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Purification and characterization of Xenobiotic detoxification enzymes in *Pachyrhizus* “yam bean” and their role in agrochemical metabolism

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LISTING OF USED ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
CDNB	1-Chloro-2,4-Dinitrobenzene
CHES	2-[N-Cyclohexylamino] ethane-sulphonic acid
cGST	Cytosolic glutathione S-Transferase
CyP	Cytochrome P450
Cys	Cysteine
DCNB	1,2 Dichloronitrobenzene
DTE	Dithioerythritol
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
FPLC	Fast Protein Liquid Chromatography
x g	Acceleration due to gravity (9,81 m s^{-2})
Glu	Glutamin
Gly	Glycine
GS-B	Glutathione bimane conjugate
GSH	Glutathione, reduced
GSSG	Glutathione, oxidised (disulphide)
GST	Glutathione S-Transferase
GT	Glucosyltransferase
hr	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrocloric acid
Hepes	4-[2-Hydroxyethyl]piperazine-1-ethanesulphonic acid
hGSH	Homoglutathione
HPLC	High Pressure Liquid Chromatography
IPU	Isoproturon
Kat	Katal
kDa	Kilo Dalton
K_m	MICHAELIS-MENTEN-constant
LRWC	Leaf relative water content
LSM	Laser scanning microscope
MBB	Monobromobimane
MCB	Monochlorbimane
MES	2-[N-Morpholino]-ethansulfonsäure
mGST	Microsomal glutathione S-Transferase
Mw	Molecular weight
NaCl	Sodium chloride
NaN_3	Sodium azide
NaOH	Sodium hydroxide
N_{liq}	Liquid nitrogen
PAGE	Polyacrylamide-gel electrophoresis

pI	Isoelectric point
PMSF	Phenylmethylsulfonylfluoride
POX	Peroxidase
PVP	Polyvinylpyrrolidone
RH	Relative humidity
r^2	Correlation coefficient
ROS	Reactive oxygen species
Rt	Retention time
sec	Seconds
SDS	Sodium dodecyl sulphate
SE	Standard error
SMC	Soil moisture content
TCP	2,4,5-trichlorophenol
TFA	Trifluoro acetic acid
Tris	N-tris-[hydroxy-methyl]-aminomethane
TFW	Tuber fresh weight
TW	Turgid weight
U	Unit of 1 μM substrate per min
V_{max}	Maximum reaction velocity
γEC	γ Glutamylcysteine

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1. INTRODUCTION

All organisms are constantly exposed to a wide range of toxic substances in potentially harmful concentrations. Owing to their sessile lifestyle, plants are exposed to far greater amounts of harmful substances and are therefore much more vulnerable. As primary producers they are even more at risk as they may be exposed to xenobiotics (foreign chemicals) either deliberately, due to their use as pesticides against crop failure, from pests and diseases or accidentally, from agriculture, industrial and other uses.

In nature, plants also encounter a combination of environmental conditions that may include stresses such as drought or excess water, exposure to low light or extremely sunny conditions, low and high UV radiation, air pollutants like ozone, or may be associated with poor soil. Such abiotic stress leads to the build up of stress and stress metabolites in cells. Stress is an unavoidable consequence of life and in an atmosphere where oxygen is produced environmental stress always leads to the accumulation of toxic reactive oxygen species (ROS) (Elstner and Osswald, 1994).

Plant growth, productivity and survival in a stressful environment is continuously faced with challenges that have to be counterbalanced. As a result, and in the course of their evolution plants have developed numerous unique adaptation and defence mechanisms to help them cope with unavoidable stresses that may be imposed upon them. One such form of a defence mechanism has been the development of an enzyme system for their protection against potentially toxic effects of xenobiotics and ROS.

Detoxification metabolism is therefore one of the most important biochemical processes in plants. By the action of these enzymes plants are able to prevent xenobiotics and their phytotoxic metabolites from entering into and disturbing the main biochemical reactions of the cell thereby maintaining metabolic homeostasis in plants.

These enzymes were found to have common features as in animal metabolism, which lead to the idea of the “green liver” concept, postulating convergent detoxification in both plants and animals (Sandermann, 1994). Numerous studies have investigated the metabolic fate of xenobiotics and have revealed a reaction detoxification sequence consisting of three phases (Coupland 1991; Sandermann, 1994; Marrs, 1996; Coleman, 1997) Figure 1.

1.1. Detoxification System

In the first detoxification phase, often considered as activation or transformation phase, xenobiotic molecules are activated by oxidation, reduction, or hydrolysis reactions catalysed by enzymes such as cytochrome P450 monooxygenases. This reaction results in the exposure, or introduction, of a functional group that makes it easier for the next phase to proceed. Thus, in many cases the process in phase 1 makes the following detox phases possible as without hydroxylation or demethylation some compounds would not be subject to metabolism at all.

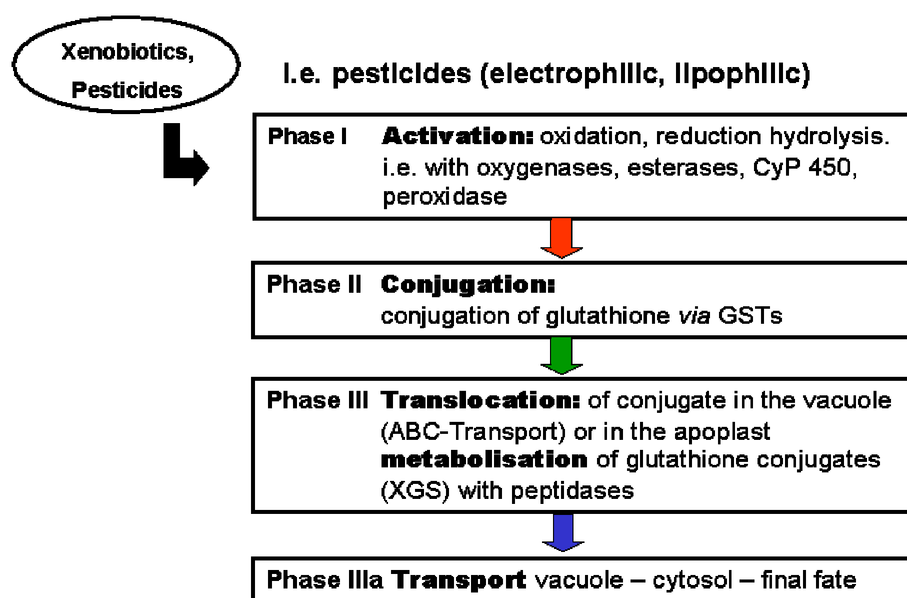


Figure 1. Three phase detoxification model of xenobiotic metabolism in plants (Sandermann *et al.*, 1983)

In phase two, classified as “conjugation metabolism” enzymes catalyze the conjugation of the activated metabolites formed in phase 1 with sugars or the tripeptide glutathione (GSH). Phase 2 is catalyzed by glucosyl transferases, or glutathione transferases. Compounds with sufficiently high electrophilicity may be conjugated without prior activation. According to Schröder (1997) detoxification in the proper sense of the word is mainly achieved by these phase 2 reactions.

In the third phase of xenobiotic metabolism, also considered as a step of secondary conjugation (Shimabukuro *et al.*, 1982) and metabolization, the inactive water soluble conjugates formed in phase 2, "tagged" with GSH or sugar are recognized by ATP-binding

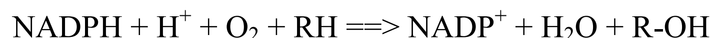
cassette transporters in the tonoplast or plasma membrane, which then transfer these conjugates into compartments, the vacuole (soluble conjugates) or in the cell wall (insoluble conjugates) (Schröder, 1997; Sandermann, 1992; Lamoureux and Rusness, 1989).

It seems to be quite clear, that from phase 1 to phase 3 toxicity is subsequently lowered and it has been proposed that the products of phase 3 are non-toxic (Coupland, 1991). During phase 2 products can be stored in the cell vacuole and thus be sequestered from the cytoplasm metabolism. Once inside this organelle, metabolites do not diffuse back to the cytosol and older literature states that they are not even actively transported back to the cytoplasm (Nakamura *et al*., 1985; Sterling and Balke, 1989). This immobility of the xenobiotic within the cell, and the lack of primary metabolic function within these organelles would effectively render these herbicide metabolites non-phytotoxic. However, recent literature states that, although it is important that these conjugates be removed from the cytosol, further metabolism proceeds in the vacuole and transport of metabolites into the cytosol may be possible (Wolf *et al*., 1996; Schröder, 1997). The products of phase 3 are often so tightly bound to biopolymers that they are able to resist the action of hydrolytic enzymes (Pillmoor *et al*., 1984). Recently, it has been proposed that phase 3 be subdivided into two distinct phases, the first of which is characterized by the mentioned transport reactions, and the second one by further cleavage or binding of the xenobiotic conjugates (Theodoulou *et al.,* 2003).
In order to get a general view of the detoxification system in *Pachyrhizus* a preliminary survey on the status of activity of representative enzymes, Cytochrome P450 (CyP 450) and peroxidase (POX) in detoxification phase 1, glucosyltransferase (GT) and glutathione S-transferase (GST) in detoxification phase 2 and glucosidase in detoxification phase 3 were investigated in this research. The main part of this thesis however deals with the major detoxification phase 2 enzymes GST.

1.1.1. Cytochrome P450

Cytochrome P450 monooxygenases (EC 1.14.14.1) also referred to as CYP constitute one of the major classes of enzymes that are responsible for detoxification of exogenous molecules in both animals and plants**.** They play an important role in plant defence and it is now clear that they form the largest class of enzymes in phase 1 detoxification system (Werck-Reichhart, *et al*., 2000). In plants they are also involved in the biosynthesis of hormones, lipids, and secondary compounds (Schuler, 1996). They represent a potentially significant metabolic sink for environmental contaminants (Sandermann, 1994), which can be used for determining herbicide tolerance and selectivity (Werck-Reichhart, 1995).

CyP 450s are heme-proteins that function as monooxygenases catalysing a variety of reactions including epoxidation, N-dealkylation, O-dealkylation, S-oxidation and hydroxylation. A typical cytochrome P450 catalysed reaction is:



$$NADPH + H^+ + O_2 + RH \Longrightarrow NADP^+ + H_2O + R\text{-}OH$$

They use electrons from NADPH to catalyse the activation of molecular oxygen and react with hydrophobic substrates to produce a more reactive and hydrophilic product. Like their animal counterparts, plants P450s are highly inducible by chemicals such as drugs or pesticides (Bolwell *et al*., 1994; Schuler, 1996) and some of them respond more strongly than others to chemical signals (Batard *et al*., 1995, 1997; Moreland *et al*., 1995; Potter *et al*., 1995).

CyP is usually located in the 'microsomal' part of the cytoplasm in the endoplasmic reticulum. Numerous isoforms of cytochrome P450 exist and are classified according to the similarities of their amino-acid sequences. They differ from plant to plant in aspects of metabolism and substrate specificity and this is one of the reasons for herbicide selectivity.

1.1.2. Peroxidase

As representative of detoxification phase 1, Peroxidases (POX, EC 1.11.1.7) are an important family of enzymes catalysing the oxidation of their substrates utilizing hydrogen peroxide at the expense of aromatic reductants, for which they appear to be relatively non-specific. They have been found in both membrane-bound and soluble fractions of cells. Several isoforms have been identified and both constitutive as well inducible forms are known (Harvey *et al*., 2000). Their activity has been used as an indicator of pollution stress in fine roots of Scotch pine (*Pinus sylvestris* L.) and in the macerated leaf tissue of forest trees (Castillo *et al*., 1987). Their natural function is to protect the plant cell constituents from damage by hydrogen peroxide produced during photosynthesis and when plants are stressed. Acidic as well as neutral POX has been described, and a large number of acidic POX has been detected in the apoplast of plant tissues. Their activity as a phase 1 detoxification enzyme in *Pachyrhizus* was determined.

