. Plant J. 90, 708-719. https://doi.org/10.1111/tpj.13461.

Choi, D.H., Ban, H.Y., Seo, B.S.*, et al.* (2016). Phenology and seed yield performance of determinate soybean cultivars grown at elevated temperatures in a temperate region. PLOS ONE 11, e0165977. https://doi.org/10.1371/journal.pone.0165977.

Choi, H., Ohyama, K., Kim, Y.Y.*, et al.* (2014). The role of *Arabidopsis* ABCG9 and ABCG31 ATP binding cassette transporters in pollen fitness and the deposition of steryl glycosides on the pollen coat. Plant Cell 26, 310-324. https://doi.org/10.1105/tpc.113.118935.

Choi, Y.J., Lee, K.S., Oh, J.W. (2021). The impact of climate change on pollen season and allergic sensitization to pollens. Immunol. Allergy Clin. North Am. 41, 97-109. https://doi.org/10.1016/j.iac.2020.09.004.

Choi, Y.J., Oh, H.R., Oh, J.W.*, et al.* (2018). Chamber and field studies demonstrate differential Amb a 1 contents in common ragweed depending on CO_2 levels. Allergy, Asthma Immunol. Res. 10, 278-282. https://doi.org/10.4168/aair.2018.10.3.278.

Choi, Y.W., Hyde, K.D., Ho, W.H. (1999). Single spore isolation of fungi. 29-38.

Chowdhary, A., Randhawa, H.S., Singh, V.*, et al.* (2011). Bipolaris hawaiiensis as etiologic agent of allergic bronchopulmonary mycosis: First case in a paediatric patient. Med. Mycol. 49, 760-765. https://doi.org/10.3109/13693786.2011.566895.

Chutipaijit, S., Cha-Um, S., Sompornpailin, K. (2009). Differential accumulations of proline and flavonoids in indica rice varieties against salinity. Pak. J. Bot. 41, 2497-2506. https://api.semanticscholar.org/CorpusID:3390419

Ciappetta, S. (2016). Study of intra and inter population variability of common ragweed (*Ambrosia artemisiifolia* L.) in relation to Amb a 1 isoforms and their allergenicity. Università degli Studi di Milano-Bicocca. https://hdl.handle.net/10281/101832

Claude, L., Yvon, J., Goursaud, D.M.A. (2007). How did common ragweed (*Ambrosia artemisiifolia* L.) spread in Québec? A historical analysis using herbarium records. J. Biogeogr. 34, 1751-1761. https://doi.org/doi:10.1111/j.1365-2699.2007.01730.x.

Codina, R., Lockey, R.F. (2017). Pollen used to produce allergen extracts. Ann. Allergy, Asthma, Immunol. 118, 148-153. https://doi.org/10.1016/j.anai.2016.02.005.

Comtois, P. (1998). Ragweed (*Ambrosia* sp.): The Phoenix of allergophytes. 6th International Congress on Aerobiology. Satellite Symposium Proceedings: Ragweed in Europe, Perugia, Italy, ALK Abelló. 3-5.

Conde, A., Chaves, M.M., Gerós, H. (2011). Membrane transport, sensing and signaling in plant adaptation to environmental stress. Plant Cell Physiol. 52, 1583-1602. https://doi.org/10.1093/pcp/pcr107.

Conway, E.M., Liu, L.L., Nowakowski, B.*, et al.* (1995). Heat Shock-sensitive Expression of Calreticulin.: *in vitro* and *in vivo* up-regulation. J. Biol. Chem. 270, 17011-17016. https://doi.org/10.1074/jbc.270.28.17011.

Cooper, G.M. (2000). The central role of enzymes as biological catalysts, The cell: A molecular approach, 2nd ed. Sinauer Associates, Sunderland (MA). https://www.ncbi.nlm.nih.gov/books/NBK9921/

Corpas, F.J., Chaki, M., Leterrier, M.*, et al.* (2009). Protein tyrosine nitration: A new challenge in plants. Plant Signal Behav. 4, 920-923. https://doi.org/10.4161/psb.4.10.9466.

Crameri, R. (1998). Recombinant *Aspergillus fumigatus* allergens: From the nucleotide sequences to clinical applications. Int. Arch. Allergy Immunol. 115, 99-114. https://doi.org/10.1159/000023889.

Cuinica, L.G., Abreu, I., Esteves-Da Silva, J. (2014). Effect of air pollutant NO_2 on *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* pollen fertility and human allergenicity. Environ. Pollut. 186, 50-55. https://doi.org/10.1016/j.envpol.2013.12.001.

D'Amato, G., Cecchi, L., Bonini, S.*, et al.* (2007). Allergenic pollen and pollen allergy in Europe. Allergy 62, 976-990. https://doi.org/10.1111/j.1398-9995.2007.01393.x.

Dahl, Å. (2018). Pollen lipids can play a role in allergic airway inflammation. Front. Immunol. 9, 2816. https://doi.org/10.3389/fimmu.2018.02816.

Dahl, Å., Galán, C., Hajkova, L.*, et al.* (2013). The onset, course and intensity of the pollen season, Allergenic pollen: A review of the production, release, distribution and health impacts, pp 29-70. https://doi.org/10.1007/978-94-007-4881-1_3.

Dai, S., Li, L., Chen, T.*, et al.* (2006). Proteomic analyses of Oryza sativa mature pollen reveal novel proteins associated with pollen germination and tube growth. Proteomics 6, 2504-2529. https://doi.org/10.1002/pmic.200401351.

Dall'antonia, F., Pavkov-Keller, T., Zangger, K.*, et al.* (2014). Structure of allergens and structure based epitope predictions. Methods 66, 3-21. https://doi.org/10.1016/j.ymeth.2013.07.024.

Damji, R., Mukherji, A., Mussani, F. (2019). Sporobolomyces salmonicolor: A case report of a rare cutaneous fungal infection. SAGE Open Med. Case Rep. 7, 2050313x19844154. https://doi.org/10.1177/2050313x19844154.

Davis, J., Gillaspie, A.G., Morris, J.B.*, et al.* (2008). Flavonoid content in different legume germplasm seeds quantified by HPLC. Plant Genet. Resour. 6, 62-69. https://doi.org/10.1017/S1479262108923807.

De-Pinto, M.C., Locato, V., Paradiso, A.*, et al.* (2015). Role of redox homeostasis in thermo-tolerance under a climate change scenario. Ann. Bot. 116, 487-496. https://doi.org/10.1093/aob/mcv071.

de Weger, L.A., Pashley, C.H., Šikoparija, B.*, et al.* (2016). The long distance transport of airborne *Ambrosia* pollen to the UK and the Netherlands from Central and

south Europe. Int. J. Biometeorol. 60, 1829-1839. https://doi.org/10.1007/s00484-016-1170-7.

Deen, W., Hunt, T., Swanton, C.J. (1998). Influence of temperature, photoperiod, and irradiance on the phenological development of common ragweed (*Ambrosia artemisiifolia*). Weed Sci. 46, 555-560. https://doi.org/10.1017/S0043174500091098.

Deen, W., Swanton, C.J., Hunt, L.A. (2001). A mechanistic growth and development model of common ragweed. Weed Sci. 49, 723-731. https://doi.org/10.1614/0043-1745(2001)049[0723:AMGADM]2.0.CO;2.

Del-Mondo, A., Sansone, C., Brunet, C. (2022). Insights into the biosynthesis pathway of phenolic compounds in microalgae. Comput. Struct. Biotechnol. J. 20, 1901-1913. https://doi.org/10.1016/j.csbj.2022.04.019.

DellaValle, C.T., Triche, E.W., Leaderer, B.P.*, et al.* (2012). Effects of ambient pollen concentrations on frequency and severity of asthma symptoms among asthmatic children. Epidemiology 23, 55-63. https://doi.org/10.1097/EDE.0b013e31823b66b8.

Denning, D.W., O'Driscoll, B.R., Hogaboam, C.M.*, et al.* (2006). The link between fungi and severe asthma: A summary of the evidence. Eur. Respir. J. 27, 615-626. https://doi.org/10.1183/09031936.06.00074705.

Diagne, N., Ngom, M., Djighaly, P.I.*, et al.* (2020). Roles of arbuscular mycorrhizal fungi on plant growth and performance: Importance in biotic and abiotic stressed regulation. Diversity 12, 370. https://doi.org/10.3390/d12100370.

Díaz-Mendoza, M., Velasco-Arroyo, B., González-Melendi, P.*, et al.* (2014). C1A cysteine protease-cystatin interactions in leaf senescence. J. Exp. Bot. 65, 3825-3833. https://doi.org/10.1093/jxb/eru043.

Dixon, R.A., Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085-1097. https://doi.org/10.1105/tpc.7.7.1085.

DPA, Local, T. (2020). Ragweed infestation in southern Germany poses allergy threat. 21.07.2020. The Local. https://www.thelocal.de/20200721/ragweed-infestation-in-southern-germany-poses-threat-to.

Dugo, P., Presti, M.L., Ohman, M.*, et al.* (2005). Determination of flavonoids in citrus juices by micro-HPLC-ESI/MS. J. Sep. Sci. 28, 1149-1156. https://doi.org/10.1002/jssc.200500053.

Eckl-Dorna, J., Klein, B., Reichenauer, T.G.*, et al.* (2010). Exposure of rye (*Secale cereale*) cultivars to elevated ozone levels increases the allergen content in pollen. J. Allergy Clin. Immunol. 126, 1315-1317. https://doi.org/10.1016/j.jaci.2010.06.012.

El-Khallal, S. (2007). Induction and modulation of resistance in tomato plants against Fusarium wilt disease by bioagent fungi (Arbuscular mycorrhiza) and/or hormonal elicitors (jasmonic acid& salicylic acid): 1- Changes in growth, some metabolic activities and endogenous hormones related to defence mechanism. Aust J Basic Appl Sci 1, 717-732.

El-Ghouizi, A., Bakour, M., Laaroussi, H.*, et al.* (2023). Bee pollen as functional food: Insights into its composition and therapeutic properties. Antioxidants 12, 557. https://doi.org/10.3390/antiox12030557.

El-Kelish, A., Zhao, F., Heller, W.*, et al.* (2014). Ragweed (*Ambrosia artemisiifolia*) pollen allergenicity: SuperSAGE transcriptomic analysis upon elevated CO_2 and drought stress. BMC Plant Biol. 14, 176. https://doi.org/10.1186/1471-2229-14-176.

Eljebbawi, A., Guerrero, Y.D.C.R., Dunand, C.*, et al.* (2021). Highlighting reactive oxygen species as multitaskers in root development. Iscience 24, 101978. https://doi.org/10.1016/j.isci.2020.101978.

Eltayeb, A.E., Kawano, N., Badawi, G.H.*, et al.* (2007). Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Planta 225, 1255-1264. https://doi.org/10.1007/s00425-006-0417-7.

Elwell, A.L., Gronwall, D.S., Miller, N.D.*, et al.* (2011). Separating parental environment from seed size effects on next generation growth and development in *Arabidopsis*. Plant, Cell Environ. 34, 291-301. https://doi.org/10.1111/j.1365-3040.2010.02243.x.

Energy, D.o., Change, C. (2013). Digest of UK energy statistics.

Engel, E., Richter, K., Obermeyer, G.*, et al.* (1997). Immunological and biological properties of Bet v 4, a novel birch pollen allergen with two EF-hand calcium-binding domains. J. Biol. Chem. 272, 28630-28637. https://doi.org/10.1074/jbc.272.45.28630.

EPA (2023). Climate change: Greenhouse gas emissions. 13.04.2023. https://www.epa.gov/ghgemissions/overview-greenhouse-gases.

Essl, F., Biró, K., Brandes, D.*, et al.* (2015). Biological flora of the British Isles: *Ambrosia artemisiifolia*. J. Ecol. 103, 1069-1098. https://doi.org/10.1111/1365-2745.12424.

Essl, F., Dullinger, S., Kleinbauer, I. (2009). Changes in the spatio-temporal patterns and habitat preferences of *Ambrosia artemisiifolia* during its invasion of Austria. Preslia 81, 119-133. http://www.preslia.cz/P092Essl.pdf

Exposito-Rodriguez, M., Laissue, P.P., Yvon-Durocher, G.*, et al.* (2017). Photosynthesis-dependent H_2O_2 transfer from chloroplasts to nuclei provides a high-light signalling mechanism. Nat. Commun. 8, 49. https://doi.org/10.1038/s41467-017-00074-w.

Fahad, S., Hussain, S., Saud, S.*, et al.* (2016). Exogenously applied plant growth regulators enhance the morpho-physiological growth and yield of rice under high temperature. Front. Plant Sci. 7, 1250. https://doi.org/10.3389/fpls.2016.01250.

Fairs, A., Wardlaw, A.J., Thompson, J.R.*, et al.* (2010). Guidelines on ambient intramural airborne fungal spores. J. Investig. Allergol. Clin. Immunol. 20, 490-498. http://www.ncbi.nlm.nih.gov/pubmed/21243933

Falk, W., Mellert, K.H. (2011). Species distribution models as a tool for forest management planning under climate change: Risk evaluation of *Abies alba* in Bavaria. J. Veg. Sci. 22, 621-634. https://doi.org/10.1111/j.1654-1103.2011.01294.x.

Farnese, F.S., Menezes-Silva, P.E., Gusman, G.S.*, et al.* (2016). When bad guys become good ones: The key role of reactive oxygen species and nitric oxide in the plant responses to abiotic stress. Front. Plant Sci. 7, 471. https://doi.org/10.3389/fpls.2016.00471.

Farooq, S., Onen, H., Ozaslan, C.*, et al.* (2019). Seed germination niche for common ragweed (*Ambrosia artemisiifolia* L.) populations naturalized in Turkey. S. Afr. J. Bot. 123, 361-371. https://doi.org/10.1016/j.sajb.2019.03.031.

Fatemi, S., Haelewaters, D., Urbina, H.*, et al.* (2022). *Sporobolomyces lactucae* sp. nov. (Pucciniomycotina, Microbotryomycetes, Sporidiobolales): An abundant component of romaine lettuce phylloplanes. J. Fungi (Basel) 8, 302. https://doi.org/10.3390/jof8030302.

Fernández-Martínez, M., Belmonte, J., Maria-Espelta, J. (2012). Masting in oaks: Disentangling the effect of flowering phenology, airborne pollen load and drought. Acta Oecol. 43, 51-59. https://doi.org/10.1016/j.actao.2012.05.006.

Ferreira, S., Hjernø, K., Larsen, M.*, et al.* (2006). Proteome profiling of *Populus euphratica* Oliv. upon heat stress. Ann. Bot. 98, 361-377. https://doi.org/10.1093/aob/mcl106.

Finlay, R.D., Rosling, A., Gadd, G.M. (2006). Integrated nutrient cycles in boreal forest ecosystems-the role of mycorrhizal fungi, Fungi in biogeochemical cycles. Cambridge University Press, pp 28-50. https://doi.org/10.1017/CBO9780511550522.003.

Forkel, S., Beutner, C., Heetfeld, A.*, et al.* (2020). Allergic rhinitis to weed pollen in Germany: Dominance by plantain, rising prevalence, and polysensitization rates over 20 years. Int. Arch. Allergy Immunol. 181, 128-135. https://doi.org/10.1159/000504297.

Foyer, C.H., Noctor, G. (2005). Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. Plant Cell 17, 1866-1875. https://doi.org/10.1105/tpc.105.033589.

Fracchia, S., Garcia-Romera, I., Godeas, A.*, et al.* (2000). Effect of the saprophytic fungus *Fusarium oxysporum* on arbuscular mycorrhizal colonization and growth of plants in greenhouse and field trials. Plant Soil 223, 177-186. https://doi.org/10.1023/A:1004848504918.

Frenz, D.A. (1999). Volumetric ragweed pollen data for eight cities in the continental United States. Ann. Allergy, Asthma, Immunol. 82, 41-46. https://doi.org/10.1016/s1081-1206(10)62658-0.

Gadermaier, G., Wopfner, N., Wallner, M.*, et al.* (2008). Array-based profiling of ragweed and mugwort pollen allergens. Allergy 63, 1543-1549. https://doi.org/10.1111/j.1398-9995.2008.01780.x.

Galveias, A., Arriegas, R., Mendes, S.*, et al.* (2021). Air pollutants NO_2- and O_3-induced *Dactylis glomerata* L. pollen oxidative defences and enhanced its allergenic potential. Aerobiologia 37, 127-137. https://doi.org/10.1007/s10453-020-09676-2.

Gao, H., Ma, K., Ji, G.*, et al.* (2022). Lipid transfer proteins involved in plant-pathogen interactions and their molecular mechanisms. Mol. Plant Pathol. 23, 1815-1829. https://doi.org/10.1111/mpp.13264.

Gard, B. (2012). Ecological and evolutionary processes influencing the colonization of common ragweed (*Ambrosia artemisiifolia* L.) in France.

Garmatiuk, T., Swoboda, I., Twardosz-Kropfmüller, A.*, et al.* (2013). Characterization of mutants of a highly cross-reactive calcium-binding protein from *Brassica* pollen for

allergen-specific immunotherapy. Immunobiology 218, 1155-1165. https://doi.org/10.1016/j.imbio.2013.04.006.

