TECHNISCHE UNIVERSITÄT MÜNCHEN

TUM School of Life Sciences

Natural variation in early developmental processes and abiotic stress tolerance between *Crassocephalum* species and establishment of tools for the genetic improvement of these orphan crops

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Vollständiger Abdruck der von der TUM School of Life Sciences der Technischen Universität München zur Erlangung des akademischen Grades einer

Doktorin der Agrarwissenschaften

genehmigten Dissertation.

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Die Dissertation wurde am 25.04.2022 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 09.08.2022 angenommen.

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Abstract

Crassocephalum crepidioides and *C. rubens* are underutilized, traditional leafy vegetables that are indigenous to tropical Africa but grow throughout tropical and sub-tropical regions of the world. They are annuals that belong to the Asteraceae, propagate rapidly and grow well even in marginal conditions. Because of their high content in nutrients, vitamins and essential oils, *C*. *crepidioides* and *C. rubens* are used as leafy vegetables and medicinal plants in Sub-Saharan Africa and Asia. Although they are of high regional importance for example in Nigeria and are considered to be among the 101 most important orphan crops of Africa, *Crassocephalum* species are not regularly cultivated but are still mainly harvested from the wild. Therefore, this thesis aimed to generate knowledge, methodology and materials for the genetic improvement and domestication of these plants.

In the first part of the work, seed development and germination capacities of *Crassocephalum* species were investigated, since the seeds exhibit a high degree of dormancy, which impairs their propagation in the field. Through comparison of different ecotypes from Africa and Asia, it could be shown that *C. crepidioides* seeds are smaller and have a higher degree of dormancy than those of *C. rubens*, which was correlated with a higher level of the plant hormone abscisic acid (ABA). Dormancy could be broken by light-treatments, but also by the application of Fluridone, an inhibitor of ABA biosynthesis. Since ABA promotes abiotic stress resistance in many plant species, responses of *C. crepidioides* and *C. rubens* to drought, heat and cold stress were compared. This showed that *C. crepidioides* was more resistant to drought and heat stress than *C. rubens*, which will benefit its cultivation, particularly in rain-fed agricultural systems. Since the increased drought tolerance was not correlated with increased ABA levels, it appeared that other modes than an increased ABA biosynthesis, account for the elevated resistance in *C. crepidioides* and this is discussed.

As a second aim of this work, protocols and resources for research and breeding with *Crassocephalum* were generated. On the one hand, an *Agrobacterium*-mediated transformation technique was developed, establishing regeneration and transformation protocols and using a *35S:GUS* reporter construct as a reference transgene. On the other hand, *C. rubens* was mutagenized with EMS, and a mutant collection for research and breeding was generated. The population was subjected to a first characterization, identifying different mutant classes, which are described.

In summary, this work generated knowledge and resources for research and a genetic improvement of *Crassocephalum* species, to facilitate the domestication of these wild crops and exploit their potential as nutritious leafy vegetable and medicinal plants.

Zusammenfassung

Crassocephalum crepidioides und *C. rubens* sind traditionelle Blattgemüsearten, die im tropischen Afrika beheimatet sind, aber in allen tropischen und subtropischen Regionen der Erde vorkommen. Sie sind einjährige Pflanzen, die zur Familie der Asteraceae gehören, sich rasch vermehren und auch unter kargen Bedingungen gut gedeihen. Aufgrund ihres hohen Gehalts an Nährstoffen, Vitaminen und ätherischen Ölen werden *C. crepidioides* und *C. rubens* in Subsahara Afrika und Asien als Blattgemüse und Heilpflanzen genutzt. Trotz ihrer hohen regionalen Bedeutung, z.B. in Nigeria, werden *Crassocephalum*-Arten aber immer noch hauptsächlich gesammelt und Ziel dieser Arbeit war es daher, Wissen, Methodik und Materialien zu generieren, die ein züchterische Erschließung und Inkulturnahme der Pflanzen ermöglichen.

Im ersten Teil der Dissertation wurde die Keimfähigkeit untersucht, da Samen von *Crassocephalum*-Arten eine starke Keimruhe aufweisen, was ihren Anbau beeinträchtigt. Durch Vergleich verschiedener Ökotypen aus Afrika und Asien konnte festgestellt werden, dass Samen von *C. crepidioides* eine höhere Dormanz aufweisen, als Samen von *C. rubens*, was mit einem höheren Gehalt des Pflanzenhormons Abscisinsäure (ABA) korrelierte. Die Samenruhe konnte durch Lichtbehandlungen, aber auch durch Behandlung mit Fluridon, einem Inhibitor der ABA-Biosynthese gebrochen werden. Da ABA bei vielen Pflanzenarten die Resistenz gegen abiotischen Stress fördert, wurden die Resistenz von *C. crepidioides* und *C. rubens* gegen Trockenheit, Hitze- und Kältestress verglichen. Dies zeigte, dass *C. crepidioides* eine höhere Toleranz gegen Trockenheit und Hitzestress aufwies, was ihrem Anbau zugutekommen sollte, insbesondere in Anbauregionen ohne Bewässerung. Die erhöhte Trockenstressresistenz war allerdings nicht mit erhöhten ABA Konzentrationen korreliert, was nahelegt, dass andere Mechanismen, als eine dynamischere Aktivierung der ABA Biosynthese, für die erhöhte Resistenz verantwortlich sind und das wird diskutiert.

Als zweites Ziel dieser Arbeit wurden Protokolle und Ressourcen für Forschung und Züchtung erstellt. Einerseits wurde eine Transformationstechnik für *Crassocephalum* entwickelt, wofür Regenerationsprotokolle etabliert und ein 35S:GUS-Reporter als Modelltransgen verwendet wurden. Andererseits wurde *C. rubens* mit EMS mutagenisiert, um eine Mutantenkollektion für Forschung und Mutationszüchtung aufzubauen. Die Population wurde grob charakterisiert und verschiedene Klassen von Mutanten wurden identifiziert, die beschrieben werden.

Zusammenfassend hat diese Arbeit Wissen und Ressourcen geschaffen, um die Erforschung und genetische Erschließung von *Crassocephalum*-Arten zu fördern, und die Domestizierung dieser Wildpflanzen voranzutreiben.

1. Introduction

1.1. *Crassocephalum crepidioides* and *C. rubens* are orphan crops

Crassochelum crepidioides and its close relative *C. rubens* are orphan crops that originate from tropical Africa but can be found in all tropical and subtropical regions of the world. They belong to the plant family Asteraceae (subfamily Asteroideae, tribe Senecioneae) (Pelser *et al.*, 2007) and are grown as vegetables and medicinal plants (Bosch, 2004; Denton, 2004; Dairo & Adanlawo, 2007). They also have some relevance as weeds in Asia, Australia, and North and South America (Ismail *et al.*, 2001; Nakamura & Hossain, 2009; Yuan & Wen, 2018). In folk nomenclature, both species are usually referred to with the same name and in Africa, these common names are Yoruba bologi, Ebolo (Nigeria) or Gbolo (Benin). English names are redflower ragleaf, thickhead and fireweed (Denton, 2004; Adjatin *et al.*, 2012).

C. crepidioides and *C. rubens* are annual erect herbs with a relatively short life cycle. *C. crepidioides* completes it in about 15 to 17 weeks, while *C. rubens* takes about 10 to 16 weeks until seed dispersal, depending on the growing season (Denton, 2004; Schramm *et al.*, 2021). Both species have lobed, irregularly serrated leaves that are spirally arranged on a stout, ripped stem and flower at approximately 6-10 weeks post-germination (Bosch, 2004; Denton, 2004; Schramm *et al.*, 2021). The seeds are cylindric-linear, ribbed achene, which are attached to a white, silky pappus for wind dispersal (Mitra & Mukherjee, 2003; Bosch, 2004; Denton, 2004).

In terms of shoot architecture, *C. crepidioides* plants are generally larger and produce more leaves than *C. rubens* (Schramm et al., 2021). *C. crepidioides* can grow up to 180 cm tall, whereas *C. rubens* reaches a size of only about 80 cm. The flower colour differs between the two species. The corolla of *C. crepidioides* is brick-red while that of *C. rubens* is purple (Denton, 2004; Adjatin *et al.*, 2013b). In addition, also the genetic make-up differs. The basic chromosome number of *Crassocephalum* plants is n=10. *Crassocephlaum crepidioides* is tetraploid with a chromosome number of 2n=40, while *C. rubens* is diploid with a chromosome number of 2n=20 (Karna *et al.*, 2013; Oyelakin & Ayodele, 2013; Rozhon *et al.*, 2018; Schramm *et al.*, 2021). In terms of genome size, *C. crepidioides* has a genome size of approximately 12.1 Gbp and *C. rubens* has a genome size of 5.8 Gbp (Schramm *et al.*, 2021).

C. crepidioides and *C. rubens* are majorly grown as leafy vegetables and are eaten as a vegetable in sauces, soups and stews. They can also be eaten raw in salads (Bosch, 2004; Denton, 2004; Adjatin *et al.*, 2012). Both *C. crepidioides* and *C. rubens* have interesting nutritional and medicinal properties. They are rich in vitamin C, minerals, essential oils and antioxidants (Yehouenou *et al.*, 2010; Joshi, 2011; Adjatin *et al.*, 2013a; Oyebode *et al.*, 2019).

Although their nutritional values are slightly different, they are both highly nutritious and contain a good amount of protein. The leaves of *C. crepidioides* contain 42.2% carbohydrates, 27.1% protein, 3.5% total lipids and 8.1% dietary fibre in % of dry matter, while *C. rubens* contains 43.1 % carbohydrates, 26.4 % protein, 2.8 % total lipids, 8 % dietary fiber. For 100 g of fresh leaves, the vitamin C content is 9.17 mg for *C. crepidioides* and 3.60 mg for *C. rubens.* In addition, *C. crepidioides* has a relatively high total dry mass as compared to *C. rubens* (Adjatin *et al.*, 2013a).

Figure 1. Photos of adult *C. crepidioides* and *C. rubens* plants at 7-8 weeks after germination.

C. crepidioides and C. rubens are also valued as medicinal plants. They are used in traditional medicine to treat various ailments such as diabetes, indigestion, anaemia, headache, stomach upset, liver problems, breast cancer, fresh wounds and sleeping sickness (Zollo *et al.*, 2000; Bosch, 2004; Denton, 2004; Adjatin *et al.*, 2012). They have also been reported to have medicinal activities such as antibiotic, antioxidant, antihelminthic, anti-inflammatory, anti-diabetic, anti-cancer, antimalaria, hepatoprotective, wound healing and blood regulation properties (Aniya *et al.*, 2005; Iwalewa *et al.*, 2005; Omotayo *et al.*, 2015; Calvin *et al.*, 2016; Ayodele *et al.*, 2019; Oyebode *et al.*, 2019; Adewale *et al.*, 2020; Ayodele *et al.*, 2020; Can & Thao, 2020; Oyebode *et al.*, 2022).

In addition to beneficial properties, there is evidence that *Crassocephalum* species can accumulate highly toxic pyrrolizidine alkaloids (PAs) (Asada *et al.*, 1985; Rozhon *et al.*, 2018). *C. crepidioides* forms substantial amounts of the PA jacobine, whereas *C rubens* appears to lack this capacity (Schramm *et al.*, 2021). The levels of jacobine in *C. crepidioides* depended heavily on the tissue type and growth conditions (Rozhon *et al.*, 2018). Young leaves of *C.*

crepidioides contained more than 200 mg/kg (fresh weight) of jacobine whereas older leaves only produce a trace amount. In addition, it was shown that nitrogen (N) deficiency strongly increased jacobine levels of *C. crepidioides* and that the capacity of the plant to form jacobine depended more strongly on the shoot than the root system (Schramm *et al.*, 2021).

1.2. Seed germination as a relevant trait in *Crassocephalum*

1.2.1. The biology of seed dormancy

Despite their value as nutritious food sources in Western and Central Africa *Crassocephalum* species are not commonly cultivated, but are still mostly collected from the wild. When cultivated and repeatedly harvested, *C. crepidioides* can produce 25–27 t/ ha of leaves and shoots per year (Denton, 2004). These impressive biomass gains are achieved even in marginal conditions, to which the plant is well-adapted (Joshi, 2011; Adjatin *et al.*, 2013b).

One of the problems that limit the cultivation of these plants is a high degree of seed dormancy, a major challenge for farmers that sow the seeds (Denton, 2004; Adebooye *et al.*, 2005). Seed dormancy is an inherent property of a seed that determines the environmental conditions in which it can germinate (Finch-Savage & Leubner-Metzger, 2006). It is defined as a temporary arrest in the germination of a viable seed under favourable conditions and is determined by genetics, with a substantial environmental impact, which is mediated, at least in part, by a sophisticated interplay of the plant hormones abscisic acid (ABA) and gibberellins (GAs) (Bewley, 1997; Finch-Savage & Leubner-Metzger, 2006; Bentsink & Koornneef, 2008). While ABA plays a key role in the induction of dormancy, GAs release dormancy, allowing seeds to initiate and complete germination (Bewley *et al.*, 2013).

Following histodifferentiation, seeds may develop the capacity to germinate. This capacity can be suppressed until development is completed, by the induction of primary dormancy. The stages in seed development and maturation at which primary dormancy sets in, differ from species to species. In most plant species, it usually occurs around mid-development and is often only completed close to full maturation (Bewley *et al.*, 2013; Chahtane *et al.*, 2017).

The onset of primary dormancy often coincides with a transient increase in ABA. Usually, ABA levels rise during the first half of seed development and decline during the maturation phase, but sometimes two peaks of ABA rise are observed, at mid-development and late maturation (Bewley *et al.*, 2013). The first peak of ABA accumulation during seed development is a result of its synthesis in both zygotic and maternal tissues. Maternal ABA may prevent precocious germination and facilitate early seed development, whereas ABA synthesized during late maturation originates from zygotic tissues and is associated with the induction and maintenance of dormancy (Bewley *et al.*, 2013). Only ABA produced by the embryo itself and

not maternal ABA is necessary to impose a lasting dormancy (Koornneef *et al.*, 1989; Bewley *et al.*, 2013).

1.2.2. Dormancy release and germination

Germination is a physiological process that culminates in the emergence of the seedling from the seed coat. It begins with the uptake of water by the seed (imbibition) and ends with the emergence of the embryonic axis, usually the radicle, through the structures surrounding it (Bewley *et al.*, 2013). Radicle protrusion is dependent on embryo growth, which is fueled by water uptake. The uptake of water by the seed is triphasic, begins with a rapid initial uptake (phase I, i.e. imbibition) and is followed by a plateau phase (phase II). A further increase in water uptake (phase III) occurs only when germination is completed, as the embryo axis elongates and breaks through its covering structures (Bewley, 1997; Bewley *et al.*, 2013).

Dormancy release is achieved by a variety of mechanisms that include complex interactions with the environment mediated by phytohormones. GAs play a key role in the release of dormancy and the promotion of germination. In developing seeds of many species, GA biosynthesis leads to the accumulation and storage of either bioinactive GA precursors or bioactive GA (Groot & Karssen, 1987; Toyomasu *et al.*, 1998; Yamaguchi *et al.*, 2001). Bioactive GA in the seed induces the synthesis of hydrolytic enzymes that facilitate the breaking of endosperm and seed coat, mobilizes seed storage reserves in the endosperm that support seedling growth, and finally promotes embryo growth and radicle protrusion (Groot & Karssen, 1987; Leubner-Metzger *et al.*, 1996). During seed germination, GA has been proposed to perform two functions: first, to stimulate the growth potential of the embryo and second, to weaken the structures surrounding the embryo (Bewley, 1997; Leubner-Metzger, 2003b, 2003a).

In many plant species, GA promotes germination and *de novo* GA biosynthesis during seed imbibition is crucial for seed germination. This is evident for example in *Arabidopsis thaliana* and *Solanum lycopersicum* (tomato) where imbibed seeds in the presence of GA biosynthesis inhibitors fail to germinate (Hilhorst & Karssen, 1988; Nambara *et al.*, 1991), showing that seed germination is influenced by the modulation of endogenous GA levels following seed imbibition (Yamaguchi & Kamiya, 2002).

Brassinosteroids (BRs) and ethylene also play roles in the regulation of seed dormancy and germination through their interactions with the ABA/GA biosynthetic and biosynthesis and/or signaling pathways. Ethylene often exhibits a promotive effect on germination and it was suggested that BRs promote germination by reducing the sensitivity of seeds to inhibition by ABA (Leubner-Metzger, 2003a; Bewley *et al.*, 2013). However, recent studies showed that

BRs can also control GA production and thereby impact germination (Tong *et al.*, 2014; Unterholzner *et al.*, 2015).

1.2.3. The impact of light on the hormonal control of seed germination

Light is an extremely important factor in the release of seed dormancy (Bewley *et al.*, 2013). Small-seeded plants such as *A. thaliana*, *Lactuca sativa* (lettuce), *Solanum lycopersicum* (cultivated tomato) and *Nicotiana tabacum* (tobacco) require light for germination. When seeds are buried and germinate well below the soil surface, seedlings with small nutrient reserves are unable to reach the surface, since they lack the required energy. As a result, seeds have evolved an ability to recognize their location and this is enabled by the detection of light.

A class of light receptors responsible for these processes are the phytochromes. They are the major photoreceptors involved in the release of photo-dormancy in most light requiring seeds (Kamiya & García-Martínez, 1999; Yamaguchi & Kamiya, 2002; Yang *et al.*, 2020). Phytochromes translate light impulses into internal cues, which then regulate physiological activities and GAs and ABA are among the internal chemical messengers recruited for this purpose. The action of light on seeds, transmitted by phytochromes, causes a rise in GA levels, which then activates a signalling pathway that enables germination to be completed (Yamaguchi & Kamiya, 2002; Bewley *et al.*, 2013).

Light-regulated seed germination was first studied in lettuce seeds, where it was found that red light (R) induced germination but far-red light (FR) reversibly inhibited it (Borthwick *et al.*, 1952). This discovery led to the identification of the R and FR photoreceptor phytochromes. Phytochromes perceive light in the R and FR spectrum. They are synthesized in two forms, the inactive form (Pr) that absorbs R light and is photo-converted to the active form (Pfr) that absorbs FR light. The Pfr state is reverted to the Pr state at this wavelength (Seo *et al.*, 2006; Bewley *et al.*, 2013).

Phytochromes regulate GA biosynthesis during seed germination and GAs can mimic the effect of R light to stimulate germination of dark-imbibed seeds (Toyomasu *et al.*, 1998; Yamaguchi *et al.*, 1998). The regulation of GA biosynthesis through phytochrome was extensively studied by Toyomasu *et al.* (1993). They reported that the level of GA_1 (but not its precursors GA_{19} and GA_{20}) in lettuce seeds was photoreversibly modulated by brief irradiation with R and/or FR light. GA_1 level increased after R light treatment. Irradiation with FR after R light treatment repressed the effect of R light on GA_1 formation. They also suggested that 3β-hydroxylation of GA_{20} is likely regulated by phytochrome.s

In lettuce and Arabidopsis, R light induces the biosynthesis of bioactive GA_1 and GA_4 in germinating seeds through induction of GA-3-oxidase encoding genes (*GA3ox1* and *GA3ox2*)

and repression of a GA-2-oxidase encoding gene (*GA2ox2*), which catalyzes the degradation of active GAs (Toyomasu *et al.*, 1993; Toyomasu *et al.*, 1998; Yamaguchi *et al.*, 1998; Kamiya & García-Martínez, 1999; Yamaguchi *et al.*, 2001; Kucera *et al.*, 2005; Bewley *et al.*, 2013). Also, seed germination of tobacco was found to be regulated by light. Photodormant seeds did not germinate in darkness but required a treatment with a R light pulse or GA, to release seed dormancy in the dark (Kretsch *et al.*, 1995; Leubner-Metzger, 2003b).

1.2.4. Hormonal interactions in the regulation of seed dormancy

The opposite effects of ABA and GAs on germination, with the former promoting seed dormancy and the latter repressing it (Bewley *et al.*, 2013), are governed by a sophisticated molecular cross-talk. The two hormones interact by affecting the metabolism and signal transduction pathways of one another with multiple layers of regulation.

On the one hand, ABA suppresses GA metabolism (Seo *et al.*, 2006; Toh *et al.*, 2008; Bewley *et al.*, 2013). In Arabidopsis, seeds of the ABA-deficient mutant *aba2-2* showed a higher expression of the GA biosynthetic genes *GA3ox* and *GA20ox* than those of wild type (Seo *et al.*, 2006; Toh *et al.*, 2008), suggesting that GA synthesis is suppressed by ABA (Bewley *et al.*, 2013). In addition, the expression of *GA2ox*, which encodes an enzyme involved in GA inactivation is reduced in *aba2-2*, providing evidence that GA catabolism is promoted by ABA (Seo *et al.*, 2006). Thus, ABA affects GA metabolism by suppressing GA biosynthesis and promoting its deactivation, resulting in less GA in seeds, which represses germination.

On the other hand, GAs also have a positive effect on ABA metabolism and signal transduction (Bewley *et al.*, 2013). For example, RGL2, a DELLA protein, and a component of GA signaling, stimulated ABA biosynthesis and signaling and repressed germination (Piskurewicz *et al.*, 2008). Thus, numerous layers of regulation ensure ABA–GA cross-talk in seeds, which act in a way that each signal is rapidly amplified. Altering the ABA–GA balance in seeds is a crucial aspect of light signal transduction for germination control (Bewley *et al.*, 2013).

1.3. The ABA biosynthetic and catabolic pathways

In addition to seed dormancy, ABA also regulates many other aspects of plant growth and development as well as plant responses to various environmental stress conditions (Seo & Koshiba, 2002; Finkelstein, 2013). ABA is a small sesquiterpene-derived carotenoid synthesized by photosynthetic organisms, as well as some non-photosynthetic bacteria and fungi through two possible routes: in fungi, it is produced directly from farnesyl pyrophosphate, whereas in plants, it is synthesized indirectly from carotenoids (Finkelstein, 2013; Izquierdo-Bueno *et al.*, 2018; Ali *et al.*, 2020).