1.1.3. Glucosyltransferase

Glucosyltransferases (GT, EC 2.4.1.-) are members of a multigene family (Mackenzie *et al*. 1997) of detoxification phase 2 enzymes responsible for the conjugation of a glycose residue from an activated sugar donor to a receptor molecule. Glycosylation can result in the formation of poly-glycosides, di-saccharides, and various mono-glycosides of non-carbohydrate moieties such as proteins, lipids, steroids, and other small molecules. GT has been identified in plants, animals, fungi, and bacteria, and also viruses (Campbell *et al*. 1997). They are characterized by utilization of UDP-activated sugar moieties as the donor molecule, and contain a conserved UGT-defining sequence motif near the C-terminus (Mackenzie *et al*. 1997). Glycosylation reactions serve to convert reactive and toxic aglycones into more stable and non-reactive storage forms. In addition, attachment of the hydrophilic glucose moiety to hydrophobic aglycones dictates increased water solubility.

Glycosylation by GT constitutes a prominent modification process and is often the last step in the biosynthesis of natural products in plants (Jones and Vogt, 2001). Glycosylation reaction is not only restricted to endogenous substrates; it is also a key step in general detoxification mechanisms for exogenous substrates (xenobiotics) in higher plants (Sandermann, 1992), thereby allowing plants to cope with environmental challenges. The biological function of the glycosylation step in plants is therefore to facilitate storage, and intra- and intercellular transport. Glycosylation also serves as a regulatory step in homeostasis of plant growth regulators, as seen for auxins, gibberellins and brassinolides.

Several studies have shown that GT enzyme activity for metabolism of xenobiotic phenols and carboxylic acids are widespread among lower and higher plants (Sandermann, 1994; Plugmacher and Sandermann, 1998). 2,4,5-trichlorophenol (TCP) is use as a model xenobiotic compound in standard assay for the enzyme activity (Plugmacher and Sandermann, 1998; Brazier *et al*., 2002). In this research the initial status of GT in *Pachyrhizus* is investigated.

1.1.4. Glutathione Conjugation

Glutathione S-transferases (GSTs, EC. 2.5.1.18) are major phase 2 enzymes comprising of a family of multifunctional detoxification enzymes found in almost all living organisms. They are catalysts of reactions in which the reduced tripeptide thiol glutathione (GSH) (Figure 2) acts as a nucleophile, conjugating to and facilitating removal or reduction of the second substrate, the xenobiotic, usually with a hydrophobic or electrophilic binding site. This conjugation results in the detoxification of these compounds, facilitating their removal from

biological tissue. Formerly studied for their role in detoxification of endogenous and xenobiotic compounds, they have since been found to have additional important roles as transport proteins, cellular response to auxins, metabolism of plant secondary products like anthocyanins and cinnamic acid and in protection against oxidative stress (Marrs, 1996).

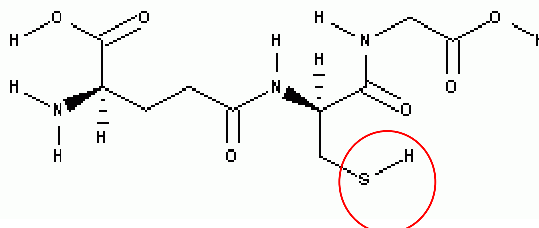


Figure 2. Chemical structure of glutathione substrate for the conjugation of xenobiotic. –SH thiol group is the most biochemically active. Reacts by nucleophilic displacement.

In any plant species, the multiple isozymes of these dimeric enzymes can be characterised and differentiated by broad and partially overlapping substrate specificities. Evidence suggests that the level of expression of GST is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals (Mannervik and Danielson, 1988). Although some GSH conjugation reaction occur non-enzymatically especially with high-activated substrates, the presence of an appropriate amount of GST is usually required (Lamoureux and Rusness, 1989). 1-chloro-2,4-dinitrobenzene (CDNB) is used as model substrate in standard assay for the determination of GST activity in cells (Figure 3).

Figure 3. Conjugation of the standard substrate CDNB, (1-chloro-2,4-dinitrobenzene) to glutathione. The assay conditions are easy to meet and measurement of conjugate absorption at 340 nm in a spectrophotometer provides fast success even for beginners in enzymology, however, the substrate does not cover all glutathione S-transferases.

The abundant low molecular weight thiol substrate of GST, glutathione (GSH), serves as a mobile pool for long-distance transport of reduced sulphur between different organs and as a reservoir of reduced sulphur that can be re-mobilized under increased demand (Rennenberg, 2001). It exists in two forms. The antioxidant "reduced glutathione" tripeptide γ–glutamyl-cysteinylglycine is conventionally called glutathione. The oxidized form is a sulfur-sulfur

linked compound, known as glutathione disulfide or GSSG. The GSSG/GSH ratio may be a sensitive indicator of oxidative stress (Foyer and Noctor, 2001).

Within the plant cell GSH has been determined to be higher in the chloroplast than in the cytoplast or vacuole. Rennenberg (1982), reports that the chloroplast may contain up to 76% of the total plant reduced GSH. Also higher levels of GSH have been accounted for in the shoots than in the roots but the highest levels of glutathione are said to occur in the seeds where they exists primarily as disulphide dimers (Rennenberg and Lamoureux, 1990; Foyer and Rennenberg, 2000). In most plant species including some leguminous species such as pea (*Pisum sativum* L.) and groundnut (*Arachis hypogaea* L.), glutathione (GSH) is the major form of free thiol. However, in other leguminous species such as soybean (*Glycine max* (L.) Merr.) and mungbean (*Vigna radiata* (L.) R. Wilcz.) homoglutathione (γ-glutamylcysteinyl-ß-alanine, hGSH) is the major from of free thiol (Klapheck, 1988; Rennenberg and Lamoureux, 1990; Klapheck et al., 1992). The significance of hGSH instead of GSH in these species is not understood, but hGSH may serve the same function as GSH (Lamoureux *et al.*, 1991). Within this research amount and distribution of hGSH in *Pachyrhizus* will be determined.

1.1.5. Structure and Classification of GSTs

Each GST subunit (Figure 4) consists of two domains, each containing two binding sites, the G site and the H site. The highly conserved G site binds the tripeptide glutathione (GSH) and is largely composed of amino acid residues found in the N-terminal domain. The H-site or electrophilic substrate binding site is more variable in structure and is largely formed from residues at the C-terminus.

In all organisms shown to have GST activity, multiple forms of the enzyme have been discovered. At least six distantly related families designated as alpha, mu, pi, sigma, tau and theta GST have been encoded in animals (Hayes and Pulford, 1995) and in plants (Edwards *et al*., 2000).

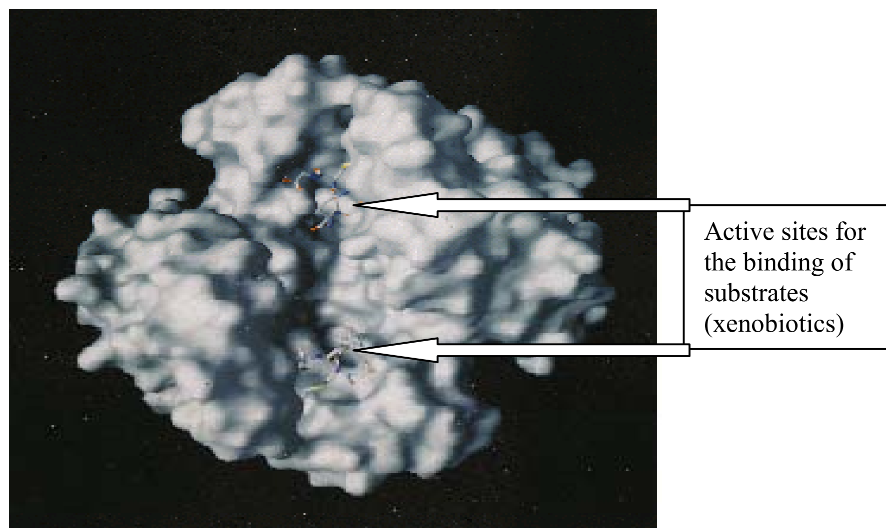


Figure 4. Structure of glutathione S-transferase. Molecular surface image showing two domain structure of subunit. The dimeric molecule is globular with a prominent large cavity formed between the two subunits. The active site is located in a cleft situated between domains I and II and each subunit can binds two molecules (Reinemer et al., 1996; Schröder 2001).

Subtypes of GST have been grouped into classes on the basis of isoelectric point, substrate and inhibitor properties, antibody recognition, and N-terminal amino acid sequence. The main cytosolic classes are alpha, mu and pi (previously known as basic, near-neutral and acidic, on the basis of isoelectric point) (Mannervik *et al*., 1992) are present only in animals and yeasts but absent in bacteria and plants (Pemble and Taylor, 1992). The very heterogeneous theta class, reported by Meyer *et al.* 1991 (Buetler and Eaton, 1992) is present in yeasts, plants, bacteria, rats, humans, chickens, salmon, and non vertebrates such as flies and apparently absent in lower animals such as molluscs, nematodes, and platyhelminthes (Taylor *et al*., 1993).

Until recently plant GSTs screened seemed to spring exclusively from the tau and theta families (Droog et al., 1993, 1995, Droog, 1997). Current classification now separates plants GSTs into four main classes, the Phi, Tau, Zeta, and Theta GSTs (Edwards *et al*., 2000). Phi (formerly Type I) and Tau (formerly Type III) are now the largest classes. They are exclusively plant-specific with important roles in herbicide detoxification (Marrs, 1996). The Theta class includes enzymes originally designated as Type IV, while the Zeta class includes those previously classified as Type II (Edwards *et al*., 2000; Sheehan *et al*., 2001).

To date all plant GSTs described are dimeric, forming homodimers or heterodimers with apparent molecular weights in the range between 23 and 29 KD (Marrs, 1996; Dixon *et al*., 2002). GST isoenzymes belonging to the same class show 50 to 60% identity in their primary structure, whereas enzymes belonging to different classes generally have less than 20% sequence identity (Mannervik and Danielson, 1988; Hayes and Pulford, 1995; Armstrong, 1997).

1.1.6. Evolution and Distribution of GSTs

Glutathione S-transferases (GSTs) in plants were first discovered in maize in 1970 (Frear and Swanson, 1970) 9 years after they were first described in animals in 1961 (Booth *et al*., 1961; Schröder, 2001). The discovery opened a new era in the practical application for agricultural development and since then several major advances in plant research on glutathione and GST have been achieved (Rennenberg, 2001). Originally identified and studied for their ability to detoxify herbicides; conferring herbicidal tolerance for most major crops they have now been implicated in numerous stress responses including those arising from pathogen attack, oxidative stress and heavy metal toxicity.

In herbicide detoxification, GSTs are a major determinant of selectivity between crops and weeds. The activities and levels of GSTs in a plant determine to a greater or lesser extent the susceptibility of that plant to various herbicides (Edwards *et al*., 2000). Using a combination of biochemical and molecular approaches large numbers of GSTs have now been cloned and characterised. Several herbicides are rapidly metabolised via GSH conjugation in crops (Gronwald and Plaisance, 1998). In contrast, many weeds contain lower activities of detoxifying GSTs and are susceptible to herbicides (Cole, 1994; Schröder, 1997).