Gayatri S, G., Ashok P, G., Vidya S, G. (2012). Gene expression profiling during wilting in chickpea caused by *Fusarium oxysporum* f. sp. *ciceri* Am. J. Plant Sci. 3, 17540. https://doi.org/10.4236/ajps.2012.32023.

Ge, Y., Cai, Y.M., Bonneau, L.*, et al.* (2016). Inhibition of cathepsin B by caspase-3 inhibitors blocks programmed cell death in *Arabidopsis*. Cell Death Differ. 23, 1493-1501. https://doi.org/10.1038/cdd.2016.34.

Geldner, N. (2004). The plant endosomal system—its structure and role in signal transduction and plant development. Planta 219, 547-560. https://doi.org/10.1007/s00425-004-1302-x.

Gelebart, P., Opas, M., Michalak, M. (2005). Calreticulin, a Ca^{2+}-binding chaperone of the endoplasmic reticulum. Int. J. Biochem. Cell Biol. 37, 260-266. https://doi.org/10.1016/j.biocel.2004.02.030.

Gentili, R., Ambrosini, R., Montagnani, C.*, et al.* (2018). Effect of soil pH on the growth, reproductive investment and pollen allergenicity of *Ambrosia artemisiifolia* L. Front. Plant Sci. 9, 1335. https://doi.org/10.3389/fpls.2018.01335.

Gentili, R., Asero, R., Caronni, S.*, et al.* (2019). *Ambrosia artemisiifolia* L. temperature-responsive traits influencing the prevalence and severity of pollinosis: A study in controlled conditions. BMC Plant Biol. 19, 155. https://doi.org/10.1186/s12870-019-1762-6.

Ghiani, A., Aina, R., Asero, R.*, et al.* (2012). Ragweed pollen collected along high-traffic roads shows a higher allergenicity than pollen sampled in vegetated areas. Allergy 67, 887-894. https://doi.org/10.1111/j.1398-9995.2012.02846.x.

Ghirardo, A., Fochi, V., Lange, B.*, et al.* (2020a). Metabolomic adjustments in the orchid mycorrhizal fungus *Tulasnella calospora* during symbiosis with *Serapias vomeracea*. New Phytol. 228, 1939-1952. https://doi.org/10.1111/nph.16812.

Ghirardo, A., Lindstein, F., Koch, K.*, et al.* (2020b). Origin of volatile organic compound emissions from subarctic tundra under global warming. Global Change Biol. 26, 1908-1925. https://doi.org/10.1111/gcb.14935.

Ghosh, B., Perry, M.P., Rafnar, T.*, et al.* (1993). Cloning and expression of immunologically active recombinant Amb a V allergen of short ragweed (*Ambrosia artemisiifolia*) pollen. J. Immunol. 150, 5391-5399. https://doi.org/10.4049/jimmunol.150.12.5391.

Ghosh, D., Mueller, G.A., Schramm, G.*, et al.* (2014). Primary identification, biochemical characterization, and immunologic properties of the allergenic pollen cyclophilin cat R 1. J. Biol. Chem. 289, 21374-21385. https://doi.org/10.1074/jbc.M114.559971.

Giegé, P., Heazlewood, J.L., Roessner-Tunali, U.*, et al.* (2003). Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. Plant Cell 15, 2140-2151. https://doi.org/10.1105/tpc.012500.

Gill, S.S., Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909-930. https://doi.org/10.1016/j.plaphy.2010.08.016.

Gilles, S., Behrendt, H., Ring, J., *et al.* (2012). The pollen enigma: Modulation of the allergic immune response by non-allergenic, pollen-derived compounds. Curr. Pharm. Des. 18, 2314-2319. https://doi.org/10.2174/138161212800166040.

Gilles, S., Fekete, A., Zhang, X., *et al.* (2011). Pollen metabolome analysis reveals adenosine as a major regulator of dendritic cell-primed T_H cell responses. J. Allergy Clin. Immunol. 127, 454-461.e9. https://doi.org/10.1016/j.jaci.2010.12.1082.

Giri, B., Saxena, B. (2017). Response of arbuscular mycorrhizal fungi to global climate change and their role in terrestrial ecosystem C and N cycling. https://doi.org/10.1007/978-3-319-53064-2_15.

Gómez-Esquivel, M.L., Guidos-Fogelbach, G.A., Rojo-Gutiérrez, M.I., *et al.* (2021). Identification of an allergenic calmodulin from *Amaranthus palmeri* pollen. Mol. Immunol. 132, 150-156. https://doi.org/10.1016/j.molimm.2021.01.031.

Gong, Z., Chen, M., Miao, J., *et al.* (2022). Calreticulin as an adjuvant *in vivo* to promote dendritic cell maturation and enhance antigen-specific T lymphocyte responses against melanoma. J. Immunol. Res. 2022, 8802004. https://doi.org/10.1155/2022/8802004.

González-Pérez, S., Quijano, C., Romero, N., *et al.* (2008). Peroxynitrite inhibits electron transport on the acceptor side of higher plant photosystem II. Arch. Biochem. Biophys. 473, 25-33. https://doi.org/10.1016/j.abb.2008.02.020.

Gordon-Weeks, R., Sugars, J.M., Antoniw, J.F., *et al.* (1997). Accumulation of a novel PR1 protein in *Nicotiana langsdorfii* leaves in response to virus infection or treatment with salicylic acid. Physiol. Mol. Plant Pathol. 50, 263-273. https://doi.org/10.1006/pmpp.1997.0085.

Gorgini-Shabankareh, H., Khorasaninejad, S., Soltanloo, H., *et al.* (2021). Physiological response and secondary metabolites of three lavender genotypes under water deficit. Sci. Rep. 11, 19164. https://doi.org/10.1038/s41598-021-98750-x.

Górny, R.L., Reponen, T., Willeke, K., *et al.* (2002). Fungal fragments as indoor air biocontaminants. Appl. Environ. Microbiol. 68, 3522-3531. https://doi.org/10.1128/AEM.68.7.3522-3531.2002.

Gouot, J.C., Smith, J.P., Holzapfel, B.P., *et al.* (2019). Grape berry flavonoids: A review of their biochemical responses to high and extreme high temperatures. J. Exp. Bot. 70, 397-423. https://doi.org/10.1093/jxb/ery392.

Gow, A.J., Farkouh, C.R., Munson, D.A., *et al.* (2004). Biological significance of nitric oxide-mediated protein modifications. Am. J. Physiol. Lung Cell. Mol. Physiol. 287, L262-L268. https://doi.org/10.1152/ajplung.00295.2003.

Gray, S.B., Brady, S.M. (2016). Plant developmental responses to climate change. Dev. Biol. 419, 64-77. https://doi.org/10.1016/j.ydbio.2016.07.023.

Grewling, Ł., Bogawski, P., Kryza, M., *et al.* (2019). Concomitant occurrence of anthropogenic air pollutants, mineral dust and fungal spores during long-distance transport of ragweed pollen. Environ. Pollut. 254, 112948. https://doi.org/10.1016/j.envpol.2019.07.116.

Grijincu, M., Huțu, I., Weber, M., *et al.* (2023). Physicochemical and immunological characterization of Amb a 12, a novel ragweed (*Ambrosia artemisiifolia*) pollen allergen. Mol. Immunol. 157, 18-29. https://doi.org/10.1016/j.molimm.2023.03.012.

Groeme, R., Airouche, S., Kopečný, D.*, et al.* (2016). Structural and functional characterization of the major allergen Amb a 11 from short ragweed pollen J. Biol. Chem. 291, 13076-13087. https://doi.org/10.1074/jbc.M115.702001.

Grudkowska, M., Zagdańska, B. (2004). Multifunctional role of plant cysteine proteinases. Acta Biochim. Pol. 51, 609-624. https://doi.org/10.18388/abp.2004_3547.

Gruijthuijsen, Y.K., Grieshuber, I., Stöcklinger, A.*, et al.* (2006). Nitration enhances the allergenic potential of proteins. Int. Arch. Allergy Immunol. 141, 265-275. https://doi.org/10.1159/000095296.

Grutsch, S., Fuchs, J.E., Ahammer, L.*, et al.* (2017). Conformational flexibility differentiates naturally occurring Bet v 1 isoforms. Int. J. Mol. Sci. 18, 1192. https://doi.org/10.3390/ijms18061192.

Grutsch, S., Fuchs, J.E., Freier, R.*, et al.* (2014). Ligand binding modulates the structural dynamics and compactness of the major birch pollen allergen. Biophys. J. 107, 2972-2981. https://doi.org/10.1016/j.bpj.2014.10.062.

Guo, X., Zhang, D., Wang, Z.*, et al.* (2023). Cold-induced calreticulin OsCRT3 conformational changes promote OsCIPK7 binding and temperature sensing in rice. EMBO J. 42, e110518. https://doi.org/10.15252/embj.2021110518.

Gupta, P.K., Chand, R., Vasistha, N.K.*, et al.* (2018). Spot blotch disease of wheat: The current status of research on genetics and breeding. Plant Pathol. 67, 508-531. https://doi.org/10.1111/ppa.12781.

Gusmao, M., Siddique, K.H.M., Flower, K.*, et al.* (2012). Water deficit during the reproductive period of grass pea (*Lathyrus sativus* L.) reduced grain yield but maintained seed size. J. Agron. Crop Sci. 198, 430-441. https://doi.org/10.1111/j.1439-037X.2012.00513.x.

Guy, C. (1999). Molecular responses of plants to cold shock and cold acclimation. J. Mol. Microbiol. Biotechnol. 1, 231-242.

Gweon, H.S., Oliver, A., Taylor, J.*, et al.* (2015). PIPITS: An automated pipeline for analyses of fungal internal transcribed spacer sequences from the Illumina sequencing platform. Methods Ecol. Evol. 6, 973-980. https://doi.org/10.1111/2041-210X.12399.

Hakan, C., Muhittin, K., Muhammet, K.*, et al.* (2017). Flavonoid accumulation behavior in response to the abiotic stress: Can a uniform mechanism be illustrated for all plants?, in: Goncalo, C.J. (Ed.), Flavonoids. IntechOpen, Rijeka, p. Ch. 8. https://doi.org/10.5772/68093.

Hall, R.M., Urban, B., Durec, N.*, et al.* (2020). Regional adjustment of management options of common ragweed (*Ambrosia artemisiifolia*) along roadside verges in Bavaria (2018-2022). Julius-Kühn-Archiv, 295-300. https://doi.org/10.5073/jka.2020.464.045.

Hämäläinen, M., Nieminen, R., Vuorela, P.*, et al.* (2007). Anti-inflammatory effects of flavonoids: Genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression

and NO production in activated macrophages. Mediators Inflamm. 2007, 45673. https://doi.org/10.1155/2007/45673.

Han, D., Yu, Z., Lai, J., *et al.* (2022). Post-translational modification: A strategic response to high temperature in plants. aBIOTECH 3, 49-64. https://doi.org/10.1007/s42994-021-00067-w.

Harohalli Masthigowda, M., Sharma, D., Khobra, R., *et al.* (2022). Pollen viability as a potential trait for screening heat-tolerant wheat (*Triticum aestivum* L.). Funct. Plant Biol. 49, 625-633. https://doi.org/10.1071/FP21096.

Hartley, A., McLachlan, D., Hetherington, A., *et al.* (2012). High temperature exposure increases plant cooling capacity. Curr. Biol. 22, R396-R397. https://doi.org/10.1016/j.cub.2012.03.044.

Hasanuzzaman, M., Nahar, K., Rahman, A., *et al.* (2016). 4 - Soybean production and environmental stresses, in: Miransari, M. (Ed.), Environmental Stresses in Soybean Production. Academic Press, San Diego, pp 61-102. https://doi.org/10.1016/B978-0-12-801535-3.00004-8.

Hasanuzzaman, M., Nahar, K., Rahman, A., *et al.* (2017). Actions of biological trace elements in plant abiotic stress tolerance, in: Naeem, M., Ansari, A.A., *et al.* (Eds.), Essential Plant Nutrients: Uptake, Use Efficiency, and Management. Springer International Publishing, Cham, pp 213-274. https://doi.org/10.1007/978-3-319-58841-4_10.

Hatfield, J.L., Prueger, J.H. (2015). Temperature extremes: Effect on plant growth and development. Weather Clim. Extreme. 10, 4-10. https://doi.org/10.1016/j.wace.2015.08.001.

Hauser, M., Roulias, A., Ferreira, F., *et al.* (2010). Panallergens and their impact on the allergic patient. Allergy, Asthma, Clin. Immunol. 6, 1. https://doi.org/10.1186/1710-1492-6-1.

Hausfather, Z. (2019). Explainer: The high-emissions 'RCP8.5' global warming scenario. 09.09.2023. https://www.carbonbrief.org/explainer-the-high-emissions-rcp8-5-global-warming-scenario/.

Hayek, B., Vangelista, L., Pastore, A., *et al.* (1998). Molecular and immunologic characterization of a highly cross-reactive two EF-hand calcium-binding alder pollen allergen, Aln g 4: Structural basis for calcium-modulated IgE recognition. J. Immunol. 161, 7031-7039. https://doi.org/10.4049/jimmunol.161.12.7031.

Hayyan, M., Hashim, M.A., AlNashef, I.M. (2016). Superoxide ion: Generation and chemical implications. Chem. Rev. 116, 3029-3085. https://doi.org/10.1021/acs.chemrev.5b00407.

He, H., Liu, D., Zhang, N., *et al.* (2014). The PR10 gene family is highly expressed in *Lilium regale* Wilson during *Fusarium oxysporum* f. sp. *lilii* infection. Genes Genomics 36, 497-507. https://doi.org/10.1007/s13258-014-0185-x.

Heinzerling, L.M., Burbach, G.J., Edenharter, G., *et al.* (2009). GA2LEN skin test study I: GA²LEN harmonization of skin prick testing: novel sensitization patterns for inhalant allergens in Europe. Allergy 64, 1498-1506. https://doi.org/10.1111/j.1398-9995.2009.02093.x.

Hemmer, W., Schauer, U., Trinca, A., *et al.* (2011). Ragweed pollen allergy in Austria: A retrospective analysis of sensitization rates from 1997 to 2007. J. Allergy Clin. Immunol. 127, AB170. https://doi.org/10.1016/j.jaci.2010.12.675.

Hepler, P.K., Rounds, C.M., Winship, L.J. (2013). Control of cell wall extensibility during pollen tube growth. Mol. Plant 6, 998-1017. https://doi.org/10.1093/mp/sst10310.1093/mp/sst103.

Hesterberg, T.W., Bunn, W.B., McClellan, R.O., *et al.* (2009). Critical review of the human data on short-term nitrogen dioxide (NO_2) exposures: Evidence for NO_2 no-effect levels. Crit. Rev. Toxicol. 39, 743-781. https://doi.org/10.3109/10408440903294945.

Hinge, V., Tidke, J., Das, B., *et al.* (2017). Air pollution exacerbates effect of allergenic pollen proteins of *Cassia siamea*: A preliminary report. Grana 56, 147-154. https://doi.org/10.1080/00173134.2016.1193221.

Hinojosa, L., Matanguihan, J.B., Murphy, K.M. (2019). Effect of high temperature on pollen morphology, plant growth and seed yield in quinoa (*Chenopodium quinoa* Willd.). J. Agron. Crop Sci. 205, 33-45. https://doi.org/10.1111/jac.12302.

Höflich, C., Balakirski, G., Hajdu, Z., *et al.* (2016). Potential health risk of allergenic pollen with climate change associated spreading capacity: Ragweed and olive sensitization in two German federal states. Int. J. Hyg. Environ. Health 219, 252-260. https://doi.org/10.1016/j.ijheh.2016.01.007.

Holtgrefe, S., Gohlke, J., Starmann, J., *et al.* (2008). Regulation of plant cytosolic glyceraldehyde 3-phosphate dehydrogenase isoforms by thiol modifications. Physiol. Plant. 133, 211-228. https://doi.org/10.1111/j.1399-3054.2008.01066.x.

Holzmeister, C., Fröhlich, A., Sarioglu, H., *et al.* (2011). Proteomic analysis of defense response of wildtype *Arabidopsis thaliana* and plants with impaired NO-homeostasis. Proteomics 11, 1664-1683. https://doi.org/10.1002/pmic.201000652.

Hoppert, M., Mayer, F. (1999). Principles of macromolecular organization and cell function in bacteria and archaea. Cell Biochem. Biophys. 31, 247-284. https://doi.org/10.1007/BF02738242.

Howlett, B.J., Vithanage, H., Knox, R.B. (1981). Pollen antigens, allergens, and enzymes. Comment. Plant Sci. 2, 191-207.

Hrabovský, M., Ščevková, J., Mičieta, K., *et al.* (2016). Expansion and aerobiology of *Ambrosia artemisiifolia* L. in Slovakia. Ann. Agric. Environ. Med. 23, 64-70. https://doi.org/10.5604/12321966.1196854.

Huang, H., Ullah, F., Zhou, D.X., *et al.* (2019). Mechanisms of ROS regulation of plant development and stress responses. Front. Plant Sci. 10, 800. https://doi.org/10.3389/fpls.2019.00800.

Huecas, S., Villalba, M., Rodrıguez, R. (2001). Ole e 9, a major olive pollen allergen is a 1, 3-β-glucanase: Isolation, characterization, amino acid sequence, and tissue specificity. J. Biol. Chem. 276, 27959-27966. https://doi.org/10.1074/jbc.M103041200.