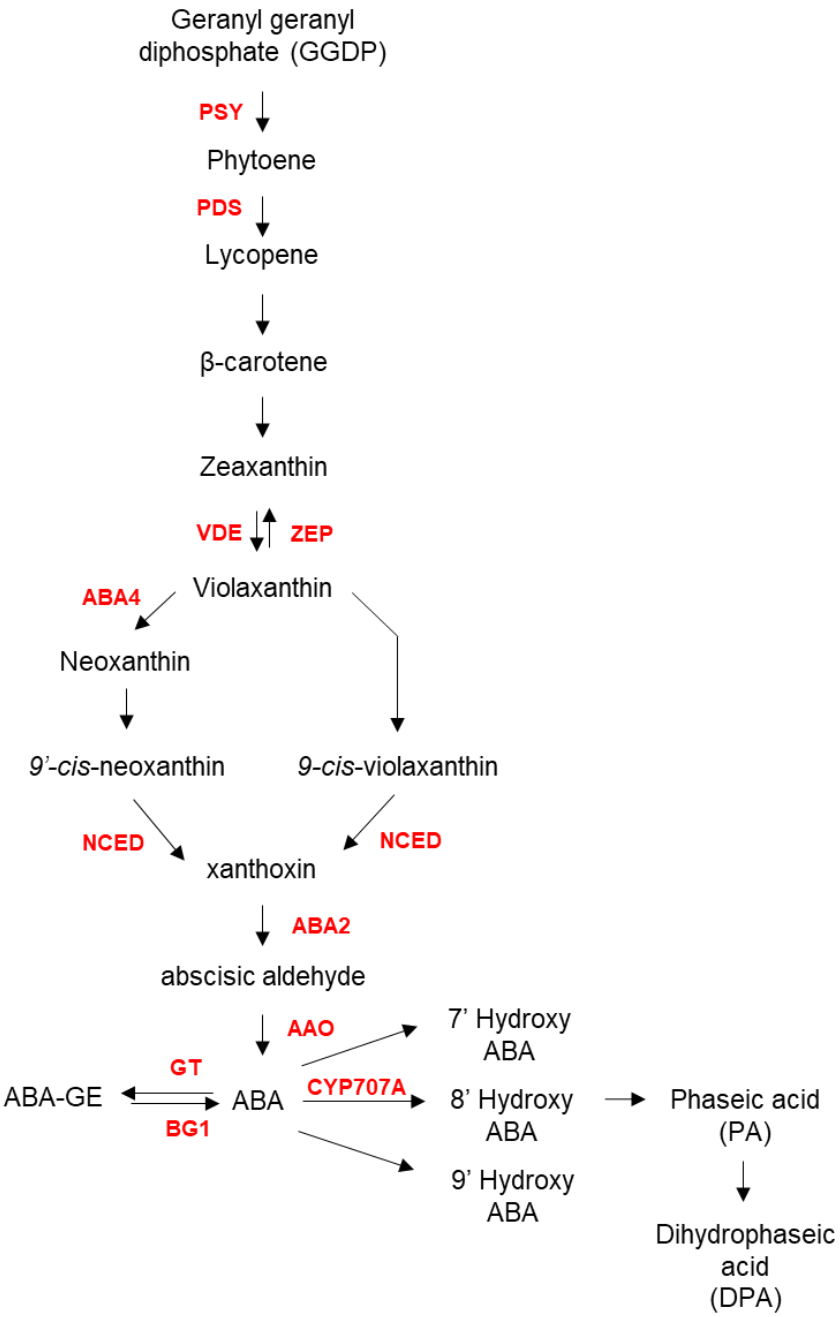

Figure 2. Illustration of the ABA biosynthesis pathway, with important roots of catabolism and conjugation shown. Characterized enzymes are marked in red.

ABA synthesis is illustrated in Figure 2 and starts from geranyl-geranyl diphosphate (GGDP), a C_{20} precursor, which undergoes a condensation reaction to form the first C_{40} carotenoid, 15-cis-phytoene. This reaction is catalyzed by phytoene synthase (PSY). Phytoene then undergoes desaturation to produce all-trans-lycopene and this reaction is catalyzed by phytoene desaturase. Phytoene is further converted to β-carotene, an important upstream substrate, which is converted to other forms of C_{40} carotenoids, including zeaxanthin and

violaxanthin. Violaxanthin is either isomerized to 9-*cis*-violaxanthin or converted to neoxanthin isomer, 9'-*cis*-neoxanthin. 9'-*cis*-neoxanthin and 9-*cis*-violaxanthin are converted to xanthoxin, a C_{15} precursor of ABA, through oxidative cleavage and it is catalyzed by 9- *cis* -epoxycarotenoid dioxygenase (NCED) which is the rate-limiting reaction. Abscisic aldehyde is synthesized from xanthoxin which is further oxidized to bioactive ABA by ABA aldehyde oxidase (AAO) (Bewley *et al.*, 2013; Seo & Marion-Poll, 2019; Ali *et al.*, 2020).

ABA homeostasis is closely monitored and adjusted through a variety of catabolic reactions, which can be either reversible or irreversible (Seo & Marion-Poll, 2019). A major route of ABA inactivation is 8′-hydroxylation catalyzed by the cytochrome P450 enzyme CYP707A (Kushiro *et al.*, 2004; Saito *et al.*, 2004; Seo & Marion-Poll, 2019). 8′-hydroxy ABA (8′-OH-ABA) is unstable and spontaneously converted to phaseic acid (PA) which is further reduced to dihydrophaseic acid (DPA) by a soluble reductase (Eggels *et al.*, 2018; Seo & Marion-Poll, 2019). ABA can also be inactivated through hydroxylation at the 7' and 9' positions. When hydroxylation occurs at the 9' positions, 9'-hydroxy-ABA is formed, which is then isomerized to neoPA (Zhou *et al.*, 2004; Okamoto *et al.*, 2011). Isomerization of 8′-OH-ABA to PA causes a reduction in its biological activity (Zhou *et al.*, 2004; Jadhav *et al.*, 2008; Kepka *et al.*, 2011). PA does, however, have a significant effect on the activation of a number of genes, including those that encode late-embryogenesis abundant (LEA) proteins. In line, it was shown that PA possesses ABA-like hormonal activity in Arabidopsis, albeit to a weaker extend (Weng *et al.*, 2016). Since DPA had no biological activity in various bioassays, it is considered to be an end product of the ABA hydroxylation pathway (Walton & Yi, 1995; Weng *et al.*, 2016).

Bioactive ABA or its pre-cursors can also be conjugated with sugar moieties or amino acids to form catabolites with altered biological activity. The most common conjugate of ABA is the glucosyl ester ABA-GE (Sauter *et al.*, 2002; Seo & Marion-Poll, 2019). ABA-GE formation is catalyzed by UDP-glucosyltransferases (UGTs), which accept various hormones as substrates (Bowles *et al*., 2006). ABA-GE formation was initially considered to be a permanent inactivation reaction, but recent studies indicated that ABA-GE can be reactivated and thus is a storage or transport form. ABA-GE accumulates in the vacuole and apoplast but is re-localized to the endoplasmic reticulum following dehydration (Lee *et al.*, 2006; Xu *et al.*, 2012). ABA-GE can be de-glucosylated and this is a possibility to increase the pool of bioactive ABA. The β-glucosidases BG1 (BGLU18: *At1g52400*) and BG2 (BGLU33: *At2g32860*), which are localized in the endoplasmic reticulum and vacuole respectively, can catalyse this reaction (Lee *et al.*, 2006; Xu *et al.*, 2012). BG1 was rapidly activated by dehydration-induced polymerization and its activity was increased by dehydration, suggesting a posttranslational activation, which triggers ABA release from inactive pools (Lee *et al.*, 2006). Since BG2 was

protected from degradation under dehydration stress conditions, it appears to function differently (Xu *et al.*, 2012).

1.4. ABA signaling

When plants are exposed to certain environmental stress types, such as drought or high salt conditions, ABA levels increase, which is an aspect of adaptive stress response reactions and has been very well documented in many plant species. But also the ABA signalling pathway is very well elucidated today. ABA perception is achieved through a subfamily of ABA receptors, which are called Pyrabactin Resistance (PYR), Pyrabactin Resistance like (PYL) or Regulatory Component of ABA Receptor (RCAR). These receptors act together with co-receptor proteins, which are phosphatases of the PP2C-type (PP2Cs). When ABA is perceived, the PP2Cs are bound, which impairs their repressive activity on signalling components that act further down-stream and in particular kinases of the SNF1-related protein kinases (SnRK2) family are targets. These kinases phosphorylate different types of transcription factors that control ABA responses via binding to ABA response elements (Fujii *et al.*, 2009; Ma *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2010; Hu *et al.*, 2015; Papacek *et al.*, 2017). Thus, ABA signaling is a phosphorylation-dependent signaling cascade in which PP2C-type phosphatases act as negative and SnRK2 kinases act as positive regulators, to regulate the expression of the ABA-controlled stress-responsive transcriptome (Fujii *et al.*, 2009; Raghavendra *et al.*, 2010).

1.5. Role of ABA in abiotic stress responses

ABA plays an important role in abiotic stress responses, which include stress types such as drought, high salinity, heat and cold (Albertos et al., 2022; Finkelstein, 2013; Wani & Kumar, 2015; Sah *et al.*, 2016). Such adverse environmental conditions occur frequently and significantly compromise plant growth and crop productivity worldwide (Lata & Prasad, 2011). Drought and salinity are serious threats to agriculture and together they can cause yield declines in major crops of more than 50% every year around the world, with the most significant effects in rain-fed agricultural systems (Bray *et al.*, 2000; Wang *et al.*, 2003).

When specific abiotic stress occurs, ABA levels increase strongly and this is primarily achieved through the activation of rate-limiting enzymes of the ABA biosynthesis pathway. Since different stresses can induce ABA biosynthesis, ABA is considered a stress hormone, although its roles in developmental processes are just as relevant (Mehrotra *et al.*, 2014; Yoshida et al., 2019). Increased ABA levels are primarily caused by stress types that cause cellular desiccation and osmotic stress. This results in stomatal closure, altered gene expression and adaptive physiological responses, all dedicated to prevent cellular damage

and increase plant survival rates (Seki *et al.*, 2002; Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007; Cutler *et al.*, 2010; Kim *et al.*, 2010).

Aside from modulating stomatal aperture, which is essential to minimize water loss from leaves during drought, ABA stimulates the expression of several genes whose products are important for stress protection, such as enzymes for osmoprotectant synthesis (Fujita *et al.*, 2011). Transcriptome studies have revealed that more than half of the genes regulated by ABA are also affected by drought or salinity, whereas the cold-regulated transcriptome has less overlap (Sah *et al.*, 2016). Out of 245 ABA-inducible genes in Arabidopsis, drought-induced 63% (155 genes), high salinity induced 54% (133 genes), and cold treatment induced 10% (25 genes) (Seki *et al.*, 2002). In rice, 73 stress-inducible genes were identified, of which 43 of them were triggered by ABA, cold-induced 36 genes, drought-induced 62 genes and high salinity induced 57 genes (Rabbani *et al.*, 2003). These findings support the notion that there is extensive interaction between ABA response and abiotic stress signalling pathways, particularly for drought and high salinity.

The role of ABA in stress response is not only established in Arabidopsis, but also very well documented in crops. In *Triticum aestivum* (wheat), *Oryza sativa* (rice), *Hordeum vulgare* (barley), *Glycine max* (soybean), *S. lycopersicum*, *Gossypium hirsutum* (cotton) and *Medicago sativa* (alfalfa), ABA contents in the leaves increased under drought and re-watering returned them to baseline levels (Bensen *et al.*, 1988; Swamy & Smith, 1999; Guóth *et al.*, 2009; Thameur *et al.*, 2011). Salt stress increased ABA in tobacco cells and alfalfa seedlings (Singh *et al.*, 1987; Luo *et al.*, 1992) and cold stress also resulted in an ABA accumulation (Daie & Campbell, 1981; Eze *et al.*, 1983; Lalk & Dörffling, 1985).

In line with its ability to induce protective reactions, exogenous application of ABA can improve a plant's adaptive capacity to certain abiotic stress types. This has been shown for a number of species exposed to drought (Waterland *et al.*, 2010; Du *et al.*, 2013; Yadegari *et al.*, 2014; Wei *et al.*, 2015), but also in *Cucumis sativus* (cucumber) and *Medicago sativa* exposed to cold stress (Flores *et al.*, 1988; Mohapatra *et al.*, 1988) and in *Phaseolus vulgaris* (common bean) and *Solanum tuberosum* (potato) exposed to salt stress (Khadri *et al.*, 2006; Etehadnia *et al.*, 2008). Moreover, the application of the ABA biosynthesis inhibitor Fluridone (Fong *et al.*, 1983; Kondhare *et al.*, 2014), decreased resistance to abiotic stress in rice (Perales *et al.*, 2005) and reduced seed dormancy in *Triticum aestivum* and *Lactuca sativa* (Rasmussen *et al.*, 1997; Yoshioka *et al.*, 1998).

Thus, in summary, ABA plays an essential role in seed dormancy and abiotic stress responses, a function very well conserved throughout the plant kingdom, which is of high

relevance for important agronomic traits, including seed germination and abiotic stress tolerance.

1.6. Aims of the project

In this work, it was aimed to generate knowledge, methodology and materials for the genetic improvement and domestication of *Crassocephalum* plants. On the one hand, traits of importance for ebolo cultivation were investigated and, in this regard, a major focus was the study of seed formation and germination, to obtain an understanding of how seed dormancy is controlled and can be broken. In addition, abiotic stress tolerance assays were carried out, since it was found that *C. crepidioides* shows a ABA hyper-responsiveness as compared to *C. rubens* and it was assessed how this impacts performance in harsh growth conditions. Moreover, I contributed to a project where jacobine accumulation in *Crassocephalum* was studied; the results of which were already published elsewhere (Schramm *et al.*, 2021).

On the other hand, it was intended to develop tools and resources that will facilitate research and breeding activities with *Crassocephalum*. Regeneration and transformation protocols were developed and an EMS-mutagenized *C. rubens* mutant collection was generated and roughly characterized, to establish if it will be suitable for mutant screens and mutation breeding in the future.

2. Material and Methods

2.1. Plant material

Crassocephalum crepidioides accession Ile-Ife (*C.c*.Ile-Ife) and Osogbo (*C.c*.Osogbo) were obtained from Nigeria. *C.c*.Ile-Ife was collected from a law patch on the Campus of Obafemi Awolowo University, Ile-Ife in 2016, and *C.c*.Osogbo was obtained from a wetland area in Agunbelewo, Osogbo in 2018. Two *C. rubens* (*C.r.*Mali and *C.r*.Burkina-Faso) and one *C. crepidioides* ecotypes (*C.c.*Nepal) were obtained from the Millenium Seed Bank (MSB) at Kew Royal Botanic Gardens (Kew, United Kingdom). *C. crepidioides* accession *C.c.*Thailand was obtained from Thailand (Schramm *et al.*, 2021).

2.2. Chemicals

Standard chemicals were purchased from Sigma-Aldrich (Steinheim, Germany), Roth (Karlsruhe, Germany), Duchefa (Haarlem, the Netherlands), Merck (Darmstadt, Germany) or VWR-Promochem (Darmstadt, Germany). Gibberellic acid (GA_3), (+)-cis, trans-Abscisic acid, Kinetin and 6-benzyaminopurine were obtained from Duchefa (Haarlem, the Netherlands). 24-epiBrassinolide was obtained from Apollo Scientific (Cheshire, United Kingdom). Fluridone was obtained from Sigma (Steinheim, Germany). Abamine was obtained from OlChemIm (OlChemIm, Olomouc, Czech Republic). Ethyl methanesulfonate was obtained from Alfa Aesar (Kandel, Germany)

2.3. Growth conditions in the soil

For standard set-ups, plants were cultivated in growth chambers (Bright Boy, CLF Plant Climatics, Wertingen) for four weeks at 25 +/- 2°C and cycles of 16 hours white light (80 µmol $m^{-2}s^{-1}$)/ 8 hours dark. For phenotyping in the adult stage, seed amplification and transformation, plants were germinated and pre-grown in small pots, filled with soil substrate C700 with Cocopor® (Stender AG, Schermbeck, Germany) in the growth chambers, using the conditions above and then transferred to larger pots and moved to the greenhouse. In the greenhouse the plants were grown at a temperature of 20 +/- 2ºC, 50% relative humidity with artificial lightning (80-100 µmol $m^{-2}s^{-1}$ for 12 hours) in winter and 23 +/- 2ºC, 50% relative humidity without artificial lightning during the summer in Freising, Germany (48° 24′ N, 11° 45′ O). Watering was done with tap water; no additional fertilizer was added.

2.4. Germination experiments

Crassocephalum seeds were sterilized using 75% commercial bleach solution containing 0.01% Triton X-100. The bleach solution was added to the seeds and they were gently shaken

on the bench for 20 minutes. Seeds were then rinsed three times with deionized water in a sterile hood and transferred to either wet filter paper (Roth, Karlsruhe, Germany) or half-strength Murashige and Skoog (1/2 MS) medium (Duchefa, Haarlem, Netherland). If different lines were compared, only seeds from mother plants grown at the same time in the same conditions were used. For assessing the impact of different hormones, hormones inhibitors or salts, the compounds were added to the medium in the indicated concentrations. Generally, the seeds were then incubated at 21 °C or 25 °C, either in the dark, a low light intensity of 30 µmol/m^2s or a higher light intensity of 80 µmol/m^2s and long-day conditions (16 hours of light/ 8 hours of darkness) for different time-points, before germination, defined as radicle emergence from the seed coat, was assessed.

2.5. Cotyledon greening analyses

For cotyledon greening assays, seeds were germinated on ABA-containing medium and greening, as defined by the presence of fully expanded, green cotyledons, was analyzed at different time points.

2.6. Phenotyping in the adult stage

Four accessions of *C. crepidioides* and two accessions of *C. rubens* were grown in the greenhouse during the 2018 Winter period. Seeds were initially germinated in the growth chambers at 25 +/- 2°C and cycles of 16 hours white light (80 µmol/m^2s) / 8 hours dark. Seedlings were transplanted to larger pots at four-week-old and were grown at a temperature of 20 +/- 2^{o}C, 50% relative humidity and artificial light (80-100 µmol/m^2s) for 12 hours daily. Plants were monitored from germination until bud formation, flower opening and subsequent seed dispersal. Seed number, as well as seed weight and overall biomass gains, were also recorded for all the accessions used.

2.7. Microscopy for phenotyping

The detailed pictures of flowers, seeds and germinating seedlings were taken with a stereomicroscope (Olympus SZX10, Tokyo, Japan) and a tabletop scanning electron microscope (Hitachi High-Tech, Tokyo, Japan).

2.8. Drought tolerance assays and detached-leaf water loss assays

The drought tolerance assays were conducted on adult plants with similar numbers of leaves. Seven-week-old plants of *C. rubens* Mali and 8-week-old plants of *C. crepidioides* Ile-Ife were subjected to progressive drought by withholding water for 7 days. Before exposing the plants to drought, the plants were subjected to comparable watering conditions and then re-

introduced water after 7 days. To determine the loss of fresh weight, a minimum of twelve plants from each accession were weighed individually and loss of fresh weight was recorded. The loss of fresh weight was calculated by dividing the post-incubation fresh weight by the initial fresh weight.

For the water loss assay, the third true leaves pair of 4-week-old plants of *C. rubens* Mali and 5-week-old plants of *C. crepidioides* Ile-Ife were detached, placed on a Petri dish and exposed to ambient conditions. The leaves were weighed at a 1 h interval for 5 h to determine the rate of water loss. The water loss (%) was calculated by dividing the difference between the initial fresh weight and the post-incubation fresh weight by the initial fresh weight.

2.9. Heat and cold stress experiments

Four-week-old plants of both *C. crepidioides* Ile-Ife and *C. rubens* Mali, grown in standard growth conditions at a temperature of 25ºC, were directly exposed to high or low temperatures ranging from 35ºC to 55ºC for 2 hours and +4ºC to -4ºC for 3 hours. Plant survival was determined after two weeks of recovery at 25ºC °C and was defined as the ability to produce new leaves from an intact shoot apical meristem.

2.10. Quantification of ABA in seeds, seedlings and drought-stressed plants

For ABA quantification from early developmental stages, seeds from *C. crepidioides* and *C. rubens* plants grown at the same time in the greenhouse were used. Dry seeds were after-ripened for 6 weeks. Imbibed seeds were generated, by seed imbibition on filter paper for 2 days at 25ºC, in darkness. Young seedling material was generated by germinating seeds on filter paper at a temperature of 25ºC in the light, using long-day growth conditions (16 hours of light/ 8 hours of darkness) and growing the seedlings for 2-3 days, until they had developed fully expanded cotyledons. For ABA quantification from *C. crepidioides* and *C. rubens* plants subjected to drought, material was harvested from plants exposed to drought stress (detailed above). Samples were taken daily for the first 3 days following water-withdrawal.

All samples were homogenized in liquid nitrogen and 150 to 200 mg of the samples were weighed into a bead beater tube, filled with ceramic balls . The weighed-in materials were then mixed with 1 ml ethyl acetate and 20 µl of internal standard. The samples were shaken and incubated for 30 min at room temperature. All samples were shock-frozen in liquid nitrogen and stored at -80ºC, before LC-MS/MS analysis was carried out by Michael Gigl from the group of Corinna Dawid at the Chair of Food Chemistry and Molecular Sensory Science of the TUM School of Life Sciences.

2.11. Establishing regeneration protocols for *Crassocephalum*

Plant regeneration for transformation purposes was tested from different tissues and using the cytokinins Kinetin and 6-Benzylamino purine (BAP). Explants were generated by germinating seeds on ½ MS media and growing them in the incubator at 21^{o}C, 80 µmol/m^{2}s and 16/8 hours of light/dark cycle for two weeks. The seedlings were dissected to obtain cotyledons, leaf disks and the shoot apical meristem, which were placed on ½ MS media supplemented with 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/l of kinetin and BAP and incubated for one month. Once shoot re-generation had occurred, the explants were transferred to fresh ½ MS medium without cytokinins and incubated for another 2 to 3 weeks, before regeneration, defined as formation callus or direct shoot formation from explant, was evaluated.

2.12. Evaluating selectable markers

The herbicide glufosinate and the antibiotic kanamycin (KAN), common selectable agents used for plant transformation, were tested for their toxicity on *Crassocephalum* plants. For this purpose, 100 seeds of both *C. crepidioides* and *C. rubens* were sterilized, plated on ½ MS media containing either 0, 5, 10 and 15 µg/ml of glufosinate ammonium (Alfa Aesar, Kandel, Germany) or 200, 250 and 300 µg/ml KAN (Roth, Karlsruhe, Germany) and incubated at 21^{o}C, 80 µmol/m^{2}s and 16/8 hours of light/dark cycle for 3 weeks, before resistance was assessed.

Figure 3. *C. crepidioides* plants after floral dipping.

2.13. Floral dipping of *Crassocephalum* with *Agrobacterium tumefaciens*

Both *C. crepidioides* Ile-Ife and *C. rubens* Burkina-Faso were used in this experiment. Twenty plants from each accession were grown in individual pots in the greenhouse until the plants started to produce inflorescences. The primary inflorescences were cut back to promote branching. Secondary inflorescences were also cut off to some extent, to increase the number of flower buds produced.