The enzyme, widely distributed in nature, has been found in all eukaryotes and in many prokaryotes (Mannervik and Danielson, 1988; Hayes and Pulford, 1995; Armstrong, 1997). GST activity has been detected and characterized in bacteria (Zablotowich *et al*., 1995; Rossjohn *et al*., 1998), fungi (Sheehan and Casey, 1993; Tamaki *et al*., 1999; Dowd and Sheehan, 1999), maize (Frear and Swanson, 1970; Edwards and Owen, 1986; Rossini *et al*., 1996; Jablonkai and Hatzios, 1991; Scarponi *et al*., 1992; Jepson *et al*., 1994; Holt *et al*., 1995; Marrs *et al*., 1995; Hatton *et al*., 1996; Dixon *et al*., 1997; Marrs and Walbot, 1997), rice (Frova *et al*., 2000), wheat (Jablonkai and Hatzios, 1991; Mauch and Dudler, 1993, Romano *et al*., 1993; Edwards and Cole, 1996; Riechers *et al*., 1997), tobacco (Droog *et al*., 1995), soybean (Ulmasov *et al*., 1995; Andrews *et al*., 1997), *Arabidopsis thaliana* (Reinemer

et al., 1996), barley (Romano *et al*., 1993; Wolf *et al*., 1996), *Setaria* spp. (Wang and Dekker, 1995), carnation (Meyer *et al*., 1991), potato (Hahn and Strittmatter, 1994), chickpea (Hunatti and Ali, 1990, 1991), pea (Frear and Swanson, 1973), peanut (Lamoureux *et al*., 1981); onion (Lamoureux and Rusness, 1980), sorghum (Gronwald *et al*., 1987; Dean *et al*., 1990), velvetleaf (Anderson and Gronwald, 1991), sugarcane (Singhal *et al*., 1991) and tree species (Balabaskaran and Muniandy, 1984; Schröder *et al*., 1990; Schröder and Rennenberg, 1992; Schröder and Berkau, 1993, Schröder and Götzberger, 1997).

Recently, in *Arabidopsis* nearly 50 gene sequences corresponding to GSTs have been identified. Ninety percent of these GSTs are classified as plant-specific while ten percent are similar to mammalian GSTs. RNA expression of some of these genes indicates that diverse signalling pathways may control the expression of this gene family in response to various treatments (Wagner *et al*. 2002). According to Lamoureux and Rusness (1993) cited by Marrs (1996) over 33 plant species have been demonstrated to possess GST activity, although in many cases the GSTs have not been purified. Each of the so far purified isozymes has a distinctive pattern of reactivity depending on the carbon skeleton or the reactive group of the substrate. However, in a given tissue, these enzymes collectively have an enormous range of catalytic capability.

It has been shown that GST activities are inducible by a variety of factors including xenobiotics (Cole, 1994; Schröder, 1997) and that numerous isoforms of GST are present in each plant (Marrs, 1996). Pickett *et al*., (1984) and Pickett and Lu (1989) reported that in common with certain other drug metabolising enzymes the level of expression of GST in many species can be increased significantly by exposure to foreign compounds; among them pesticides, organic chemicals and herbicide antidotes, suggesting that they form part of an adaptive response to chemical stress. The level of GST and concentration of GSH are important factors to be taken into account in considering the role of GSH conjugation in herbicide metabolism, selectivity and tolerance (Edwards and Owen, 1986; Debus and Schröder, 1990; Dean *et al*., 1991; Fuerst *et al*., 1993; Cole, 1994).

Additionally, GSTs relative expression may be controlled spatially or temporally (Cole *et al*., 1994). They can account for up to 1% of the soluble protein in plants and they have been found both in the cytosol and more recently in the microsomes (Belford and Schröder, 1996). Whereas cytosolic GSTs have been extensively investigated, there have been relatively few studies examining the occurrence, diversity and functional aspects of membrane GSTs in

plants. Plant microsomal GST has been detected in spruce (Schröder and Belford, 1996), in onion (Schröder and Stampfl, 1999) and in numerous other plant species (Pflugmacher *et al*., 2000).

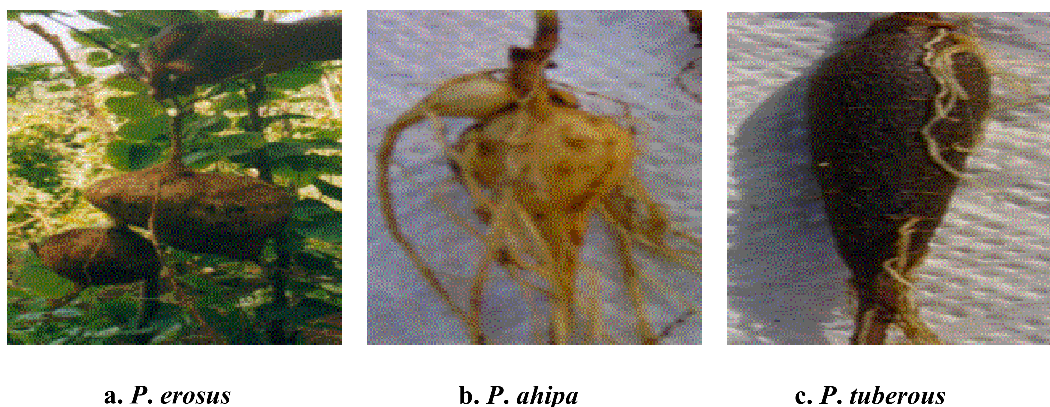
1.1.7. Glucosidases

Glucosidases (EC 3.2.1.21) are phase 3 detoxification enzymes that catalyse the cleavage of glycosides, formed by glycosyl-transferases in phase 2. The aglyca may then either regain their xenobiotic activity or be further conjugated to biomolecules in the cell wall. Several types of glucosidase are known, depending on the type of sugar moiety and binding type. Some glucosidase are described to be apoplastic. They play an important role in rendering compounds unavailable for metabolism.

1.2. Yam bean (*Pachyrhizus*)

The present research on detoxification enzymes focuses on the tolerance of the tropical tuber-forming legume Yam bean (*Pachyrhizus* (Rich. ex) DC, (Vietmeyer, 1986)), to pesticides and environmental stress with a view of its possible establishment and large-scale production in Sierra Leone and other developing countries.

Originating from Central America and central South America the genus comprises of five species (Sørensen, 1988), of which three, *Pachyrhizus erosus* (L.) Urban, *Pachyrhizus ahipa* (Wedd.) Parodi and *Pachyrhizus tuberous* (Lam.) Sprengel are actively cultivated to date. *Pachyrhizus erosus* is most widely cultivated, more as a reason of its higher yield than lack of agriculturally attractive features in *P. ahipa* and *P. tuberous* (Sørensen, 1996).



a. *P. erosus* **b. *P. ahipa*** **c. *P. tuberous***

Figure 4. Tubers of "yam bean" *Pachyrhizus*. (a) *P. erosus* (b) *P. ahipa* and (c) *P. tuberous*

The plants have been described as a valuable vegetable with a number of attractive characteristics. Most importantly, *Pachyrhizus* is a source of high nutritive value. The fleshy tuber, which can be eaten raw or cooked, supplies a high amount of carbohydrates with low protein content much higher in nutritive value than that observed for other tubers (Zinsou, 1994). Biochemical analysis of *Pachyrhizus erosus* tuber carried out by Fernandez *et al.*, (1997) obtained values of dry matter as 16.19-22.28%, protein 1.11-1.6%, fat 0.553-0.867%, crude fibre 0.3048-0.3943% and ash 0.669-1.089%. The *P. erosus* tubers could provide three to five times the amount of protein when compared with traditional root crops (sweet potato and cassava). Recent research has also indicated that the young pods and seeds from all three varieties have a higher protein and lipids/oils quality than soybean and other oil seeds (Sales *et al*. 1990; Ratanadilok and Thanisawanyangkura, 1994; Santos *et al*., 1996). Mature pods are not recommended for consumption since they contain the insecticidal compound rotenone ($C_{23}H_{22}O_6$).

This crop is also attractive for several other qualities, such as its natural insecticidal (Sørensen, 1990) and pesticidal (Esquivel *et al.*, 1992) properties. It develops high yield with and without reproductive pruning (Nielsen, 1996; Csatellanos *et al*., 1996; Belford *et al*., 2001) with low nitrogen fertilizer (Kjaer, 1992, Belford *et al*., 2001). It can also be used as green manure and its biological nitrogen fixation ability (Kjaer, 1992) makes it an interesting crop for many farming systems for soil protection and improvement. The aerial parts of the crop can also be used as fodder, and its adaptability to a wide range of climatic and edaphic conditions is rather unique (Annerose and Diouf, 1994; Vieira da Silva, 1995; Ørting *et al*., 1996; Sørensen *et al*., 1996). Furthermore, it could contribute to agricultural diversity, increasing biodiversity and be a source of production of new and alternative products.

Due to its high yield capacity and low input requirements, yam bean is considered a sustainable crop and could be grown extensively in most tropical and subtropical countries. Therefore, in order to ensure stable supplies of nutritionally adequate food production, to achieve better natural resource management and environmental protection in Sierra Leone, the large-scale cultivation of yam bean, an underexploited crop of potential economic importance, should be welcomed.

Though a promising crop with lots of potential it has some limitations, and as a cultivated crop it’s vulnerable to attack from pests and disease, and exposure to abnormal climatic

conditions. The greatest problem posed on the plant survival is that faced by the seeds from pests attack. As a rule the plant is seed propagated and a severe pest attack will mean the loss of a whole harvest. Several pests, especially insect (bruchids) and fungi, have been reported to cause severe damage in all *Pachyrhizus spp.* (Sørensen, 1996). Thus, pesticide application is necessary. Currently farmers do add fungicides to seeds as a control of fungi during storage. However, data on the reaction of the plant after application of agrochemicals i.e. fungicides, herbicides and insecticides is still lacking.

1.2.1. Agrochemicals

Therefore the capability of these plants to tolerate chemical stress should be evaluated. Hence, the status of the major detoxification enzymes for agrochemicals metabolism, i.e. glutathione S-transferases, was determined in roots and shoots of this plant after seed treatment with, Captan (fungicide), Fenoxaprop (herbicide) and Imidacloprid (insecticide), isolated, and purified for characterization.

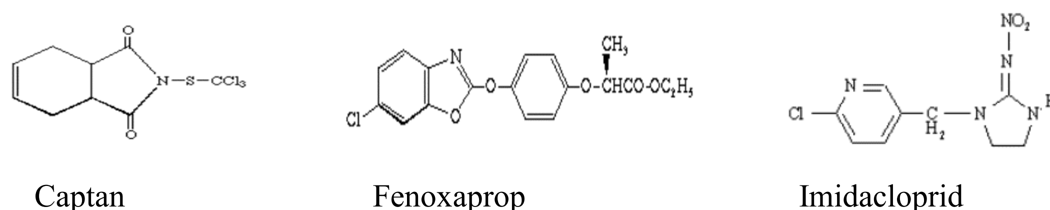


Figure 5. Chemical structure of captan (fungicide) fenoxaprop (herbicide) and imidacloprid (insecticide) used as pesticides to determine the catalytic activity of GST for glutathione substrate conjugation of xenobiotics.

Captan

Captan ($C_9H_8Cl_3NO_2S$) is a non-systemic phthalimide fungicide with broad-spectrum activity effective against a large variety of pathogenic fungi especially rot and leaf spot of many fruits and vegetable crops. It inhibits mycelial growth from germinating fungus spores. As a result, it has effective protection action, although it will not eradicate a preexisting infection.

Captan is also used as a preservative for leather, as a post-harvest root dip and seed treatment particularly on peas, and as a pre-planting soil fungicide. It has been used as a growth regulator to increase size of oranges and tangeloes.

Imidacloprid

Imidacloprid ($C_9H_{10}ClN_5O_2$) is a systemic, chloronicotinyl insecticide with soil, seed and foliar uses for the control of sucking insects including rice hoppers, aphids, thrips, whiteflies, termites, turf insects, soil insects and some beetles. It is most commonly used on rice, cereal, maize, potatoes, vegetables, sugar beets, fruit, cotton, hops and turf, and is especially systemic when used as a seed or soil treatment.