Hugouvieux-Cotte-Pattat, N., Condemine, G., Shevchik, V.E. (2014). Bacterial pectate lyases, structural and functional diversity. Environ. Microbiol. Rep. 6, 427-440. https://doi.org/10.1111/1758-2229.12166.

IPCC (2013). Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA. https://doi.org/10.1017/CBO9781107415324.

IPCC (2022). Cliamte Change 2022: Mitigation of Cimate Change. Contribution of Working Group III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. doi: 10.1017/9781009157926.

Ipsen, H., Løwenstein, H. (1983). Isolation and immunochemical characterization of the major allergen of birch pollen (*Betula verrucosa*). J. Allergy Clin. Immunol. 72, 150-159. https://doi.org/10.1016/0091-6749(83)90523-7.

Ischebeck, T., Valledor, L., Lyon, D.*, et al.* (2014). Comprehensive cell-specific protein analysis in early and late pollen development from diploid microsporocytes to pollen tube growth. Mol. Cell. Proteomics 13, 295-310. https://doi.org/10.1074/mcp.M113.028100.

Ischiropoulos, H. (1998). Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. Arch. Biochem. Biophys. 356, 1-11. https://doi.org/10.1006/abbi.1998.0755.

Ischiropoulos, H. (2003). Biological selectivity and functional aspects of protein tyrosine nitration. Biochem. Biophys. Res. Commun. 305, 776-783. https://doi.org/10.1016/s0006-291x(03)00814-3.

Iskusnykh, I.Y., Popova, T.N., Agarkov, A.A.*, et al.* (2013). Expression of glutathione peroxidase and glutathione reductase and level of free radical processes under toxic hepatitis in rats. J. Toxicol. 2013, 870628. https://doi.org/10.1155/2013/870628.

Ivanciuc, O., Garcia, T., Torres, M.*, et al.* (2009). Characteristic motifs for families of allergenic proteins. Mol. Immunol. 46, 559-568. https://doi.org/10.1016/j.molimm.2008.07.034.

Ivanciuc, O., Mathura, V., Midoro-Horiuti, T.*, et al.* (2003). Detecting potential IgE-reactive sites on food proteins using a sequence and structure database, SDAP-food. J. Agric. Food Chem. 51, 4830-4837. https://doi.org/10.1021/jf034218r.

Iwuala, E., Odjegba, V., Unung, O.*, et al.* (2020). Expression of stress responsive β-1,3-glucanase and chitinase genes in *Arachis hypogaea* seedlings against *Macrophomina phaseolina*. Gene Rep. 20, 100693. https://doi.org/10.1016/j.genrep.2020.100693.

Jäger, S. (2000). Ragweed (*Ambrosia*) sensitisation rates correlate withthe amount of inhaled airborne pollen. A 14-year studyin Vienna, Austria. Aerobiologia 16, 149-153. https://doi.org/10.1023/A:1007603321556.

Jahn-Schmid, B., Hauser, M., Wopfner, N.*, et al.* (2012). Humoral and cellular cross-reactivity between Amb a 1, the major ragweed pollen allergen, and its mugwort homolog Art v 6. J. Immunol. 188, 1559-1567. https://doi.org/10.4049/jimmunol.1102445.

Jaleel, C.A., Riadh, K., Gopi, R.*, et al.* (2009). Antioxidant defense responses: Physiological plasticity in higher plants under abiotic constraints. Acta Physiol. Plant. 31, 427-436. https://doi.org/10.1007/s11738-009-0275-6.

Jamloki, A., Bhattacharyya, M., Nautiyal, M.C., *et al.* (2021). Elucidating the relevance of high temperature and elevated CO_2 in plant secondary metabolites (PSMs) production. Heliyon 7, e07709. https://doi.org/10.1016/j.heliyon.2021.e07709.

Járai-Komlódi, M., Juhász, M. (1993). *Ambrosia elatior* (L.) in Hungary (1989–1990). Aerobiologia 9, 75-78. https://doi.org/10.1007/BF02311373.

Jia, X.Y., He, L.H., Li, R.Z. (2009). Calreticulin: Conserved protein and diverse functions in plants. Physiol. Plant. 136, 127-138. https://doi.org/10.1111/j.1399-3054.2009.1223.x.

Jo, S.H., Park, H.J., Lee, A., *et al.* (2022). The Arabidopsis cyclophilin CYP18-1 facilitates PRP18 dephosphorylation and the splicing of introns retained under heat stress. Plant Cell 34, 2383-2403. https://doi.org/10.1093/plcell/koac084.

Jones, N.R., Agnew, M., Banic, I., *et al.* (2019). Ragweed pollen and allergic symptoms in children: Results from a three-year longitudinal study. Sci. Total Environ. 683, 240-248. https://doi.org/10.1016/j.scitotenv.2019.05.284.

Jung, S., Estrella, N., Pfaffl, M.W., *et al.* (2021). Impact of elevated air temperature and drought on pollen characteristics of major agricultural grass species. PLOS ONE 16, e0248759. https://doi.org/10.1371/journal.pone.0248759.

Kandasamy, M.K., Burgos-Rivera, B., McKinney, E.C., *et al.* (2007). Class-specific interaction of profilin and ADF isovariants with actin in the regulation of plant development. Plant Cell 19, 3111-3126. https://doi.org/10.1105/tpc.107.052621.

Kang, Y., Han, Y., Torres-Jerez, I., *et al.* (2011). System responses to long-term drought and re-watering of two contrasting alfalfa varieties. Plant J. 68, 871-889. https://doi.org/10.1111/j.1365-313X.2011.04738.x.

Kanter, U., Heller, W., Durner, J., *et al.* (2013). Molecular and immunological characterization of ragweed (*Ambrosia artemisiifolia* L.) pollen after exposure of the plants to elevated ozone over a whole growing season. PLOS ONE 8, e61518. https://doi.org/10.1371/journal.pone.0061518.

Karagiannidis, N., Bletsos, F., Stavropoulos, N. (2002). Effect of *Verticillium wilt* (*Verticillium dahliae* Kleb.) and mycorrhiza (*Glomus mosseae*) on root colonization, growth and nutrient uptake in tomato and eggplant seedlings. Sci. Hortic. 94, 145-156. https://doi.org/10.1016/S0304-4238(01)00336-3.

Karamloo, F., Wangorsch, A., Kasahara, H., *et al.* (2001). Phenylcoumaran benzylic ether and isoflavonoid reductases are a new class of cross-reactive allergens in birch pollen, fruits and vegetables. Eur. J. Biochem. 268, 5310-5320. https://doi.org/10.1046/j.0014-2956.2001.02463.x.

Karle, A.C., Oostingh, G.J., Mutschlechner, S., *et al.* (2012). Nitration of the pollen allergen Bet v 1.0101 enhances the presentation of Bet v 1-derived peptides by HLA-DR on human dendritic cells. PLOS ONE 7, e31483. https://doi.org/10.1371/journal.pone.0031483.

Karrer, G. (2016). Germination and viability of ragweed seeds. Julius-Kühn-Archiv 455, 13. https://doi.org/10.5073/jka.2016.455.03.

Kasprzyk, I., Myszkowska, D., Grewling, Ł., *et al.* (2011). The occurrence of *Ambrosia* pollen in Rzeszów, Kraków and Poznań, Poland: Investigation of trends and possible

transport of *Ambrosia* pollen from Ukraine. Int. J. Biometeorol. 55, 633-644. https://doi.org/10.1007/s00484-010-0376-3.

Kaur, A. (2021). Assessment of airborne fungal allergens: Biochemical and immunoproteomic approach : A review. Plant Archives 21, 2428-2432. https://doi.org/10.51470/PLANTARCHIVES.2021.v21.S1.396.

Kaur, G., Singh, S.K., Singh, H.*, et al.* (2015). Characterization of peptidyl-prolyl *Cis-Trans* isomerase- and calmodulin-binding activity of a cytosolic *Arabidopsis thaliana* cyclophilin AtCyp19-3. PLOS ONE 10, e0136692. https://doi.org/10.1371/journal.pone.0136692.

Kaur, H., Sirhindi, G., Bhardwaj, R.*, et al.* (2018). 28-homobrassinolide regulates antioxidant enzyme activities and gene expression in response to salt- and temperature-induced oxidative stress in *Brassica juncea*. Sci. Rep. 8, 8735. https://doi.org/10.1038/s41598-018-27032-w.

Kawahara, Y., De-La Bastide, M., Hamilton, J.P.*, et al.* (2013). Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. Rice (N. Y.) 6, 4. https://doi.org/10.1186/1939-8433-6-4.

Kaya, H., Nakajima, R., Iwano, M.*, et al.* (2014). Ca^{2+}-activated reactive oxygen species production by *Arabidopsis* RbohH and RbohJ is essential for proper pollen tube tip growth. Plant Cell 26, 1069-1080. https://doi.org/10.1105/tpc.113.120642.

Kazerooni, E.A., Maharachchikumbura, S.S.N., Al-Sadi, A.M.*, et al.* (2022). *Actinomucor elegans* and *Podospora bulbillosa* positively improves endurance to water deficit and salinity stresses in tomato plants. J. Fungi (Basel) 8, 785. https://doi.org/10.3390/jof8080785.

Kelish, A.E., Winkler, J.B., Lang, H.*, et al.* (2014). Effects of ozone, CO_2 and drought stress on the growth and pollen production of common ragweed (*Ambrosia artemisiifolia*). Julius-Kühn-Archiv 445, 139-147. https://doi.org/10.5073/jka.2013.445.017.

Kelly, F.J. (2014). Influence of air pollution on respiratory disease. EMJ Respir. 2, 96-103. https://doi.org/10.33590/emjrespir/10312112.

Khan, A., Pan, X.D., Najeeb, U.*, et al.* (2018). Coping with drought: Stress and adaptive mechanisms, and management through cultural and molecular alternatives in cotton as vital constituents for plant stress resilience and fitness. Biol. Res. 51, 47. https://doi.org/10.1186/s40659-018-0198-z.

Khan, N., Bano, A., Babar, M.A. (2019). Metabolic and physiological changes induced by plant growth regulators and plant growth promoting rhizobacteria and their impact on drought tolerance in *Cicer arietinum* L. PLOS ONE 14, e0213040. https://doi.org/10.1371/journal.pone.0213040.

Khanna-Chopra, R., Chauhan, S. (2015). Wheat cultivars differing in heat tolerance show a differential response to oxidative stress during monocarpic senescence under high temperature stress. Protoplasma 252, 1241-1251. https://doi.org/10.1007/s00709-015-0755-z.

Kim, B.J., Lee, S.Y., Kim, H.B.*, et al.* (2014). Environmental changes, microbiota, and allergic diseases. Allergy, Asthma Immunol. Res. 6, 389-400. https://doi.org/10.4168/aair.2014.6.5.389.

Kim, K.R., Han, M.J., Oh, J.W. (2021a). Forecast for pollen allergy: A review from field observation to modeling and services in Korea. Immunol. Allergy Clin. North Am. 41, 127-141. https://doi.org/10.1016/j.iac.2020.09.011.

Kim, T.Y., Ku, H., Lee, S.Y. (2020). Crop enhancement of cucumber plants under heat stress by shungite carbon. Int. J. Mol. Sci. 21, 4858. https://doi.org/10.3390/ijms21144858.

Kim, Y.J., Kim, M.H., Hong, W.J.*, et al.* (2021b). OsMTD2-mediated reactive oxygen species (ROS) balance is essential for intact pollen-tube elongation in rice. Plant J. 107, 1131-1147. https://doi.org/10.1111/tpj.15373.

Kimata, M., Inagaki, N., Nagai, H. (2000). Effects of luteolin and other flavonoids on IgE-mediated allergic reactions. Planta Med. 66, 25-29. https://doi.org/10.1055/s-2000-11107.

Kofler, S., Asam, C., Eckhard, U.*, et al.* (2012). Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen Bet v 1. J. Mol. Biol. 422, 109-123. https://doi.org/10.1016/j.jmb.2012.05.016.

Kolek, F., Plaza, M.D.P., Leier-Wirtz, V.*, et al.* (2021). Earlier flowering of *Betula pendula* Roth in Augsburg, Germany, due to higher temperature, NO_2 and urbanity, and relationship with *Betula spp.* pollen season. Int. J. Environ. Res. Public Health 18, 10325. https://doi.org/10.3390/ijerph181910325.

Kõljalg, U., Nilsson, R.H., Abarenkov, K.*, et al.* (2013). Towards a unified paradigm for sequence-based identification of fungi. Mol. Ecol. 22, 5271-5277. https://doi.org/10.1111/mec.12481.

Komosinska-Vassev, K., Olczyk, P., Kaźmierczak, J.*, et al.* (2015). Bee pollen: Chemical composition and therapeutic application. Evid. Based Complement Alternat. Med. 2015, 297425. https://doi.org/10.1155/2015/297425.

Kosová, K., Vítámvás, P., Prášil, I.T.*, et al.* (2021). Plant proteoforms under environmental stress: Functional proteins arising from a single gene. Front. Plant Sci. 12, 793113. https://doi.org/10.3389/fpls.2021.793113.

Kovacs, I., Lindermayr, C. (2013). Nitric oxide-based protein modification: Formation and site-specificity of protein S-nitrosylation. Front. Plant Sci. 4, 229. https://doi.org/10.3389/fpls.2013.00137.

Krichevsky, A., Kozlovsky, S.V., Tian, G.W.*, et al.* (2007). How pollen tubes grow. Dev. Biol. 303, 405-420. https://doi.org/10.1016/j.ydbio.2006.12.003.

Krishnan, P., Ramakrishnan, B., Reddy, K.R.*, et al.* (2011). Chapter three - High-Temperature Effects on Rice Growth, Yield, and Grain Quality, in: Sparks, D.L. (Ed.), Adv. Agron. Press, Academic Advances in Agronomy, pp 87-206. https://doi.org/10.1016/B978-0-12-387689-8.00004-7.

Krumova, K., Cosa, G. (2016). Overview of reactive oxygen species, in: Nonell, S., Flors, C.*, et al.* (Eds.), Singlet Oxygen: Applications in Biosciences and Nanosciences. The Royal Society of Chemistry, pp 1-21. https://doi.org/10.1039/9781782622208-00001.

Kumar, S., Gupta, D., Nayyar, H. (2012). Comparative response of maize and rice genotypes to heat stress: Status of oxidative stress and antioxidants. Acta Physiol. Plant. 34, 75-86. https://doi.org/10.1007/s11738-011-0806-9.

Kunert, K.J., Van-Wyk, S.G., Cullis, C.A., *et al.* (2015). Potential use of phytocystatins in crop improvement, with a particular focus on legumes. J. Exp. Bot. 66, 3559-3570. https://doi.org/10.1093/jxb/erv211.

Kurepa, J., Shull, T.E., Smalle, J.A. (2016). Quercetin feeding protects plants against oxidative stress F1000Research 5, 2430. https://doi.org/10.12688/f1000research.9659.1.

Lai, H.Y., Tam, M.F., Tang, R.B., *et al.* (2002). cDNA cloning and immunological characterization of a newly identified enolase allergen from *Penicillium citrinum* and *Aspergillus fumigatus*. Int. Arch. Allergy Immunol. 127, 181-190. https://doi.org/10.1159/000053862.

Lake, I.R., Jones, N.R., Agnew, M., *et al.* (2017). Climate change and future pollen allergy in Europe. Environ. Health Perspect. 125, 385-391. https://doi.org/10.1289/EHP173.

Lamb, C., Dixon, R.A. (1997). The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 251-275. https://doi.org/10.1146/annurev.arplant.48.1.251.

Lavola, A., Nybakken, L., Rousi, M., *et al.* (2013). Combination treatment of elevated UVB radiation, CO_2 and temperature has little effect on silver birch (*Betula pendula*) growth and phytochemistry. Physiol. Plant. 149, 499-514. https://doi.org/10.1111/ppl.12051.

Ledesma, A., Villalba, M., Batanero, E., *et al.* (1998). Molecular cloning and expression of active Ole e 3, a major allergen from olive-tree pollen and member of a novel family of Ca^{2+}-binding proteins (polcalcins) involved in allergy. Eur. J. Biochem. 258, 454-459. https://doi.org/10.1046/j.1432-1327.1998.2580454.x.

Ledesma, A., Villalba, M., Rodríguez, R. (2000). Cloning, expression and characterization of a novel four EF-hand Ca^{2+}-binding protein from olive pollen with allergenic activity. FEBS Lett. 466, 192-196. https://doi.org/10.1016/s0014-5793(99)01790-1.

Lei, L., Xue, Y., Liu, Z., *et al.* (2015). Coumarin derivatives from *Ainsliaea fragrans* and their anticoagulant activity. Sci. Rep. 5, 13544. https://doi.org/10.1038/srep13544.

Leiblein-Wild, M.C., Tackenberg, O. (2014). Phenotypic variation of 38 European *Ambrosia artemisiifolia* populations measured in a common garden experiment. Biol. Invasions 16, 2003-2015. https://doi.org/10.1007/s10530-014-0644-y.

Léonard, R., Wopfner, N., Pabst, M., *et al.* (2010). A new allergen from ragweed (*Ambrosia artemisiifolia*) with homology to Art v 1 from mugwort. J. Biol. Chem. 285, 27192-27200. https://doi.org/10.1074/jbc.M110.127118.