For transformation *Agrobacterium tumefaciens* strain GV3101, carrying a pGWR8 binary vector with a kanamycin selection marker for both bacteria and plant selection (Rozhon *et al.*, 2010), which contained a 35S:GUS construct (Yin *et al.*, 2002), was used. A single colony of the strain was inoculated in 20 ml MLB medium containing 20 µl of kanamycin (KAN) and grown on a shaker at 28ºC for approximately 20 h. The culture was then transferred into a 300 ml MLB medium containing 300 µl of KAN and grown at 28ºC with good aeration until an OD_{600} of 0.8 was reached. The bacteria were harvested by centrifugation and re-suspended in 300 ml 5% sucrose. The bacteria suspension was transferred into a beaker, and 150 µl Silwet-L77 (Obermeier, Bad Berleburg, Germany) was added to it.

The floral buds were dipped in the suspensions for 30 to 60 seconds. The dipped plants were covered with plastic bags to maintain a high humidity and kept in the dark overnight. The plastic bags were removed the following day, and the plants were allowed to continue to grow until the seeds were set. Inflorescences in this, but also in all other experiments, were wrapped up in vegetable nets (Inspirion, Bremen, Germany) to save all seeds (Figure 3).

Seeds were harvested manually, dried and stored in paper bags. Fifteen thousand seeds of both *C. rubens* and *C. crepidioides* were screened. The seeds were sterilized with 75% bleach solution and then plated on KAN selection plates. Transformants were identified based on their KAN resistance.

2.14. GUS staining

Leaves of adult plants and young seedlings were immersed in GUS staining buffer containing 1mM X-Gluc (5-Bromo-4-Chloro-3-Indoyl-Beta-D-Glucuronide), 100mM phosphate buffer pH 7.0, 0.1% Triton X-100, 10mM EDTA, 0.5mM $K_4[Fe(CN)_6]$ and 0.5mM $K_3[Fe(CN)_6]$. The immersed tissues were incubated for 2 hours at 37 ºC and destained with 70% ethanol.

2.15. Generation of an EMS mutagenized population of *C. rubens*

To generate a *Crassocephalum* mutant population, *C. rubens* Burkina-Faso seeds were treated with the mutagen Ethyl methanesulfonate (EMS). For a kill-curve analysis, 500 seeds were incubated in 0.0, 0.2, 0.4, or 0.6 % EMS, with gentle shaking, for 15 hours. Seeds were

then rinsed up to 20x with distilled water, were sown immediately on soil in square pots (9x9 cm; Poppelmann, Germany), with 25 seeds per pot, and were incubated in standard growth conditions in the incubator. After 4 weeks, the plants that had emerged from the 25 seeds (approx. 10-20) were transplanted together into containers and grown in the greenhouse until the seeds had set (Figure 4). The seeds of the plants growing together in containers were also harvested together, generating seed pools of 10-20 plants each. From these pools, seeds were sown for a rough characterization, determining seed germination, seedling survival, leaf area, hypocotyl length and seed yield.

The kill-curve analysis established that a concentration of 0.1 and 0.2 produced about 50% survival rates and these concentrations were then used for a larger trial in which seeds were treated with 0.0, 0.1 and 0.2 % EMS solution for 8 hours and then handled in the same manner as described above. Finally, to upscale the experiment and generate a good-sized mutant population, 3,000 seeds were mutagenized with 0.2% EMS and pools of seeds from 10-20 M1 plants were generated by self-fertilization, which were ready for the screening of M2 plants.

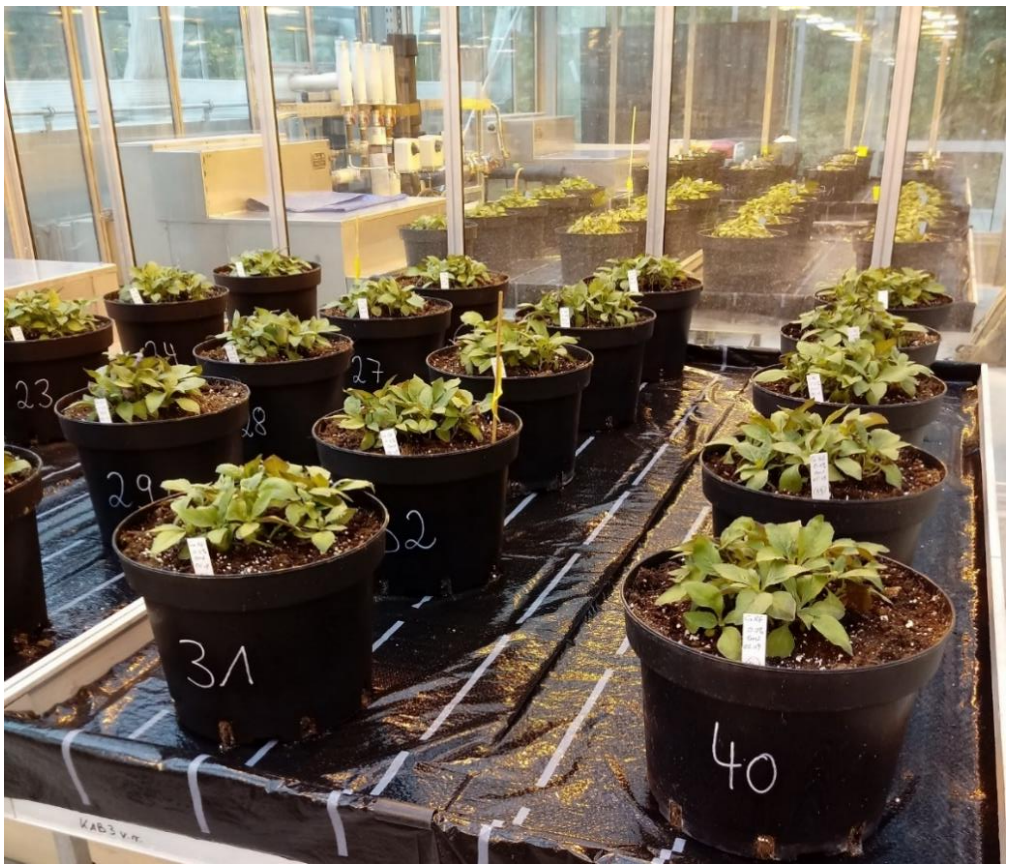

Figure 4. *C. rubens* mutants mutagenized with 0.0, 0.2, 0.4 and 0.6% EMS growing in the greenhouse.

2.16. *C. rubens* mutant screen

Five hundred seeds from the 0.2% EMS mutagenized population of *C. rubens* were sown directly on soil and incubated at 25^{o}C, 80 µmol/m^{2}s and 16/8 h of light/dark cycle for four weeks. Seedlings were transplanted into bigger pots and grown in the greenhouse at 25^{o}C ± 2 with artificial lightning at long-day growth conditions of 16 hours light/ 8 hours dark cycles. The phenotypes of adult plants were evaluated visually at 7 weeks after germination and a set of leave morphology mutants and dwarfs was isolated for a further characterization.

3. Results

3.1. Characterizing differences in development and abiotic stress responses between *C. crepidioides* and *C. rubens*

3.1.1. Tetraploid *C. crepidioides* has higher biomass gains and forms more but smaller seeds than diploid *C. rubens*

Tetraploid *C. crepidioides* is known to grow more vigorously than its diploid relative *C. rubens* in the field, where it can reach a biomass of 175.12 g (Adjatin *et al.*, 2013b). To investigate the differences in the growth and development of *C. crepidioides* and C. *rubens* in more detail, four accessions of *C. crepidioides* (*C.c*.Ile-Ife, *C.c*.Osogbo, *C.c*.Nepal, *C.c*.Thailand) and two accessions of *C. rubens* (*C.r.*Mali, *C.r*.Burkina Faso) were grown to the adult stage in the greenhouse and different traits were evaluated, including flower development, time to flowering and seed dispersal, the number and weight of seeds and overall biomass gains.

The phenotyping was started with a rough characterization of flower development. *C. crepidioides* and *C. rubens* form composite flowers (named capitula) that contained disc florets, which are surrounded by involucral bracts (Sakpere *et al.,* 2013). Flower development in both species began with the formation of capitula buds (Figure 5A), which opened when the florets were fully developed. At anthesis, the flower head of *C. crepidioides* had a characteristic brick-red colour whereas those of *C. rubens* had a purple colouration (Figure 5A).

Interestingly, the *C. crepidioides* accessions from Nigeria took significantly longer from germination until bolting than the other two *C. crepidioides* accessions (from Asia) and the two *C. rubens* ecotypes (Figure 5B). After bolting (first capitula bud development), all lines took approximately another 20 days to flower opening. However, *C. rubens* required a significant longer period of time from flower opening to seed dispersal. Moreover, *C*. *rubens* formed significantly fewer seeds (Figure 5C), which had an increased seed weight as compared to *C. crepidioides* (Figure 5D, E).

In the growth conditions that were applied (20 +/- 2^{o}C, 50% relative humidity and artificial light (80-100 µmol $m^{-2}s^{-1}$) for 12 hours), fertilization was reduced in *C. rubens* as compared to *C. crepidioides* (Figure 5D). Seed formation occurred in all the *C. crepidioides* accessions about 9 days after the opening of the capitula, whereas it took about 16 days in the case of *C. rubens* (Figure 5D).

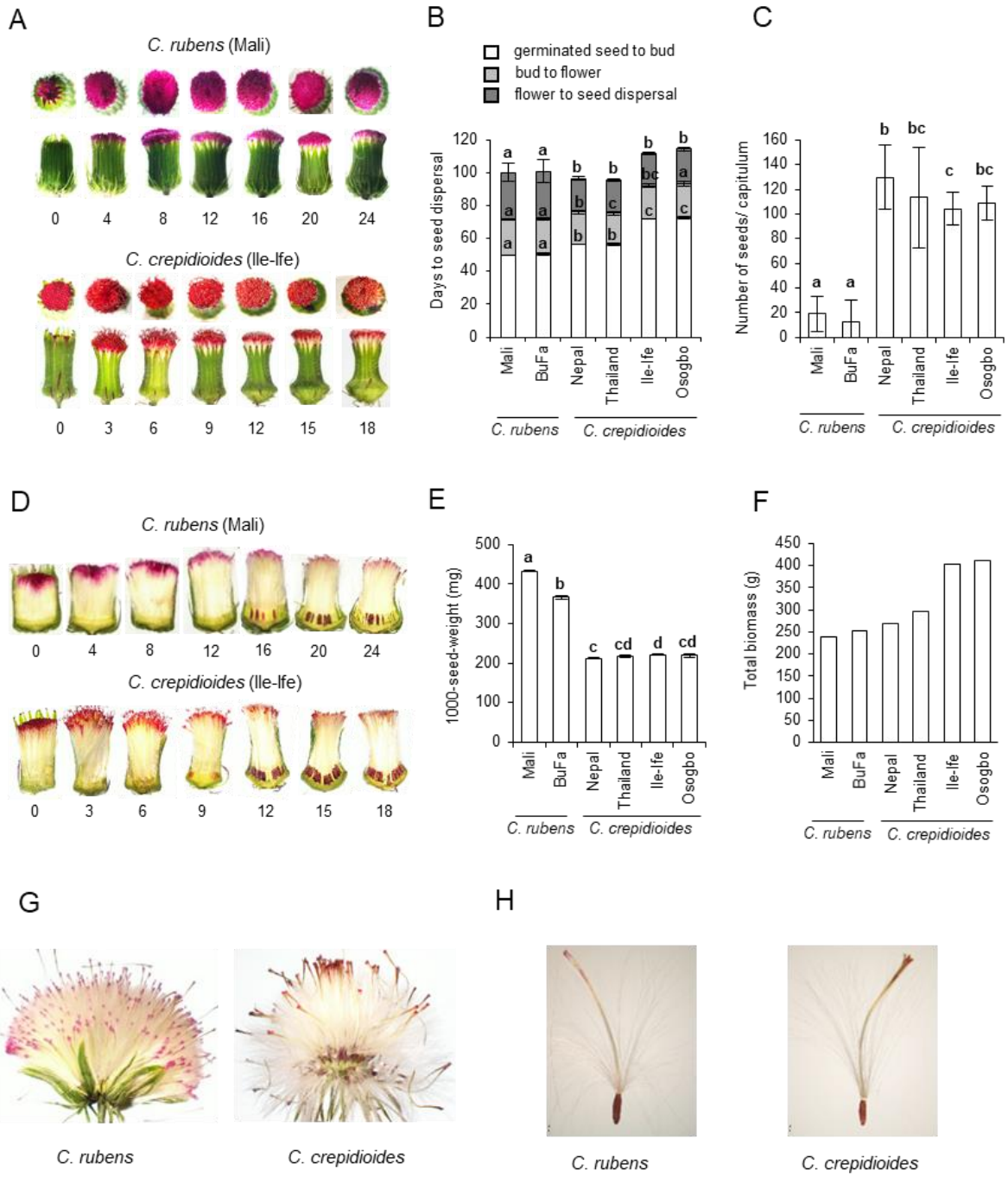

Figure 5. *C. crepidioides* has higher biomass gains and forms more, but smaller seeds than *C. rubens*.

(A) Photos of capitula of *Crassocephalum* species at the different developmental stages. The plants were grown in the greenhouse at a temperature of 20±2ºC and artificial light (80-100 µmol/m^{2}s) for 12 hours daily. (B) Evaluation of the time from germination to flowering. Plants were grown as in A and the days to first bud development, first flower development and first seed dispersal were counted. The average and standard deviation of 10 plants is shown. (C) Evaluation of seed produced per capitulum. Twenty-five capitula were harvested at maturity and the number of seeds were counted. Data shown are the average ± SD of 25 capitula. (D) Photos of capitula of *Crassocephalum* species dissected to show the developmental pattern of seeds. (E) Evaluation of seed weight of *Crassocephalum* species. One thousand seeds from each accession were counted and weighed. Data shown are mean ± SD of four biological replicates. (F) Total biomass gains. Ten plants were bulked together to determine the overall biomass gain. (G) *Crassocephalum* species flower at dispersal. (H) *C. crepidioides* and *C. rubens* seeds attached to its style and pappus. Statistically significant difference at $P \leq 0.01$ between ecotypes is indicated with different letters and was determined with one-way ANOVA with a post-hoc Tukey HSD test.

The delayed flowering was correlated with higher biomass gains in the two Nigerian *C. crepidioides* accessions, which showed a total biomass of about 400 g in the growth conditions used (Figure 5F). *C. crepidioides* and *C. rubens* seeds are dispersed with the aid of wind (Denton, 2004; Bosch, 2004; Sakpere *et al.,* 2013). At maturity, the phyllaries surrounding the capitulum break open and the receptacle reflex back thereby exposing the seeds for dispersal (Figure 5G). Following the reflexing of the receptacle, the seeds are dispersed away and it is similar for both *C. crepidioides* and *C. rubens*. *Crassocephalum* seeds have a characteristic dark-brown colour (Figure 5H) and are attached to a translucent to white pappus.

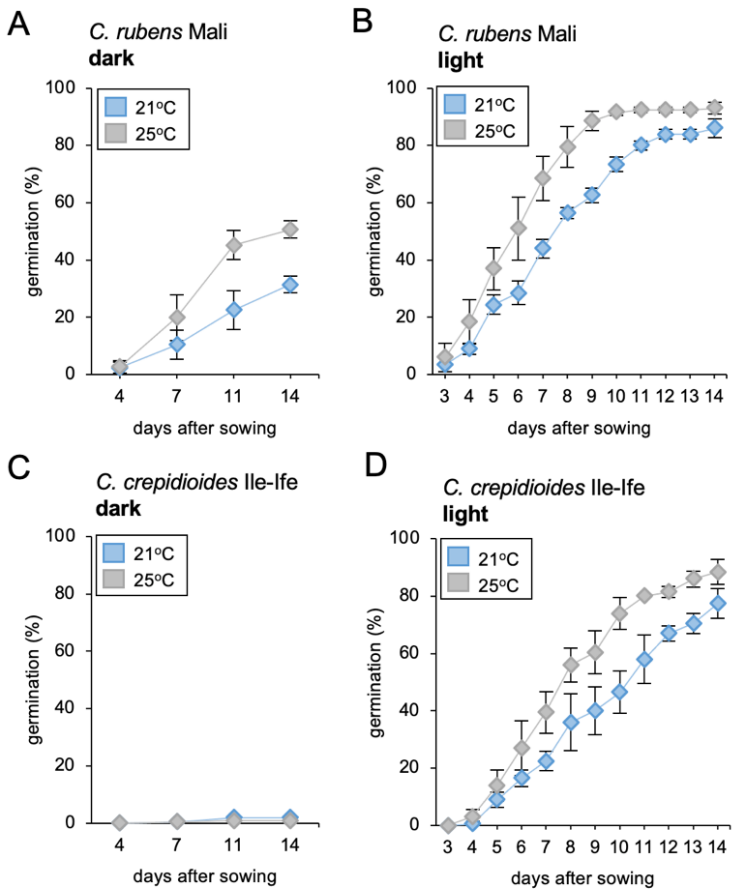

Figure 6. *C. crepidioides* has a higher level of seed dormancy than *C. rubens*.

(A, B) Germination of *C. rubens* seeds in the dark (A) or in the light (B) and at a temperature of 21ºC or 25ºC. (C, D) Germination of *C. crepidioides* seeds in the dark (C) or in the light (D) and at a temperature of 21ºC or 25ºC. In all experiments, 50 seeds were plated on ½ MS medium and incubated at a low light intensity of 30 µmol/m^2s in the representative conditions. Germination, defined as radicle emergence from the seed coat, was assessed at the indicated time points. Data shown are mean ± SD of four biological replicates.

3.1.2. *C. crepidioides* has a higher level of seed dormancy than *C. rubens*

C. crepidioides had previously been reported to have a light requirement for germination (Chen *et al.*, 2009; Nakamura & Hossain, 2009; Sakpere *et al.*, 2013; Yuan & Wen, 2018). To verify

this, germination experiments were carried out with the *C. crepidioides* ecotype Ile-Ife and the *C. rubens* ecotype Mali, using seeds with the same growth history. This showed that both species exhibited a high degree of seed dormancy in the dark. Whereas *C. crepidioides* seeds had no germination capacity in darkness, *C. rubens* seeds germinated with approximately 30% efficiency (Figure 6A, C). Light promoted germination, with stronger effects on *C. rubens* seeds, which reached 50% germination already after 7 days, whereas *C. crepidioides* seeds took 10 days at 21°C. Increasing the temperature to 25°C also promoted germination in both species, yielding close to 100% germination after 10 days in the case of *C. rubens* and after 14 days in the case of *C. crepidioides* (Figure 6B, D).

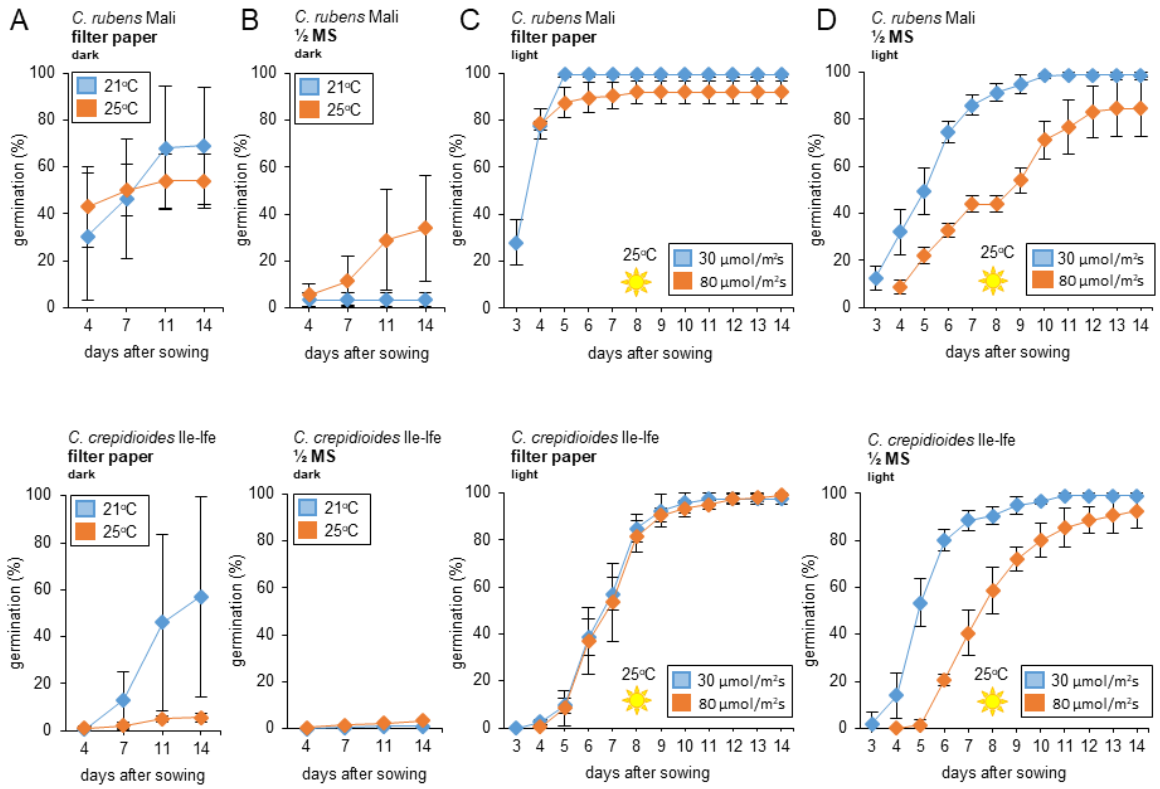

Figure 7. Germination of *Crassocephalum* seeds is facilitated by light and inhibited by MS salts.

(A, B) Germination of *C. crepidioides* and *C. rubens* seeds on filter paper (A) or on ½ MS medium (B) in the dark and at a temperature of 21ºC or 25ºC. (C, D) Germination of *C. crepidioides* and *C. rubens* seeds on filter paper (C) or on ½ MS medium (D), in light intensities of 30 µmol/m^{2}s or 80 µmol/m^{2}s and at a temperature of 25ºC. In all experiments 50 seeds were incubated in the representative conditions and for the indicated periods of time and germination, defined as radicle emergence from the seed coat, was assessed. Data shown are mean ± SD of four biological replicates.

In addition to temperature and light, also the germination medium had a significant influence on seed germination of both *C. rubens* and *C. crepidioides* (Figure 7A, B). When compared with ½ MS, germination was faster for both *C. crepidioides* and *C. rubens* when seeds were germinated on filter paper (Figure 7C, D). On ½ MS, a low light intensity increased the speed of germination (Figure 7C, D).