It causes a blockage in a type of neural pathway that is more abundant in insects than in warm-blooded animals, leading to an accumulation of acetylcholine, a neurotransmitter, and resulting in the insect's paralysis and eventual death. It is effective on contact and via stomach action. It has been shown to be compatible with fungicides when used as a seed treatment to control insect pests (Pike *et al*., 1993). It has been tested in a variety of application and crop types, and is metabolized following the same pathways. The most important steps were loss of the nitro group, hydroxylation at the imidazolidine ring, hydrolysis to 6- chloronicotinic acid and formation of conjugates (Kidd and James, 1994). In the soil, imidacloprid is degraded stepwise to the primary metabolite 6-chloronicotinic acid, which eventually breaks down into carbon dioxide (Hellpointer, 1994).

Fenoxaprop

Fenoxaprop ($C_{16}H_{12}ClNO_5$) is a systemic postemergence herbicide that inhibits fatty acid synthesis essential for the production of plant lipids. Broadleaf plants are tolerant to these herbicide families and almost all perennial and annual grasses are susceptible. Injury symptoms are slow to develop and appear first on new leaves emerging from the whorl of the grass plant, rotten growing point, leading to death of plant. Newer leaf tissue will be yellow (chlorotic) or brown (necrotic) and the leaves in the leaf whorl can be easily separated from the rest of the plant. It is use against annual and perennial grass weeds in potatoes, beans, soybeans, beets, vegetables and groundnuts.

1.2.2. Environmental Conditions

Environmental condition is a major limiting factor in crop production. Crop yields fall far short of potential yields because plants usually grow under environmental stress. Most crops are highly sensitive to climatic change and even those that have innate capability to tolerate stress cannot withstand prolong abnormal environmental conditions.

Atmospheric carbon dioxide (CO_2) concentration has increased and is expected to continue to rise at a rate of about 1.6 ppm per annum for the foreseeable future (Colls, 1997) as a result of fossil fuel combustion and forest clearance (Hertstein *et al*., 1995). It is predicted to reach about 550 ppm by the middle of the present century (Houghton *et al*., 1992; Schimel *et al*., 1996). Plants grown in CO_2 enriched air nearly always exhibit increased photosynthetic rates and biomass production relative to plants grown at the current ambient CO_2 concentration.

Ozone, a highly reactive form of oxygen is a powerful oxidizing agent that can destroy leaf tissues of plants. Tropospheric ozone (O_3) concentrations are also rising at an annual rate of about 0.5% (Hertstein *et al*., 1995). O_3 is not only a “greenhouse gas” but is regarded as the most important phytotoxic air pollutant (Ashmore and Bell, 1991). The effects on plants are most pronounced when soil moisture and nutrients are adequate and ozone concentrations are high. Under good soil moisture and nutrient conditions the ozone will enter through openings into the leaf and damage the cells that produce the food for the plants. Once the ozone is absorbed into the leaf, some plants spend energy to produce bio-chemicals that can neutralize a toxic effect from the ozone. Other plants will suffer from a toxic effect, and growth loss in which visible symptoms may occur.

In contrast to CO_2, plants exposed to elevated ozone concentrations typically display reductions in photosynthesis and growth in comparison with plants grown at the current ambient ozone concentration. Hertstein *et al*. (1995) estimated that yield losses resulting from O_3 exposure have increased from 0.1% in pre-industrial times to 32.6% at present-day levels, although the losses are highly species dependent.

Both CO_2 and O_3 gases have direct effects on the physiology and productivity of crops (Bosac *et al*., 1998; Mulholland *et al*., 1998). By contrast, studies of the effects of O_3 on species such wheat (Finnan *et al*., 1998; Pleijel *et al*., 2000; Olleren shaw and Lyons, 1999), potato (Pell *et al*., 1988), soybean (Fiscus *et al*., 1997) and radish (Barnes and Porrmann, 1992) and trees (Langebartels *et al*., 1998) have shown that growth and reproductive development are adversely affected.

Drought is one of the most serious worldwide problems for agriculture especially in arid or semi-arid regions. It is the main cause of low plant productivity and particularly of seasonally erratic productivity. Transient droughts can cause death of livestock, famine and social

dislocation. In a drought the plant has to rely on the stored reserves for a longer period of time thus reducing stored nutrients for future use and increasing the plants susceptibility to damage in extended periods of drought.

Although drought, CO_2 and O_3 have been extensively studied, little is known about how the molecular reponses of detoxifaction enzymes on plants tolerance to withstand these stressors. To understand a little more about *Pachyrhizus* responses to the environment, the status of the major detoxification enzyme GSTs, on responses to oxidative stress, on the exposure of the plant to elevated levels of carbon dioxide CO_2 and ozone O_3 and water stress, drought, was determined, the respective enzymes isolated, and partially purified for characterization.

2. MATERIALS AND METHODS

2.1. Plant Materials

Experiments were carried out with the three cultivated varieties of ***Pachyrhizus***.
Pachyrhizus ahipa (Wedd.) Parodi. Also known as Andean yam bean, Manioc bean.
Pachyrhizus erosus (L.) Urban. Mexican yam bean, yam bean, Manioc bean, and
Pachyrhizus tuberous (Lam.) Spreng. Jícama, manioc bean, potato bean, tuberous gram.

Seeds of *P. ahipa* AC 102 and *P. tuberous* TC 361 were supplied by Dr Martin Sørensen (KVL, Denmark). AC 102 seeds were obtained from the local market in the Tarija Province of Bolivia whilst those of TC 361 were product of multiplication in Conta Manillo, PERU. *Pachyrhizus erosus* EC 550 seeds were from Freetown, Sierra Leone, product of seed multiplication from the Department of Biological Sciences, Fourah Bay College, University of Sierra Leone.

2.2. Treatment of Plants under Environmental Stress Conditions

Experiments on environmental stress were designed to determine the effects of elevated carbon dioxide (CO_2), ozone (O_3), and water stress (induced drought) on *Pachyrhizus* GST activity under greenhouse conditions, and for the isolation and purification of enzyme for characterization of stress condition of the plant.

2.2.1. Climatic Conditions and Cultivation

Pachyrhizus plants were cultivated in cabin 5 of the greenhouse of the Department of Experimental Environmental Simulation, in the National Research Centre for Environment and Health, Neuherberg, Germany under experimental number 1034.

Plants were grown in pots (5 L) filled with a loamy sand-soil mixture in a composition of 50% humus, 30% sand and 20% gravel. During the period of investigation temperature range was maintained between 23 to 26°C during the 9 hours daylight, and 15 to 18°C during the dark period. The light intensity range was 400 to 800 mol m^{-2} s^{-1}. The relative humidity (RH) was maintained between 65 to 80%. Carbon dioxide and ozone concentration were at current ambient levels of 330 ppm and 20 ppb respectively.

Five seeds were sown in each pot at a depth not below 2 cm. Thirty-six pots were planted, with each accession (variety) *P. ahipa*, *P. erosus* and *P. tuberous* having 12 pots; 3 pots for control and 3 pots each for carbon dioxide, ozone and drought treatments. Plants were allowed to develop under well-watered conditions for three weeks up to vegetative developmental stage three (V3), (Grum, 1990 and Sørensen *et al*., 1993) i.e. when the first trifoliate leaf opens.

During the fourth week the pots were separated into four groups with each of the three accessions having three pots in a group. Group one was designed as the control and remained in cabin 5. The second group of pots was transferred to cabin 4 for carbon dioxide exposure and the third group transferred to cabin 8 for ozone exposure. Group four remained in cabin 5 and was subjected to water stress (Table 1).

Table 1. Environmental stress treatment of *Pachyrhizus* plants

Pots	Conditions
Group 1	Control (ambient air)
Group 2	Elevated carbon dioxide (700 ppm)
Group 3	Elevated ozone (200 ppb)
Group 4	Drought/water stress

2.2.2. Exposure of Plants to Elevated Level of Carbon Dioxide

Pachyrhizus plants were exposed to a stream of air with elevated (700 ppm) carbon dioxide in cabin 4 of the GSF greenhouse for eight weeks. The environmental growth conditions of temperature, daylight hours, light intensity and relative humidity in the cabin were the same as those maintained for the control plants in cabin 5 except for the change in CO_2 concentration which was 400 ppm above ambient levels. After CO_2 treatment and three months after sowing the leaves and roots were rapidly harvested.

Harvest and Measurements of Yield

For GST enzyme extraction, assay and characterization leaves and roots were quickly harvested from a sample of 6 plants, separated and immediately frozen in liquid nitrogen. They were then stored at -80°C until analysed. Between 6–10 leaves were harvested, avoiding the most immature leaves and the older ageing leaves.

For yield measurements, root/tubers were harvested from a representative sample of 5 plants. They were weighed immediately to determine their fresh weight.

2.2.3. Exposure of Plants to Elevated Level of Ozone

Three to 4 weeks old *Pachyrhizus* plants were transferred to ozone fumigation in a cabin operating under similar environmental growth conditions as that maintained for control plants except for the O_3 treatment.

The plants were exposed to a stream of air with 200 ppb O_3 in Plexiglas boxes (730 l) for 6 hours per day for up to 30 days. Ozone was generated by electrical discharge in dry oxygen, and the desired O_3 concentration was computer-controlled together with the photometer ozone analyzer CSI 3100 (Columbia Scientific Industries, Austin, TX; Langebartels *et al*., 1991). The ozone concentration in the ambient air control chambers was monitored and varied from 0 to 30 ppb over the course of these experiments. After O_3 treatment, the plants were removed from the cabin and harvested in pollutant-free air.

For enzyme extraction, assay and characterization leaves and roots were quickly harvested from a sample of 6 plants, separated and immediately frozen in liquid nitrogen. They were then stored at –80°C until use. Between 6–10 leaves were harvested, avoiding the most immature leaves, cotyledons, and the first two true leaves. Yield measurements was carried out as previously described for CO_2.

2.2.4. Water Stress Experiments

Pachyrhizus plants in the fourth group, in the same cabin as the control plants, under the same environmental conditions were subjected to drought by withholding irrigation for 21 days during the seventh week after sowing.

Water Stress Measurements

Leaf relative water content (LRWC) and soil moisture content (SMC) were determined every third day throughout the period of stress at 13.00 hours.

Leaf Relative Water Content

The leaf relative water content (LRWC) was determined by the gravimetric method. Measurements were obtained from the third youngest fully expanded trifoliate leaf. Hewlett and Kramer (1963) and Kramer (1983) found entire leaves more satisfactory than disks for some species. After being excised from the stem, the leaf was quickly placed into a petri dish and the fresh weight (FW) of the leaf was immediately recorded. Submerging in distilled water for two hours then rehydrated the leaf sample, after which the turgid weight (TW) was measured. The same leaf sample was then allowed to dry in an oven at 80°C for 24 hours and the dry weight (DW) measured. The leaf relative water content was calculated as LRWC = ((FW-DW)/(TW-DW)) x100.

Soil Moisture Content

The intentional moisture stress in the soil profile, soil moisture content (SMC) was measured by gravimetric methods. This involves collecting a sample, weighing it, drying it, and then reweighing it. Soil sample measurements were made periodically every third day until plants were harvested. Samples from each pot were collected from different parts and depths in order to obtain a homogenous sample. They were immediately weighed and the FW were recorded. The samples were then oven dried for 24 hours at 80°C, cooled and then reweighed to obtain the soil DW. Soil moisture content was calculated as SMC = ((FW-DW)/FW) x100. The moisture level was monitored for real-time evaluation.
Plants were harvested three months after sowing, 21 days after water stress. All measurements for enzyme extraction and yield were carried out immediately as described above.

2.2.5. Treatment of Plants with Agrochemicals

This experiment was designed for the determination of Pachyrhizus GST enzyme activity, purification and characterization after pesticide treatment.