Leru, P.M., Anton, V.F., Eftimie, A.M., *et al.* (2022). Biologic pollution due to *Ambrosia* (ragweed) pollen in urban environment of Bucharest. Int. J. Environ. Res. Public Health 19, 10613. https://doi.org/10.3390/ijerph191710613.

Leru, P.M., Eftimie, A.M., Anton, V.F., *et al.* (2019). Five-year data on pollen monitoring, distribution and health impact of allergenic plants in Bucharest and the Southeastern region of Romania. Medicina 55, 140. https://doi.org/10.3390/medicina55050140.

Leskovsek, R., Datta, A., Simoncic, A.*, et al.* (2012). Influence of nitrogen and plant density on the growth and seed production of common ragweed (*Ambrosia artemisiifolia* L.). J. Pest Sci. 85, 527-539. https://doi.org/10.1007/s10340-012-0433-2.

Levetin, E. (2021). Aeroallergens and climate change in tulsa, oklahoma: Long-term trends in the south central United States. Front. Allergy 2, 726445. https://doi.org/10.3389/falgy.2021.726445.

Li, W.Q., Zhang, X.Q., Xia, C.*, et al.* (2010). MALE GAMETOPHYTE DEFECTIVE 1, encoding the FAd subunit of mitochondrial F1F0-ATP synthase, is essential for pollen formation in *Arabidopsis thaliana*. Plant Cell Physiol. 51, 923-935. https://doi.org/10.1093/pcp/pcq066.

Li, Y., Niu, L., Wu, X.*, et al.* (2021). Genome-wide identification and comparison of cysteine proteases in the pollen coat and other tissues in maize. Front. Plant Sci. 12, 709534. https://doi.org/10.3389/fpls.2021.709534.

Lim, S.H., Chew, F.T., Sim, S.M.*, et al.* (1995). Allergens of *Bipolaris* species. Asian Pac. J. Allergy Immunol. 13, 101-105.

Lindermayr, C., Saalbach, G., Bahnweg, G.*, et al.* (2006). Differential inhibition of *Arabidopsis* methionine adenosyltransferases by protein S-nitrosylation. J. Biol. Chem. 281, 4285-4291. https://doi.org/10.1074/jbc.M511635200.

Lippmann, R., Babben, S., Menger, A.*, et al.* (2019). Development of wild and cultivated plants under global warming conditions. Curr. Biol. 29, R1326-R1338. https://doi.org/10.1016/j.cub.2019.10.016.

Liu, F., Zhang, X., Lu, C.*, et al.* (2015). Non-specific lipid transfer proteins in plants: Presenting new advances and an integrated functional analysis. J. Exp. Bot. 66, 5663-5681. https://doi.org/10.1093/jxb/erv313.

Liu, H., Hu, M., Wang, Q.*, et al.* (2018a). Role of papain-like cysteine proteases in plant development. Front. Plant Sci. 9, 1717. https://doi.org/10.3389/fpls.2018.01717.

Liu, Q., Luo, L., Zheng, L. (2018b). Lignins: Biosynthesis and biological functions in plants. Int. J. Mol. Sci. 19. https://doi.org/10.3390/ijms19020335.

Liu, S., Zenda, T., Tian, Z.*, et al.* (2023). Metabolic pathways engineering for drought or/and heat tolerance in cereals. Front. Plant Sci. 14, 1111875. https://doi.org/10.3389/fpls.2023.1111875.

Liu, X.X., Guo, Q.H., Xu, W.B.*, et al.* (2022). Rapid regulation of alternative splicing in response to environmental stresses. Front. Plant Sci. 13, 832177. https://doi.org/10.3389/fpls.2022.832177.

Locato, V., Gadaleta, C., De-Gara, L.*, et al.* (2008). Production of reactive species and modulation of antioxidant network in response to heat shock: A critical balance for cell fate. Plant, Cell Environ. 31, 1606-1619. https://doi.org/10.1111/j.1365-3040.2008.01867.x.

Lopez, M., Salvaggio, J.E. (1985). Mold-sensitive asthma. Clin. Rev. Allergy 3, 183-196.

Lorenzo, C., Marco, M., Paola, D.M.*, et al.* (2006). Long distance transport of ragweed pollen as a potential cause of allergy in central Italy. Ann. Allergy, Asthma, Immunol. 96, 86-91. https://doi.org/10.1016/S1081-1206(10)61045-9.

Loucks, D.P. (2021). Chapter 2 - Impacts of climate change on economies, ecosystems, energy, environments, and human equity: A systems perspective, in: Letcher, T.M. (Ed.), The Impacts of Climate Change. Elsevier, pp 19-50. https://doi.org/10.1016/B978-0-12-822373-4.00016-1.

Lowry, C.J., Smith, R.G. (2018). Chapter 5 - Weed control through crop plant manipulations, in: Jabran, K., Chauhan, B.S. (Eds.), Non-Chemical Weed Control. Academic Press, pp 73-96. https://doi.org/10.1016/B978-0-12-809881-3.00005-X.

Lu, J., Pollard, T.D. (2001). Profilin binding to poly-L-proline and actin monomers along with ability to catalyze actin nucleotide exchange is required for viability of fission yeast. Mol. Biol. Cell 12, 1161-1175. https://doi.org/10.1091/mbc.12.4.1161.

Lu, S., Yao, C., Zhou, S., et al. (2020). Studies on relationships between air pollutants and allergenicity of Humulus Scandens pollen collected from different areas of Shanghai. J. Environ. Sci. 95, 43-48. https://doi.org/10.1016/j.jes.2020.03.037.

Lucas, J.A., Gutierrez-Albanchez, E., Alfaya, T., et al. (2019). Oxidative stress in ryegrass growing under different air pollution levels and its likely effects on pollen allergenicity. Plant Physiol. Biochem. 135, 331-340. https://doi.org/10.1016/j.plaphy.2018.12.018.

Lundgren, M.R., Des-Marais, D.L. (2020). Life history variation as a model for understanding trade-offs in plant–environment interactions. Curr. Biol. 30, R180-R189. https://doi.org/10.1016/j.cub.2020.01.003.

Luo, W., Hu, H., Tang, W., et al. (2019). Allergen sensitization pattern of allergic adults and children in southern China: A survey based on real life data. Allergy, Asthma, Clin. Immunol. 15, 1-9. https://doi.org/10.1186/s13223-019-0357-y.

Luo, X., Xie, C., Dong, J., et al. (2014). Interactions between Verticillium dahliae and its host: Vegetative growth, pathogenicity, plant immunity. Appl. Microbiol. Biotechnol. 98, 6921-6932. https://doi.org/10.1007/s00253-014-5863-8.

Ma, P., Huang, H.C., Kokko, E.G., et al. (2000). Infection of cotton pollen by Verticillium dahliae. Plant Pathol. Bull. 9, 93-98.

MacKay, J., Kotanen, P.M. (2008). Local escape of an invasive plant, common ragweed (Ambrosia artemisiifolia L.), from above-ground and below-ground enemies in its native area. J. Ecol. 96, 1152-1161. https://doi.org/10.1111/j.1365-2745.2008.01426.x.

Madkour, L.H. (2019). Function of reactive oxygen species (ROS) inside the living organisms and sources of oxidants. Pharm. Sci. Anal. Res. J. 2, 180023.

Magyar, D., Krasznai, B., Tóth, M.D. (2022). Microscopic fungi and other contaminants on airborne pollen grains of ragweed (Ambrosia artemisiifolia L.). Aerobiologia 38, 217-231. https://doi.org/10.1007/s10453-022-09743-w.

Majd, A., Chehregani, A., Moin, M., et al. (2004). The effects of air pollution on structures, proteins and allergenicity of pollen grains. Aerobiologia 20, 111-118. https://doi.org/10.1023/B:AERO.0000032950.12169.38.

Makra, L., Juhász, M., Béczi, R., et al. (2005). The history and impacts of airborne Ambrosia (Asteraceae) pollen in Hungary. Grana 44, 57-64. https://doi.org/10.1080/00173130510010558.

Makra, L., Matyasovszky, I., Deák, Á.J. (2014). Ragweed in Eastern Europe, Invasive Species and Global Climate Change. https://doi.org/10.1079/9781780641645.0117.

Makra, L., Matyasovszky, I., Hufnagel, L., *et al.* (2015). The history of ragweed in the world. Appl. Ecol. Env. Res. 13, 489-512. https://doi.org/10.15666/aeer/1302_489512.

Makra, L., Pálfi, S., Gál, A., *et al.* (2007). Long-distance transport of ragweed pollen to Southern Hungary. Epidemiology 18, S8. https://doi.org/10.1097/01.ede.0000276427.39068.8e.

Mammoto, A., Mammoto, T., Ingber, D.E. (2012). Mechanosensitive mechanisms in transcriptional regulation. J. Cell Sci. 125, 3061-3073. https://doi.org/10.1242/jcs.093005.

Mangena, P. (2020). Phytocystatins and their potential application in the development of drought tolerance plants in soybeans (*Glycine max* L.). Protein Pept. Lett. 27, 135-144. https://doi.org/10.2174/0929866526666191014125453.

Manirajan, B.A., Maisinger, C., Ratering, S., *et al.* (2018). Diversity, specificity, co-occurrence and hub taxa of the bacterial–fungal pollen microbiome. FEMS Microbiol. Ecol. 94, fiy112. https://doi.org/10.1093/femsec/fiy112.

Manirajan, B.A., Ratering, S., Cardinale, M., *et al.* (2019). The microbiome of flower pollen and its potential impact on pollen-related allergies. Environ. Sci., 65-72. https://api.semanticscholar.org/CorpusID:215807679

Manzano, C., Pallero-Baena, M., Casimiro, I., *et al.* (2014). The emerging role of reactive oxygen species signaling during lateral root development   Plant Physiol. 165, 1105-1119. https://doi.org/10.1104/pp.114.238873.

Marin-Felix, Y., Groenewald, J.Z., Cai, L., *et al.* (2017). Genera of phytopathogenic fungi: GOPHY 1. Stud. Mycol. 86, 99-216. https://doi.org/10.1016/j.simyco.2017.04.002.

Marín-Rodríguez, M.C., Orchard, J., Seymour, G.B. (2002). Pectate lyases, cell wall degradation and fruit softening. J. Exp. Bot. 53, 2115-2119. https://doi.org/10.1093/jxb/erf089.

Marinkovich, V.A. (2004). Fungal hypersensitivity: Pathophysiology, diagnosis, therapy. Adv. Appl. Microbiol. 55, 289-307. https://doi.org/10.1016/s0065-2164(04)55011-6.

Marino, D., Andrio, E., Danchin, E.G., *et al.* (2011). A *Medicago truncatula* NADPH oxidase is involved in symbiotic nodule functioning. New Phytol. 189, 580-592. https://doi.org/10.1111/j.1469-8137.2010.03509.x.

Mascarenhas, J.P. (1993). Molecular mechanisms of pollen tube growth and differentiation. Plant Cell 5, 1303-1314. https://doi.org/10.1105/tpc.5.10.1303.

Mase, K., Tsukagoshi, H. (2021). Reactive oxygen species link gene regulatory networks during *Arabidopsis* root development. Front. Plant Sci. 12, 660274. https://doi.org/10.3389/fpls.2021.660274.

Matyasovszky, I., Makra, L., Csépe, Z., *et al.* (2015). Plants remember past weather: A study for atmospheric pollen concentrations of *Ambrosia*, Poaceae and *Populus*. Theor. Appl. Climatol. 122, 181-193. https://doi.org/10.1007/s00704-014-1280-2.

McGoey, B.V., Stinchcombe, J.R. (2021). Introduced populations of ragweed show as much evolutionary potential as native populations. Evol. Appl. 14, 1436–1449. https://doi.org/10.1111/eva.13211.

McKenna, O.E., Asam, C., Araujo, G.R., *et al.* (2016). How relevant is panallergen sensitization in the development of allergies? Pediatr. Allergy Immunol. 27, 560-568. https://doi.org/10.1111/pai.12589.

Meissner, M., Stark, K., Cresnar, B., *et al.* (1999). Volvox germline-specific genes that are putative targets of RegA repression encode chloroplast proteins. Curr. Genet. 36, 363-370. https://doi.org/10.1007/s002940050511.

Méndez, J., Comtois, P., Iglesias, I. (2005). *Betula* pollen: One of the most important aeroallergens in Ourense, Spain. Aerobiological studies from 1993 to 2000. Aerobiologia 21, 115-124. https://doi.org/10.1007/s10453-005-4181-0.

Menzel, A., Ghasemifard, H., Yuan, Y., *et al.* (2021). A first pre-season pollen transport climatology to Bavaria, Germany. Front. Allergy 2, 627863. https://doi.org/10.3389/falgy.2021.627863.

MeŞE, Ö., Turfan, N. (2019). Effects of air pollution on some chemical compounds of Cherry Laurel (*Prunus Laurocerasus* L.) in Kastamonu. Bartın Orman Fakültesi Dergisi 21, 486-494. https://doi.org/10.24011/barofd.535984.

Metzler, W.J., Valentine, K., Roebber, M., *et al.* (1992). Proton resonance assignments and three-dimensional solution structure of the ragweed allergen Amb a V by nuclear magnetic resonance spectroscopy. Biochemistry 31, 8697-8705. https://doi.org/10.1021/bi00152a003.

Meyer, R.C., Steinfath, M., Lisec, J., *et al.* (2007). The metabolic signature related to high plant growth rate in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. 104, 4759-4764. https://doi.org/10.1073/pnas.0609709104.

Mhamdi, A., Van-Breusegem, F. (2018). Reactive oxygen species in plant development. Development 145, dev164376. https://doi.org/10.1242/dev.164376.

Midoro-Horiuti, T., Brooks, E.G., Goldblum, R.M. (2001). Pathogenesis-related proteins of plants as allergens. Ann. Allergy, Asthma, Immunol. 87, 261-271. https://doi.org/10.1016/S1081-1206(10)62238-7.

Midoro-Horiuti, T., Goldblum, R.M., Kurosky, A., *et al.* (2000). Variable expression of pathogenesis-related protein allergen in mountain cedar (*Juniperus ashei*) pollen. J. Immunol. 164, 2188-2192. https://doi.org/10.4049/jimmunol.164.4.2188.

Mihajlovic, L., Radosavljevic, J., Burazer, L., *et al.* (2015). Composition of polyphenol and polyamide compounds in common ragweed (*Ambrosia artemisiifolia* L.) pollen and sub-pollen particles. Phytochemistry 109, 125-132. https://doi.org/10.1016/j.phytochem.2014.10.022.

Milakovic, I., Fiedler, K., Karrer, G., *et al.* (2014). Management of roadside populations of invasive *Ambrosia artemisiifolia* by mowing. Weed Res. 54, 256-264. https://doi.org/10.1111/wre.12074.

Misas-Villamil, J.C., van der Hoorn, R.A.L., Doehlemann, G. (2016). Papain-like cysteine proteases as hubs in plant immunity. New Phytol. 212, 902-907. https://doi.org/10.1111/nph.14117.

Mlcek, J., Jurikova, T., Skrovankova, S., *et al.* (2016). Quercetin and its anti-allergic immune response. Molecules 21, 623. https://doi.org/10.3390/molecules21050623.

Mohammadi, K., Khalesro, S., Sohrabi, Y., *et al.* (2011). A review: Beneficial effects of the mycorrhizal fungi for plant growth. J. Appl. Environ. Biol. Sci. 1, 310-319.

Mohammadrezakhani, S., Hajilou, J., Rezanejad, F. (2018). Evaluation of phenolic and flavonoid compounds in pollen grains of three *Citrus* species in response to low temperature. Grana 57, 214-222. https://doi.org/10.1080/00173134.2017.1358763.

Mohan, J.E., Cowden, C.C., Baas, P., *et al.* (2014). Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: Mini-review. Fungal Ecol. 10, 3-19. https://doi.org/10.1016/j.funeco.2014.01.005.

Mollet, J.C., Leroux, C., Dardelle, F., *et al.* (2013). Cell wall composition, biosynthesis and remodeling during pollen tube growth Plants (Basel) 2, 107-47. https://doi.org/10.3390/plants2010107.

Mølmann, J.A., Steindal, A.L., Bengtsson, G.B., *et al.* (2015). Effects of temperature and photoperiod on sensory quality and contents of glucosinolates, flavonols and vitamin C in broccoli florets. Food Chem. 172, 47-55. https://doi.org/10.1016/j.foodchem.2014.09.015.

Monika, P., Bikash-Lal, S., Dharmendra, K., *et al.* (2020). Prevalence of aeroallergens in allergic rhinitis in a tertiary care hospital. J. Nepal Med. Assoc. 58, 866–870. https://doi.org/10.31729/jnma.5218.

Montagnani, C., Gentili, R., Smith, M., *et al.* (2017). The worldwide spread, success, and impact of ragweed (*Ambrosia* spp.). Crit. Rev. Plant Sci. 36, 139-178. https://doi.org/10.1080/07352689.2017.1360112.

Moraes, A.H., Asam, C., Almeida, F.C.L., *et al.* (2018). Structural basis for cross-reactivity and conformation fluctuation of the major beech pollen allergen Fag s 1. Sci. Rep. 8, 10512. https://doi.org/10.1038/s41598-018-28358-1.