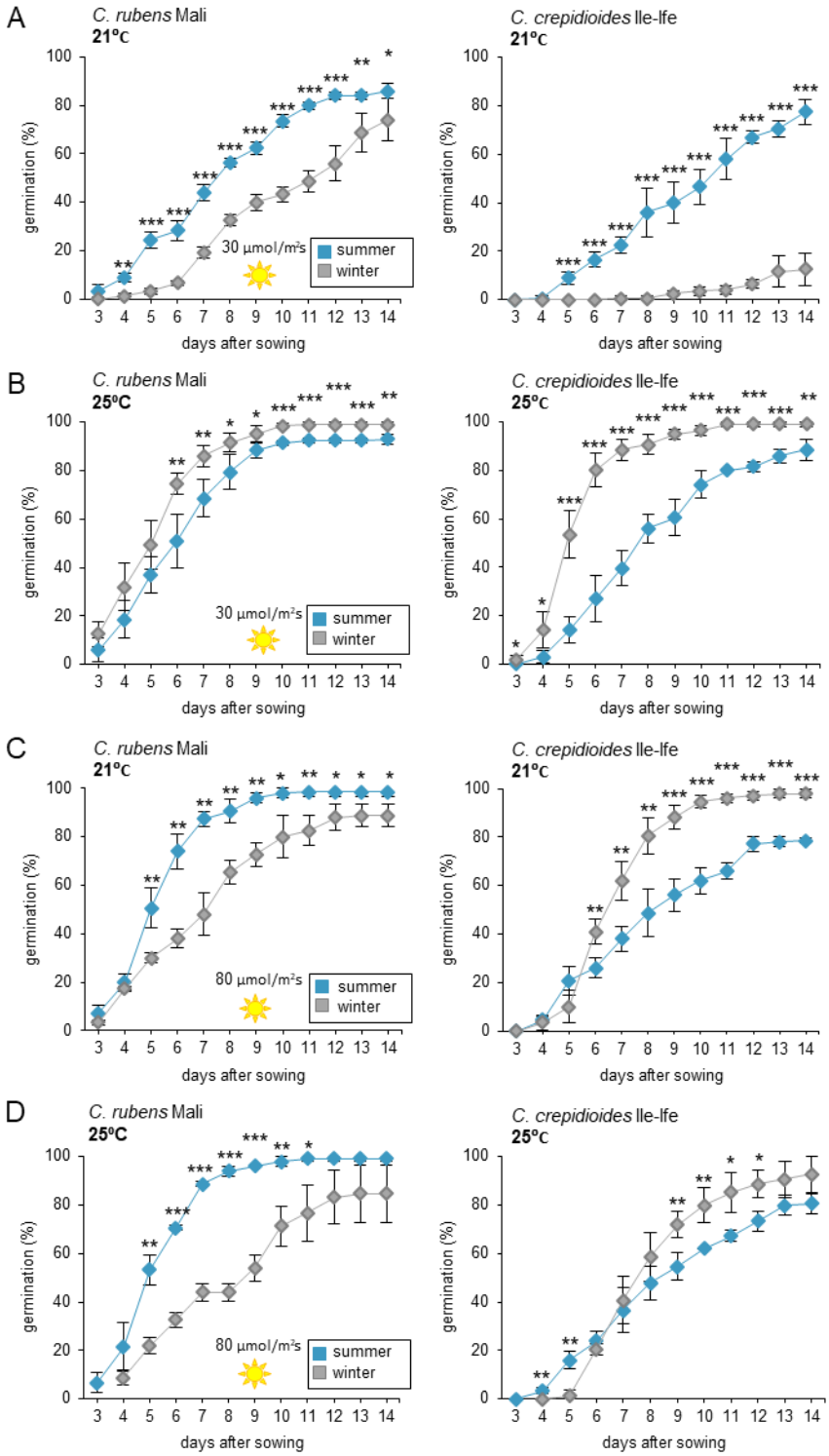

Figure 8. Time of planting influences the dormancy level of *Crassocephalum* seed.

Germination of *C. crepidioides* and *C. rubens* seeds from mother plants grown in winter or summer on ½ MS medium and incubated either at a light intensity of 30 µmol/m^{2}s and a temperature of 21°C (A) or 25°C (B) or at a light intensity of 80 µmol/m^{2}s and a temperature of 21°C (C) or 25°C (D). In all experiments, 50 seeds were plated on ½ MS medium and incubated in the representative conditions and for the indicated periods of time and germination, defined as radicle emergence from the seed coat, was assessed. Data shown are mean ± SD of four biological replicates. Statistically significant difference at $P \leq 0.05$ between seasons is shown in asterisks and was determined with one-way ANOVA with a post-hoc Tukey HSD test.

3.1.3. Seasonal differences in the acquisition of seed dormancy occur

When comparing the germination of two different seed batches, harvested from *C. crepidioides* and *C. rubens* mother plants grown either during the 2018/19 winter period or during the 2020 summer period in the greenhouse, differences in germination outcomes were observed. Both *C. rubens* and *C. crepidioides* seeds that came from mother plants grown during winter were more dormant than those from mother plants grown during summer (Figure 8A). This trait was apparent when seeds were germinated at a low light intensity of 30 µmol/$m^{-2}s^{-1}$ and a temperature of 21°C, a condition where seeds of *C. crepidioides* harvested during winter showed a strongly reduced germination capacity (Figure 8A).

This increased dormancy could be released by germination at a higher temperature of 25°C or at a higher light intensity of 80 µmol/$m^{-2}s^{-1}$ (Figure 8B-D). Surprisingly, *C. crepidioides* seeds from winter-grown plants exhibited an even larger germination capacity at higher temperatures and light levels, than those from summer-grown plants, an observation which is difficult to explain. In *C. rubens,* seeds from mother plants grown in summer generally germinated better than those from plants grown in winter, which was independent of the germination environments applied.

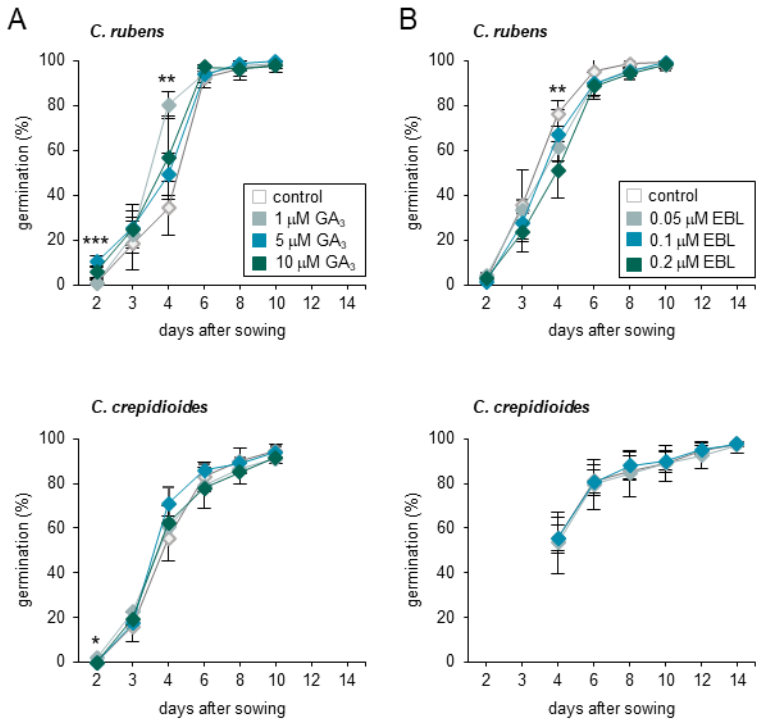

Figure 9. GA_3 or 24-epiBL don't promote germination of *C. crepidioides* and *C. rubens* seeds.

Germination of *C. rubens* and *C. crepidioides* seeds in presence of GA_3 (A) or epiBL (B). 50 seeds were plated on ½ MS containing the indicated concentrations of GA_3 and 24-epiBL and incubated at a temperature of 25°C and light intensity of 30 µmol/m^2s in long days. Germination, defined as radicle emergence from the seed coat, was assessed at the indicated time points. Data shown are mean ± SD of four biological replicates. Statistically significant difference at P ≤ 0.05 between concentrations is shown in asterisks and was determined with one-way ANOVA with a post-hoc Tukey HSD test.

3.1.4. A higher level of seed dormancy of *C. crepidioides* seeds is correlated with increased levels of ABA

Light and temperature effects on the growth and development of plants are signaled by hormones and to investigate, if hormones may impact the germination of *Crassocephalum* seeds, the seeds were treated with the synthetic GAs and BRs GA_3 and 24-epi brassinolide (epiBL) and ABA. Interestingly, neither GA_3 nor epiBL had a significant impact on germination at the concentrations used (Figure 9). However, ABA suppressed germination of both *C. rubens* and *C. crepidioides* seeds, with much stronger effects on the latter. At 0.1 µM ABA, light-induced seed germination of *C. crepidioides* was suppressed below 20% at 14 days post-incubation (Figure 10A).

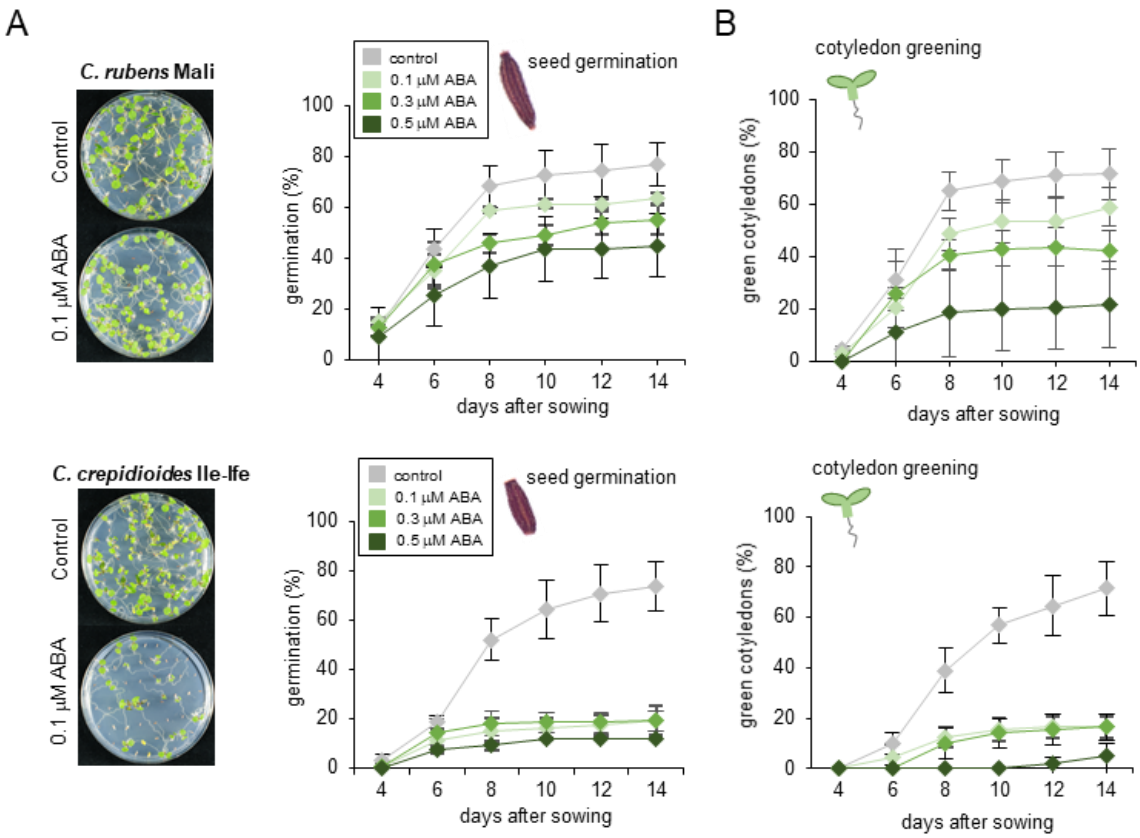

Figure 10. *C. crepidioides* is hypersensitive to ABA during germination and early seedling development.

Germination (A) and cotyledon greening (B) of *C. crepidioides* and *C. rubens* in the presence of ABA. 50 seeds were plated on ½ MS medium containing 0; 0,1; 0,3 and 0,5 µM ABA and incubated at a temperature of 25°C and a light intensity of 30 µmol/m^2s in long days. Germination, defined as radicle emergence from the seed coat, and cotyledon greening, defined as fully developed, green cotyledons, were assessed at the indicated time points. Data shown are mean ± SD of four biological replicates.

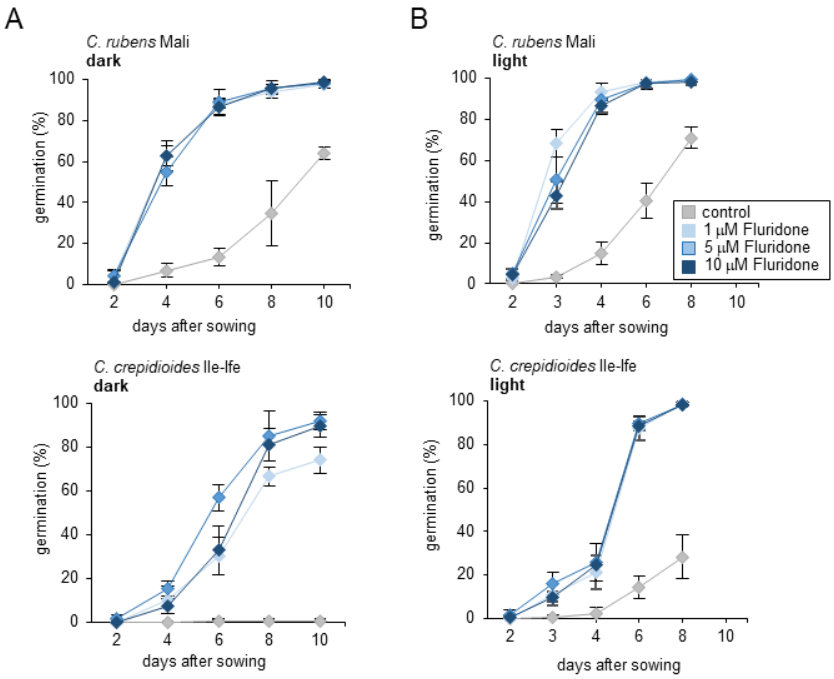

Figure 11. Fluridone treatment releases dormancy of *C. rubens* and *C. crepidioides* seeds.

Germination of *C. crepidioides* and *C. rubens* seeds in presence of Fluridone in the dark (A) and in the light (B). 50 seeds were plated on ½ MS containing 0, 1, 5 and 10 µM Fluridone and incubated at a temperature of 25°C and light intensity of 30 µmol/m^{2}s in long days. Germination, defined as radicle emergence from the seed coat, was assessed at the indicated time points. Data shown are mean ± SD of four biological replicates.

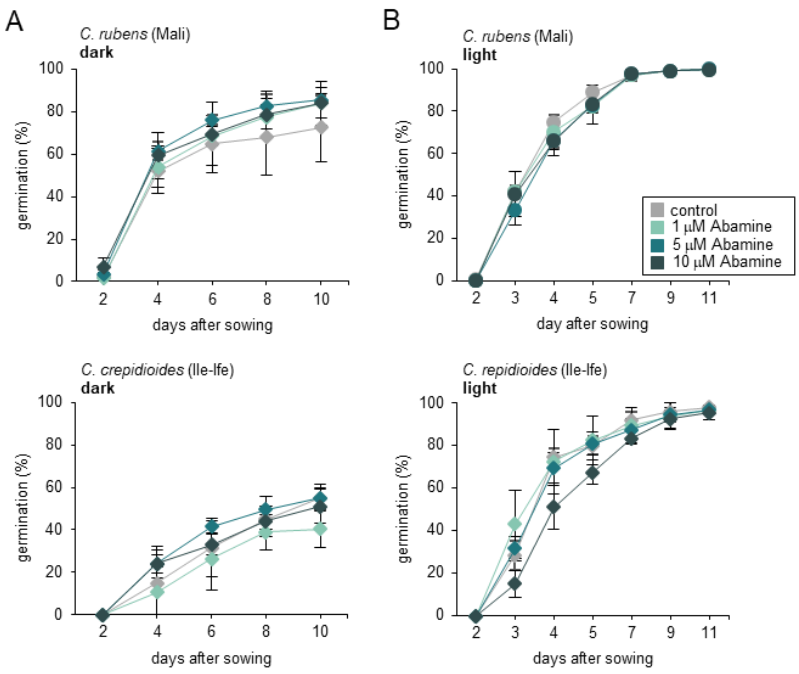

Figure 12. Abamine treatment does not release dormancy of *C. rubens* and *C. crepidioides* seeds.

Germination of *C. crepidioides* and *C. rubens* seeds in presence of Abamine in the dark (A) and in the light (B). 50 seeds were plated on ½ MS containing 0, 1, 5 and 10 µM Abamine and incubated at a temperature of 25°C and light intensity of 30 µmol/m^{2}s in long days. Germination, defined as radicle emergence from the seed coat, was assessed at the indicated time points. Data shown are mean ± SD of four biological replicates.

In addition, I noted that ABA impaired cotyledon greening, which is a common ABA response of seedlings (Lopez-Molina *et al.*, 2001) and to verify this, cotyledon greening was quantified. This showed that *C. crepidioides* was extremely sensitive to ABA, with the greening of *C. crepidioides* Ile-Ife cotyledons being reduced to 20% already at a concentration of 0,1 µM ABA, an amount where the cotyledons of *C. rubens* Mali had a greening rate of 60% (Figure 10B). This suggested that in *C. crepidioides,* ABA responses are increased which may explain aspects of the low germination capacities of their seeds, especially in the dark. To further verify this, I supplemented ½ MS medium with inhibitors of ABA biosynthesis, namely Fluridone (Flu; Fong *et al.*, 1983; Kondhare *et al.*, 2014) and Abamine (Aba; Han *et al.*, 2004) to evaluate their impact on seed germination in the light and dark. Interestingly, Flu released the delayed germination in both growth conditions (Figure 11), with particularly striking effects in the case of *C. crepidioides*, where it completely restored germination in the dark. Aba however, had no significant influence on germination in both conditions (Figure 12).

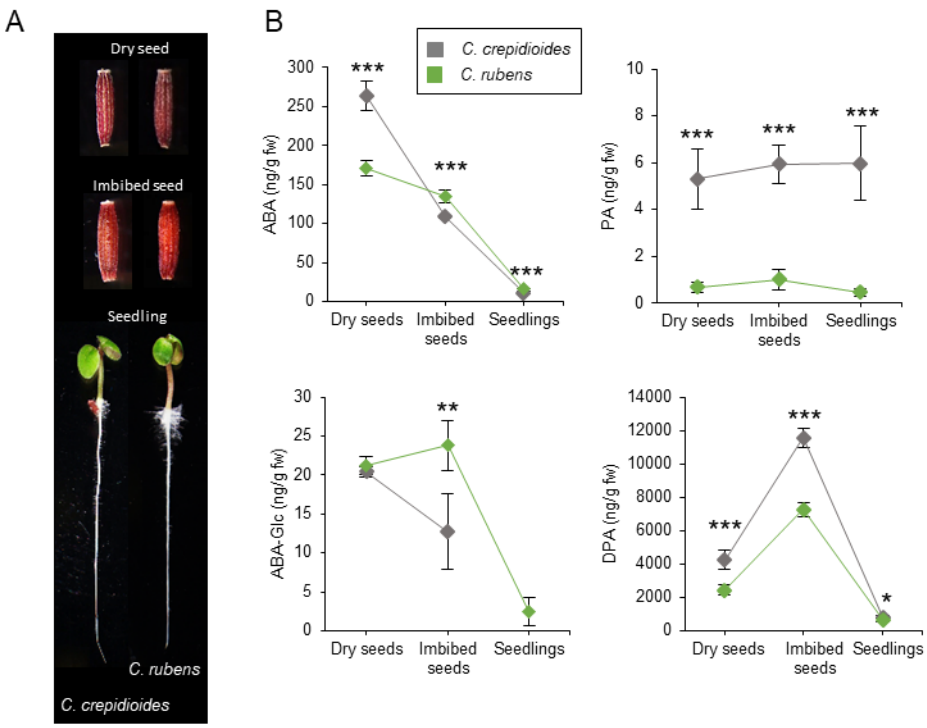

Figure 13. *C. crepidioides* contains a high level of ABA, which is rapidly catabolized during seed imbibition.

(A) Photographs of dry seeds, imbibed seeds and young seedlings of *C. crepidioides* and *C. rubens*, which were used in the analysis. (B) Levels of ABA and its catabolites in dry seeds, imbibed seeds and young seedlings of *C. crepidioides* and *C. rubens* measured by LC-MS/MS by Dr. Michael Gigl, TUM Chair of Food Chemistry and Molecular Sensory Science. The mean and standard deviation of 5 biological replicates is shown. The asterisks indicate significant differences between *C. crepidioides* and *C. rubens*, calculated by Student *t*- test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

The ability of Flu to restore germination in *C. crepidioides* seeds suggested that ABA level may be increased. To test this, concentrations of ABA and its catabolites phaseic acid (PA),

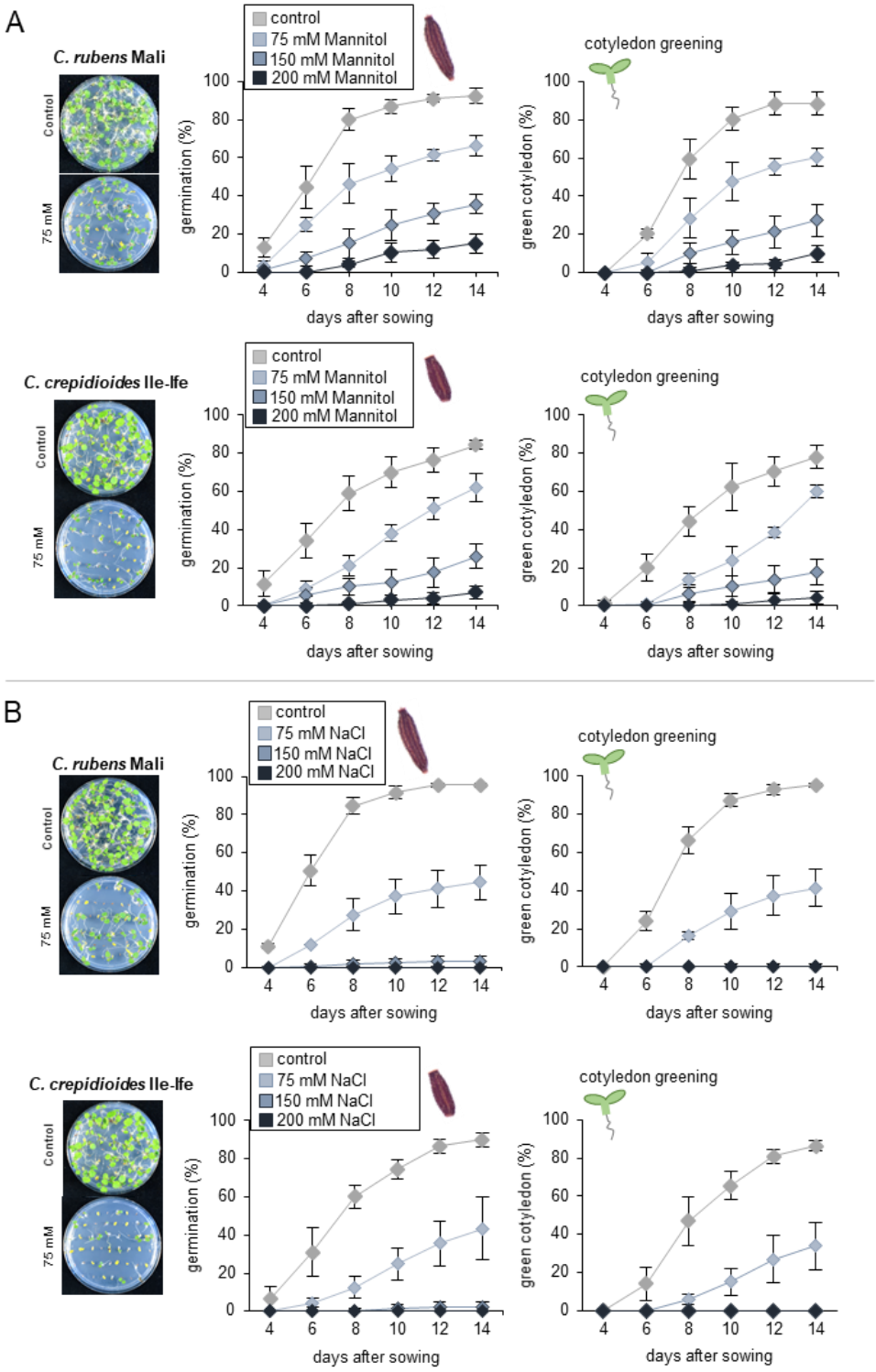

Figure 14. *C. crepidioides* has an increased sensitivity to Mannitol as compared to *C. rubens*.