Pesticide Application and Cultivation

For the determination of the effect of various pesticides on *Pachyrhizus* GST activity *Pachyrhizus* plants were cultivated in cabin 5 of the greenhouse of the National Research Centre for Environment and Health, Neuherberg, Germany. Plants were grown in pots (5 L) filled with a loamy sand-soil mixture in a composition of 50% humus, 30% sand and 20% gravel. During the period of investigation the temperature range was maintained between 23

to 26°C during the 9 hours daylight, and 15 to 18°C during the dark period. The light intensity range was 400 to 800 µmol m^{-2} s^{-1}. The relative humidity was maintained between 65 to 80%.

Five seeds were sown in each pot at a depth not below 2 cm. Twenty four pots were planted, with each of the three variety (*P. ahipa*, *P.erosus* and *P.tuberous*) having 12 pots; 2 pots for control and 2 pots each for Captan, Fenoxaprop and Imidacloprid treatments. The first group-designed control received no pre-treatment of seeds with agrochemicals (Table 2). For the second group the seeds were pre-treated, soaked in a solution of the fungicide Captan (2.5 g/kg). The third group was pre-treated, soaked in a solution of the herbicide Fenoxaprop (2.5 g/kg) and the fourth group was pre-treated, soaked in the solution insecticide of Imidacloprid (2.5 g/kg). The seeds were incubated in their respective pre-treatment solution for 12 hours, and then dried on filter paper before sowing. Incubation time in their respective pre-treated solution was 12 hours after which the seeds were dried on filter paper before sowing.

Table 2. Pesticide treatment of *Pachyrhizus* plants before sowing

Pots	**Seed treatment**
Group 1	Untreated (control)
Group 2	Captan 2.5g/kg
Group 3	Fenoxaprop 2.5g/kg
Group 4	Imidacloprid 2.5g/kg

Plants were allowed to develop under well-watered conditions for four weeks up to vegetative phase three (V3) (Grum, 1990 and Sørensen *et al*., 1993) when the second trifoliate leaves were fully opened. The plants were then harvested, separated into leaves and roots. The samples were immediately immersed in liquid nitrogen and stored at –80°C until use.

2.3. Preparation of Cytosolic and Microsomal Extracts for the Determination of GST Activities from Stress Induced Plants

2.3.1. Extraction of Microsomal and Cytosolic Enzymes

Frozen samples of leaves and roots of *Pachyrhizus* plants exposed to elevated levels of carbon dioxide and ozone, water stress and controls, weighing between 3 to 10g, were crushed and ground with a mortar and pestle in liquid nitrogen until the mixture was a smooth paste. Immediately, 10% insoluble polyvinylpyrrolidone (PVP K 90) were added to the fine powder followed by five volumes (v/w) of 0.1 M Tris-HCl buffer (pH 7.8; buffer A) containing 2 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerine, and freshly prepared 0.25 M saccharose, 5 mM dithioerythritol (DTE) and 1 mM phenylmethylsulfonylfluoride (PMSF), Table 3. The mixture was homogenized at 13,000 rpm with Ultra Turax (T8, IkA Werke, 9219 Staufen) for 2 x 90 seconds. The homogenate was pressed through a double layer of miracloth (Calbiochem) and centrifuged (Beckman & Coulter, JA 25.50) at 10,000 x g at 4°C for 5 minutes. The supernatant was transferred to ultracentrifuge tubes and ultra-centrifuged (LE-70, Beckman & Coulter, 56 T1 SER) at 105,000 x g at 4°C for 1 hour. The resulting pellet is the microsomal fraction and the supernatant (designated as crude extract) is the cytosolic fraction (Figure 5).

Table 3. Composition of extraction buffer for microsomal GST isolaton

Tris-HCl buffer 0,1M pH 7.8	+ 10 % PVP K90
	+ 10 % glycerine + 2 mM EDTA + 0.25 M Saccharose + 5 mM DTE + 1 mM PMSF

2.3.2. Production of Microsomal Extract

The pellet containing the microsomal fraction was re-suspended, using a Pasteur pipette, in 1:1 volume of 25 mM Tris-HCl buffer (pH 7.8; buffer B) containing 2 mM EDTA, and 20% glycerol. The mixture was ultra-centrifuged (LE-70, Beckman & Coulter, 56 T1 SER), at 105,000 x g at 4°C for 1 hour. The pellet obtained was re-dissolved in buffer B, 50 µl ml^{-1} of discarded supernatant. The mixture was homogenized with a glass potter and a pistil. This

microsomal preparation serves for the measurements of both cytochrome P450 and GST activity.

2.3.3. Isolation and Partial Purification of Cytosolic GST

Solid ammonium sulphate was added to the supernatant (designated as crude extract) obtained after first ultra-centrifugal step to give a saturation of 40%. After stirring for 30 min in ice, precipitated proteins were removed by centrifugation at 39,200 × g at 4°C for 30 min (Beckman & Coulter, JA 25.50). The supernatant was decanted and adjusted to 80% ammonium sulphate saturation. After stirring for 30 min the solution was centrifuged as described for the first step. The resulting pellet was re-suspended in 2.5 ml, 25 mM Tris-HCl, buffer (pH 7.8; buffer B).

The extract was desalted by gel filtration through Sephadex G25 material, PD 10 column (Amersham Pharmacia), which had been preconditioned (equilibrated) with buffer B. After loading the protein extract, the column was rinsed with 3.5 ml buffer B. The total fraction of eluted-purified GST enzymes was 3.5 ml separated in seven tubes, each containing 0.5 ml and stored at –80°C until use. This fraction serves only for the determination of GST activity.

Figure 5 on the opposite page summarizes the extraction scheme for *Pachyrhizus* detoxification enzymes.

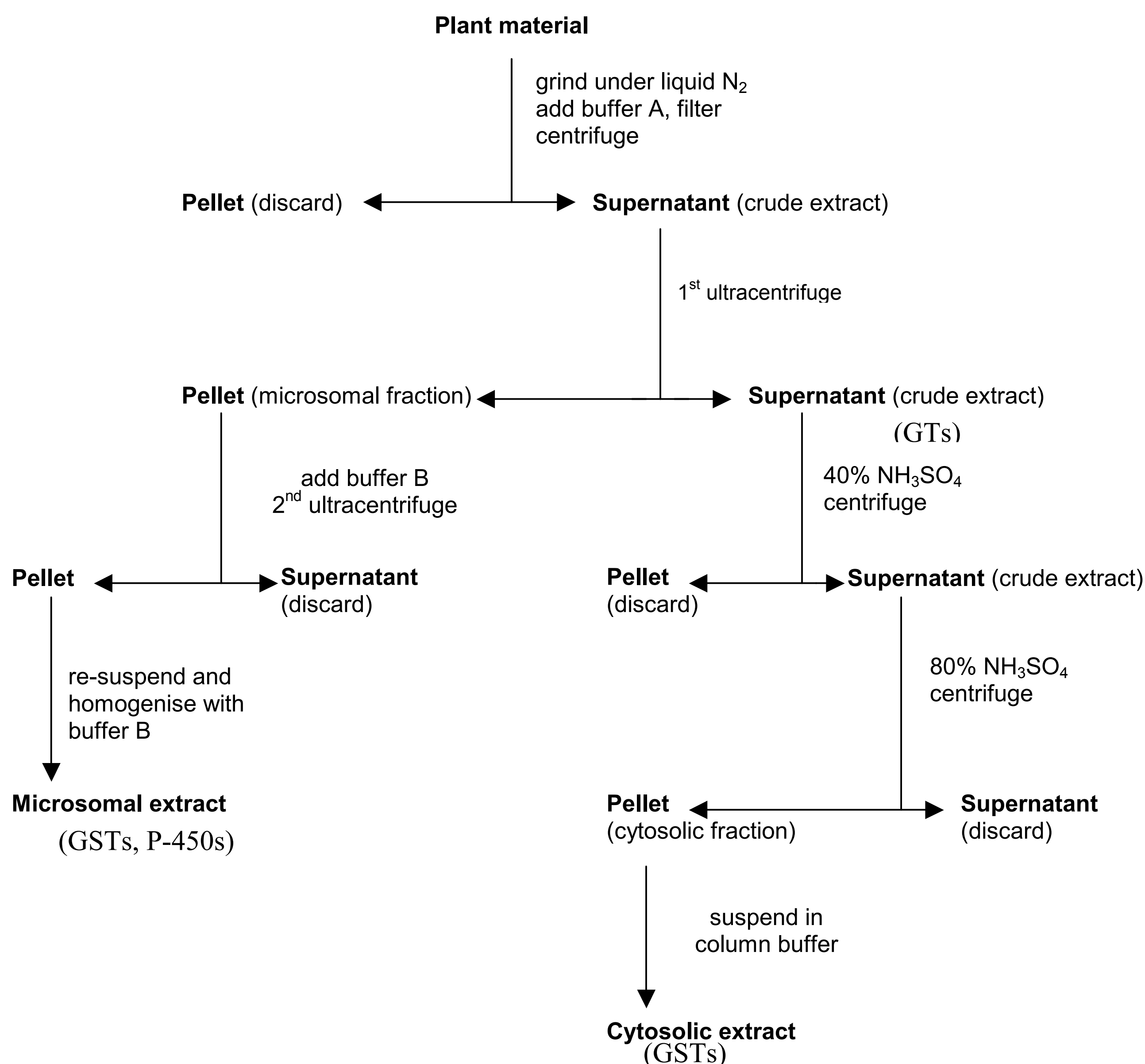


Figure 5. From plant material to enzymes extracts. Schematic model for the preparation of microsomal and cytosolic extracts.

2.3.4. Isolation of Chlorophyll Pigments from Stress Induced Plants

Extraction

Chlorophyll extraction was performed as according to Lichtenthaler (1987). Freeze dried samples of leaves from control and treated (CO_2, O_3 and drought) *Pachyrhizus erosus* EC 550 plants were pulverized under liquid nitrogen with a mortar and pestle in dim light. Aliquot, 0.5g of the resulting powder was suspended in 10 ml of 80% cold aqueous acetone. The

suspension mixture was centrifuged at 39,200 × g at 4°C for 30 min (Beckman & Coulter, JA 25.50). The resulting supernatant was collected as the first portion of chlorophyll extract. It was kept in ice and in the dark. The resulting pellet was re-suspended in 10 ml of 80% cold aqueous acetone and centrifuged as above. To ensure complete extraction the re-suspension of pellet and centrifugal step was repeated three times. The supernatants from the three extractions were combined bringing the total volume collected to 30 ml.

Determination of Concentrations of Chlorophyll Pigments

Quantitative determination of pigments was carried out immediately after extraction as according to Lichtenthaler (1987). Pigments absorbance values in (mg/ml) were measured spectrophotometrically (Beckman, DU 600) monitoring the changes in absorbance from λ_{max} in the blue to λ_{max} in the red at 663.2, 646.8 and 470 nm at room temperature.

The actual concentrations of the pigments, chlorophyll *a* (C_a), chlorophyll *b* (C_b), total chlorophyll (C_{a+b}) and of total carotenoids (C_{x+c}) in *Pachyrhizus* leaves by extraction in 80% (v/v) acetone were determined using the following equations:

$$C_a = 12.25A_{663.2} - 2.79A_{646.8}$$
$$C_b = 21.50A_{646.8} - 5.10A_{663.2}$$
$$C_{a+b} = 7.15A_{663.2} + 18.71A_{646.8}$$
$$C_{x+c} = 1000A_{470} - 1.82C_a - 85.02\ C_b$$

The equations are based on the specific absorption coefficients as determined by Lichtenthaler (1987).

2.4. Preparation of Cytosolic Extracts for the Determination of GST Activities from Pesticide Treated Plants

2.4.1. Extraction of Cytosolic Enzymes

Enzyme extraction was performed as previously described with minor modifications. Frozen samples of leaves and roots of *Pachyrhizus* plants germinated from seeds treated with pesticides (Captan, Fenoxaprop and Imidacloprid) and controls, weighing between 3 to 10g, were pulverized under liquid nitrogen with a mortar and pestle.