Morales-Amparano, M.B., Huerta-Ocampo, J.A., Pastor-Palacios, G., *et al.* (2021). The role of enolases in allergic disease. J. Allergy Clin. Immunol. Pract. 9, 3026-3032. https://doi.org/10.1016/j.jaip.2021.04.005.

Morales, S., Castro, A.J., Jimenez-Lopez, J.C., *et al.* (2012a). A novel multiplex method for the simultaneous detection and relative quantitation of pollen allergens. Electrophoresis 33, 1367-1374. https://doi.org/10.1002/elps.201100667.

Morales, S., Castro, A.J., Rodríguez-García, M.I., *et al.* (2012b). Involvement of climatic factors in the allergen expression in olive pollen, in: Jimenez-Lopez, J.C. (Ed.), Current Insights in Pollen Allergens. IntechOpen. https://doi.org/10.5772/50077.

Motaung, T.E., Peremore, C., Wingfield, B., *et al.* (2020). Plant-associated fungal biofilms—knowns and unknowns. FEMS Microbiol. Ecol. 96, fiaa224. https://doi.org/10.1093/femsec/fiaa224.

Moya, R., González-Ruiz, A., Beitia, J.M., *et al.* (2021). Ole e 3, a candidate for *in vivo* diagnosis of polcalcin sensitization. Int. Arch. Allergy Immunol. 182, 465-473. https://doi.org/10.1159/000512303.

Mueller, G.A., Thompson, P.M., DeRose, E.F., *et al.* (2016). A metabolomic, geographic, and seasonal analysis of the contribution of pollen-derived adenosine to

allergic sensitization. Metabolomics 12, 187. https://doi.org/10.1007/s11306-016-1130-6.

Muhlemann, J.K., Younts, T.L.B., Muday, G.K. (2018). Flavonols control pollen tube growth and integrity by regulating ROS homeostasis during high-temperature stress. Proc. Natl. Acad. Sci. 115, E11188. https://doi.org/10.1073/pnas.1811492115.

Muller, J., Mettbach, U., Menzel, D., *et al.* (2007). Molecular dissection of endosomal compartments in plants. Plant Physiol. 145, 293-304. https://doi.org/10.1104/pp.107.102863.

Nasr, J., Todtmann, F., Kuhlmey, F., *et al.* (2020). Air quality 2019: Trend in NO_2 decline continues, in: A number of cities still exceeding NO_2 limit – concentrations of particulates appear in compliance, high ozone peaks. The Umweltbundesamt For our environment. https://www.umweltbundesamt.de/en/press/pressinformation/air-quality-2019-trend-in-no2-decline-continues.

Nawrath, S., Alberternst, B. (2014). Aktivitäten der Bundesländer zur Verhinderung der Ausbreitung der Beifuß-*Ambrosie* (*Ambrosia artemisiifolia*) in Deutschland, in: Starfinger, U., Sölter, U., *et al.* (Eds.), *Ambrosia* in Deutschland - lässt sich die Invasion aufhalten? Julius Kühn-Institut, Berlin, Deutschland, pp. 47-69. https://doi.org/10.5073/jka.2013.445.006.

Negrini, A.C. (1992). Pollens as allergens. Aerobiologia 8, 9-15. https://doi.org/10.1007/BF02291321.

Nickel, U.T., Weikl, F., Kerner, R., *et al.* (2018). Quantitative losses vs. qualitative stability of ectomycorrhizal community responses to 3 years of experimental summer drought in a beech–spruce forest. Global Change Biol. 24, e560-e576. https://doi.org/10.1111/gcb.13957.

Niculaes, C., Morreel, K., Kim, H., *et al.* (2014). Phenylcoumaran benzylic ether reductase prevents accumulation of compounds formed under oxidative conditions in poplar xylem. Plant Cell 26, 3775-3791. https://doi.org/10.1105/tpc.114.125260.

Niederberger, V., Hayek, B., Vrtala, S., *et al.* (1999). Calcium-dependent immunoglobulin E recognition of the apo- and calcium-bound form of a cross-reactive two EF-hand timothy grass pollen allergen, Phl p 7. FASEB J. 13, 843-856. https://doi.org/10.1096/fasebj.13.8.843.

Niederberger, V., Pauli, G., Grönlund, H., *et al.* (1998). Recombinant birch pollen allergens (rBet v 1 and rBet v 2) contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen: A quantitative IgE inhibition study with sera from different populations. J. Allergy Clin. Immunol. 102, 579-591. https://doi.org/10.1016/s0091-6749(98)70273-8.

Niessen, M., Krause, K., Horst, I., *et al.* (2012). Two alanine aminotranferases link mitochondrial glycolate oxidation to the major photorespiratory pathway in *Arabidopsis* and rice. J. Exp. Bot. 63, 2705-2716. https://doi.org/10.1093/jxb/err453.

Nieves-Cordones, M., Ródenas, R., Lara, A., *et al.* (2019). The combination of K^+ deficiency with other environmental stresses: What is the outcome? Physiol. Plant. 165, 264-276. https://doi.org/10.1111/ppl.12827.

Nievola, C.C., Carvalho, C.P., Carvalho, V., *et al.* (2017). Rapid responses of plants to temperature changes. Temperature 4, 371-405. https://doi.org/10.1080/23328940.2017.1377812.

Nilamyani, A.N., Auliah, F.N., Moni, M.A., *et al.* (2021). PredNTS: Improved and robust prediction of nitrotyrosine sites by integrating multiple sequence features. Int. J. Mol. Sci. 22, 2704. https://doi.org/10.3390/ijms22052704.

Nilsen, B.M., Paulsen, B.S. (1990). Isolation and characterization of a glycoprotein allergen, Art v II, from pollen of mugwort (*Artemisia vulgaris* L.). Mol. Immunol. 27, 1047-1056. https://doi.org/10.1016/0161-5890(90)90128-m.

NOAA (2023). State of the cliamte: Global climate report for 2022. https://www.ncei.noaa.gov/access/monitoring/monthly-report/global/202213.

Noir, S., Bräutigam, A., Colby, T., *et al.* (2005). A reference map of the *Arabidopsis thaliana* mature pollen proteome. Biochem. Biophys. Res. Commun. 337, 1257-1266. https://doi.org/10.1016/j.bbrc.2005.09.185.

Nokthai, P., Lee, V.S., Shank, L. (2010). Molecular modeling of peroxidase and polyphenol oxidase: Substrate specificity and active site comparison. Int. J. Mol. Sci. 11, 3266-3276. https://doi.org/10.3390/ijms11093266.

Noman, E., Al-Gheethi, A.A., Rahman, N.K., *et al.* (2018). Single spore isolation as a simple and efficient technique to obtain fungal pure culture. IOP Conf. Ser.: Earth Environ. Sci. 140, 012055. https://doi.org/10.1088/1755-1315/140/1/012055.

O'riordain, G., Radauer, C., Hoffmann-Sommergruber, K., *et al.* (2002). Cloning and molecular characterization of the *Hevea brasiliensis* allergen Hev b 11, a class I chitinase. Clin. Exp. Allergy 32, 455-462. https://doi.org/10.1046/j.1365-2222.2002.01312.x.

Oberhuber, C., Ma, Y., Wopfner, N., *et al.* (2008). Prevalence of IgE-binding to Art v 1, Art v 4 and Amb a 1 in mugwort-allergic patients. Int. Arch. Allergy Immunol. 145, 94-101. https://doi.org/10.1159/000108134.

Obersteiner, A., Gilles, S., Frank, U., *et al.* (2016). Pollen-associated microbiome correlates with pollution parameters and the allergenicity of pollen. PLOS ONE 11, e0149545. https://doi.org/10.1371/journal.pone.0149545.

Oeder, S., Alessandrini, F., Wirz, O.F., *et al.* (2015). Pollen-derived nonallergenic substances enhance Th2-induced IgE production in B cells. Allergy 70, 1450-1460. https://doi.org/10.1111/all.12707.

Oh, J.W. (2018). Pollen allergy in a changing world, Pollen allergy in a changing world. Springer. https://doi.org/10.1007/978-981-10-5499-0.

Oh, J.W. (2022). Pollen allergy in a changing planetary environment. Allergy, Asthma Immunol. Res. 14, 168-181. https://doi.org/10.4168/aair.2022.14.2.168.

Onen, H., Farooq, S., Gunal, H., *et al.* (2017). Higher tolerance to abiotic stresses and soil types may accelerate common ragweed (*Ambrosia artemisiifolia*) invasion. Cell Death Differ. 65, 115-127. https://doi.org/10.1614/WS-D-16-00011.1.

Orcutt, D.M., Nilsen, E.T. (2000). Physiology of plants under stress: Soil and biotic factors. John Wiley & Sons.

Organization, W.H. (2022). Home page. 01.01.2023. https://www.who.int/en/news-room/fact-sheets/detail/ambient-(outdoor)-air-quality-and-health.

Organization, W.H., Societies, I.U.o.I. (1994). Allergen nomenclature. www.allergen.org.

Oteros, J., Bartusel, E., Alessandrini, F., *et al.* (2019). *Artemisia* pollen is the main vector for airborne endotoxin. J. Allergy Clin. Immunol. 143, 369-377.e5. https://doi.org/10.1016/j.jaci.2018.05.040.

Pacchierotti, F., Spanò, M. (2015). Environmental impact on DNA methylation in the germline: State of the art and gaps of knowledge. Biomed Res. Int. 2015, 123484. https://doi.org/10.1155/2015/123484.

Palevitz, B.A., Liu, B., Joshi, H.C. (1994). γ-Tubulin in tobacco pollen tubes: Association with generative cell and vegetative microtubules. Sex. Plant Reprod. 7, 209-214. https://doi.org/10.1007/BF00232739.

Pan, H., Wang, D. (2017). Nodule cysteine-rich peptides maintain a working balance during nitrogen-fixing symbiosis. Nat. Plants 3, 17048. https://doi.org/10.1038/nplants.2017.48.

Pant, B.D., Oh, S., Lee, H.K., *et al.* (2020). Antagonistic regulation by CPN60A and CLPC1 of TRXL1 that regulates MDH activity leading to plant disease resistance and thermotolerance. Cell Rep. 33, 108512. https://doi.org/10.1016/j.celrep.2020.108512.

Park, J.W., Kim, K.S., Jin, H.S., *et al.* (2002). Der p 2 isoallergens have different allergenicity, and quantification with 2-site ELISA using monoclonal antibodies is influenced by the isoallergens. Clin. Exp. Allergy 32, 1042-1047. https://doi.org/10.1046/j.1365-2222.2002.01421.x.

Parliament, E., Union, C.o.t.E. (2008). Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. http://data.europa.eu/eli/dir/2008/50/oj.

Pasqualini, S., Tedeschini, E., Frenguelli, G., *et al.* (2011). Ozone affects pollen viability and NAD(P)H oxidase release from *Ambrosia artemisiifolia* pollen. Environ. Pollut. 159, 2823-2830. https://doi.org/10.1016/j.envpol.2011.05.003.

Pastorello, E.A., Vieths, S., Pravettoni, V., *et al.* (2002). Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. J. Allergy Clin. Immunol. 109, 563-570. https://doi.org/10.1067/mai.2002.121946.

Patel, D., Franklin, K.A. (2009). Temperature-regulation of plant architecture. Plant Signal Behav. 4, 577-579. https://doi.org/10.4161/psb.4.7.8849.

Paupière, M.J., Müller, F., Li, H., *et al.* (2017). Untargeted metabolomic analysis of tomato pollen development and heat stress response. Plant Reprod. 30, 81-94. https://doi.org/10.1007/s00497-017-0301-6.

Pazmandi, K., Kumar, B.V., Szabo, K., *et al.* (2012). Ragweed subpollen particles of respirable size activate human dendritic cells. PLOS ONE 7, e52085. https://doi.org/10.1371/journal.pone.0052085.

Pedrosa, M., Prieto-García, A., Sala-Cunill, A., *et al.* (2014). Management of angioedema without urticaria in the emergency department. Ann. Med. 46, 607-618. https://doi.org/10.3109/07853890.2014.949300.

Pękal, A., Pyrzynska, K. (2014). Evaluation of aluminium complexation reaction for flavonoid content assay. Food Anal. Methods 7, 1776-1782. https://doi.org/10.1007/s12161-014-9814-x.

Pemberton, T.J. (2006). Identification and comparative analysis of sixteen fungal peptidyl-prolyl cis/trans isomerase repertoires. BMC Genomics 7, 244. https://doi.org/10.1186/1471-2164-7-244.

Pereira, S., Fernández-González, M., Guedes, A., *et al.* (2021). The strong and the stronger: The effects of increasing ozone and nitrogen dioxide concentrations in pollen of different forest species. Forests 12, 88. https://doi.org/10.3390/f12010088.

Petibon, C., Malik-Ghulam, M., Catala, M., *et al.* (2021). Regulation of ribosomal protein genes: An ordered anarchy. Wiley Interdiscip. Rev.: RNA 12, e1632. https://doi.org/10.1002/wrna.1632.

Picard, M., McEwen, B.S., Epel, E.S., *et al.* (2018). An energetic view of stress: Focus on mitochondria. Front. Neuroendocrinol. 49, 72-85. https://doi.org/10.1016/j.yfrne.2018.01.001.

Pichler, U., Hauser, M., Wolf, M., *et al.* (2015). Pectate lyase pollen allergens: Sensitization profiles and cross-reactivity pattern. PLOS ONE 10, e0120038. https://doi.org/10.1371/journal.pone.0120038.

Pinke, G., Karácsony, P., Botta-Dukát, Z., *et al.* (2013). Relating *Ambrosia artemisiifolia* and other weeds to the management of Hungarian sunflower crops. J. Pest Sci. 86, 621-631. https://doi.org/10.1007/s10340-013-0484-z.

Plaxton, W.C. (1996). The organization and regulation of plant glycolysis. Annu. Rev. Plant Biol. 47, 185-214. https://doi.org/10.1146/annurev.arplant.47.1.185.

Plundrich, N.J., Bansode, R.R., Foegeding, E.A., *et al.* (2017). Protein-bound *Vaccinium* fruit polyphenols decrease IgE binding to peanut allergens and RBL-2H3 mast cell degranulation *in vitro*. Food Funct. 8, 1611-1621. https://doi.org/10.1039/c7fo00249a.

Poidevin, L., Forment, J., Unal, D., *et al.* (2021). Transcriptome and translatome changes in germinated pollen under heat stress uncover roles of transporter genes involved in pollen tube growth. Plant, Cell Environ. 44, 2167-2184. https://doi.org/10.1111/pce.13972.

Pomés, A., Davies, J.M., Gadermaier, G., *et al.* (2018). WHO/IUIS Allergen Nomenclature: Providing a common language. Mol. Immunol. 100, 3-13. https://doi.org/10.1016/j.molimm.2018.03.003.

Ponce de León, I., Hamberg, M., Castresana, C. (2015). Oxylipins in moss development and defense. Front. Plant Sci. 6, 483. https://doi.org/10.3389/fpls.2015.00483.

Pontaroli, A.C., Camadro, E.L., Babinec, F.J., *et al.* (2000). Responses of *Asparagus officinalis* pollen to the culture filtrate of *Fusarium oxysporum* f. sp. *asparagi*. Sci. Hortic. 84, 349-356. https://doi.org/10.1016/S0304-4238(99)00113-2.

Pooja, Munjal, R. (2019). Oxidative Stress and Antioxidant Defense in Plants Under High Temperature, Reactive Oxygen, Nitrogen and Sulfur Species in Plants, pp 337-352. https://doi.org/10.1002/9781119468677.ch14.

Popescu, F.D. (2015). Cross-reactivity between aeroallergens and food allergens. World J. Methodol. 5, 31-50. https://doi.org/10.5662/wjm.v5.i2.31.

Porter, A.S., Evans-Fitz Gerald, C., McElwain, J.C.*, et al.* (2015). How well do you know your growth chambers? Testing for chamber effect using plant traits. Plant Methods 11, 44. https://doi.org/10.1186/s13007-015-0088-0.

Poschl, U., Shiraiwa, M. (2015). Multiphase chemistry at the atmosphere-biosphere interface influencing climate and public health in the anthropocene. Chem. Rev. 115, 4440-4475. https://doi.org/10.1021/cr500487s.

Potocký, M., Jones, M.A., Bezvoda, R.*, et al.* (2007). Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. New Phytol. 174, 742-751. https://doi.org/10.1111/j.1469-8137.2007.02042.x.

Poudel, M., Mendes, R., Costa, L.A.S.*, et al.* (2021). The role of plant-associated bacteria, fungi, and viruses in drought stress mitigation. Front. Microbiol. 12, 743512. https://doi.org/10.3389/fmicb.2021.743512.

Powers, H.J., Corfe, B.M., Nakano, E. (2012). Riboflavin in development and cell fate. Subcell Biochem. 56, 229-245. https://doi.org/10.1007/978-94-007-2199-9_12.

Prank, M., Chapman, D.S., Bullock, J.M.*, et al.* (2013). An operational model for forecasting ragweed pollen release and dispersion in Europe. Agric. For. Meteorol. 182-183, 43-53. https://doi.org/10.1016/j.agrformet.2013.08.003.