Germination and cotyledon greening of *C. crepidioides* and *C. rubens* in presence of Mannitol (A) or NaCl (B). 50 seeds were plated on ½ MS medium containing the indicated concentrations of mannitol or NaCl and were incubated at a temperature of 25°C and a light intensity of 30 µmol/m^{2}s in long days. Germination, defined as radicle emergence from the seed coat, and cotyledon greening, defined as fully developed, green cotyledons, were assessed at the indicated time-points. Data shown are mean ± SD of four biological replicates.

dihydrophaseic acid (DPA) and ABA glucose ester (ABA-GE) were measured by GC-MS in dry seeds, imbibed seeds and young seedlings of both *C. crepidioides* and *C. rubens* (Figure 13A). The LC-MS/MS analyses were done by Dr. Michael Gigl from the group of Prof. Dr. Corinna Dawid at the TUM Chair of Food Chemistry and Molecular Sensory Science. The results showed that ABA level were indeed higher in seeds of *C. crepidioides* than in *C. rubens* (Figure 13B). *C. crepidioides* seeds had about 260 ng/g of ABA while *C. rubens* had about 170 ng/g of ABA in its dry seed. In addition, also the levels of PA were clearly increased in *C. crepidioides* seeds in all stages of germination (Figure 13B). DPA and ABA-GE concentrations were not consistently altered.

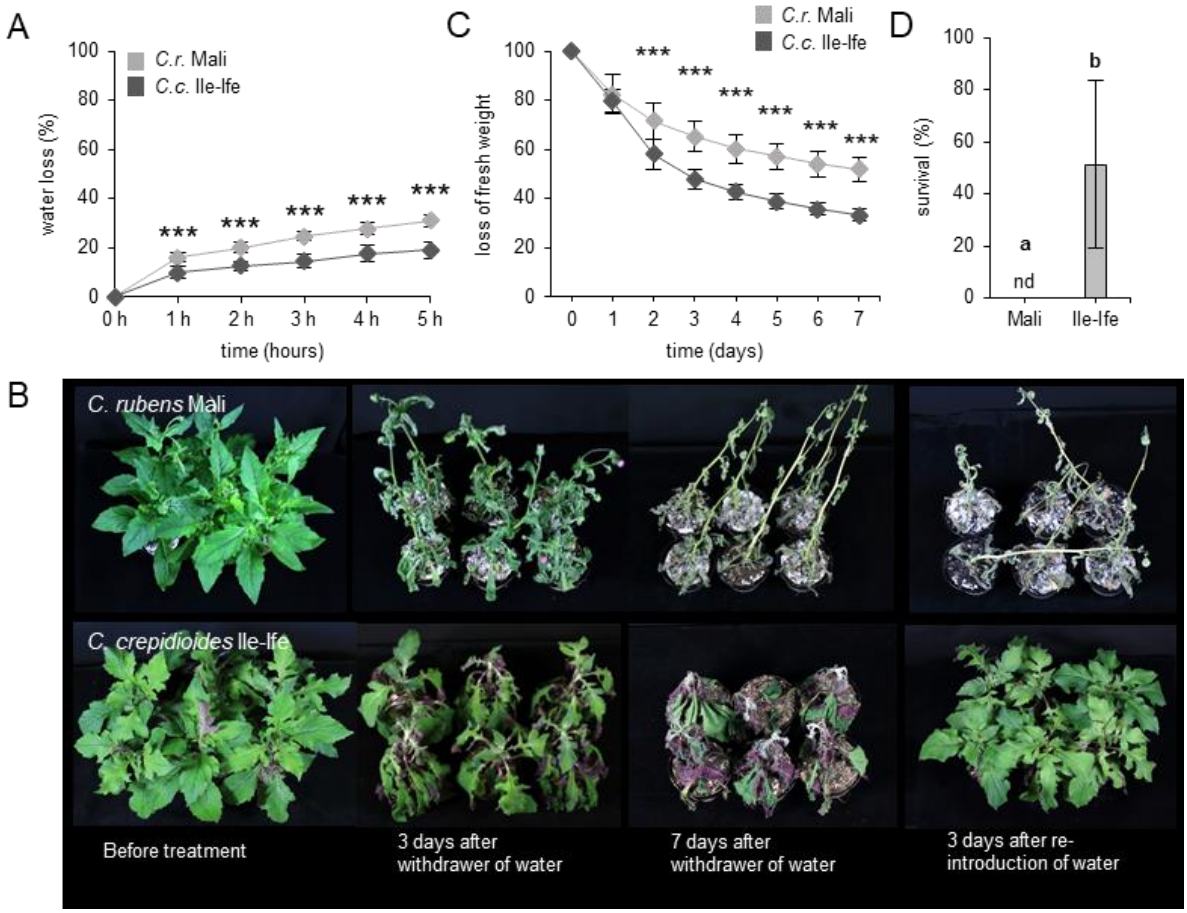

Figure 15. *C. crepidioides* shows a higher resistance to drought stress than *C. rubens*.

(A) Percentage of water loss measured in detached leaves of *C. rubens* Mali and *C. crepidioides* Ile-Ife. The third true leaves pair of 4-week-old plants of *C. rubens* Mali and 5-week-old plants of *C. crepidioides* Ile-Ife were detached, placed on a Petri's dish and exposed to ambient conditions. Data shown are mean ± SD of 10 leaves (B) Pictorial representation of *C. rubens* Mali and *C. crepidioides* Ile-Ife at different stages of exposure to drought stress. Seven-week-old plants of *C. rubens* Mali and 8-week-old plants of *C. crepidioides* Ile-Ife were subjected to progressive drought by withholding water for 7 days. Water was re-introduced 7 days after exposing the plants to drought. Data shown are mean ± SD of 16 plants. (C) Loss of fresh weight measured in whole plants of *C. rubens* and *C. crepidioides.* Data shown are mean ± SD of 16 plants (D) Survival percentage *C. rubens* and *C. crepidioides* after subjecting to drought stress. Mean is the average of 3 biological replicates. Significant differences between *C. crepidioides* and *C. rubens* are shown in asterisks and different letters ($P \leq 0.05$, calculated by one-way ANOVA with a post-hoc Tukey HSD test).

3.1.5. *C. crepidioides* shows a higher resistance against drought than *C. rubens*

In addition to dormancy, ABA plays a vital role in abiotic stress responses of plants (Nakashima and Yamaguchi-Shinozaki, 2013) and thus we asked if the increased ABA responses of *C. crepidioides* may benefit abiotic stress tolerance of this species. To do this, the responses of *Crassocephalum* seeds to high salt and osmotic stress were studied.

C. crepidioides and *C. rubens* seeds were germinated on ½ MS media supplemented with various concentrations of sodium chloride (NaCl) and D-Mannitol, which are used to mimic high salt and osmotic stress conditions respectively (Verslues *et al.*, 2006; Claeys *et al.*, 2014; Van den Broeck *et al.*, 2017). Both *C. crepidioides* and *C. rubens* responded similarly to NaCl. However, *C. crepidioides* was more sensitive to Mannitol when compared to *C. rubens* with especially noticeable differences at 75 mM (Figure 14).

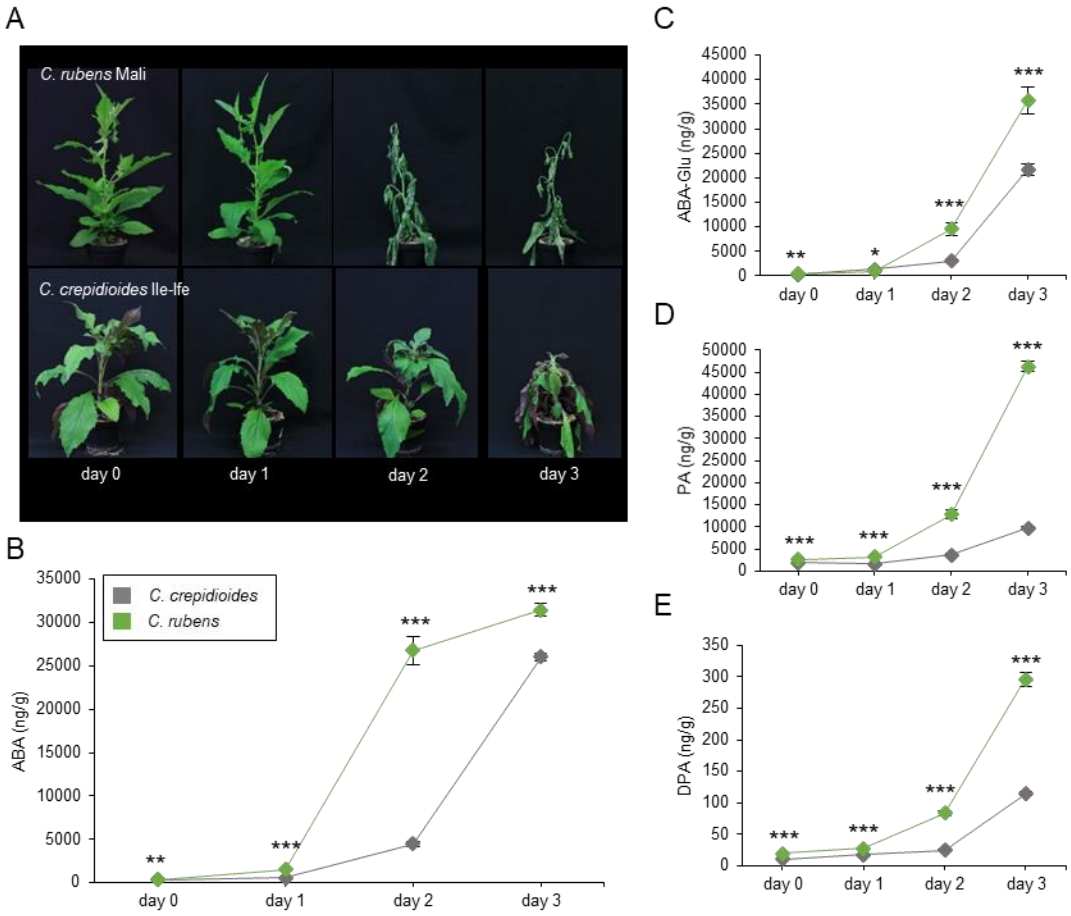

Figure 16. Drought induction increases the level of ABA in *Crassocephalum* plants.

(A) Pictorial representation of *C. rubens* Mali and *C. crepidioides* Ile-Ife different stages of exposure to drought stress. Seven-week-old plants of *C. rubens* Mali and 8-week-old plants of *C. crepidioides* Ile-Ife were exposed to progressive drought by withholding water for 3 days. (B) ABA (C) ABA-GE (D) PA and (E) DPA levels in drought-stressed plants of *C. crepidioides* and *C. rubens* measured by LC-MS/MS by Michael Gigl, TUM Chair of Food Chemistry and Molecular Sensory Science. The mean and standard deviation of 5 biological replicates is shown. The asterisks indicate significant differences between *C. crepidioides* and *C. rubens*, calculated by Student *t*- test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

Since ABA can also confer drought resistance, the responses of *Crassocephalum* plants to drought stress were investigated. This was done by conducting whole plant assays in which water was withheld from adult plants of both *C. crepidioides* (8-week-old plants) and *C. rubens* (7-week-old plants) for seven days. After this period, water was re-introduced and the plants were grown for another 3 days.

In a first analysis, the rate of water loss from the leaves of *C. crepidioides* and *C. rubens* was studied, by determining the fresh weight changes of detached leaves over a period of time. This showed that excised leaves of *C. crepidioides* lost water significantly slower than leaves of *C. rubens* (Figure 15A). In line, *C. crepidioides* showed an increased resistance to drought stress as compared to *C. rubens*. The plants exhibited delayed wilting and after rewatering, about half of the *C. crepidioides* plants recovered, whereas none of *C. rubens* survived (Figure 15B). We also analysed the rate of water loss from whole plants of *C. crepidioides* and *C. rubens* by determining the fresh weight changes of the whole plants over a period of time. Interestingly, when the rate of water loss from whole plants of both *C. crepidioides* and *C. rubens* was measured, *C. crepidioides* plants lost water more rapidly than *C. rubens* (Figure 15C). At the end of 7 days, *C. crepidioides* plants had lost more than 50% of their fresh weight compared to *C. rubens*. And this was correlated with clearly increased survival rates (Figure 15D).

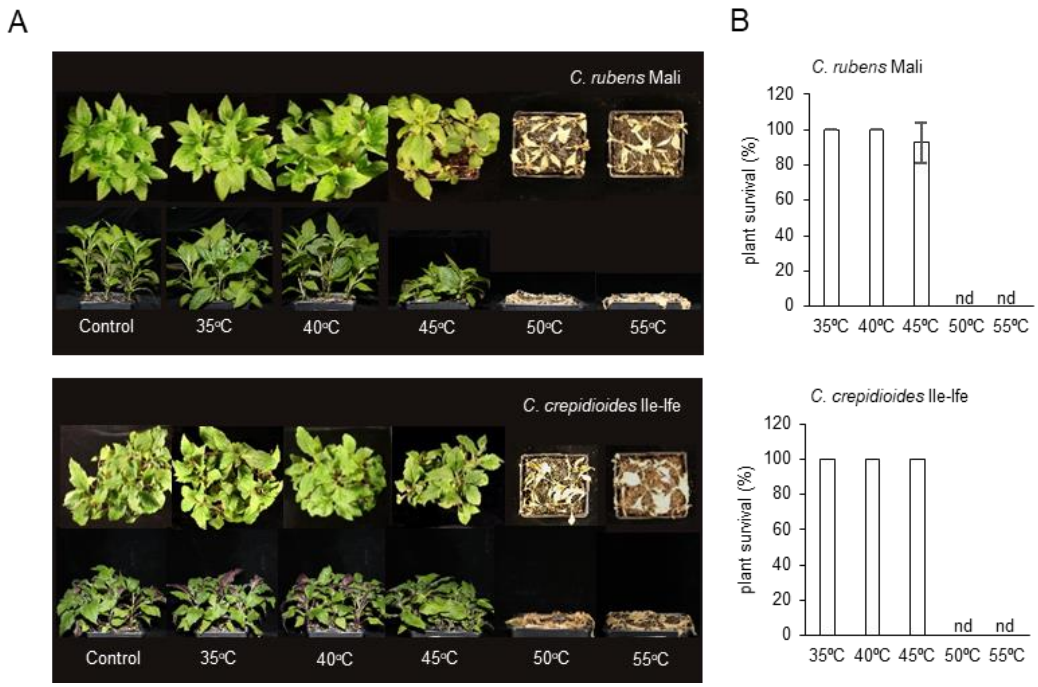

Figure 17. *C. crepidioides and C. rubens* are both resistant to heat shock up to 45 °C.

(A) Phenotype of *C. crepidioides* and *C. rubens* plants exposed to high temperature and (B) quantification of survival rates. Four-week-old plants of *C. crepidioides* Ile-Ife and *C. rubens* Mali were exposed to a temperature from 35 °C to 55 °C for two hours. Plant evaluation was performed after two weeks of recovery in standard growth conditions. . Survival was defined as the ability to produce new leaves from an intact shoot apical meristem. The graph shows the mean ± SD of 6 biological replicates.

ABA play a prominent role in preventing water loss during drought exposure (Kim *et al.*, 2010; Sah *et al.*, 2016; Bharath *et al.*, 2021) and thus it was of interest to test, if the increased drought tolerance of *C. crepidioides* may be correlated with altered capacities to form ABA. To investigate this, the levels of ABA and its catabolites PA, DPA and ABA-GE were measured in leaves of both *C. crepidioides* and *C. rubens*. Water was withheld from adult plants of *C. crepidioides* and *C. rubens* for 3 days (Figure 16A) and leaves samples were taken daily starting on day 1. The LC-MS/MS measurements were done by Dr. Michael Gigl from the group of Prof. Dr. Corinna Dawid at the TUM Chair of Food Chemistry and Molecular Sensory Science and the results showed that drought strongly increased ABA contents in both species (Figure 16B). However, interestingly the increase was significantly delayed in *C. crepidioides*. In line, also the formation of the ABA catabolites was delayed in *C. crepidioides* (Figure 16C, D & E) speaking for a delayed ABA response in this species.

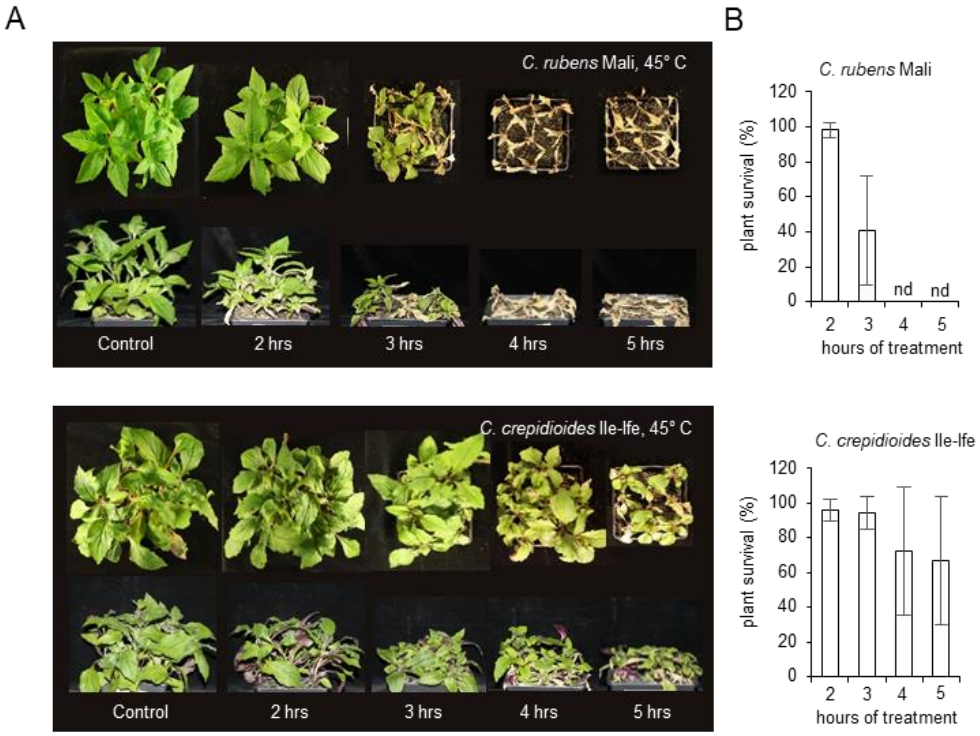

Figure 18. *C. crepidioides* shows a higher heat stress resistance than *C. rubens*.

(A) Phenotype of *C. crepidioides* and *C. rubens* plants exposed to 45 °C and (B) quantification of survival rates. Four-week-old plants of *C. crepidioides* Ile-Ife and *C. rubens* Mali were exposed to a temperature of 45°C for a range of 2 to 8 hours. Plant evaluation was performed after two weeks of recovery in standard growth conditions. Survival was defined as the ability to produce new leaves from an intact shoot apical meristem.

3.1.6. *C. crepidioides* shows a higher resistance against heat than *C. rubens*

To test additional types of stress, four-week-old plants of *C. crepidioides* and *C. rubens* were exposed to high temperatures ranging from 30°C to 55°C for two hours and then re-covered at normal ambient temperatures before survival rates were assessed. Both *C. crepidioides*

and *C. rubens* fully survived temperature of up to 45ºC for 2 hours, although *C. rubens* showed a stronger growth repression than *C. crepidioides* at 45°C (Figure 17A, B). The 2 hours treatments at 50°C and 55°C however killed all plants (Figure 17). To assess potential differences between the two species, the experiment was modified and plants were exposed to a temperature of 45°C for a range of 2 to 8 hours. This revealed clear differences: whereas *C. crepidioides* could endure 5 hours of 45°C treatment, survival rates of *C. rubens* were significantly reduced already after 3 hours (Figure 18). Thus *C. crepidioides* also has a higher capacity to survive heat stress.

Since *Crassocephalum* originated from regions with subtropical climates, it was of interest to investigate if it has a certain level of frost tolerance. To do so, both *C. crepidioides* and *C. rubens* were exposed to temperatures ranging from +4ºC to -4ºC for 3 hours of time and survival rates were assessed after 2 weeks of recovery at ambient temperatures. This showed that, somewhat surprisingly, both species tolerated frost quite well, suffering damage only at a temperature of -4°C (Figure 19) but with a similar degree.