The powder was suspended in ten volumes (v/w) of 0.1 M Tris-HCl buffer (pH 7.8; buffer A) containing 5 mM dithioerythritol (DTE), 5 mM EDTA, 1% Nonidet P40 and 10 mg ml^{-1} soluble polyvinylpyrrolidone (PVP K 30), Table 4. The suspension mixture was homogenized at 13,000 rpm with Ultra Turax for 2 x 90 seconds. The homogenate was filtered through a double layer of miracloth (Calbiochem). After a short incubation of ten minutes on ice, the homogenized mixture was centrifuged at 39,200 × g at 4°C for 30 min (Beckman & Coulter, JA 25.50). The resulting supernatant was collected and used as crude enzyme extract.

Table 4. Composition of extraction buffer for cytosolic GST isolaton

Tris-HCl buffer 0,1M pH 7.8	+ 1 % PVP K30
	+ 5 mM EDTA + 5 mM DTE + 1 % Nonidet

2.4.2. Partial Purification of GST

Bulk proteins in crude extracts were precipitated with ammonium sulphate in two steps of 40 to 80% saturation. After stirring for 30 min, the first precipitated proteins at 40% saturation were removed by centrifugation at 39,200 × g for 30 min. The supernatant was decanted and adjusted to 80% ammonium sulphate saturation. After stirring for 30 min the solution was centrifuged as described for the first step.

Ammonium sulphate precipitation in steps, from 0 to 40% and from 40% to 80% saturation to separate the GST enzymes from bulk proteins:

$$\textbf{Ammonium sulphate [g]} = \frac{V*1.77*[S-s]}{3.54-S}$$

Where: V = Volume of solution (ml)

S = Final concentration of ammonium sulphate (in decimal below one)

s = Initial concentration of ammonium sulphate (in decimal below one)

The resulting pellet was re-suspended in 2.5 ml, 25 mM Tris-HCl, buffer (pH 7.8; buffer B). The extract was desalted by gel filtration through Sephadex G25 material, PD 10 columns (Amersham Pharmacia), which had been preconditioned (equilibrated) with buffer B. After loading the protein extract, the column was rinsed with 3.5 ml buffer B. The total fraction of

eluted-purified GST enzymes was 3.5 ml separated in seven tubes, each containing 0.5 ml and stored at –80°C until use.

2.5. Biochemical Assays

2.5.1. GST Enzyme Assays and Protein Determination

GST activity was monitored at all stages of the purification. Activity was determined spectrophotometrically using CDNB (1-chloro-2,4-dinitrobenzene) as a model substrate according to Habig and Jakoby (1981) and using the assay method of Schröder *et al*. (1990).

All measurements were made in a 96 well plate spectrophotometer (SPECTRAmax, 384 PLUS, Molecular Devices) monitoring the change in absorbance at 340 nm (extinction coefficient = 9.6 cm^{-1} mM^{-1}) at 30 °C. The reaction mixture in each well consisted of 190 µl 0.1 M Tris-HCl buffer (pH 6.4; buffer C), 7 µl 30 mM CDNB in ethanol, 3.5 µl 60 mM GSH and 10 µl GST enzyme extract. The reaction was started by the addition of GST enzyme extract. One unit of activity given in katal was the amount of GST required to produce 1 mol of glutathione conjugate per seconds. Measurements were made over a duration of 5 min at ambient temperature and the determined absorption units against a blank run.

Activity with DCNB (1,2-dichloro-4-nitrobenzene) was tested in the final preparations of the purified enzyme. DCNB assay followed the assay method of Schröder *et al*. (1990). Measurements were made in a 96 well plate spectrophotometer monitoring the change in absorbance at 345 nm (extinction coefficient = 8.5 cm^{-1} mM^{-1}) at 30°C. The reaction mixture in each well consisted of 190 µl 0.1 M Tris-HCl buffer (pH 7.5; buffer D), 7 µl 30 mM DCNB in ethanol, 3.5 µl 60 mM GSH and 10 µl GST enzyme extract. The reaction was started by the addition of GST enzyme extract.

All measurements were made in triplicate and corrected for non-enzymatic conjugation rates. Enzyme activity per sample is expressed as µkat $min^{-1}g^{-1}$ for partially purified enzymes and as µkat $min^{-1}mg^{-1}$ for all purified enzymes.

Samples were also retained from all steps of the purification for measurement of protein content. Protein contents were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Cytochrome P450, Peroxidase, glucosyltransferase and glucosidase activities were determined from crude extract to get a general overview of detoxification phase 1, 2 and 3 enzymes distribution in *Pachyrhizus*. The main part of this thesis deals with GST the major detoxification phase 2 enzyme.

Calculating enzyme activity

Specific factor:

$$F = V \times 1000 / (e \times d \times t \times v)$$

V: Final volume of the enzyme assay (µl)
e: Extinction coefficient of the substrate ($mM^{-1}cm^{-1}$)
d: Thickness of the cuvette (cm)
t: Measuring time in seconds (e.g. Rates/min = 60s)
v: Volume of the enzyme sample in the assay (µl)

Formula for calculating enzymatic activity in µkat/mg protein:

$$\text{Enzymatic activity} = R \times F / P$$

R: Rate/min
F: specific factor
P: Protein concentration in mg/ml

A unit of GST activity is defined as µkat/mg glutathione conjugated product formed per minute, molar extinction coefficient given in Table 5.

Table 5. Extinction coefficient of substrates

Substrate	Extinction coefficient
Chlorodinitrobenzol CDNB	9,6 ($mM^{-1}cm^{-1}$)
Dichloronitrobenzol DCNB	8,5 ($mM^{-1}cm^{-1}$)

2.5.2. Cytochrome P450 Enzyme Assay

Measurement of Cytochrome P450 activity from microsomal extract was done according to Salaün *et al*., (1989), Mougin *et al*., (1991) and Haas, (1997). The reaction was started by the addition of buffered microsomal enzyme to the substrate ^{14}C-ring labelled isoproturon IPU, (specific radioactivity = 507 Bq μg^{-1}, radiochemical purity > 98%), and pre-incubated for 5 minutes. Hydrolysis occurred in the presence of 2mM NADPH plus a regenerating system containing 6.7mM glucose-6-phosphate, 0.4U glucose-6-phosphate dehydrogenase. The reaction mixture with a volume of 250µl was further incubated at 25 °C for 1 hour in a shaker.

The reaction was stopped by the addition of 100µl cold acetone. The resulting mixture was centrifuged for 10 minutes at 13,000rpm.

HPLC Determination of Cytochrome P450 Activity

Aliquots of reaction mixture obtained from organic phase of supernatant was analysed by high pressure liquid chromatography (HPLC) using a reversed-phase C-18 column (LiChrospher 100, 5 µm, 250 x 4 mm, Merck). Separations of metabolites proceed with a linear gradient of acetonitrile from 5 to 60% at a flow rate of 0.80 ml/min. Both radioactive substrate and metabolic products were quantified from peak areas monitored by UV-VIS detector 240 nm using standard spectrum reference as control for the calibration of the retention times.

2.5.3. Peroxidase Enzyme Assay

Measurement of the peroxidase (POX) using guajacol as substrate was performed spectrophotometrically as described by Bergmeyer (1983-86) and Drotar *et al*. (1985). All measurements were made in a 96 well plate spectrophotometer (SPECTRAmax, 384 PLUS, Molecular Devices) monitoring the change in absorbance at 420 nm (extinction coefficient = 26,6 cm^{-1} mM^{-1}) at 30 °C. The reaction mixture in each well consisted of 190 µl 0.05 M Tris-HCl buffer pH 6.0, 7 µl 3.4 mM guajacol, 3.5 µl 0.9 mM H_2O_2 and 10 µl enzyme extract. Measurements were made over a duration of 5 min at ambient temperature and the determined absorption units were corrected for a blank run.

2.5.4. Glucosyltransferase Enzyme Assay

UDP-glucose-dependent glucosyltransferase (UGT; EC 2.4.1. -) activity was determined by high-pressure liquid chromatography (HPLC) for TCP-conjugates using a RP C-18 column on an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany).

Assay mixtures contained 15 µl 0.5 M Tris–HCl (pH 7.5), 7.5 µl 50 mM UDP-glucose, 3.75 µl 50 mM nitrophenyl glucopyranoside, 3.75 µl 50 mM salicin, 27 µl H_2O, 3 µl 5 mM 2,4,5-trichlorophenol (TCP) and 15 µl of enzyme extract. The reacting mixture having a final volume of 75µl and was incubated for 60 min at 30 °C. The reaction was stopped by addition of 25 µl of 1 M H_3PO_4 and centrifuged at 15,000 x g for 4 min.

Separation of glucose conjugates from their aglycones was achieved with a linear gradient of acetonitrile from 10 to 100% against 0,025% H_3PO_4 at a flow rate of 1 ml min^{-1}. Both

substrate and degradation product were quantified from peak areas at 340 nm using a TCP control standard as reference for the calibration of the retention times.

2.5.5. Glucosidase

Measurement of glucosidase activity was determined by incubating the reaction mixture containing 0.1 M Tris-HCl buffer pH 5.4, 150 mM nitrophenyl-ß-D-glucopyranoside (PNPG) solution and 40 µl of enzyme solution at room temperature for 5 minutes before measurement.

Measurement was performed spectrophotometrically in a 96 well plate spectrophotometer (SPECTRAmax, 384 PLUS, Molecular Devices) monitoring the change in absorbance at 410 nm (extinction coefficient = 0.4448 cm^{-1} mM^{-1}) at 30 °C. The reaction mixture in each well consisted of 190 µl 0.1 M Tris-HCl buffer pH 5.4, 7 µl 150 mM PNPG, 3.5 µl 0.9 mM H_2O_2 and 10 µl enzyme extract. Measurements were made over duration of 10 min at ambient temperature and the determined absorption units against a blank run.

2.5.6. Measurement of Cytosolic Glutathione in *Pachyrhizus*

The levels of the low molecular weight thiols glutathione (GSH) and homoglutathione (hGSH) in *Pachyrhizus* plants were determined as described by Klapheck (1988), Schupp and Rennenberg (1988) with minor modifications.

Extraction

Frozen samples of leaves and roots of *Pachyrhizus* plants germinated from seeds from control plants, weighing between 2 to 3g, were pulverized under liquid nitrogen with a mortar and pestle. 0.4g of the powder was weighed out in a cool beaker and suspended in 5 ml of 0.1 M HCl containing 0.5g of soluble polyvinylpyrrolidone (PVP K 30), Table 6. For the determination of the recovery within each sample 0.1 ml of a standard solution containing 1 mM GSH, 0.2 mM cysteine (Cys) and 0.1 mM γ-glutamyl-cysteine (γ-EC) were added to the mixture.

Table 6. Composition of extraction solution for cytosolic thiols determination

Extraction solution	0.1 M HCl
	0.5g PVP
Standard solution	1 mM GSH 0.2 mM cysteine 0.1 mM γ-glutamyl-cysteine

The mixture was homogenized at 13,000 rpm with Ultra Turax (T8, IkA Werke, 9219 Staufen) for 30 seconds and centrifuged at 39,200 × g at 4°C for 30 min (Beckman & Coulter, JA 21). The resulting supernatant was collected and diluted by 50% with 0.1 M HCl.

Reduction and Derivatization of GSB Conjugate

For each sample aliquots of 10 and 50 µl were removed from the supernatant and brought to a final volume of 700 µl by the addition of 10 mM HCl solution containing 1 mM EDTA.