Prasad, P.V.V., Djanaguiraman, M., Perumal, R.*, et al.* (2015). Impact of high temperature stress on floret fertility and individual grain weight of grain sorghum: Sensitive stages and thresholds for temperature and duration. Front. Plant Sci. 6, 820. https://doi.org/10.3389/fpls.2015.00820.

Prasad, P.V.V., Pisipati, S.R., Mutava, R.N.*, et al.* (2008). Sensitivity of grain sorghum to high temperature stress during reproductive development. Crop Sci. 48, 1911-1917. https://doi.org/10.2135/cropsci2008.01.0036.

Protocols, C.S.H. (2006). Recipe for LB (Luria-Bertani) liquid medium. Cold Spring Harbor Protocols. https://doi.org/10.1101/pdb.rec8141.

Qayyum, A., Al-Ayoubi, S., Sher, A.*, et al.* (2021). Improvement in drought tolerance in bread wheat is related to an improvement in osmolyte production, antioxidant enzyme activities, and gaseous exchange. Saudi J. Biol. Sci. 28, 5238-5249. https://doi.org/10.1016/j.sjbs.2021.05.040.

Rabbani, M.A., Maruyama, K., Abe, H.*, et al.* (2003). Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol. 133, 1755-1767. https://doi.org/10.1104/pp.103.025742.

Radauer, C., Breiteneder, H. (2006). Pollen allergens are restricted to few protein families and show distinct patterns of species distribution. J. Allergy Clin. Immunol. 117, 141-147. https://doi.org/10.1016/j.jaci.2005.09.010.

Radauer, C., Bublin, M., Wagner, S.*, et al.* (2008). Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J. Allergy Clin. Immunol. 121, 847-52.e7. https://doi.org/10.1016/j.jaci.2008.01.025.

Radauer, C., Nandy, A., Ferreira, F*., et al.* (2014). Update of the WHO/IUIS Allergen Nomenclature Database based on analysis of allergen sequences. Allergy 69, 413-419. https://doi.org/10.1111/all.12348.

Radi, R. (2004). Nitric oxide, oxidants, and protein tyrosine nitration. Proc. Natl. Acad. Sci. 101, 4003-4008. https://doi.org/10.1073/pnas.0307446101.

Raghavan, M., Wijeyesakere, S.J., Peters, L.R*., et al.* (2013). Calreticulin in the immune system: Ins and outs. Trends Immunol. 34, 13-21. https://doi.org/10.1016/j.it.2012.08.002.

Randriamanana, T.R., Lavola, A., Julkunen-Tiitto, R. (2015). Interactive effects of supplemental UV-B and temperature in European aspen seedlings: Implications for growth, leaf traits, phenolic defense and associated organisms. Plant Physiol. Biochem. 93, 84-93. https://doi.org/10.1016/j.plaphy.2015.03.001.

Rao, M.V., Paliyath, G., Ormrod, D.P. (1996). Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis* thaliana. Plant Physiol. 110, 125-136. https://doi.org/10.1104/pp.110.1.125.

Rasmussen, K., Thyrring, J., Muscarella, R*., et al.* (2017). Climate-change-induced range shifts of three allergenic ragweeds (*Ambrosia* L.) in Europe and their potential impact on human health. PeerJ 5, e3104. https://doi.org/10.7717/peerj.3104.

Rauer, D., Gilles, S., Wimmer, M*., et al.* (2021). Ragweed plants grown under elevated CO_2 levels produce pollen which elicit stronger allergic lung inflammation. Allergy 76, 1718-1730. https://doi.org/10.1111/all.14618.

Rehman, A., Khan, I., Farooq, M. (2023). Secondary metabolites mediated reproductive tolerance under heat stress in plants. J. Plant Growth Regul., 1-19. https://doi.org/10.1007/s00344-023-11161-2.

Reinmuth-Selzle, K., Kampf, C.J., Lucas, K*., et al.* (2017). Air pollution and climate change effects on allergies in the anthropocene: Abundance, interaction, and modification of allergens and adjuvants. Environ. Sci. Technol. 51, 4119-4141. https://doi.org/10.1021/acs.est.6b04908.

Resentini, F., Orozco-Arroyo, G., Cucinotta, M*., et al.* (2023). The impact of heat stress in plant reproduction. Front. Plant Sci. 14, 1271644. https://doi.org/10.3389/fpls.2023.1271644.

Rezanejad, F. (2009). Air pollution effects on structure, proteins and flavonoids in pollen grains of *Thuja orientalis* L. (Cupressaceae). Grana 48, 205-213. https://doi.org/10.1080/00173130902949417.

Reznik, S.Y. (2009). Common ragweed (*Ambrosia artemisiifolia* L.) in Russia: Spread, distribution, abundance, harmfulness and control measures. Ambroisie First Int. Ragweed Rev. 26, 88. https://api.semanticscholar.org/CorpusID:52206237

Riahi, K., Rao, S., Krey, V*., et al.* (2011). RCP 8.5—A scenario of comparatively high greenhouse gas emissions. Clim. Change 109, 33-57. https://doi.org/10.1007/s10584-011-0149-y.

Riboni, M., Galbiati, M., Tonelli, C*., et al.* (2013). *GIGANTEA* enables drought escape response via abscisic acid-dependent activation of the florigens and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS*. Plant Physiol. 162, 1706-19. https://doi.org/10.1104/pp.113.217729.

Rihs, H.P., Chen, Z., Rozynek, P.*, et al.* (2001). Allergenicity of rHev b 10 (manganese-superoxide dismutase). Allergy 56, 85-86. https://doi.org/10.1034/j.1398-9995.2001.00931.x.

Roberts, I.N., Caputo, C., Criado, M.V.*, et al.* (2012). Senescence-associated proteases in plants. Physiol. Plant. 145, 130-139. https://doi.org/10.1111/j.1399-3054.2012.01574.x.

Robles, C., Greff, S., Pasqualini, V.*, et al.* (2003). Phenols and flavonoids in *Aleppo* pine needles as bioindicators of air pollution. J. Environ. Qual. 32, 2265-2271. https://doi.org/10.2134/jeq2003.2265.

Rodinkova, V., Palamarchuk, O., Kremenska, L. (2012). The most abundant *Ambrosia* pollen count is associated with the Southern, Eastern and the Northern-Eastern Ukraine. Allergol. Immunopath. 9, 181.

Roebber, M., Hussain, R., Klapper, D.G.*, et al.* (1983). Isolation and properties of a new short ragweed pollen allergen, Ra6. J. Immunol. 131, 706-711. https://doi.org/10.4049/jimmunol.131.2.706.

Roebber, M., Marsh, D.G. (1991). 739 Isolation and characterization of allergen Amb a VII from short ragweed pollen. J. Allergy Clin. Immunol. 87, 324.

Rogers, H., Munné-Bosch, S. (2016). Production and scavenging of reactive oxygen species and redox signaling during leaf and flower senescence: Similar but different. Plant Physiol. 171, 1560-1568. https://doi.org/10.1104/pp.16.00163.

Rogers, H.J. (2012). Is there an important role for reactive oxygen species and redox regulation during floral senescence? Plant, Cell Environ. 35, 217-233. https://doi.org/10.1111/j.1365-3040.2011.02373.x.

Rojo, J., Picornell, A., Oteros, J.*, et al.* (2021). Consequences of climate change on airborne pollen in Bavaria, Central Europe. Reg. Environ. Change 18, 1331. https://dx.doi.org/10.1007/s10113-020-01729-z.

Romano, M.L.G., Gallego, M.T., Berrens, L. (1996). Extraordinary stability of IgE-binding *Parietaria* pollen allergens in relation to chemically bound flavonoids. Mol. Immunol. 33, 1287-1293. https://doi.org/10.1016/S0161-5890(96)00106-X.

Rubel, F., Brugger, K., Haslinger, K.*, et al.* (2017). The climate of the European Alps: Shift of very high resolution Köppen-Geiger climate zones 1800–2100. Meteorol. Z. 26, 115-125. https://doi.org/10.1127/metz/2016/0816.

Ruëff, F., Przybilla, B., Walker, A.*, et al.* (2012). Sensitization to common ragweed in southern Bavaria: Clinical and geographical risk factors in atopic patients. Int. Arch. Allergy Immunol. 159, 65-74. https://doi.org/10.1159/000335192.

Sachdev, S., Ansari, S.A., Ansari, M.I.*, et al.* (2021). Abiotic stress and reactive oxygen species: Generation, signaling, and defense mechanisms. Antioxidants 10, 277. https://doi.org/10.3390/antiox10020277.

Saldajeno, M.G.B., Chandanie, W.A., Kubota, M.*, et al.* (2008). Effects of interactions of arbuscular mycorrhizal fungi and beneficial saprophytic mycoflora on plant growth and disease protection. https://doi.org/10.1007/978-1-4020-8770-7_9.

Salo, P.M., Arbes, S.J., Jaramillo, R.*, et al.* (2014). Prevalence of allergic sensitization in the United States: Results from the National Health and Nutrition

Examination Survey (NHANES) 2005-2006. J. Allergy Clin. Immunol. 134, 350-359. https://doi.org/10.1016/j.jaci.2013.12.1071.

San Segundo-Acosta, P., Oeo-Santos, C., Benedé, S., *et al.* (2019). Delineation of the olive pollen proteome and its allergenome unmasks cyclophilin as a relevant cross-reactive allergen. J. Proteome Res. 18, 3052-3066. https://doi.org/10.1021/acs.jproteome.9b00167.

Sasaki, K., Yoshida, H. (2015). Organelle autoregulation-stress responses in the ER, Golgi, mitochondria and lysosome. J. Biochem. 157, 185-95. https://doi.org/10.1093/jb/mvv010.

Sato, S. (2008). The effects of moderately elevated temperature stress due to global warming on the yield and the male reproductive development of tomato (*Lycopersicon esculentum* Mill.). HortResearch 60, 85–89.

Sato, S., Peet, M.M., Thomas, J.F. (2000). Physiological factors limit fruit set of tomato (*Lycopersicon esculentum* Mill.) under chronic, mild heat stress. Plant, Cell Environ. 23, 719-726. https://doi.org/10.1046/j.1365-3040.2000.00589.x.

Savchenko, T., Tikhonov, K. (2021). Oxidative stress-induced alteration of plant central metabolism. Life 11, 304. https://doi.org/10.3390/life11040304.

Scevkova, J., Vaskova, Z., Sepsiova, R., *et al.* (2020). Relationship between Poaceae pollen and Phl p 5 allergen concentrations and the impact of weather variables and air pollutants on their levels in the atmosphere. Heliyon 6, e04421. https://doi.org/10.1016/j.heliyon.2020.e04421.

Schaffner, U., Steinbach, S., Sun, Y., *et al.* (2020). Biological weed control to relieve millions from *Ambrosia* allergies in Europe. Nat. Commun. 11, 1745. https://doi.org/10.1038/s41467-020-15586-1.

Schindler, L.W. (1993). The immune system: How it works. US Department of Health and Human Services, Public Health Service, National ….

Schlenker, W., Roberts, M.J. (2009). Nonlinear temperature effects indicate severe damages to U.S. crop yields under climate change. Proc. Natl. Acad. Sci. 106, 15594-15598. https://doi.org/10.1073/pnas.0906865106.

Schmidt, C.W. (2016). Pollen overload: Seasonal allergies in a changing climate. Environ. Health Perspect. 124, A70-A75. https://doi.org/10.1289/ehp.124-A70.

Schmidt, S., Zietz, M., Schreiner, M., *et al.* (2010). Genotypic and climatic influences on the concentration and composition of flavonoids in kale (*Brassica oleracea var. sabellica*). Food Chem. 119, 1293-1299. https://doi.org/10.1016/j.foodchem.2009.09.004.

Seidler, C.A., Zeindl, R., Fernández-Quintero, M.L., *et al.* (2024). Allergenicity and conformational diversity of allergens. Allergies 4, 1-16. https://doi.org/10.3390/allergies4010001.

Selinski, J., Scheibe, R. (2014). Pollen tube growth: Where does the energy come from? Plant Signal Behav. 9, e977200. https://doi.org/10.4161/15592324.2014.977200.

Seth, D., Bielory, L. (2021). Allergenic pollen season variations in the past two decades under changing climate in the United States. Immunol. Allergy Clin. North Am. 41, 17-31. https://doi.org/10.1016/j.iac.2020.09.006.

Seutter-Von Loetzen, C., Hoffmann, T., Hartl, M.J*., et al.* (2014). Secret of the major birch pollen allergen Bet v 1: Identification of the physiological ligand. Biochem. J. 457, 379-390. https://doi.org/10.1042/bj20130413.

Shaar-Moshe, L., Hayouka, R., Roessner, U*., et al.* (2019). Phenotypic and metabolic plasticity shapes life-history strategies under combinations of abiotic stresses. Plant Direct 3, e00113. https://doi.org/10.1002/pld3.113.

Shahali, Y., Pourpak, Z., Moin, M*., et al.* (2009). Impacts of air pollution exposure on the allergenic properties of Arizona cypress pollens. J. Phys.: Conf. Ser. 151, 012027. https://doi.org/10.1088/1742-6596/151/1/012027.

Sharma, P., Dubey, R.S. (2005). Drought induces oxidative stress and enhances the activities of antioxidant enzymes in growing rice seedlings. Plant Growth Regul. 46, 209-221. https://doi.org/10.1007/s10725-005-0002-2.

Sharma, V., Gupta, R., Jhingran, A*., et al.* (2006). Cloning, recombinant expression and activity studies of a major allergen "enolase" from the fungus *Curvularia lunata*. J. Clin. Immunol. 26, 360-369. https://doi.org/10.1007/s10875-006-9032-4.

Sheng, Y.W., Abreu, I.A., Cabelli, D.E*., et al.* (2014). Superoxide dismutases and superoxide reductases. Chem. Rev. 114, 3854-3918. https://doi.org/10.1021/cr4005296.

Shi, D.Q., Yang, W.C. (2010). Pollen Germination and Tube Growth, Plant Developmental Biology - Biotechnological Perspectives, pp 245-282. https://doi.org/10.1007/978-3-642-02301-9_13.

Shiina, T., Shimizu, Y. (2020). Temperature-dependent alternative splicing of precursor mRNAs and its biological significance: A review focused on post-transcriptional regulation of a cold shock protein gene in hibernating mammals. Int. J. Mol. Sci. 21, 7599. https://doi.org/10.3390/ijms21207599.

Shim, J.S., Jang, G. (2020). Environmental signal-dependent regulation of flowering time in rice. Int. J. Mol. Sci. 21, 6155. https://doi.org/10.3390/ijms21176155.

Sikorski, M.M., Biesiadka, J., Kasperska, A.E*., et al.* (1999). Expression of genes encoding PR10 class pathogenesis-related proteins is inhibited in yellow lupine root nodules. Plant Sci. 149, 125-137. https://doi.org/10.1016/S0168-9452(99)00148-X.

Simard, M.J., Nurse, R.E., Page, E.R*., et al.* (2020). Common ragweed (*Ambrosia artemisiifolia*) seed shattering in wheat, corn, and soybean. Weed Sci. 68, 510-516. https://doi.org/10.1017/wsc.2020.51.

Simon-Nobbe, B., Denk, U., Pöll, V*., et al.* (2008). The spectrum of fungal allergy. Int. Arch. Allergy Immunol. 145, 58-86. https://doi.org/10.1159/000107578.

Simon-Nobbe, B., Probst, G., Kajava, A.V*., et al.* (2000). IgE-binding epitopes of enolases, a class of highly conserved fungal allergens. J. Allergy Clin. Immunol. 106, 887-895. https://doi.org/10.1067/mai.2000.110799.

Sina, C., Ion, T., Violeta, N. (2015). Chemical composition and antioxidant activity of walnut pollen samples. Not. Bot. Horti Agrobot. Cluj-Napoca 43, 361. https://doi.org/10.15835/nbha43210090.

Singer, B.D., Ziska, L.H., Frenz, D.A*., et al.* (2005). Research note: Increasing Amb a 1 content in common ragweed (*Ambrosia artemisiifolia*) pollen as a function of rising

atmospheric CO$_2$ concentration. Funct. Plant Biol. 32, 667-670. https://doi.org/10.1071/fp05039.

Singh, A., Mehta, S., Yadav, S.*, et al.* (2022). How to cope with the challenges of environmental stresses in the era of global climate change: An update on ROS stave off in plants. Int. J. Mol. Sci. 23, 1995. https://doi.org/10.3390/ijms23041995.

Singh, P., Arif, Y., Bajguz, A.*, et al.* (2021). The role of quercetin in plants. Plant Physiol. Biochem. 166, 10-19. https://doi.org/10.1016/j.plaphy.2021.05.023.

Sinha, M., Singh, R.P., Kushwaha, G.S.*, et al.* (2014). Current overview of allergens of plant pathogenesis related protein families. Sci. World J. 2014, 543195. https://doi.org/10.1155/2014/543195.

Sinkevičienė, J., Tarasevičienė, Ž., Tamutis, V. (2023). *Fusarium* fungi and mycotoxins in bee pollen collected in Lithuania. Appl. Sci. 13, 1571. https://doi.org/10.3390/app13031571.

Sircar, G., Bhowmik, M., Sarkar, R.K.*, et al.* (2020). Molecular characterization of a fungal cyclophilin allergen Rhi o 2 and elucidation of antigenic determinants responsible for IgE-cross-reactivity. J. Biol. Chem. 295, 2736-2748. https://doi.org/10.1074/jbc.RA119.011659.