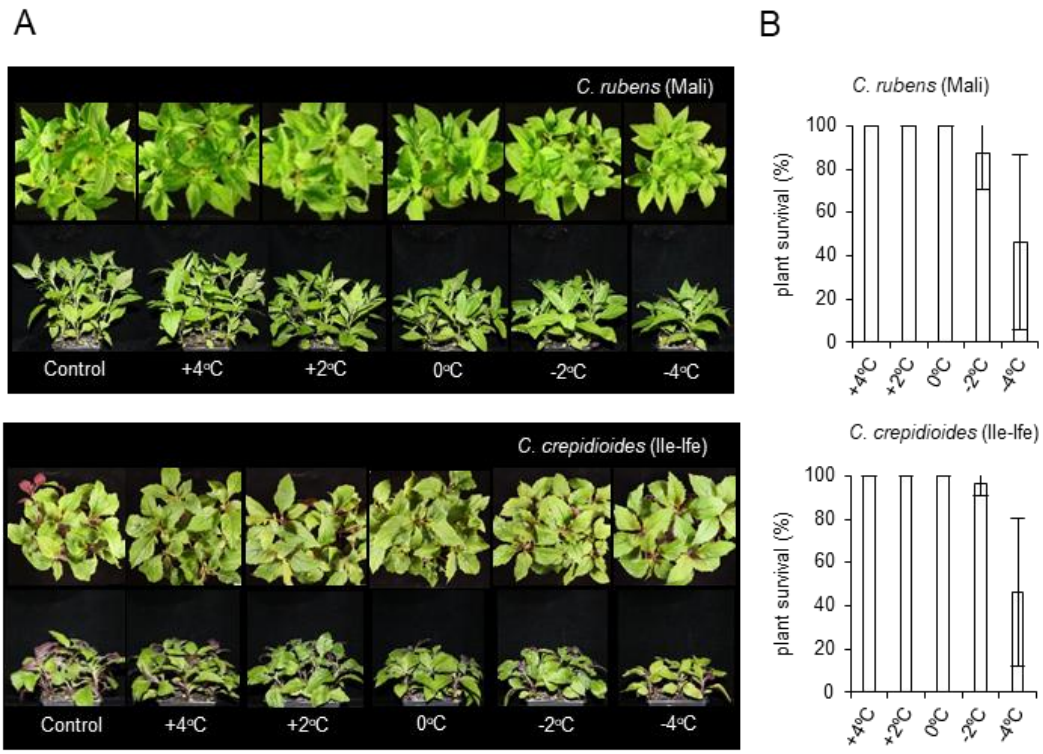

Figure 19. *C. crepidioides* and *C. rubens* have a similar degree of frost tolerance.

(A) Phenotype of *C. crepidioides* and *C. rubens* plants exposed to freezing temperature and (B) quantification of survival rates. Four-week-old plants of *C. crepidioides* Ile-Ife and *C. rubens* Mali were exposed to a temperature from +4 °C to -4 °C for 3 hours. Plant evaluation was performed after two weeks of recovery in standard growth conditions. Survival was defined as the ability to produce new leaves from an intact shoot apical meristem. The graph shows the mean ± SD of 6 biological replicates.

3.2. Establishing tools of molecular genetics for *Crassocephalum*

3.2.1. Developing methodology for *Crassocephalum* transformation

3.2.1.1. Testing markers for transformant selection in *Crassocephalum*

The ability to transform plants with transgenes is a highly important research tool since it allows to generate gain- and loss-of-function mutants for biological studies. In addition, procedures such as genome editing may be relevant for a fast-track genetic improvement of *Crassocephalum* in the future, which is why it was aimed to establish transformation protocols.

Transformation can be an inefficient technique and thus selection markers are needed, which enable a selection of the few transgenic organisms in the sea of non-transgenic ones (Wilmink & Dons, 1993). Selection agents are usually toxins and for plant selection, the herbicide glufosinate and the antibiotic KAN are commonly used and it was therefore tested if they are applicable for *Crassocephalum*.

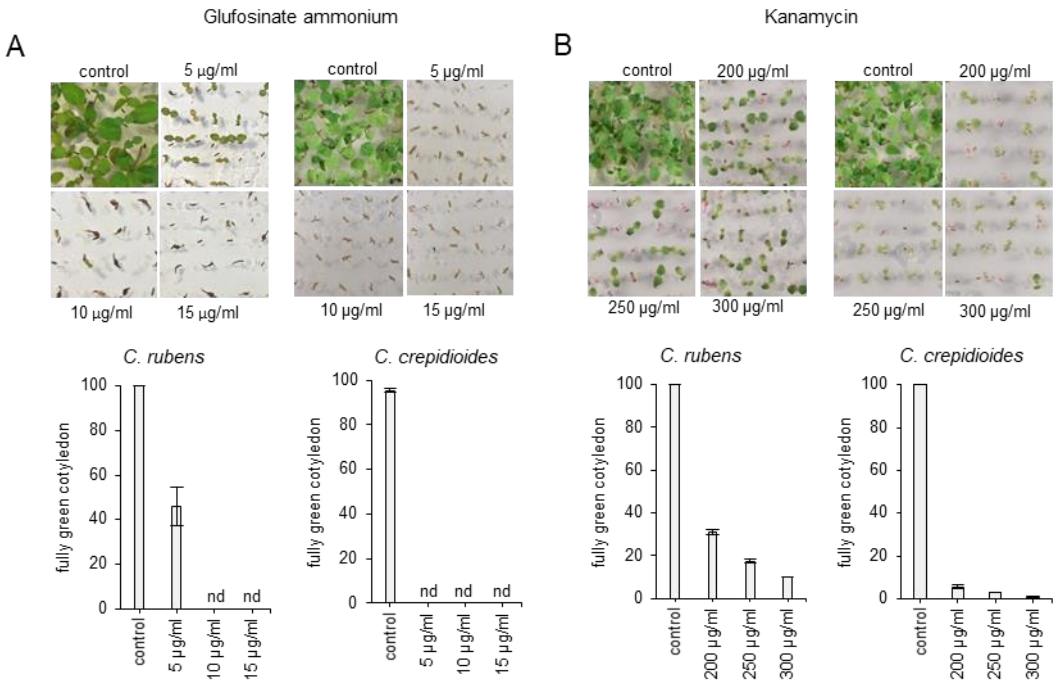

Figure 20. Glufosinate and KAN impaired seedling development of *Crassocephalum* species.

Effect of Glufosinate ammonium (A) and Kanamycin (B) on seedling development of *C. crepidioides* and *C. rubens.* 100 seeds were incubated on ½ MS media supplemented with indicated concentrations of glufosinate and kanamycin for 3 weeks. Seeds were incubated at 21°C, 80 µmol/m^{2}s and 16/8 hours of light/dark cycle and graph shown are mean ± SD of 4 independent replicates.

To assess this, their impact on germination and early seedling development of *C. crepidioides* and *C. rubens* was determined. For this purpose, seeds were plated on ½ MS medium containing different concentrations of glufosinate and KAN and incubated for 3 weeks.

This showed that glufosinate greatly impaired seedling development in both species, with a clear impact already at the lowest concentration of 5µg/ml for *C. crepidioides* (Figure 20A). *C. rubens* was slightly more resistant, surviving 5µg/ml to some extent, but being clearly impaired at the higher levels (Figure 20A). KAN also impaired seedling growth of both *C. crepidioides* and *C. rubens* but at higher concentrations (Figure 20B). *C. rubens* was more resistant to KAN compared to *C. crepidioides*. At 200µg/ml KAN, *C. crepidioides* growth was already clearly restricted (Figure 20B). However, *C. rubens* required an additional 100µg/ml KAN for its growth to be impaired (Figure 20B). Concentrations below 200µg/ml did not have any significant effects on *Crassocephalum* seedling growth. Therefore, glufosinate is more suitable for *Crassocephalum* selection than KAN.

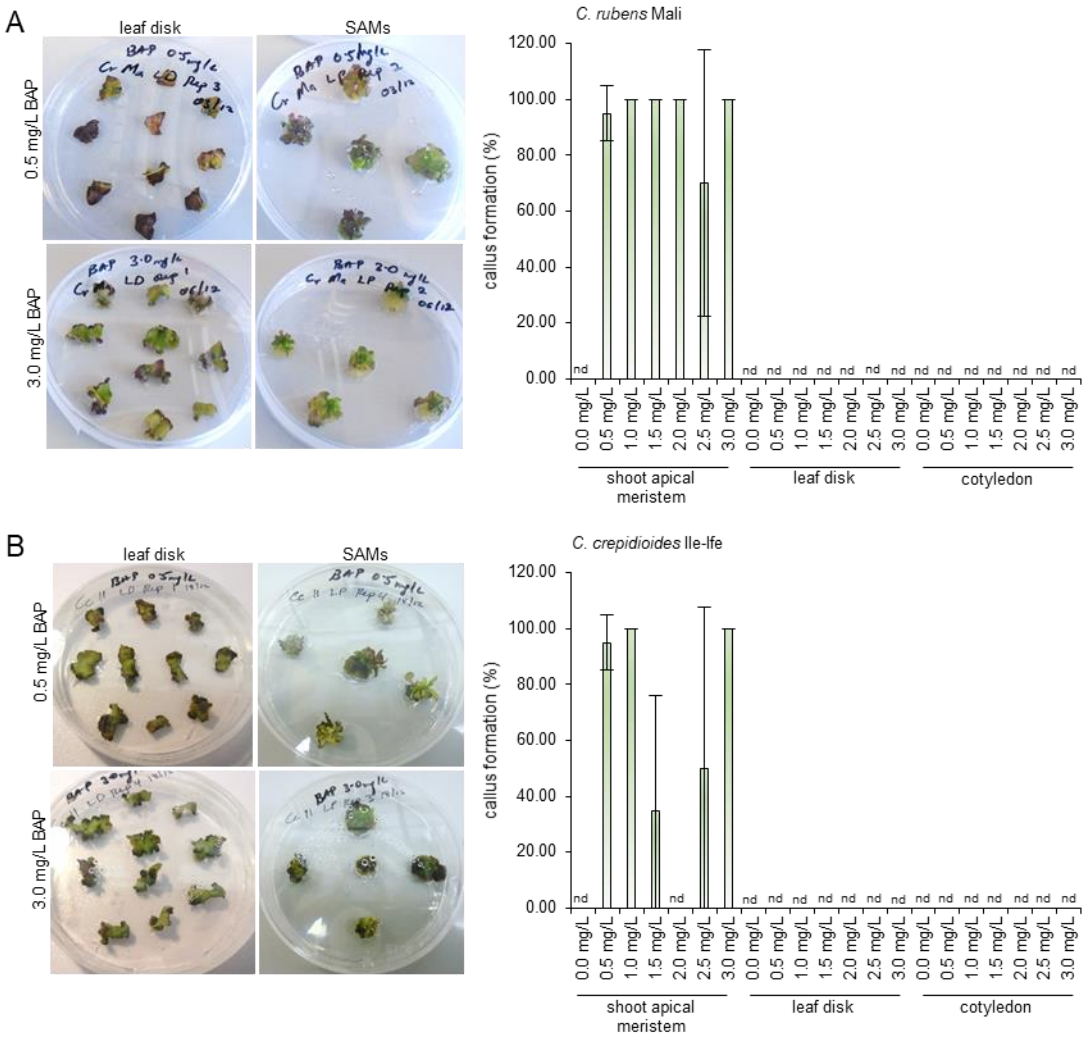

Figure 21. BAP induces callus formation in SAM explants of *C. crepidioides* and *C. rubens.*

Right: photos of representative plates, showing the effect of different concentrations of BAP on callus formation from shoot apical meristem, cotyledon and leaf disk explants. Left: quantification of callus formation, as defined by group or mass of cells with or without elongated shoot. Shoot apical meristems, cotyledons and leaf disks were dissected and plated on ½ MS media supplemented with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l of BAP for one month. Explants were incubated at 21°C, 80 µmol/m^{2}s and 16/8 hours of light/dark cycle, before callus formation was assessed.

3.2.1.2. Developing regeneration protocols for *Crassocephalum*

A common method of plant transformation employs the plant pathogen *Agrobacterium tumefaciens* (now *Rhizobium radiobacter*) as a vector, to stably introduce DNA into the plant genome. *A. tumefaciens* can transform both vegetative (callus) and generative cells (Chumakov, 2007) and for callus transformation regeneration protocols are needed, that allow to regenerate whole plants from a single transformed cell. A common method of regeneration uses shoot-induction-medium, which contains the plant hormone cytokinin (Miller *et al.*, 1955; Miller *et al.*, 1956; Kieber & Schaller, 2018) and here it was tested if cytokinin can induce shoot formation in *Crassocephalum*. For this purpose, three different types of explants were generated from *C. crepidioides and C. rubens* plants, namely leaf disk, cotyledons and shoot apical meristems (SAMs), which were dissected from sterilely grown two-weeks-old seedlings. These were plated on ½ MS media supplemented with 0.5 - 3.0 mg/l of the cytokinin 6-Benzylamino purine (BAP) or without BAP as a control and incubated for one month before callus formation was assessed.

The result showed that in the case of both *C. crepidioides and C. rubens,* BAP was able to induce callus formation from the SAM explants. However, no callus was formed from the leaf disks or cotyledons (Figure 21A, B). In the case of *C. crepidioides*, callus formation was concentration-dependent, since, at 2.0 mg/l of BAP, no callus was formed, whereas the other concentrations were quite effective.

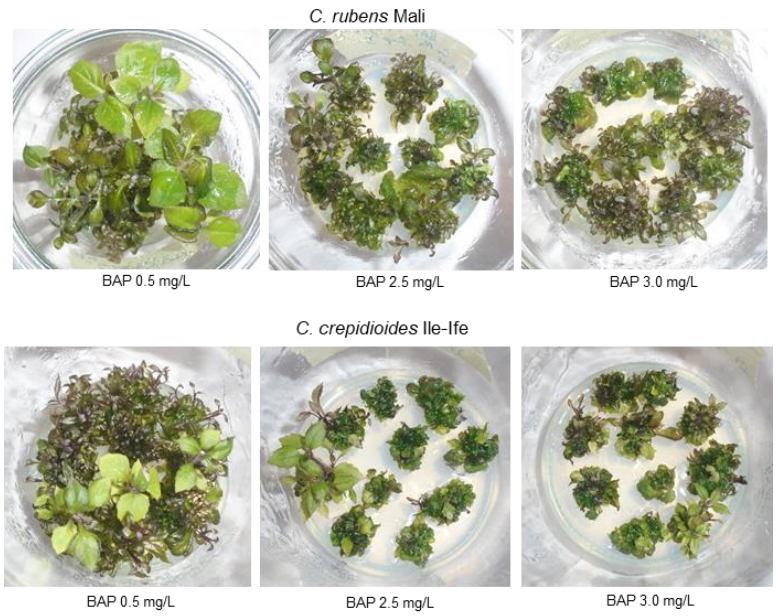

Figure 22. Low concentration of 6-Benzylamino purine (BAP) causes direct shoot regeneration from shoot apical meristem explants.

Effect of different concentrations of BAP on shoot regeneration of *C. rubens* and *C. crepidioides* from SAM explants. The SAMs were plated on ½ MS media supplemented with 0.5, 2.5 and 3.0 mg/l of BAP for one month. Calli were then transferred to fresh ½ MS media, without BAP, for another 2 to 3 weeks. Explants were incubated at 21°C, 80 µmol/m^2s and 16/8 hours of light/dark cycle.

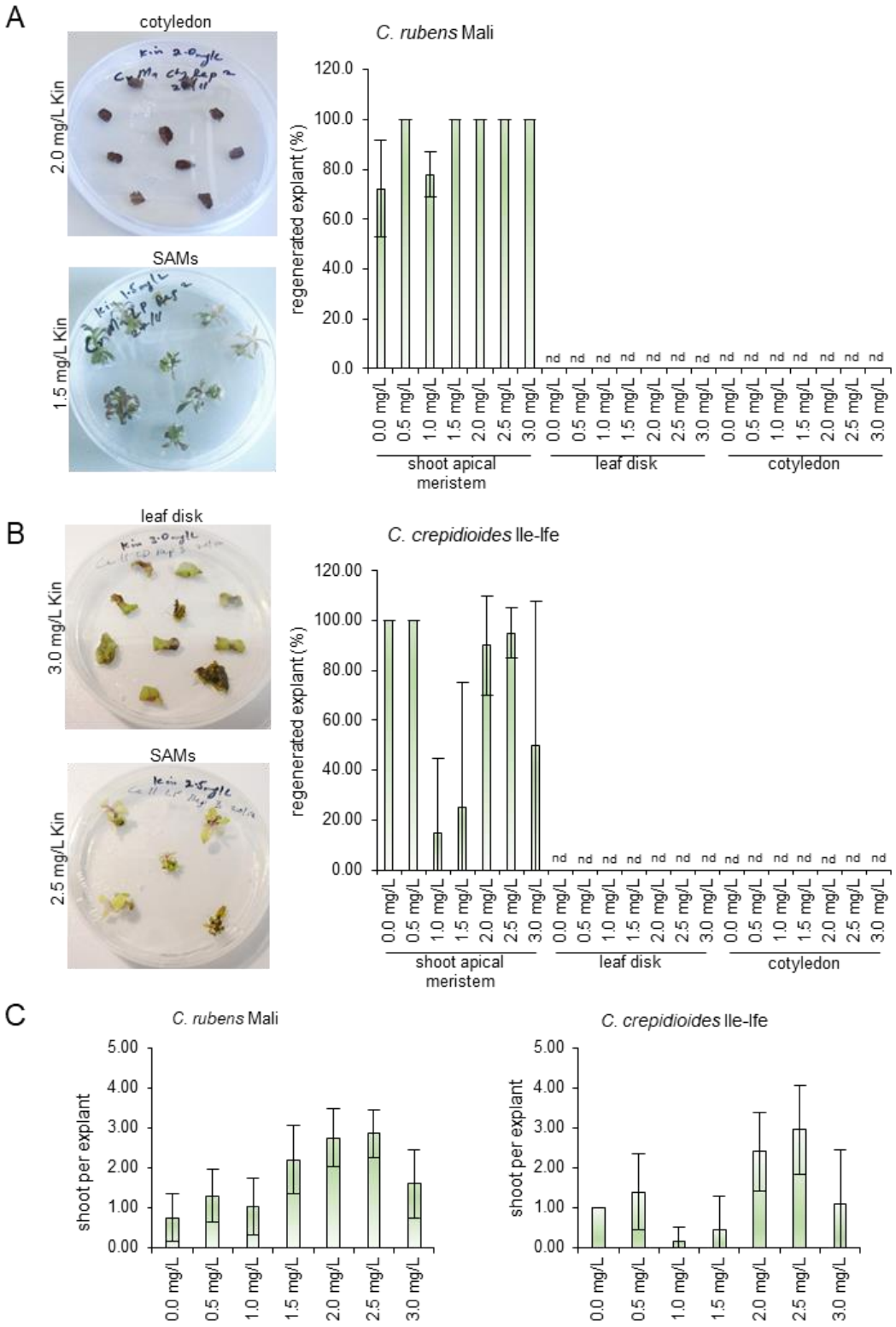

Figure 23. Kinetin induces shoot regeneration from SAM explants of *C. crepidioides* and *C. rubens.*

(A, B) Right: photos of representative plates, showing the effect of different concentrations of kinetin on callus formation from shoot apical meristem, cotyledon and leaf disk explants. Left: quantification of callus formation, as defined by group or mass of cells with or without elongated shoot. (C) Shoot explants produced from SAM at different concentrations of kinetin. Shoot apical meristems, cotyledons and leaf disks were dissected and plated on ½ MS media supplemented with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l of kinetin for one month. Explants were incubated at 21^{o}C, 80 µmol/m^{2}s and 16/8 hours of light/dark cycle, before callus formation was assessed.

Since no intact shoots were formed, the procedure was optimized. SAMs were plated on ½ MS containing 0.5, 2.5 and 3.0 mg/l of BAP for one month and then transferred to ½ MS medium without BAP for another 2-3 weeks. This showed that at a concentration of 0.5 mg/l BAP, elongated shoots developed in both *Crassocephalum* species (Figure 22).

At higher concentrations, shoots were formed but did not properly elongate, which would impair regeneration of whole plants. Thus, when SAMs were plated on a concentration of 0.5 mg/l BAP and transferred back to ½ MS medium for another 2-3 weeks, nicely elongated shoots could be efficiently generated for both species.

In addition to BAP, also the cytokinin kinetin was tested, by using the same procedures and kinetin concentrations of 0.5-3.0 mg/l. Irrespective of the kinetin concentrations used, no callus or shoots were formed from the leaf disk or cotyledons, but only from the SAM explants (Figure 23). The re-generation of both callus and shoots from SAMs was dose-dependent with 2.0-2.5 mg/l of kinetin having the strongest effects

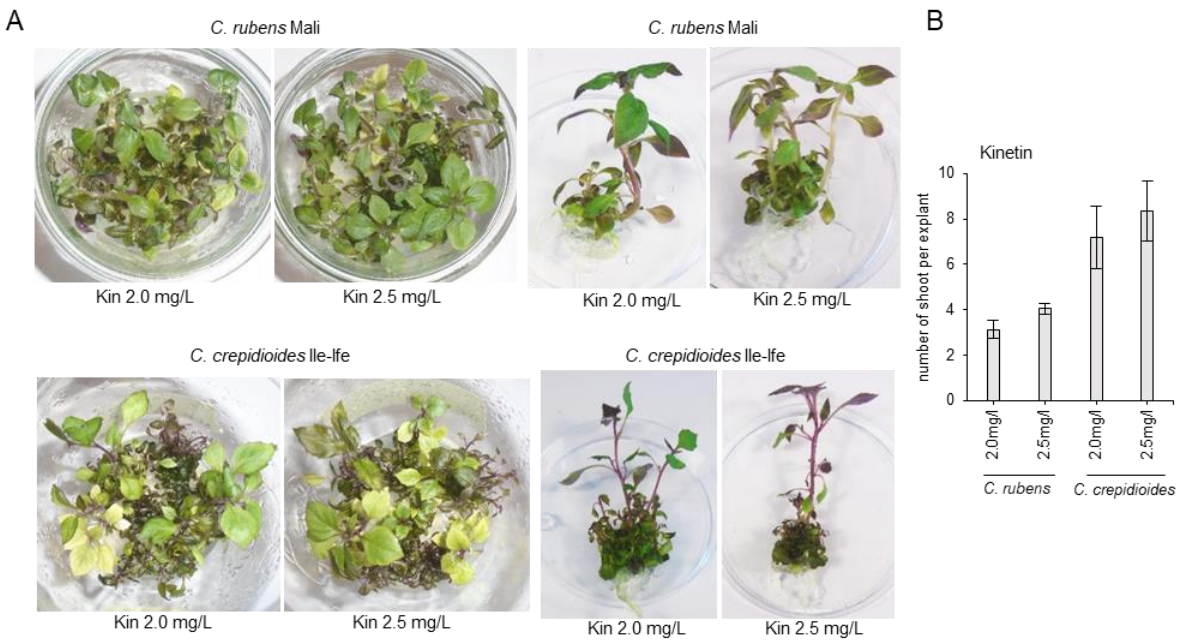

Figure 24. Kinetin causes direct shoot and root regeneration from SAM explants of *C. rubens* and *C. crepidioides*.