For the reduction, 0.4 ml of the stock solution was treated with 0.6 ml of 0.2 M Ches buffer pH 9.6 and 0.1 M dithio-dierythritol (DTT). The mixture was shaken on an auto shaker and incubated at room temperature in the dark for 60 min. In a parallel experiment DTT was omitted from the mixture. Reduction was terminated by the addition of monobromobimane (MBB) used for derivatization. 0.4 ml were removed from the reduced mixture and treated with 15 µl of 15 mM MBB. This mixture was incubated for 15 min at room temperature in the dark. The reaction was finally stopped by the addition of 0.3 ml of 5% acetic acid. Aliquots of standard solution (1 mM GSH and 0.2 mM cysteine) were also subjected to the same reduction and derivatization procedure and used as control.

HPLC Analysis of GSH and hGSH

The composition of bimane-labelled thiol derivatives from the extracts were analysed by reverse phase high-pressure liquid chromatography (HPLC) as described by Schröder and Rennenberg (1992), Schupp and Rennenberg (1992).

The samples were centrifuged (Microfuge Lite, Beckman, 98E SER) at 6,000 x g for 5 minutes. Aliquots of 50 µl were injected on the HPLC high precision pump system (Model 114, Beckman). The MBB derivatives were resolved on a RP C-18 column (250 × 40 mm; 4 µm, Bischoff Chromatography). They were eluted with a linear gradient of methanol/water at 1 ml/min. The solution was developed through a gradient flow (Table 7) of solvent A, consisting of 10% methanol and 0.25% acetic acid (pH 3.9) to solvent B, consisting of 90% methanol and 0.25% acetic acid (pH 3.9) detected by fluorescence HPLC monitor (RF 1002, Gynkotek) with excitation at 380 nm and emission at 480 nm using standards as reference for the calibration of the retention times.

The HPLC system was connected to an analysis unit (Varian star chromatography workstation). Data were analysed with Varian star chromatography software version 5,52.

Table 7. HPLC Gradient solution for the elution of thiol derivatives (%)

Solvent A		Solvent B	
Methanol	10	Methanol	90
Acetic acid	0.25	Acetic acid	0.25
H_2O_{dd}	85	H_2O_{dd}	8
Adjust to pH 3.9 with NaOH		Adjust to pH 3.9 with NaOH	

2.5.7. Fluorescence Microscopy

The conjugation of xenobiotics to glutathione (GSH) enhanced by GSTs has been shown to be a reliable method in the determination of GSH content in intact tissue using monochlorobimane (MCB) as a model substrate (Coleman *et al*., 1997). Monochlorobimane reacts with glutathione to form a shinning fluorescent bimane conjugate thus making it possible to trace the glutathione dependent pathway.

To complement the observed *Pachyrhizus* mGST activities determined in sub cellular organelles and further confirm GSH conjugation in the microsomes, the catalytic formation of the bimane-glutathione adducts in the microsomal fraction was examined by fluorescence microscopy.

Enzyme Assay and Microscopic Measurement

All fluorescence microscopic work was carried out in co-operation with A. Stampfl in the Institute for Toxicology, GSF. Microscopy procedure for the fluorescence excitation of the GSH conjugate followed the method of Schröder and Stampfl (1999). Aliquots of 30 µl from microsomal fraction of leaves of *P. erosus* EC 550 were transferred to clean microscopic cover slide and suspended in 50 µl, 1% Phytagel (Sigma). 10 µl of 20 mM GSH was added followed by 10 µl of 50 mM monochlorobimane. The assay was allowed to incubate for 5 min at room temperature. The slide was covered with a cover slip and mounted in a special measuring chamber. The chamber was fixed on the stage of an inverse fluorescence microscopy (Axiovert 100, Objective: Flat Neofluar 20x/0,50,Zeiss, Goettingen). The GSH–MCB adduct was measured in a Fluorescent Monochromator (Till vision, Munich) reader with excitation at 380 nm and emission measured at 510 nm. The fluorescence measurements were made over a period from 40 min. Three to five attempts was repeated.

2.6. Enzymology

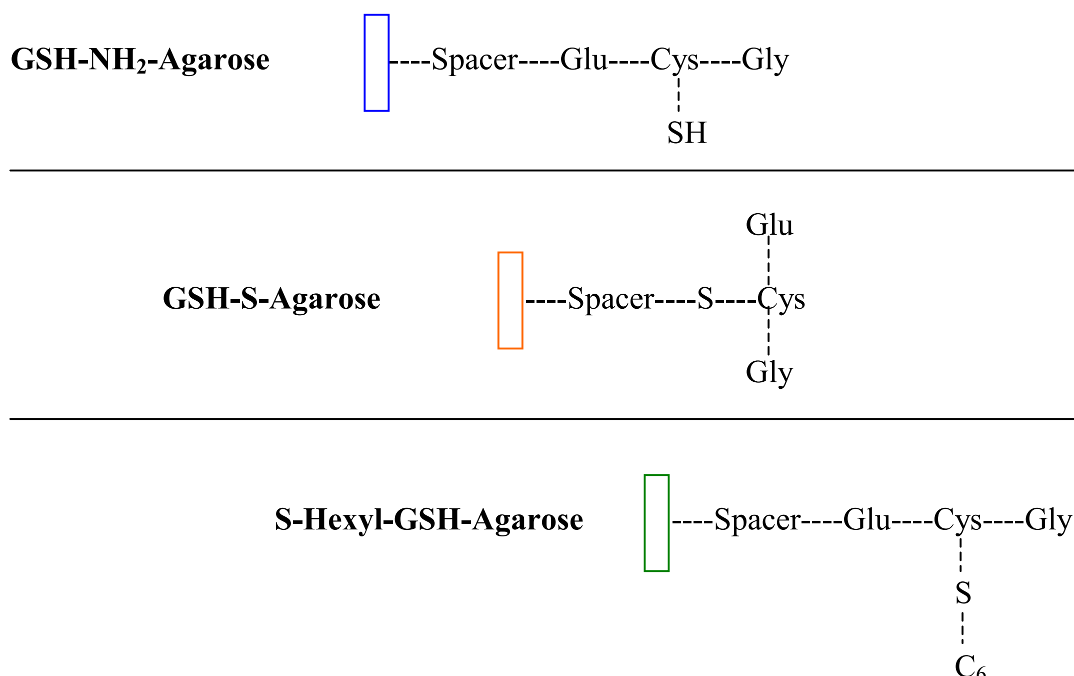
2.6.1. Affinity Chromatography

Preliminary Trials: Choice of Affinity Matrix

Affinity chromatography is widely acknowledged as one of the most desirable protein purification method devised to date. Since careful choice of affinity matrices is necessary for optimum separation, purification and isolation of desired proteins, three chromatographic bed materials of epoxy-activated 4% crossed-linked beaded agarose with three different ligands that covalently bind for GST; GSH-NH_2-agarose, GSH-S-agarose and S-hexyl-gluthatione agarose (Sigma) Table 8, were evaluated to determine which column offered the highest protein binding capacity and highest degree of purification and for the isolation of GST proteins. The technique relies on the inherent ability of most catalytically active proteins to recognise and bind to substrates or products in a specific and reversible manner.

Aliquots (1 ml) of crude enzyme extracts of non-induced plants of *Pachyrhizus* were loaded separately to the three different affinity chromatography columns for GST isolation. The columns were initially equilibrated in binding buffer (25 mM Tris-HCl, pH 7.8; buffer B). They were washed with 10 x 1ml of buffer B. GST active fractions were eluted with 10 x 1ml of the same buffer containing a linear gradient of 1 to 16mM GSH and 0.1 to 0.3 M NaCl. The columns were finally washed with 2 x 1ml buffer B and 5 x 1ml of the buffer B containing 0.3 M NaCl. Protein concentration and GST activity of fractions were determined and analysed for column choice.

Table 8. Affinity chromatographic bed materials: matrix, spacer and ligand



Purification of GSTs

Desalted extracts of partially purified *Pachyrhizus* GSTs from control and treated plants were applied to an S-hexylglutathione agarose immobilized 4% beaded affinity columns (Sigma), previously equilibrated in 25 mM Tris-HCl buffer (pH 7.8; buffer B). The same procedure was followed as that for the preliminary trials but with slight modification in the gradient.

Aliquots of 1 ml were loaded on the column. The column was initially washed with 10 x 1 ml of buffer B. Bound GST enzyme was eluted with linear gradients of 0.1 to 0.5 M NaCl and 1 to 16mM GSH in buffer B. 10 x 1 ml fraction (Table 9). The column was further washed with linear gradient of 0.6 to 1M NaCl and reconditioned with buffer B for repeated use. Protein concentration and GST activity were determined. Fractions with GST activity were pooled desalted and lyophilised.

Table 9. Gradient concentration for GSH/NaCl fraction for affinity chromatography

GSH-NaCl concentration in Tris-HCl buffer [25 mM, pH 7.8]										
Fraction	11	12	13	14	15	16	17	18	19	20
GSH [mM]	1	1	2	2	4	4	8	8	16	16
NaCl [M]	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5	0.5

2.6.2. Fast Protein Liquid Chromatography

Ion Exchange Chromatography: Mono Q

The *Pachyrhizus* GST subunits were further purified by fast protein liquid chromatography (FPLC) conducted using the procedure according to Dean *et al*. (1990) and of Fuerst *et al*. (1993). GSTs enriched fractions from S-hexylglutathione agarose affinity columns were pooled, desalted and lyophilised.

The process was performed on an FPLC system (Bio-Rad, Germany, Figure 6) at 4°C equipped with an Econo-Pac High Q cartridge. This anion exchange matrix (polystyrene) also regarded as the stationary phase has a bed volume of 5 ml and is specific for proteins with a negative charge surface as described in Figure 7.
The column was equilibrated at a flow rate of 6.50 ml min^{-1}, with degassed 25 mM Tris-HCl buffer pH 7.5, as mobile phase A, followed by the same buffer containing 1 M sodium chloride (NaCl) as mobile phase B and then again by phase A (Table 10).

Table 10. FPLC gradient solutions for the elution of GST subunits

Mobile Phase A	Mobile Phase B
Low salt buffer	High salt buffer
Tris-HCl buffer 25 mM pH 7.5	Tris-HCl buffer 25 mM pH 7.5 + 1 M NaCl

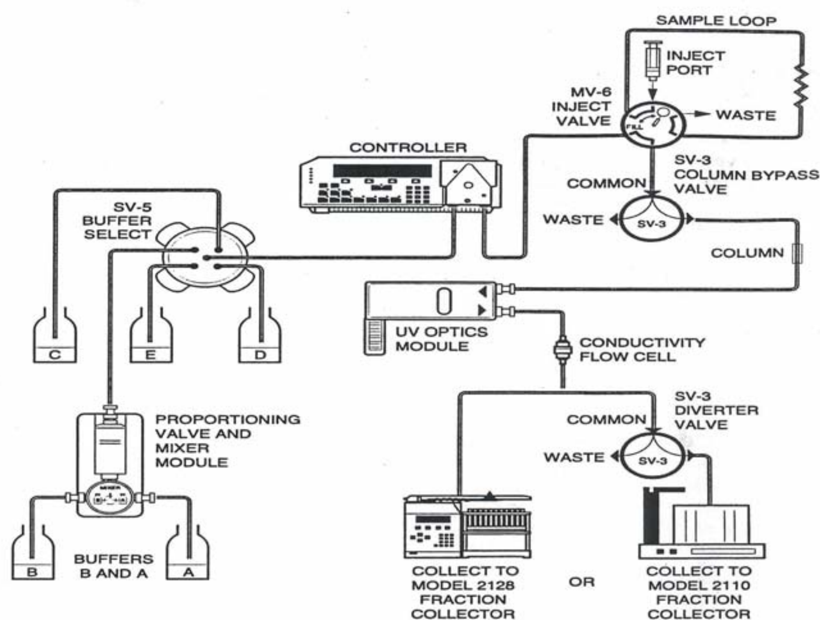


Figure 6. Summary of FPLC-Plumbing System (Bio-Rad)

2 ml of the re-constituted pooled protein fractions were then applied to the Econo Q column. GST activity was eluted with a 50 ml linear gradient of NaCl from 0 to 1.0 M in the mobile phase B. The flow rate was 1 ml min^{-1}. Fractions of 1 ml were collected in a fraction collector (Model 2128, Bio-Rad).