Skálová, H. (2019). Performance of *Ambrosia artemisiifolia* and its potential competitors in an experimental temperature and salinity gradient and implications for management. Manag. Biol. Invasion 10, 359-376. https://doi.org/10.3391/mbi.2019.10.2.10.

Slieker, R. (2024). Transport proteins in the movement of ion channels, molecules and cellular homeostasis. J. Glycomics Lipidomics 13. https://doi.org/10.35248/2153-0637.24.13.364.

Smiljanic, K., Apostolovic, D., Trifunovic, S.*, et al.* (2017). Subpollen particles are rich carriers of major short ragweed allergens and NADH dehydrogenases: Quantitative proteomic and allergomic study. Clin. Exp. Allergy 47, 815-828. https://doi.org/10.1111/cea.12874.

Smith, L.M., Kelleher, N.L. (2013). Proteoform: A single term describing protein complexity. Nat. Methods 10, 186-187. https://doi.org/10.1038/nmeth.2369.

Smith, L.M., Kelleher, N.L. (2018). Proteoforms as the next proteomics currency. Science 359, 1106-1107. https://doi.org/10.1126/science.aat1884.

Smole, U., Balazs, N., Hoffmann-Sommergruber, K.*, et al.* (2010). Differential T-cell responses and allergen uptake after exposure of dendritic cells to the birch pollen allergens Bet v 1.0101, Bet v 1.0401 and Bet v 1.1001. Immunobiology 215, 903-909. https://doi.org/10.1016/j.imbio.2009.11.003.

Somssich, I.E., Schmelzer, E., Kawalleck, P.*, et al.* (1988). Gene structure and in situ transcript localization of pathogenesis-related protein 1 in parsley. Mol. Gen. Genet. 213, 93-98. https://doi.org/10.1007/BF00333403.

Song, L.F., Zou, J.J., Zhang, W.Z.*, et al.* (2009). Ion transporters involved in pollen germination and pollen tube tip-growth. Plant Signal Behav. 4, 1193-1195. https://doi.org/10.4161/psb.4.12.10063.

Songnuan, W. (2013). Wind-pollination and the roles of pollen allergenic proteins. Asian Pac. J. Allergy Immunol. 31, 261-270. https://doi.org/10.12932/AP0287.31.4.2013.

Sousa, R., Duque, L., Duarte, A.J., *et al.* (2012). *In vitro* exposure of *Acer negundo* pollen to atmospheric levels of SO_2 and NO_2: Effects on allergenicity and germination. Environ. Sci. Technol. 46, 2406-2412. https://doi.org/10.1021/es2034685.

Souza, J.M., Daikhin, E., Yudkoff, M., *et al.* (1999). Factors determining the selectivity of protein tyrosine nitration. Arch. Biochem. Biophys. 371, 169-178. https://doi.org/10.1006/abbi.1999.1480.

Speranza, A., Scoccianti, V. (2012). New insights into an old story. Plant Signal Behav. 7, 994-998. https://doi.org/10.4161/psb.20674.

Springate, D.A., Kover, P.X. (2014). Plant responses to elevated temperatures: A field study on phenological sensitivity and fitness responses to simulated climate warming. Global Change Biol. 20, 456-465. https://doi.org/10.1111/gcb.12430.

Stach, A., Smith, M., Skjøth, C.A., *et al.* (2007). Examining *Ambrosia* pollen episodes at Poznań (Poland) using back-trajectory analysis. Int. J. Biometeorol. 51, 275-286. https://doi.org/10.1007/s00484-006-0068-1.

Stadler, M.B., Stadler, B.M. (2003). Allergenicity prediction by protein sequence. FASEB J. 17, 1141-1143. https://doi.org/10.1096/fj.02-1052fje.

Stanley, R.G. (1971). Pollen chemistry and tube growth, Pollen. Elsevier, pp 131-155. https://doi.org/10.1016/B978-0-408-70149-5.50019-1.

Steinhorst, L., Kudla, J. (2013). Calcium - a central regulator of pollen germination and tube growth. Biochim. Biophys. Acta 1833, 1573-1581. https://doi.org/10.1016/j.bbamcr.2012.10.009.

Stella, T., Webber, H., Olesen, J.E., *et al.* (2021). Methodology to assess the changing risk of yield failure due to heat and drought stress under climate change. Environ. Res. Lett. 16, 104033. https://doi.org/10.1088/1748-9326/ac2196.

Stępalska, D., Myszkowska, D., Piotrowicz, K., *et al.* (2020). High *Ambrosia* pollen concentrations in Poland respecting the long distance transport (LDT). Sci. Total Environ. 736, 139615. https://doi.org/10.1016/j.scitotenv.2020.139615.

Subburaj, S., Zhu, D., Li, X., *et al.* (2017). Molecular characterization and expression profiling of *Brachypodium distachyon* L. cystatin genes reveal high evolutionary conservation and functional divergence in response to abiotic stress. Front. Plant Sci. 8, 743. https://doi.org/10.3389/fpls.2017.00743.

Sun, J.L., Li, J.Y., Wang, M.J., *et al.* (2021). Protein quality control in plant organelles: Current progress and future perspectives. Mol. Plant 14, 95-114. https://doi.org/10.1016/j.molp.2020.10.011.

Sun, Q.H., Miao, C.Y., Hanel, M., *et al.* (2019). Global heat stress on health, wildfires, and agricultural crops under different levels of climate warming. Environ. Int. 128, 125-136. https://doi.org/10.1016/j.envint.2019.04.025.

Sunyer, R., Trepat, X., Fredberg, J.J., *et al.* (2009). The temperature dependence of cell mechanics measured by atomic force microscopy. Phys. Biol. 6, 025009. https://doi.org/10.1088/1478-3975/6/2/025009.

Swanson, S., Gilroy, S. (2010). ROS in plant development. Physiol. Plant. 138, 384-392. https://doi.org/10.1111/j.1399-3054.2009.01313.x.

Swindle, E.J., Metcalfe, D.D. (2007). The role of reactive oxygen species and nitric oxide in mast cell-dependent inflammatory processes. Immunol. Rev. 217, 186-205. https://doi.org/10.1111/j.1600-065X.2007.00513.x.

Swoboda, I., Scheiner, O., Kraft, D., *et al.* (1994). A birch gene family encoding pollen allergens and pathogenesis-related proteins. Biochim. Biophys. Acta 1219, 457-464. https://doi.org/10.1016/0167-4781(94)90072-8.

Takahashi, M., Morikawa, H. (2014). Nitrogen dioxide is a positive regulator of plant growth. Plant Signal Behav. 9, e28033. https://doi.org/10.4161/psb.28033.

Takahashi, M., Morikawa, H. (2019a). Nitrate, but not nitrite, derived from nitrogen dioxide accumulates in *Arabidopsis* leaves following exposure to ^{15}N-labeled nitrogen dioxide. Plant Signal Behav. 14, 1559579. https://doi.org/10.1080/15592324.2018.1559579.

Takahashi, M., Morikawa, H. (2019b). Nitrogen dioxide at ambient concentrations induces nitration and degradation of PYR/PYL/RCAR receptors to stimulate plant growth: A hypothetical model. Plants (Basel) 8, 198. https://doi.org/10.3390/plants8070198.

Takahashi, M., Nakagawa, M., Sakamoto, A., *et al.* (2005). Atmospheric nitrogen dioxide gas is a plant vitalization signal to increase plant size and the contents of cell constituents. New Phytol. 168, 149-154. https://doi.org/10.1111/j.1469-8137.2005.01493.x.

Takahashi, M., Sakamoto, A., Ezura, H., *et al.* (2011). Prolonged exposure to atmospheric nitrogen dioxide increases fruit yield of tomato plants. Plant Biotechnol. 28, 485-487. https://doi.org/10.5511/plantbiotechnology.11.0819a.

Tan, Y., Yang, Y., Li, C., *et al.* (2017). Overexpression of *MpCYS4*, a phytocystatin gene from *Malus prunifolia* (Willd.) Borkh., delays natural and stress-induced leaf senescence in apple. Plant Physiol. Biochem. 115, 219-228. https://doi.org/10.1016/j.plaphy.2017.03.025.

Tang, D., Chen, M., Huang, X., *et al.* (2023). SRplot: A free online platform for data visualization and graphing. PLOS ONE 18, e0294236. https://doi.org/10.1371/journal.pone.0294236.

Taylor, A.O. (1965). Some effects of photoperiod on the biosynthesis of phenylpropane derivatives in *Xanthium*. Plant Physiol. 40, 273-280. https://doi.org/10.1104/pp.40.2.273.

Taylor, J.L., Fritzemeier, K.H., Haeuser, I., *et al.* (1990). Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. Mol. Plant-Microbe Interact. 3, 72-77. https://api.semanticscholar.org/CorpusID:21965530

Taylor, P.E., Esch, R., Flagan, R.C., *et al.* (2006). Identification and possible disease mechanisms of an under-recognized fungus, *Aureobasidium pullulans*. Int. Arch. Allergy Immunol. 139, 45-52. https://doi.org/10.1159/000089522.

Tedersoo, L., Anslan, S., Bahram, M., *et al.* (2015). Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in

metabarcoding analyses of fungi. MycoKeys 10, 1-43. https://doi.org/10.3897/MYCOKEYS.10.4852.

Tegart, L.J., Johnston, F.H., Borchers Arriagada, N*., et al.* (2021). 'Pollen potency': The relationship between atmospheric pollen counts and allergen exposure. Aerobiologia 37, 825-841. https://doi.org/10.1007/s10453-021-09726-3.

Toole, E.H., Brown, E. (1946). Final results of the Duvel buried seed experiment. J. Agric. Res. 72, 201-210. https://api.semanticscholar.org/CorpusID:82629244

Torres, N., Hilbert, G., Luquin, J*., et al.* (2017). Flavonoid and amino acid profiling on *Vitis vinifera* L. cv Tempranillo subjected to deficit irrigation under elevated temperatures. J. Food Compos. Anal. 62, 51-62. https://doi.org/10.1016/j.jfca.2017.05.001.

Traidl-Hoffmann, C., Jakob, T., Behrendt, H. (2009). Determinants of allergenicity. J. Allergy Clin. Immunol. 123, 558-566. https://doi.org/10.1016/j.jaci.2008.12.003.

Traidl-Hoffmann, C., Kasche, A., Jakob, T*., et al.* (2002). Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes. J. Allergy Clin. Immunol. 109, 831-838. https://doi.org/10.1067/mai.2002.124655.

Vaghela, B., Vashi, R., Rajput, K*., et al.* (2022). Plant chitinases and their role in plant defense: A comprehensive review. Enzyme Microb. Technol. 159, 110055. https://doi.org/10.1016/j.enzmictec.2022.110055.

Vaidyanathan, H., Sivakumar, P., Chakrabarty, R*., et al.* (2003). Scavenging of reactive oxygen species in NaCl-stressed rice (*Oryza sativa* L.)—differential response in salt-tolerant and sensitive varieties. Plant Sci. 165, 1411-1418. https://doi.org/10.1016/j.plantsci.2003.08.005.

Vakili-Moghaddam, M., Fallahpour, M., Mohammadi, M*., et al.* (2019). Identification of polcalcin as a novel allergen of *Amaranthus retroflexus* pollen. Allergol. Immunopathol. (Madr) 47, 357-364. https://doi.org/10.1016/j.aller.2018.12.006.

Valenta, R., Hayek, B., Seiberler, S*., et al.* (1998). Calcium-binding allergens: From plants to man. Int. Arch. Allergy Immunol. 117, 160-166. https://doi.org/10.1159/000024005.

Van-Der Linde, K., Hemetsberger, C., Kastner, C*., et al.* (2012a). A maize cystatin suppresses host immunity by inhibiting apoplastic cysteine proteases. Plant Cell 24, 1285-1300. https://doi.org/10.1105/tpc.111.093732.

Van-Der Linde, K., Mueller, A.N., Hemetsberger, C*., et al.* (2012b). The maize cystatin CC9 interacts with apoplastic cysteine proteases. Plant Signal Behav. 7, 1397-1401. https://doi.org/10.4161/psb.21902.

Van-Der Vliet, A. (2011). Nox enzymes in allergic airway inflammation. Biochim. Biophys. Acta 1810, 1035-1044. https://doi.org/10.1016/j.bbagen.2011.03.004.

Van-Loon, L.C., Pierpoint, W.S., Boller, T.H*., et al.* (1994). Recommendations for naming plant pathogenesis-related proteins. Plant Mol. Biol. Rep. 12, 245-264. https://doi.org/10.1007/BF02668748.

Van-Loon, L.C., Rep, M., Pieterse, C.M. (2006). Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. 44, 135-162. https://doi.org/10.1146/annurev.phyto.44.070505.143425.

Van-Loon, L.C., Van-Strien, E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. 55, 85-97. https://doi.org/10.1006/pmpp.1999.0213.

Van-Vliet, A.J.H., Overeem, A., De-Groot, R.S*., et al.* (2002). The influence of temperature and climate change on the timing of pollen release in the Netherlands. Int. J. Climatol. 22, 1757-1767. https://doi.org/10.1002/joc.820.

Verdino, P., Westritschnig, K., Valenta, R*., et al.* (2002). The cross-reactive calcium-binding pollen allergen, Phl p 7, reveals a novel dimer assembly. EMBO J. 21, 5007-5016. https://doi.org/10.1093/emboj/cdf526.

Verkman, A.S. (2002). Solute and macromolecule diffusion in cellular aqueous compartments. Trends Biochem. Sci. 27, 27-33. https://doi.org/10.1016/S0968-0004(01)02003-5.

Vidali, L., Augustine, R.C., Fay, S.N*., et al.* (2009). Rapid screening for temperature-sensitive alleles in plants   Plant Physiol. 151, 506-514. https://doi.org/10.1104/pp.109.143727.

Vidali, L., McKenna, S.T., Hepler, P.K. (2001). Actin polymerization is essential for pollen tube growth. Mol. Biol. Cell 12, 2534-2545. https://doi.org/10.1091/mbc.12.8.2534.

Vignais, P.V. (2002). The superoxide-generating NADPH oxidase: Structural aspects and activation mechanism. Cell. Mol. Life Sci.   59, 1428-1459. https://doi.org/10.1007/s00018-002-8520-9.

Vo, T.S. (2020). Natural products targeting FcεRI receptor for anti-allergic therapeutics. J. Food Biochem. 44, e13335. https://doi.org/10.1111/jfbc.13335.

Volcheck, G.W. (2009). Environmental Allergens, in: Volcheck, G.W. (Ed.), Clinical Allergy: Diagnosis and Management. Humana Press, Totowa, NJ, pp 41-77. https://doi.org/10.1007/978-1-59745-315-8_2.

Vranová, E., Inzé, D., Van-Breusegem, F. (2002). Signal transduction during oxidative stress. J. Exp. Bot. 53, 1227-1236. https://doi.org/10.1093/jexbot/53.372.1227.

Wagner, S., Breiteneder, H., Simon-Nobbe, B*., et al.* (2000). Hev b 9, an enolase and a new cross-reactive allergen from *hevea* latex and molds. Purification, characterization, cloning and expression. Eur. J. Biochem. 267, 7006-7014. https://doi.org/10.1046/j.1432-1327.2000.01801.x.

Wagner, S., Radauer, C., Bublin, M*., et al.* (2008). Naturally occurring hypoallergenic Bet v 1 isoforms fail to induce IgE responses in individuals with birch pollen allergy. J. Allergy Clin. Immunol. 121, 246-252. https://doi.org/10.1016/j.jaci.2007.08.006.

Wagner, S., Sowka, S., Mayer, C*., et al.* (2001). Identification of a *Hevea brasiliensis* latex manganese superoxide dismutase (Hev b 10) as a cross-reactive allergen. Int. Arch. Allergy Immunol. 125, 120-127. https://doi.org/10.1159/000053805.

Wahid, A., Gelani, S., Ashraf, M*., et al.* (2007). Heat tolerance in plants: An overview. Environ. Exp. Bot. 61, 199-223. https://doi.org/10.1016/j.envexpbot.2007.05.011.

Wang, H.J., Huang, J.C., Jauh, G.Y. (2010). Pollen Germination and Tube Growth, in: Kader, J.C., Delseny, M. (Eds.), Advances in Botanical Research. Academic Press, pp 1-52. https://doi.org/10.1016/S0065-2296(10)54001-1.

Wang, H.J., Schippers, J.H.M. (2019). The role and regulation of autophagy and the proteasome during aging and senescence in plants. Genes 10, 267. https://doi.org/10.3390/genes10040267.

Wang, J., Gao, Z.P., Qin, S., *et al.* (2017). Calreticulin is an effective immunologic adjuvant to tumor-associated antigens. Exp. Ther. Med. 14, 3399-3406. https://doi.org/10.3892/etm.2017.4989.

Wang, W., Vinocur, B., Shoseyov, O., *et al.* (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 9, 244-252. https://doi.org/10.1016/j.tplants.2004.03.006.

Wang, W., Zhang, X., Zhu, L., *et al.* (2020). Investigation of allergic sensitization pattern in 4203 children in Northern China. Int. Arch. Allergy Immunol. 182, 455-458. https://doi.org/10.1159/000511976.