(A) Effect of different concentrations of kinetin on shoot regeneration of *C. rubens* and *C. crepidioides* from SAM explants and (B) Quantification of shoot explants produced at the indicated concentrations. The SAMs were plated on ½ MS media supplemented with 2.0 and 2.5 mg/l of kinetin for one month. Calli were then transferred to fresh ½ MS media, without BAP, for another 2 weeks. Explants were incubated at 21°C, 80 µmol/m^{2}s and 16/8 hours of light/dark cycle.

To further test the effect of kinetin on shoot regeneration from SAMs, SAMs were plated on ½ MS supplemented with 2.0 and 2.5 mg/l of kinetin for one month and then transferred to ½ MS medium without kinetin for another two weeks. This gave a very nice shoot regeneration and elongation, showing that kinetin is very effective for this application (Figure 24).

3.2.1.3. Floral dipping for *Crassocephalum* transformation

In addition to the transformation of callus cells, also egg cells can be transformed with *A. tumefaciens*. If this occurs right after fertilization, before the first cell division events, the originating embryo will be fully transgenic (Chumakov, 2007). Thus, it is a way of transformation, which avoids the drawbacks of tissue culture.

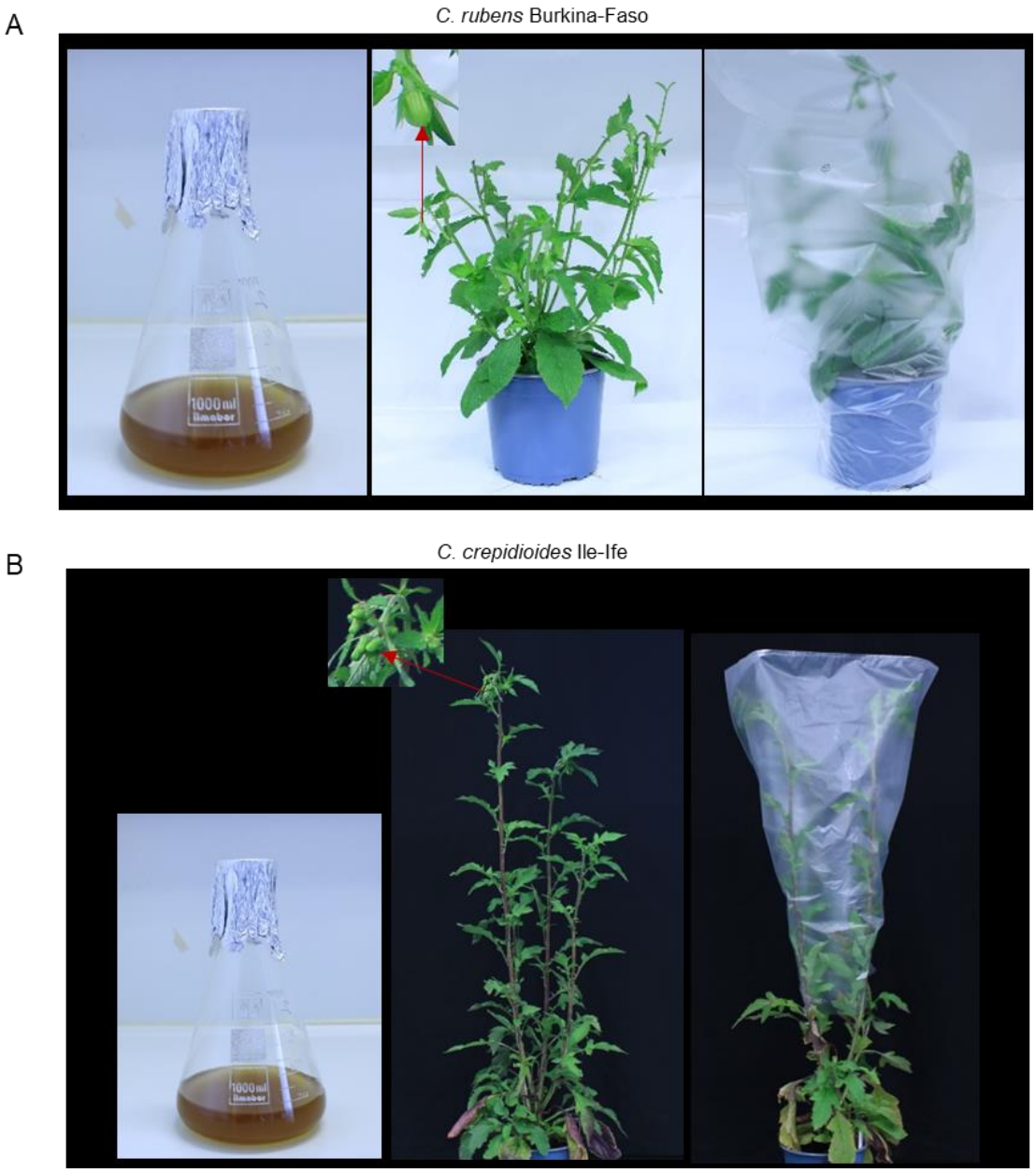

Figure 25. Floral dipping of *C. rubens* and *C. crepidioides*.
Shown are plants before dipping, with a magnification of the buds, and after dipping, wrapped in plastic bags.

The floral dipping technique was originally developed for the transformation of *A. thaliana*, but can also be adopted for other species (Clough & Bent, 1998; Curtis & Nam, 2001; Yasmeen *et al.*, 2009; Zale *et al.*, 2009; Li *et al.*, 2010; Mu *et al.*, 2012; Rod-in *et al.*, 2014) and it was thus of interest to test, if it may also be suitable for *Crassocephalum* transformation. To do so, flowering plants of *C. rubens* Burkina-Faso and *C. crepidioides* Ile-Ife were produced (Figure 25) and dipped into a suspension of *A. tumefaciens*, transformed with a vector containing a 35S:GUS construct, which expresses a KAN resistance marker for plant selection.

The dipped plants were covered with plastic bags to maintain a high humidity and kept in the dark overnight (Figure 25). The plastic bags were removed the following day, and the plants were allowed to continue to grow until the seeds were set. After floral dipping and seed development, sterilized seeds were plated on KAN selection plates and transformants were identified based on their KAN resistance. Using this approach, 15,000 seeds of both *C. rubens* and *C. crepidioides* were screened and one transformant of *C. rubens* was identified based on its KAN resistance (Figure 26A). The line was amplified and its progeny was GUS stained, which showed that GUS was expressed at high levels in seedlings (Figure 26B). This proves that the GUS gene is expressed and is stably inherited to the next generation. While this shows that *Crassocephalum* plants can be transformed in principle using floral dipping, the protocol will have to be optimized, to increase the transformation efficiency.

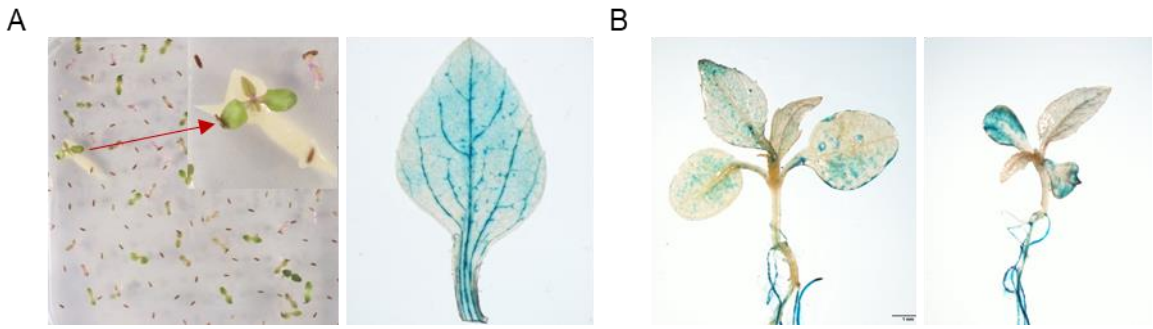

Figure 26. *C. rubens* can be transformed with *Agrobacterium tumefaciens*-mediated floral dipping.

(A) Transformed seedling of *C. rubens.* (B) *C. rubens* transformant expressing the GUS gene. A leave of an adult plant was stained using X-Gluc (5-Bromo-4-Chloro-3-Indoyl-Beta-D-Glucuronide) and destained with 70% Ethanol. GUS activity is visualized by blue pigment formation.

3.2.2. Assembling a *C. rubens* mutant collection

Mutant screens are an important tool of forward molecular genetics, where plants are selected based on interesting phenotypes and a characterization of their molecular nature can yield information about loci that account for them (Bado *et al.*, 2015; FAO/IAEA, 2018). Moreover, the generation of mutants is a means of breeding, where natural mutation frequencies are

increased with mutagens, to generate new lines with interesting traits, that can serve as breeding material, a technique called mutation breeding (Bado *et al.*, 2015; FAO/IAEA, 2018). Therefore, to facilitate research and breeding with *Crassocephalum*, it was aimed to generate a mutant collection, using the chemical mutagen ethyl methane sulfonate (EMS) and diploid *C. rubens* accession Burkina Faso, which the AOCC plans to sequence.

3.2.2.1. Establishing EMS concentrations which yield 50% survival rates

When plants are mutagenized, survival rates of about 50% in the M1 generation are usually aimed for, since this ensures a high mutation frequency (Jander *et al.*, 2003; Hohmann *et al.*, 2005; Arisha *et al.*, 2014). To determine a suitable EMS concentration for *C. rubens*, 500 seeds of the *C. rubens* ecotype Burkina Faso were treated with three different EMS concentrations and a control (0.0, 0.2, 0.4, and 0.6% v/v) for 15 hours and the seeds were dried. They were then sown in soil and the phenotypic outcomes were quantified in the M1 plants.

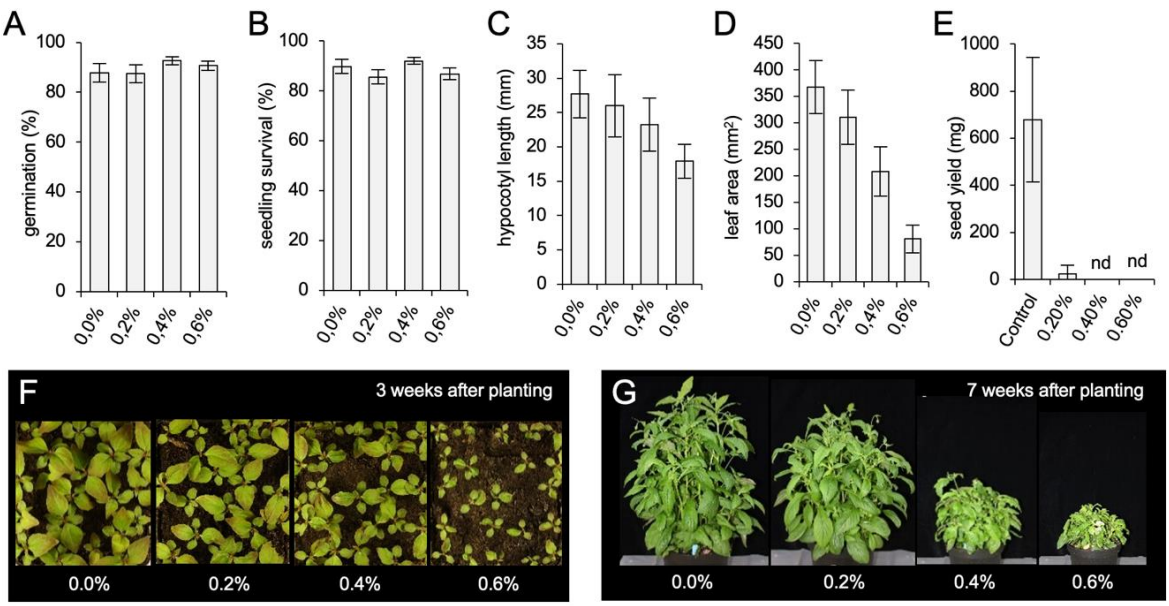

Figure 27. EMS effects on plant growth and fertility in *C. rubens*.

500 seeds of *C. rubens,* treated with different concentrations of EMS, were planted on soil and 3 weeks after sowing, germination efficiency (A), seedling survival (B), seedling hypocotyl elongation (C) and leaf area (D) were quantified. Moreover, total seed yield was determined from adult plants (E). Photographs show the effect of EMS on growth and development of *C. rubens* M1 plants 3 weeks (F) and 7 weeks (G) after planting.

This showed that seed germination and seedling survival were not affected by these EMS concentrations (Figure 27A, B). However, seedling hypocotyl length and leave area, calculated using the Olympus CellSens imaging software (Olympus SZX10, Tokyo, Japan), were negatively correlated with increasing EMS concentrations, with clear effects at 0.4 and 0.6% (Figure 27C, D). Interestingly the effects only became very pronounced in the adult stage,

where plants treated with 0.4 and 0.6% EMS were infertile and even 0.2% EMS strongly reduced fertility (Figure 27E). Visually, growth was seen to be clearly compromised starting at a concentration of 0.4% in the seedling stage (Figure 27F) and at 0.2% in the adult stage (Figure 27G).

To optimize the EMS treatment, in a next step, *C. rubens* seeds were exposed to 0.1 and 0.2% v/v EMS, as well as to a control treatment, for a shorter period of time, namely 8 hours, and the outcomes on plant development were again assessed. This showed that an 8-hour exposure to EMS concentrations of 0.1 and 0.2% had no significant effect on seed germination, seedling survival, seedling hypocotyl length or leaf area in M1 plants (Figure 28A-E). However, these EMS concentrations reduced seed yield of *C. rubens* with 0.1% EMS concentration having weaker effects than 0.2% as compared to the control (Figure 28F).

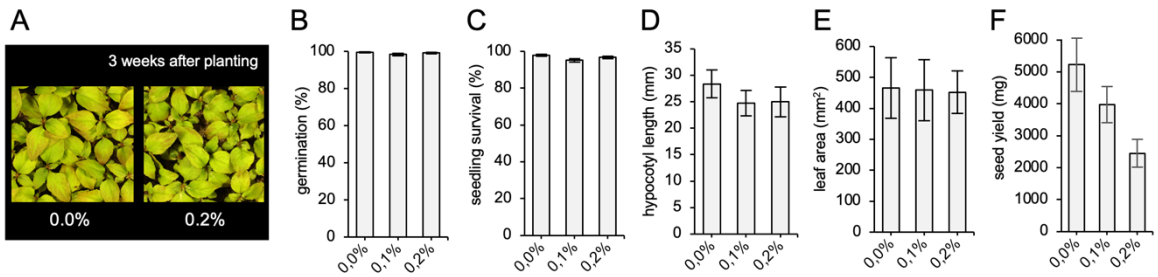

Figure 28. Low EMS concentration don't impair plant growth and fertility of *C. rubens*.

(A) Photos of representative *C. rubens* seedlings treated with 0.0 or 0.2% EMS. (B-E) 500 seeds of *C. rubens,* treated with 0.1 and 0.2% of EMS, were planted on soil and 3 weeks after sowing, germination efficiency (B), seedling survival (C), seedling hypocotyl elongation (D) and leaf area (E) were quantified. (F) Moreover, total seed yield was determined from adult plants.

3.2.2.2. First characterization of the *C. rubens* mutant population

Since an 8-hour treatment with 0.2% EMS was a condition, where growth was already affected, but seeds were still produced in reasonable amounts, this set-up was used to mutagenize more plants. About 3,000 seeds of *C. rubens* Burkina Faso were treated and were harvested in pools of 10-20 M1 plants, which were allowed to self-fertilize, generating M2 seed pools. These seed pools can now be used for screening and for a first characterization, 500 plants of M2 generation were grown in the greenhouse to the adult stage and mutants with altered leave morphology and growth patterns were selected.

Various alterations in leave shapes were observed, including leaves with more or less serration, longer or shorter petioles and narrower or broader leaf blades. Also, as compared to wild-type, increased or decreased sizes, altered tropic growth responses and defective branching occurred and flowering time was promoted or delayed (Figure 29).

In addition, also other interesting developmental defects occurred. One mutant showed a hyper-active SAM, which remained in a vegetative state and continuously produced leaves instead of switching to generative development. This resulted in larger, sterile plants with much more leaves than wild-type (Figure 29D; details in Figure 30A).

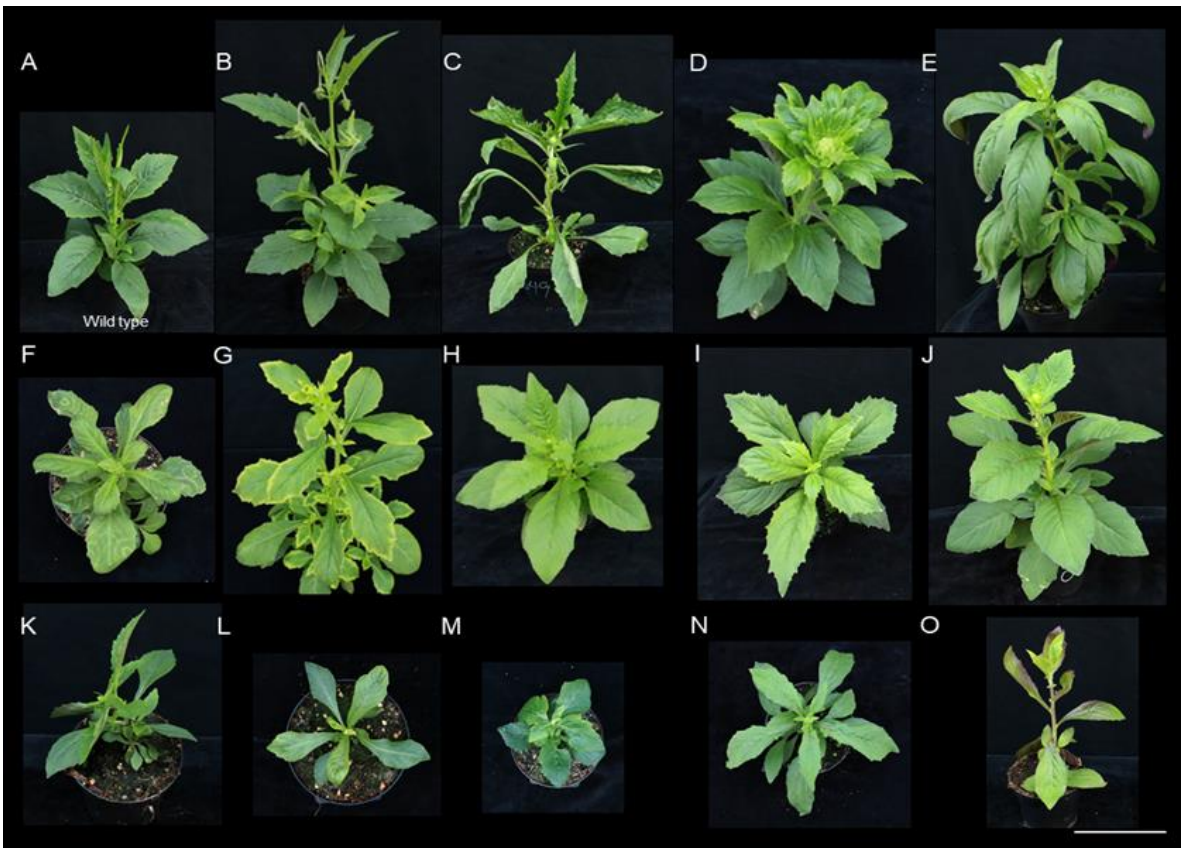

Figure 29. Phenotypes of some *C. rubens* mutants isolated in a first, small-scale screen.

The plants were grown in soil, in the greenhouse for 7 weeks and photos were taken. Shown are (A) wild-type and mutants with early flowering (B), curved leaves (C), defective flower development and more leaves (D), outwardly curving, epinastic leaves (E), spontaneous cell death (F), variegated leaves (G), broader leaves (H), more serrated leaves (I), less serrated leaves (J), defective tropic growth responses (K), narrower, non-serrated leaves (L), dwarf growth, with dark-green round leaves (M), malformed leaves (N), or reduced branching (O). White bar = 10 cm.

One mutant showed concentric, necrotic rings, indicating increased spontaneous cell death events (Figure 29F; details in Figure 30B). But also strong semi-dwarfs (Figure 29H, I) and strong dwarfs (Figure 29K-M) were isolated, the former having rounder leaves, with shorter petioles and shorter internodes, leading to a reduced plant size (Figure 30C), resembling mutant with reduced GA or BR responses in *A. thaliana* (Sun, 2008; Clouse, 2011). The last mutant class to be mentioned here were taller and had outwardly curving epinastic leaves (Figure 29E; details in Figure 30D), resembling mutants with hyper-active BR signalling in *A. thaliana* (Mora-García *et al.*, 2004; Poppenberger *et al.*, 2011).

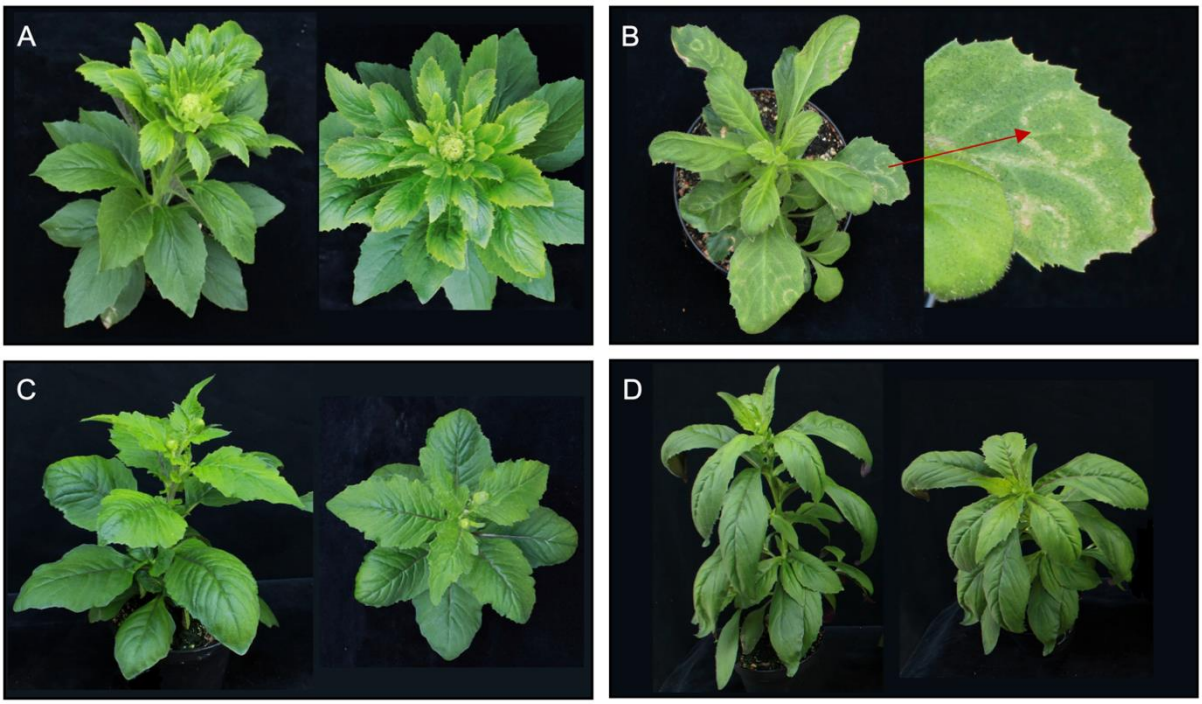

Figure 30. Side and top views of *C. rubens* mutant with developmental and stress response defects.