Wang, X.L., Takai, T., Kamijo, S., *et al.* (2009). NADPH oxidase activity in allergenic pollen grains of different plant species. Biochem. Biophys. Res. Commun. 387, 430-434. https://doi.org/10.1016/j.bbrc.2009.07.020.

Wang, Y., Branicky, R., Noë, A., *et al.* (2018). Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J. Cell Biol. 217, 1915-1928. https://doi.org/10.1083/jcb.201708007.

Wang, Y., Liu, M., Wang, X., *et al.* (2021). A novel β-1,3-glucanase Gns6 from rice possesses antifungal activity against Magnaporthe oryzae. J. Plant Physiol. 265, 153493. https://doi.org/10.1016/j.jplph.2021.153493.

Wang, Y., Teng, Z.Y., Zhang, X.L., *et al.* (2019). Research progress on the effects of atmospheric nitrogen dioxide on plant growth and metabolism. Chin. J. Appl. Ecol. 30, 316-324. https://doi.org/10.13287/j.1001-9332.201901.028.

Wang, Z., Xiao, Y., Chang, H., *et al.* (2023). The regulatory network of sweet corn (*Zea mays* L.) seedlings under heat stress revealed by transcriptome and metabolome analysis. Int. J. Mol. Sci. 24, 10845. https://doi.org/10.3390/ijms241310845.

Waraich, E.A., Ahmad, R., Ashraf, M.Y., *et al.* (2011). Improving agricultural water use efficiency by nutrient management in crop plants. Acta Agric. Scand., Sect. B 61, 291-304. https://doi.org/10.1080/09064710.2010.491954.

Waraich, E.A., Ahmad, R., Halim, A., *et al.* (2012). Alleviation of temperature stress by nutrient management in crop plants: A review. J. Soil Sci. Plant Nutr. 12, 221-244. http://dx.doi.org/10.4067/S0718-95162012000200003

Warner, S.A.J., Scott, R., Draper, J. (1993). Isolation of an asparagus intracellular *PR* gene (*AoPR1*) wound-responsive promoter by the inverse polymerase chain reaction and its characterization in transgenic tobacco. Plant J. 3, 191-201. https://doi.org/10.1046/j.1365-313X.1993.t01-11-00999.x.

Webster, T.M., Nichols, R.L. (2012). Changes in the prevalence of weed species in the major agronomic crops of the Southern United States: 1994/1995 to 2008/2009. Weed Sci. 60, 145-157. https://doi.org/10.1614/WS-D-11-00092.1.

Wei, D., Ci, X., Chu, X., *et al.* (2012). Hesperidin suppresses ovalbumin-induced airway inflammation in a mouse allergic asthma model. Inflammation 35, 114-121. https://doi.org/10.1007/s10753-011-9295-7.

Wei, H., Dhanaraj, A.L., Arora, R., *et al.* (2006). Identification of cold acclimation-responsive Rhododendron genes for lipid metabolism, membrane transport and lignin biosynthesis: Importance of moderately abundant ESTs in genomic studies. Plant Cell Environ. 29, 558-570. https://doi.org/10.1111/j.1365-3040.2005.01432.x.

Wei, M., Yu, C., Ge, B., *et al.* (2022). The phenylcoumaran benzylic ether reductase gene *PtPCBER* improves the salt tolerance of transgenic poplar through lignan-mediated reactive oxygen species scavenging. Environ. Exp. Bot. 201, 104946. https://doi.org/10.1016/j.envexpbot.2022.104946.

Wessel, D., Flügge, U.I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138, 141-143. https://doi.org/10.1016/0003-2697(84)90782-6.

Wetterdienst, D.D. (2016). The climatological period of 1981-2010 in Munich and Würzburg areas. 25.02.2016. https://opendata.dwd.de/climate_environment/CDC/observations_germany/climate/daily/kl/historical/.

White, J.F., Bernstein, D.I. (2003). Key pollen allergens in North America. Ann. Allergy, Asthma, Immunol. 91, 425-435. https://doi.org/10.1016/S1081-1206(10)61509-8.

White, T.J., Bruns, T.D., Lee, S., *et al.* (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc.: Guide Methods Appl. 31, 315-322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1.

Williams, P.B., Barnes, C.S., Portnoy, J.M., *et al.* (2016). Innate and adaptive immune response to fungal products and allergens. J. Allergy Clin. Immunol. Pract. 4, 386-395. https://doi.org/10.1016/j.jaip.2015.11.016.

Wimmer, M., Alessandrini, F., Gilles, S., *et al.* (2015). Pollen-derived adenosine is a necessary cofactor for ragweed allergy. Allergy 70, 944-954. https://doi.org/10.1111/all.12642.

Wolf, M., Twaroch, T.E., Huber, S., *et al.* (2017). Amb a 1 isoforms: Unequal siblings with distinct immunological features. Allergy 72, 1874-1882. https://doi.org/10.1111/all.13196.

Woo, H.H., Kuleck, G., Hirsch, A.M., *et al.* (2002). Flavonoids: Signal Molecules in Plant Development, in: Buslig, B.S., Manthey, J.A. (Eds.), Flavonoids in Cell Function. Springer US, Boston, MA, pp 51-60. https://doi.org/10.1007/978-1-4757-5235-9_5.

Wopfner, N., Bauer, R., Thalhamer, J., *et al.* (2007). Natural and recombinant Amb a 1: Immunologic analysis of IgE and monoclonal antibody epitopes. J. Allergy Clin. Immunol. 119, S106. https://doi.org/10.1016/j.jaci.2006.11.395.

Wopfner, N., Gadermaier, G., Egger, M., *et al.* (2005). The spectrum of allergens in ragweed and mugwort pollen. Int. Arch. Allergy Immunol. 138, 337-346. https://doi.org/10.1159/000089188.

Wopfner, N., Gruber, P., Wallner, M., *et al.* (2008). Molecular and immunological characterization of novel weed pollen pan-allergens. Allergy 63, 872-881. https://doi.org/10.1111/j.1398-9995.2008.01635.x.

Wopfner, N., Jahn-Schmid, B., Schmidt, G., *et al.* (2009). The alpha and beta subchain of Amb a 1, the major ragweed-pollen allergen show divergent reactivity at the IgE and T-cell level. Mol. Immunol. 46, 2090-2097. https://doi.org/10.1016/j.molimm.2009.02.005.

Xia, X.J., Zhou, Y.H., Shi, K., *et al.* (2015). Interplay between reactive oxygen species and hormones in the control of plant development and stress tolerance. J. Exp. Bot. 66, 2839-2856. https://doi.org/10.1093/jxb/erv089.

Xie, D.L., Zheng, X.L., Zhou, C.Y., *et al.* (2022). Functions of redox signaling in pollen development and stress response. Antioxidants 11, 287. https://doi.org/10.3390/antiox11020287.

Xie, Y., Luo, X., Li, Y., *et al.* (2018). DeepNitro: Prediction of protein nitration and nitrosylation sites by deep learning. Genomics, Proteomics Bioinf. 16, 294-306. https://doi.org/10.1016/j.gpb.2018.04.007.

Xu, C., Sullivan, J.H., Garrett, W.M., *et al.* (2008). Impact of solar Ultraviolet-B on the proteome in soybean lines differing in flavonoid contents. Phytochemistry 69, 38-48. https://doi.org/10.1016/j.phytochem.2007.06.010.

Xu, P., Jiang, L., Wu, J., *et al.* (2014). Isolation and characterization of a pathogenesis-related protein 10 gene (*GmPR10*) with induced expression in soybean (*Glycine max*) during infection with *Phytophthora sojae*. Mol. Biol. Rep. 41, 4899-4909. https://doi.org/10.1007/s11033-014-3356-6.

Xu, S., Li, J., Zhang, X., *et al.* (2006). Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. Environ. Exp. Bot. 56, 274-285. https://doi.org/10.1016/j.envexpbot.2005.03.002.

Yang, B., Liu, H., Yang, J., *et al.* (2018). New insights on bioactivities and biosynthesis of flavonoid glycosides. Trends Food Sci. Technol. 79, 116-124. https://doi.org/10.1016/j.tifs.2018.07.006.

Yang, H., Zhang, Y., Pöschl, U. (2010). Quantification of nitrotyrosine in nitrated proteins. Anal. Bioanal. Chem. 397, 879-886. https://doi.org/10.1007/s00216-010-3557-3.

You, J., Chan, Z. (2015). ROS regulation during abiotic stress responses in crop plants. Front. Plant Sci. 6, 165102. https://doi.org/10.3389/fpls.2015.01092.

Yuan, G., Gao, H., Yang, T. (2023). Exploring the role of the plant actin cytoskeleton: From signaling to cellular functions. Int. J. Mol. Sci. 24, 15480. https://doi.org/10.3390/ijms242015480.

Yuan, Y., Liu, Y., Wu, C., *et al.* (2012). Water deficit affected flavonoid accumulation by regulating hormone metabolism in *Scutellaria baicalensis* Georgi roots. PLOS ONE 7, e42946. https://doi.org/10.1371/journal.pone.0042946.

Yue, X., Gao, X., Wang, F., *et al.* (2014). Transcriptional evidence for inferred pattern of pollen tube-stigma metabolic coupling during pollination. PLOS ONE 9, e107046. https://doi.org/10.1371/journal.pone.0107046.

Yun, Z., Gao, H., Liu, P., *et al.* (2013). Comparative proteomic and metabolomic profiling of citrus fruit with enhancement of disease resistance by postharvest heat treatment. BMC Plant Biol. 13, 44. https://doi.org/10.1186/1471-2229-13-44.

Zahid, S.M., Farooq, M., Yasmin, M*., et al.* (2022). Alternative splicing plays a vital role in regulating pollen allergen (Ole e 1) P19963 protein in *Gossypium arboreum*. Plant Gene 31, 100362. https://doi.org/10.1016/j.plgene.2022.100362.

Zandi, P., Schnug, E. (2022). Reactive oxygen species, antioxidant responses and implications from a microbial modulation perspective. Biology (Basel) 11, 155. https://doi.org/10.3390/biology11020155.

Zaynab, M., Peng, J.F., Sharif, Y*., et al.* (2021). Expression profiling of pathogenesis-related Protein-1 (PR-1) genes from Solanum tuberosum reveals its critical role in phytophthora infestans infection. Microb. Pathog. 161, 105290. https://doi.org/10.1016/j.micpath.2021.105290.

Zeevaart, A.J. (1976). Some effects of fumigating plants for short periods with NO_2. Environ. Pollut. 11, 97-108. https://doi.org/10.1016/0013-9327(76)90022-7.

Zenda, T., Wang, N., Dong, A*., et al.* (2022). Reproductive-stage heat stress in cereals: Impact, plant responses and strategies for tolerance improvement. Int. J. Mol. Sci. 23, 6929. https://doi.org/10.3390/ijms23136929.

Zeng, J.K., Li, X., Zhang, J*., et al.* (2016). Regulation of loquat fruit low temperature response and lignification involves interaction of heat shock factors and genes associated with lignin biosynthesis. Plant Cell Environ. 39, 1780-1789. https://doi.org/10.1111/pce.12741.

Zeng, W., Zhao, H., Liu, R*., et al.* (2020). Association between NO_2 cumulative exposure and influenza prevalence in mountainous regions: A case study from southwest China. Environ. Res. 189, 109926. https://doi.org/10.1016/j.envres.2020.109926.

Zhang, H., Zhao, Y., Zhu, J. (2020a). Thriving under stress: How plants balance growth and the stress response. Dev. Cell 55, 529-543. https://doi.org/10.1016/j.devcel.2020.10.012.

Zhang, H., Zhou, Z., An, J. (2019). Pollen release dynamics and daily patterns of pollen-collecting activity of honeybee *Apis mellifera* and bumblebee *Bombus lantschouensis* in solar greenhouse. Insects 10, 216. https://doi.org/10.3390/insects10070216.

Zhang, Q.H., Nie, F.H., Wang, X.N*., et al.* (2018). Effect of flavonoids from *Eupatorium odoratum* L. on immunoglobulins and cecal microflora in broilers. Pak. Vet. J. 38, 296-300. https://doi.org/10.29261/pakvetj/2018.068.

Zhang, T., Hu, Z., Cheng, Y*., et al.* (2020b). Changes in allergenicity of ovalbumin *in vitro* and *in vivo* on conjugation with quercetin. J. Agric. Food Chem. 68, 4027-4035. https://doi.org/10.1021/acs.jafc.0c00461.

Zhang, W., Wang, S., Yang, J*., et al.* (2022). Glycosylation of plant secondary metabolites: Regulating from chaos to harmony. Environ. Exp. Bot. 194, 104703. https://doi.org/10.1016/j.envexpbot.2021.104703.

Zhang, X.N., Mount, S.M. (2009). Two alternatively spliced isoforms of the Arabidopsis SR45 protein have distinct roles during normal plant development. Plant Physiol. 150, 1450-1458. https://doi.org/10.1104/pp.109.138180.

Zhang, Y., Bielory, L., Mi, Z., *et al.* (2015). Allergenic pollen season variations in the past two decades under changing climate in the United States. Global Change Biol. 21, 1581-1589. https://doi.org/10.1111/gcb.12755.

Zhang, Y., Ji, T.T., Li, T.T., *et al.* (2020c). Jasmonic acid promotes leaf senescence through MYC2-mediated repression of *CATALASE2* expression in Arabidopsis. Plant Sci. 299, 110604. https://doi.org/10.1016/j.plantsci.2020.110604.

Zhang, Y.X., Steiner, A.L. (2022). Projected climate-driven changes in pollen emission season length and magnitude over the continental United States. Nat. Commun. 13, 1234. https://doi.org/10.1038/s41467-022-28764-0.

Zhao, F., Durner, J., Winkler, J.B., *et al.* (2017a). Pollen of common ragweed (*Ambrosia artemisiifolia* L.): Illumina-based de novo sequencing and differential transcript expression upon elevated NO_2/O_3. Environ. Pollut. 224, 503-514. https://doi.org/10.1016/j.envpol.2017.02.032.

Zhao, F., Elkelish, A., Durner, J., *et al.* (2016). Common ragweed (*Ambrosia artemisiifolia* L.): Allergenicity and molecular characterization of pollen after plant exposure to elevated NO_2. Plant, Cell Environ. 39, 147-164. https://doi.org/10.1111/pce.12601.

Zhao, F., Heller, W., Stich, S., *et al.* (2017b). Effects of NO_2 on inflorescence length, pollen/seed amount and phenolic metabolites of common ragweed (*Ambrosia artemisiifolia* L.). Am. J. Plant Sci. 8, 2860-2870. https://doi.org/10.4236/ajps.2017.811194.

Zheng, C., Wang, Y., Ding, Z., *et al.* (2016). Global transcriptional analysis reveals the complex relationship between tea quality, leaf senescence and the responses to cold-drought combined stress in *Camellia sinensis*. Front. Plant Sci. 7, 1858. https://doi.org/10.3389/fpls.2016.01858.

Zheng, R.H., Su, S.D., Xiao, H., *et al.* (2019). Calcium: A critical factor in pollen germination and tube elongation. Int. J. Mol. Sci. 20, 420. https://doi.org/10.3390/ijms20020420.

Zhou, S., Wang, X., Lu, S., *et al.* (2021). Characterization of allergenicity of *Platanus* pollen allergen a 3 (Pla a 3) after exposure to NO_2 and O_3. Environ. Pollut. 278, 116913. https://doi.org/10.1016/j.envpol.2021.116913.

Zhu, J., Thompson, C.B. (2019). Metabolic regulation of cell growth and proliferation. Nat. Rev. Mol. Cell Biol. 20, 436-450. https://doi.org/10.1038/s41580-019-0123-5.

Zhu, Y., Chen, J., Bi, X., *et al.* (2020). Spatial and temporal representativeness of point measurements for nitrogen dioxide pollution levels in cities. Atmos. Chem. Phys. 20, 13241-13251. https://doi.org/10.5194/acp-20-13241-2020.

Ziska, L., Knowlton, K., Rogers, C., *et al.* (2011). Recent warming by latitude associated with increased length of ragweed pollen season in central North America. Proc. Natl. Acad. Sci. U. S. A. 108, 4248-4251. https://doi.org/10.1073/pnas.1014107108.

Ziska, L.H., Bunce, J.A., Goins, E.W. (2004). Characterization of an urban-rural CO_2/temperature gradient and associated changes in initial plant productivity during secondary succession. Oecologia 139, 454-458. https://doi.org/10.1007/s00442-004-1526-2.

Ziska, L.H., Gebhard, D.E., Frenz, D.A.*, et al.* (2003). Cities as harbingers of climate change: Common ragweed, urbanization, and public health. J. Allergy. Clin. Immunol. 111, 290-295. https://doi.org/10.1067/mai.2003.53.

Ziska, L.H., Makra, L., Harry, S.K.*, et al.* (2019). Temperature-related changes in airborne allergenic pollen abundance and seasonality across the northern hemisphere: A retrospective data analysis. Lancet Planet. Health 3, e124-e131. https://doi.org/10.1016/S2542-5196(19)30015-4.

Zlatev, Z.S., Lidon, F.C., Ramalho, J.C.*, et al.* (2006). Comparison of resistance to drought of three bean cultivars. Biol. Plant. 50, 389-394. https://doi.org/10.1007/s10535-006-0054-9.

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