The plants were grown as in Figure 29 for 8 weeks and photos were taken. Shown are a mutant with defective flower development (A), a mutant with concentric, necrotic rings (B), a semi-dwarf plant with round leaves (C) and a taller plant, with outwardly curving, epinastic leaves (D).

In summary, a mutant population of *C. rubens* could be generated and an initial characterization showed that it contains a large variety of interesting mutants, with defects indicative of altered development or stress responses. Thus, this collection will be a valuable tool for research and breeding.

4. Discussion and Conclusion

4.1. *C. crepidioides* has a longer vegetative growth phase than *C. rubens*

C. crepidioides and *C. rubens* are orphan crops with high potential to improve crop diversity and nutrition in the countries of Subsaharan Africa. The plants are still mainly collected from the wild, but shall be taken into cultivation and an important aim in the domestication of these species is to improve traits that are relevant for crop production and food safety. In the context of food safety, it has previously been shown that *C. crepidioides* can form the highly toxic PA jacobine, an ability that *C. rubens* lacked, at least under the tested conditions (Schramm *et al.*, 2021). Given the presence of this anti-nutritional factor, it is relevant to ask, if *C. crepidioides* is a sensible choice for food production, or if it should rather be *C. rubens*, on which our activities should be focused on. Therefore, in this work the two species were compared, to obtain more information about potential differences in relevant traits, such as seed germination, yield quantity and yield stability, focusing on abiotic stress resistance.

C. crepidioides is a tetraploid species and polyploids can have competitive advantages over their diploid relatives (Comai, 2005). Such advantages include the ability to form a richer spectrum of secondary metabolites and in line, *C. crepidioides* formed more jacobine than *C. rubens* but also accumulated more anthocyanins in stressful conditions, such as under nitrogen starvation and when grafted (Schramm *et al.*, 2021). In addition, polyploids can show higher biomass gains and seed yields (Comai, 2005; Mayfield *et al.*, 2011; Wei *et al.*, 2019), traits that are beneficial for plant production, and here it is shown that *C. crepidioides* has delayed flowering and thus a prolonged vegetative growth phase, resulting in larger biomass gains as compared to *C. rubens*. Interestingly, the increased vegetative growth was particularly obvious for the two Nigerian accessions, which formed almost twice the amount of biomass when compared to *C. rubens* in the same growth conditions. This is in line with a report by Adjatin *et al.* (2013b) who also reported a higher total biomass and consumable biomass for *C. crepidioides*, when growth was assessed on the field.

In addition to increased biomass gains, also seed yield was clearly increased in *C. crepidioides*. The tetraploid species formed approximately 100 seeds per capitulum, whereas diploid *C. rubens* formed only about 20 seeds. This was due to an impaired embryo development since in capitula of *C. rubens* many embryos arrested and did not develop into seeds, an effect that may be caused by the growth conditions in the greenhouse, since in other studies differences in seed yield between the two species had not been reported (Adjatin *et al.*, 2013b). While fewer seeds were formed, the weight of the individual seeds was much higher in the case of *C. rubens*, with approximately 0.40 g (0.36 to 0.43 g and for the two accessions), whereas it was only approximately 0.22 g for all *C. crepidioides* accessions, the

latter being in line with previous results (Denton, 2004; Chen *et al.*, 2009; Adjatin *et al.*, 2013b; Sakpere *et al.*, 2013).

Seed formation in *C. rubens* also took longer than in *C. crepidioides* and this was observed in all the accessions. In *C. crepidioides*, seeds were fully developed approximately 9 days after flower opening, whereas it took 16 days in *C. rubens*. In line, also the total time it took from bud formation to seed release differed between *C. rubens* and *C. crepidioides. C. crepidioides* took approximately 40 days, whereas *C. rubens* took approximately 50 days in the conditions that I applied.

4.2. Seed dormancy in *Crassocephalum* is mediated by ABA

Another trait that requires attention is seed viability and germination capacities, which can limit the cultivation of *C*. *crepidioides* (Sakpere *et al.*, 2013). The plant is a pioneer species that rapidly colonize open areas through wind-dispersal of its seeds (Denton, 2004) and there was first evidence that *C. crepidioides*, like other pioneer plants, has a light-requirement for germination (Chen *et al.*, 2009; Nakamura & Hossain, 2009; Sakpere *et al.*, 2013; Yuan & Wen, 2018). To address this, the Nigerian *C. crepidioides* ecotype C.c.Ile-Ife and the *C. rubens* ecotype C.r.Mail were compared, which confirmed that on ½ MS medium, *C. crepidioides* was unable to germinate in the dark, whereas *C. rubens* showed some germination capacities, albeit to a very low extend. Interestingly, on filter paper, both species did germinate in darkness to some extent, indicating that an MS component may impair germination.

Light very efficiently promoted germination of *C. crepidioides* and *C. rubens* seeds, with the effects being stronger in the case of *C. rubens*, which reached about 50 % germination already after 7 days, whereas *C. crepidioides* took 10 days at 21°C. Increasing the temperature to 25°C promoted germination in both species, with clearer effects for *C. rubens*, which achieved close to 100 % germination already after 10 days, whereas *C. crepidioides* took 14 days. On filter paper, higher germination rates were also observed at higher temperatures, with *C. rubens* achieving close to 100 % germination after 4 days and *C. crepidioides* after 8 days.

Germination outcomes are highly controlled by diverse environmental cues, with light playing a key role. Light is perceived by photoreceptors that specifically detect certain wave lengths, and for red/far-red light perception the phytochromes are utilized, which release photo-dormancy in most light-requiring seeds (Borthwick *et al.*, 1952; Shinomura *et al.*, 1994; Kretsch *et al.*, 1995; Shinomura *et al.*, 1996). Phytochromes are present in two forms: an inactive form that absorbs red light (called Pr) and a bioactive form that absorbs far-red light (called Pfr), which is implicated in light-stimulated germination (Borthwick *et al.*, 1952; Li *et al.*, 2011; Yang *et al.*, 2020). In the dark, the Pfr reverts to the Pr form, and dormant seeds contain Pr, which require red-light perception for Pfr formation and dormancy-release (Bewley *et al.*, 2013).

Phytochromes perceive light to activate hormone biosynthetic and signaling pathways, which convey physiological responses and during seed germination in particular the role of GA is very well established. Phytochromes activate seed germination through induction of GA metabolism, which is achieved through the expression of the GA biosynthetic enzyme 3β-hydroxylase (Toyomasu *et al.*, 1998; Yamaguchi *et al.*, 1998). An increase in GA levels in the imbibed seeds leads to an activation of the GA signal transduction pathway through degradation of the DELLA RGL2 (Ariizumi & Steber, 2007), which acts as a negative regulator of GA responses. Thereby GA responsive genes are activated, which encode proteins required for germination. RGL2 also indirectly induces ABA biosynthesis and the ABA signaling component ABI5, thereby promoting seed dormancy (Piskurewicz *et al.*, 2008; Liu *et al.*, 2016) and thus RGL2 degradation also releases repressive ABA effects.

In line with GAs acting down-stream of light perception, GA application promotes germination in many plants, even if the light environment is not ideal. Moreover, GA can also release dormancy in seeds that require after-ripening or cold stratification (Baskin and Baskin, 2004). However, in *Crassocephalum*, application of the GA GA_3 had no impact on seed germination, even at a relatively high concentration of 10 µM. This could indicate that GAs don't play a major role in seed germination in *Crassocephalum*. Alternatively, and more likely, the GA_3 concentration may have been too low, GA_3 may not have been taken up, or may not be able to efficiently activate GA responses in these species. Also, the BR 24-epiBL, which can promote germination in other species (Tong *et al.*, 2014; Unterholzner *et al.*, 2015), showed no effect on *C. rubens* or *C. crepidioides* germination capacities, which may have similar reasons.

While GA_3 and 24-epiBL did not have a significant impact, ABA suppressed seed germination in both *C. crepidioides* and *C. rubens*, with stronger effects on *C. crepidioides*. In addition, C. *crepidioides* was also hyper-responsive to ABA in terms of its ability to suppress cotyledon greening. Moreover, an application of the ABA biosynthesis inhibitor Flu markedly improved germination of both *C. rubens* and *C. crepidioides.* This activity was particularly impressive in *C. crepidioides*, where the lack of germination capacities in the dark was completely restored with Flu. Abamine, another ABA biosynthesis inhibitor, which specifically targets 9-*cis*-epoxycarotenoid dioxygenase (NCED) activity (Han *et al.*, 2004), had no impact on seed germination in *Crassocephalum*. However, this is not too surprising, since NCED inhibitors have been reported to be relatively inefficient in inducing germination also in other plants (Creelman *et al.*, 1992; Han *et al.*, 2004; Awan *et al.*, 2017). Conclusive evidence for an altered ABA biosynthesis came from LC-MS/MS measurements of ABA in dry seeds, imbibed seeds and 3-day-old seedlings. ABA was higher in dry seeds of *C. crepidioides* than in those of *C. rubens*, but dropped to similar levels after imbibition.

In other plant species it is well established that seed dormancy is released upon imbibition through ABA degradation (Gubler *et al.*, 2005) and this was also correlated well in *Crassocephalum*. After seed imbibition, the amount of ABA accumulated in dry seed rapidly declines and this is achieved through catabolism of ABA to form hydroxylated or glucosylated ABA (Seo & Marion-Poll, 2019). ABA-GE levels rapidly dropped during imbibition in both *C. crepidioides* and *C. rubens*, whereas PA levels didn't clearly change, neither during imbibition nor during early seedling growth. PA is further catabolized to DPA, and DPA levels clearly increased in imbibed seeds of both *C. crepidioides* and *C. rubens*, but without obvious differences. Thus, there is clear evidence that *C. crepidioides* has an increased seed dormancy as compared to *C. rubens*, which correlates with increased ABA levels and a hyper-responsiveness to ABA. Thus, light, as well as Flu, can break seed dormancy in *Crassocephalum*, which is a relevant finding for plant production.

Another interesting observation made was that the season of harvesting impacted seed germination in *Crassocephalum*. Seeds from plants grown in the greenhouse in winter were more dormant than those from plants grown in summer, with stronger effects in *C. crepidioides*. When winter-harvested *C. crepidioides* seeds were germinated at low temperatures of 21°C and low light levels, they hardly germinated, an effect that could be released by warmer germination conditions and higher light levels. This is in line with reports from other plants, which had also shown that autumn- or winter-matured seeds germinated significantly better at higher temperatures and in continuous light than spring or summer-matured seeds (Meyer *et al.*, 1989; Gutterman, 1991; El-Keblawy & Al-Rawai, 2006; El-Keblawy *et al.*, 2009).

4.3. *Crepidioides* shows a high drought and heat stress tolerance

Since ABA plays a vital role in abiotic stress responses of plants, it was investigated if the increased ABA responses of *C. crepidioides* may benefit abiotic stress tolerance of this species. Germination and cotyledon greening experiments in the presence of NaCl or Mannitol showed that while NaCl responses were comparable, *C. crepidioides* was more sensitive to Mannitol. Thus, there was evidence that resistance against stress types that induce cellular desiccation is increased. To study this further, detached leaves water loss assay was conducted and showed that *C. rubens* lost water faster and also wilted earlier than *C. crepidioides,* indicating that the latter has an increased stomatal guard cell conductivity. Because stomatal conductance is controlled by ABA (Pantin *et al.*, 2013; Bharath *et al.*, 2021; Hsu *et al.*, 2021), ABA was measured in drought-stressed plants, which showed that the reduced water loss in *C. crepidioides* was not correlated with enhanced ABA formation, but rather with a less rapid increase in the ABA contents in the leaves. Also, formation of the

analysed ABA catabolites was delayed in *C. crepidioides*, indicating that the stress response was delayed, potentially due to the fact that the plants were less damaged by drought. To verify this, it was tested if the recovery potential after 7 days of drought-exposure differed between the two species, by re-watering the plants. This revealed that whereas *C*. *rubens* was unable to recover and died off, *C. crepidioides* showed an impressive ability to survive this period of water withdrawal. This is relevant since in assays, where whole plant and soil water loss was measured in combination, *C. crepidioides* consumed water more rapidly than *C. rubens*, which may not be surprising, given that it produces more biomass. Therefore, while *C. crepidioides* produces more biomass, it can survive with less amount of water than *C. rubens*, at least for a limited period of time, another very relevant trait for plant production, in particular in rain-fed cropping systems.

In addition to increasing drought tolerance, ABA can promote cold stress tolerance (Lee & Seo, 2015; Eremina *et al.*, 2016), but appears to repress resistance to heat (Albertos *et al.*, 2022) and it was therefore interesting to investigate, how well *Crassocephalum* species can cope with temperature extremes. Both species survived freezing temperatures of -4°C for 2 hours, which is surprising given that they originate from subtropical zones. *C. crepidioides* however displayed a higher heat resistance, surviving 5 hours of 45°C, a temperature that *C. rubens* could not survive, again something that's beneficial for plant production.

4.4. Towards transformation protocols for *Crassocephalum*

C. crepidioides, and in particular the Nigerian accessions, showed multiple traits that are very interesting for plant production, including increased biomass gains and seed yield, as well as elevated drought and heat stress tolerance. Thus, these ecotypes are interesting for breeding. However, since they also accumulate the toxin jacobine (Schramm *et al.*, 2021), a first step in their genetic improvement would have to be jacobine removal. In theory, this is doable, since it is known that jacobine synthesis requires Homospermidine Synthase (HSS), which catalyses an essential step in the PA biosynthetic pathway (Ober & Hartmann, 1999) and thus if HSS is removed, jacobine will not be formed.

To do this in practice, for a targeted removal of HSS from the Nigerian *C. crepidioides* accessions, two breeding methods could be applied: (1.) genome editing using CRISPR/Cas9 (Doudna & Charpentier, 2014) and (2.) mutation breeding (Bado *et al.*, 2015; FAO/IAEA, 2018) in which mutant populations are generated and screened for individuals with relevant traits (absence of HSS), and for both approaches methodology was developed.

For genome editing, transformation protocols are required, which also allow for important research approaches such as forward genetics. Therefore, it was aimed to develop a

transformation protocol for *Crassocephalum* and the use of the plant pathogen *Agrobacterium tumefaciens* as a vector was tested for this purpose. *Agrobacterium* can transform both vegetative (callus) and generative cells (Chumakov, 2007) and thus, either the development of reliable tissue culture shoot regeneration systems or an efficient generative cell (embryo) transformation technique is needed. Callus transformation requires a regeneration protocol and the cytokinins kinetin and BAP were tested for shoot regeneration from different types of explants of *C.rubens* and *C. crepidioides*. This showed that in particular kinetin, at a concentration of 2.0 to 2.5 mg/l was efficient in regenerating shoots from SAMs. No callus or shoots were formed from leaf disks or cotyledon explants, which makes SAMs the only suitable explants.

For embryo transformation, a method called floral dipping can be applied, which was first developed for *A. thaliana* and is applicable particularly for plants that are not too large and produce high amounts of seeds (Clough & Bent, 1998; Curtis & Nam, 2001; Yasmeen *et al.*, 2009; Zale *et al.*, 2009; Li *et al.*, 2010; Mu *et al.*, 2012; Rod-in *et al.*, 2014). Since *Crassocephalum* meets these requirements, floral dipping was tried but yielded only one transformant for *C. rubens*. While this shows that the technique can work in principle, it is much too inefficient in the form tested and will thus have to be optimized to be fit for application.

Transformation also requires selection procedures, which use selecting agents for the elimination of non-transgenics and in plants usually herbicides such as glufosinate or antibiotics such as KAN are used (Wilmink & Dons, 1993). *C. crepidioides* and *C. rubens* were both highly resistant to KAN, being impaired in seedling development only at concentrations above 200 g/ml and thus KAN is not a good choice. Glufosinate however impaired growth already at a concentration of 5 µg/ml, with *C. rubens* being slightly more resistant, making this herbicide an excellent selection agent for *Crassocephalum*.

4.5. A *C. rubens* mutant population for breeding and forward genetic screens

Mutation breeding is another possibility to introduce novel traits into a breeding population and for this purpose mutagenized plants are generated and screened. Chemical mutagens such as EMS can be applied and here it was used, to generate a *Crassocephalum* mutant population. Diploid *C. rubens* was employed, since loss of function phenotypes can be expected at much higher rates in the M2 progeny, than in tetraploid *C. crepidioides*.

EMS concentration above 0.2% v/v had a significant impact on plant growth and development and reduced fertility in *C. rubens*, but 0.1-0.2% proved to be suitable. Since the impact of a 15-hour exposure to 0.2% EMS on seed yield in M1 plants was too strong, the treatment time was reduced to 8 hours, which was less damaging in the M1, but still efficient in generating

mutants at high frequency. This was tested through a small-scale screen of M2 progeny of a population of approximately 3000 independent M1 plants. 500 M2 plants were analyzed among which various very interesting morphological mutants were identified. The developmental defects included defective leaf development, defective developmental-phase transitions (delayed flowering), increased SAM activity, defective branching, or increased or decreased elongation growth. In addition, mutants with spontaneous cell death were isolated, which indicates defective biotic stress signaling. Thus, a *C. rubens* mutant population was developed, which can be used for mutant screens in the future, benefiting research and breeding activities with this plant.

4.6. Conclusion

In conclusion, *C. crepidioides* outperformed *C. rubens* in a number of traits, including biomass gains, seed yield, drought and heat stress tolerance, which may relate to its tetraploid nature. A high degree of seed dormancy and drought tolerance was correlated with an ABA hyper-sensitivity and this may contribute to the higher survival potential of *C. crepidioides* in stressful conditions. In particular, the high drought and heat stress tolerance will profit *C. crepidioides* production in rain-fed agriculture. Thus, although it accumulates PAs, this tetraploid species warrants further work, and as a first, essential step in its domestication PAs would require genetic removal, to ensure a safe use. This could be achieved through targeted removal of HSS, by genome editing, which would need transformation techniques. Floral dipping with *A. tumefaciens* as a vector system, while working in principle, was very inefficient and thus more work is needed to enable *Crassocephalum* transformation. As an alternative, mutation breeding could be considered, and while it may be challenging to generate HSS null mutants of tetraploid *C. crepidioides*, it was shown here that EMS mutagenesis is applicable for *Crassocephalum*, using *C. rubens* as a model. The *C. rubens* mutant collection, which was generated in this work, contains a large number of mutants with defects in developmental processes and stress responses and will be a highly valuable resource to screen for lines with beneficial traits, such as defective hairy pappy formation, for decreased seed dispersal, or improved growth characteristics.

Thus, significant progress was made in understanding important traits and developing resources for *Crassocephalum*, which will benefit approaches that aim to exploit the potential of these plants for combating malnutrition and increasing the resilience of marginal cropping systems in Africa.

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Acknowledgements

My heartfelt and profound gratitude goes to my supervisor, Prof. Dr. Brigitte Poppenberger for giving me the opportunity to work in her institute and for her continuous support in making this project a success. You gave me the freedom to work at my own pace and offer my own ideas while keeping me motivated in challenging situations. Under your guidance, I have experienced both professional and personal changes, and I am forever grateful.

Furthermore, I would like to specially thank Prof. Dr. Patrick Bienert and apl. Prof. Dr. Alexander Christmann for their participation as members of my examination board.

I would also like to extend my appreciation to Prof. Dr. Wilfried Rozhon and Sebastian Schramm for integrating me into the ebolo project. I would also want to express my gratitude to Dr. Tobias Sieberer for his invaluable contribution and support throughout my time at the institute.

In addition, I want to thank Prof. Dr. Corinna Dawid, the chair of the Food Chemistry and Molecular Sensory Science at the TUM and Dr. Michael Gigl for the LC-MS/MS measurement of ABA in *Crassocephalum* plants.

Moreover, I would like to acknowledge all the lab members and colleagues for a pleasant working environment. I am specially thankful to Mrs Irene Ziegler for her technical assistance, as well as Veronica Ramirez and Dr. Pablo Albertos for their scientific and moral support. Also, my appreciation goes to the entire TUM GHL-Team for their technical support.

Special thanks go to the Deutscher Akademischer Austauschdienst (DAAD) for funding this project.

Finally, my heartfelt gratitude goes out to my loving, supportive, patient and motivating family: my parents, parents-in-law, siblings and most importantly, my husband and son, Ruhullah and Abdulmujeeb. Thank you for your love.

Publications

Adedeji-Badmus, A.N., Schramm, S., Gigl, M., Iwebema, W., Albertos, P., Dawid, C. Sieberer, T., and Poppenberger, B. (2022) Species-specific variation in abscisic acid homeostasis and responses impacts important traits in *Crassocephalum* orphan crops. Submitted

Schramm, S., Rozhon, W., **Adedeji-Badmus, A.N.**, Liang, Y., Nayem, S., Winkelmann, T., and Poppenberger, B. (2021). The Orphan Crop *Crassocephalum crepidioides* Accumulates the Pyrrolizidine Alkaloid Jacobine in Response to Nitrogen Starvation. *Frontier in Plant Science* 12: 702985

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Presentations at Conferences or Symposia

Adedeji-Badmus A.N., and Poppenberger B. (2020) Natural variation in seed development and germination capacities of Crassocephalum species and their implication for the domestication of these orphan crops. Poster**.** *International Symposium of the Society for Plant Breeding.* 11-13 February, 2020, Tulln, Austria.

Adedeji-Badmus A.N. (2020) Establishing tools and resources for the domestication of Crassocephalum species, orphan crops with high importance in sub-Saharan Africa. *6th HEFagrar PhD Symposium.* 28 April, 2020, Freising, Germany.

Freising, 14th of April 2022

Aspects of this thesis were published in:

Articles

Adedeji-Badmus, A.N., Schramm, S., Gigl, M., Iwebema, W., Albertos, P., Dawid, C. Sieberer, T., and Poppenberger, B. (2022) Species-specific variation in abscisic acid homeostasis and responses impacts important traits in *Crassocephalum* orphan crops. Submitted

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