The distribution of proteins in the eluent was monitored at 280 nm using a dual pen recorder (LKB 2210, Bromma). GST enzyme activity was measured and peak fractions were pooled and desalted by elution against 25 mM Tris-HCl buffer (pH 7.8; buffer B) through Sephadex G-25 material, (PD 10 column, Amersham Pharmacia), which had been preconditioned (equilibrated) with buffer B. The fractions were lyophilised and reconstituted for chromatofocusing.

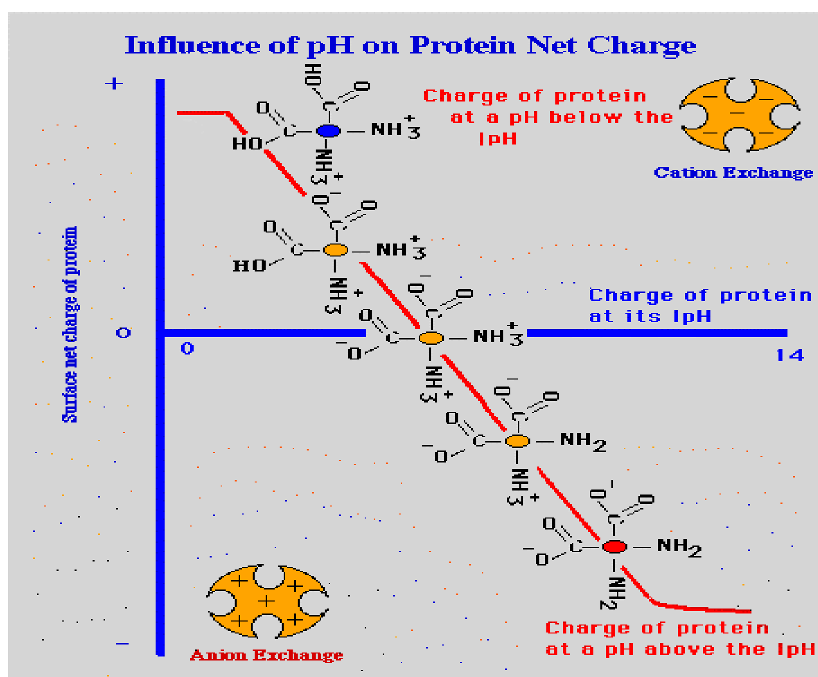


Figure 7. Selectivity of ion exchange column as is influence by pH. Ion exchange separates biomolecules on basis of differences in charge characteristics (cationic/anionic). It depends on the pH of the buffer and the isoelectric points of the biomolecules. Adapted from Natural Toxins Research Center at Texas A&M University – Kingsville.

2.6.3. Chromatofocusing: Mono P

Determination of Isoelectric Point

To achieve a very fine separation and increased purification of *Pachyrhizus* GST, combined active fractions obtained after Mono Q (FPLC) were subjected to chromatofocusing on Mono P system (Pharmacia, Amersham) to determine the isoelectric points (pI).

This method takes advantage of the buffering exchanger and generates a pH gradient across the column developed by a mixture of buffers called the "polybuffer exchanger", PBE. The high-resolution column, HR 5/5, Polystyrene (Amersham) was initially washed with the start buffer A, (25 mM Bis-Tris/HCL, pH 7.4) (Roth, Germany) followed by 1 M NaCl and then again with the Start buffer A. The mobile phase B contains the polybuffer 74 (Amersham Biosciences) diluted in the ratio (1:8).

Table 11. Mono P gradient solutions for the elution of GST isoenzymes

Mobile Phase A	Mobile Phase B
(Start buffer)	(Elution buffer)
Bis-Tris-HCl buffer 25 mM pH 7.4	Polybuffer 74-HCl pH 4.0

The column was equilibrated with the Start buffer A followed by the injection of 1 ml 5 N NaOH. 5 ml of eluent buffer, polybuffer 74-HCl pH 4.0 was first applied to the column followed by the sample in the same buffer. Proteins were then eluted by a decreasing pH gradient of eluent buffer in mobile phase B. The flow rate was 1 ml min^{-1}. Samples of 1 ml were collected in a fraction collector in ice. Protein concentration, enzyme activity and the pH were determined in these fractions.

2.7. Characterization of Catalytic Parameters

2.7.1. Kinetic Measurements K_m and V_{max}

Enzyme kinetic constants for substrate affinity (K_m) and the maximal velocity (V_{max}) of GSH and CDNB according to Michaelis-Menten were determined for the purified *Pachyrhizus* isozymes from Lineweaver-Burk plots, Eadie-Hofstee and Direct linear plots. The measurements were performed according to the standard assay procedures cited for CDNB above Habig and Jakoby (1981), Schröder *et al*. (1990) at a constant GSH concentration (60 mM). The concentrations of the CDNB substrates were varied in a range of 0.0625 to 2 mM. GST activity was measured in triplicate at five different substrate concentrations. The values were calculated with the Sigma plot enzyme kinetics software (Sigma) and rejected when correlation coefficients for the fits were smaller than 0.8. Protein contents were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.7.2. Physicochemical Enzyme Properties

Determination of Optimal pH

The optimum pH for *Pachyrhizus* GST isoenzyme activity was assessed over a pH gradient range from 3 to 9 for final purified isoenzymes extract obtained from chromatofocusing.

GST activity with CDNB as substrate was measured using the method of Habig and Jakoby (1981) and Schröder *et al*. (1990) as earlier described. The assay measures the formation of a conjugate between GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) at 340 nm (extinction coefficient = 9.6 cm^{-1} mM^{-1}) in different buffers at the following pH (Table 12).

Table 12. pH ranges of selected biological buffers (0.1M, 25°C)

Buffer	Useful pH range	pH
Citric	4.2–5.2	4
KH_2PO_4	4.0–6.0	5
Mes	5.5–6.7	6
K_2HPO_4	6.0–8.0	6.4
Hepes	6.8–8.2	7
Tris/HCl	7.0–9.0	7.5
Tris/HCl	7.0–9.0	8
Ches	8.6–10.0	9

Table 13. pH of selected active buffers suitable for *Pachyrhizus* GST stability over the active pH range (0.1M, 25°C)

Buffer	pH
Citric	4
KH_2PO_4	5
Tris/HCl	6
K_2HPO_4	6.4
Tris/HCl	7
Tris/HCl	7.5
Tris/HCl	8
Ches	9

Temperature Optima and Activation Energy (E_a)

In a separate experiment the optimal temperatures dependence of purified *Pachyrhizus* GST isoenzymes activity for CDNB reduction were assessed over the range from 10°C up to 70° C in steps of 10°C. The final purified isoenzymes extract obtained after chromatofocusing were used. GST activity was measured using the method of Habig and Jakoby (1981) and Schröder *et al*. (1997) as earlier described. Temperature effects were analysed using an Arrhenius plot of log V versus 1/° K. The activation energy (E_a) of the purified enzyme was determined as defined by the Arrhenius equation as follow:

$$\mathbf{K = A * exp^{(-E_a/R*T)}}$$

Where k is the rate coefficient,
A is a constant,
E_a is the activation energy,
R is the universal gas constant, and
T is the temperature (in degrees Kelvin).
R has the value of 8.314×10^{-3} kJ $mol^{-1}K^{-1}$

2.7.3. Electrophoresis

Gel electrophoresis was performed to yield information about fraction purity, molecular weight and isoelectric point of GST isoenzymes.

Estimation of Molecular Mass: One-dimensional SDS

Molecular weights of *Pachyrhizus* isoenzymes were determined by one-dimensional Sodium dodecyl sulphate (Lauryl Sulphate, Sigma), polyacrylamide gel electrophoresis (SDS-PAGE) as described by Flury *et al*., (1995), using the buffer system of Laemmli (1970).

The process was performed essentially by the method of Weber & Osborn (1969). The polyacrylamide solution (Roth) was 40% consisting of 30% (w/v) acrylamide to bisacry1amide in a ratio 29:1 respectively. The process was carried out on a discontinuous gel system comprising of a stacking and a resolving gel (Table 14,15,16).

The non-restrictive large pore gels, the stacking gels were made of 4% (w/v) polyacrylamide in 0.5 M Tris-HCL buffer pH 6.8. The resolving gels were made up of 12% (w/v) polyacrylamide in 1.5 M Tris-HCL buffer pH 8.8. After casting the gels were equilibrated

with the running buffer 0.25 M Tris-HCl buffer pH 8.3 containing 1% SDS and 1.92 M glycine.

Samples (15 to 20 µl) at protein concentration of approximately 50 µg/ml were diluted in the ratio 1:1 with loading buffer, 0.1 M Tris-HCl pH 6.8 containing 4% SDS, 20% glycerol, 0.2 M EDTA, 20% β-mercaptoethanol and 0,1% bromophenol blue. Proteins were denatured, by heating at 95°C for 3 min on a sand block. Samples (15 to 20µl) and low molecular weight (Mw) markers (10µl) (Rainbow, Amersham; Precision plus, Bio-Rad) were loaded into wells on the stacking gel in a gel running apparatus (Mini vertical unit SE 250, Hoefer) and run at 200 volts, 200 mA and 300 W for approximately 1hr 45 min.

After electrophoresis, gels were fixed and resolved protein bands were silver stained following the method of Blum *et al*., (1987) with slight variation. The low molecular weight (Mw) marker was used as standard reference for determination of GST subunit molecular weight. Apparent molecular mass was determined by interpolating linear plots of log $_{Mw}$ of makers versus ratio of marker dye front.

Table 14. Gel electrophoresis composition of Upper and Lower Gel buffers

10% SDS stock	Upper Gel buffer pH 8.8	Lower Gel buffer pH 6.8
SDS (10%)	Tris base (0.,5 M)	Tris base (1.,5 M)
Tris (1 M)	H_2O_{dd} (400 ml)	H_2O_{dd} (400 ml)
EDTA (0.2 M)	10% SDS stock solution (0.4 %)	10% SDS stock solution (0.4 %)
	Adjust to pH 8.8 with HCl (12 M)	Adjust to pH 6.8 with HCl (12 M)
	Bring to (500 ml) with H_2O_{dd}	Bring to (500 ml) with H_2O_{dd}
	Store in dark	Store in dark

Table 15. Gel electrophoresis composition of Loading and Sampling buffer

Loading buffer pH 8.3	Sampler Buffer pH 6.8
SDS (1 %)	10% SDS stock solution (4 %)
Tris (0.25 M)	Tris (1 M)
Glycine (1.92 M)	Glycerine (20 %)
H_2O_{dd} (1 L)	EDTA (0.2 M)
	β-Mercaptoethanol (20 %)
	H_2O_{dd} (1 ml)
	Bromphenol Blue (0.1 %)

Table 16. Gel electrophoresis composition of Stacking and Resolving Gels

Stacking Gel		Resolving Gel	
Upper Gel buffer	2.5 ml	Upper Gel buffer	2.5 ml
2-D Stock Acrylamide	1.1 ml	2-D Stock Acrylamide	1.1 ml
Glycerine	1.5 ml	Glycerine	1.5 ml
H_2O_{dd}	6.3 ml	H_2O_{dd}	6.3 ml
Degas for 8 min		Degas for 15 min	
Ammonium persulphate	75 µl	Ammonium persulphate	400 µl
Temed	10 µl	Temed	20 µl

The following scheme gives an overview about the complete set of methods applied to purify *Pachyrhizus* GSTs (Fig. 8).

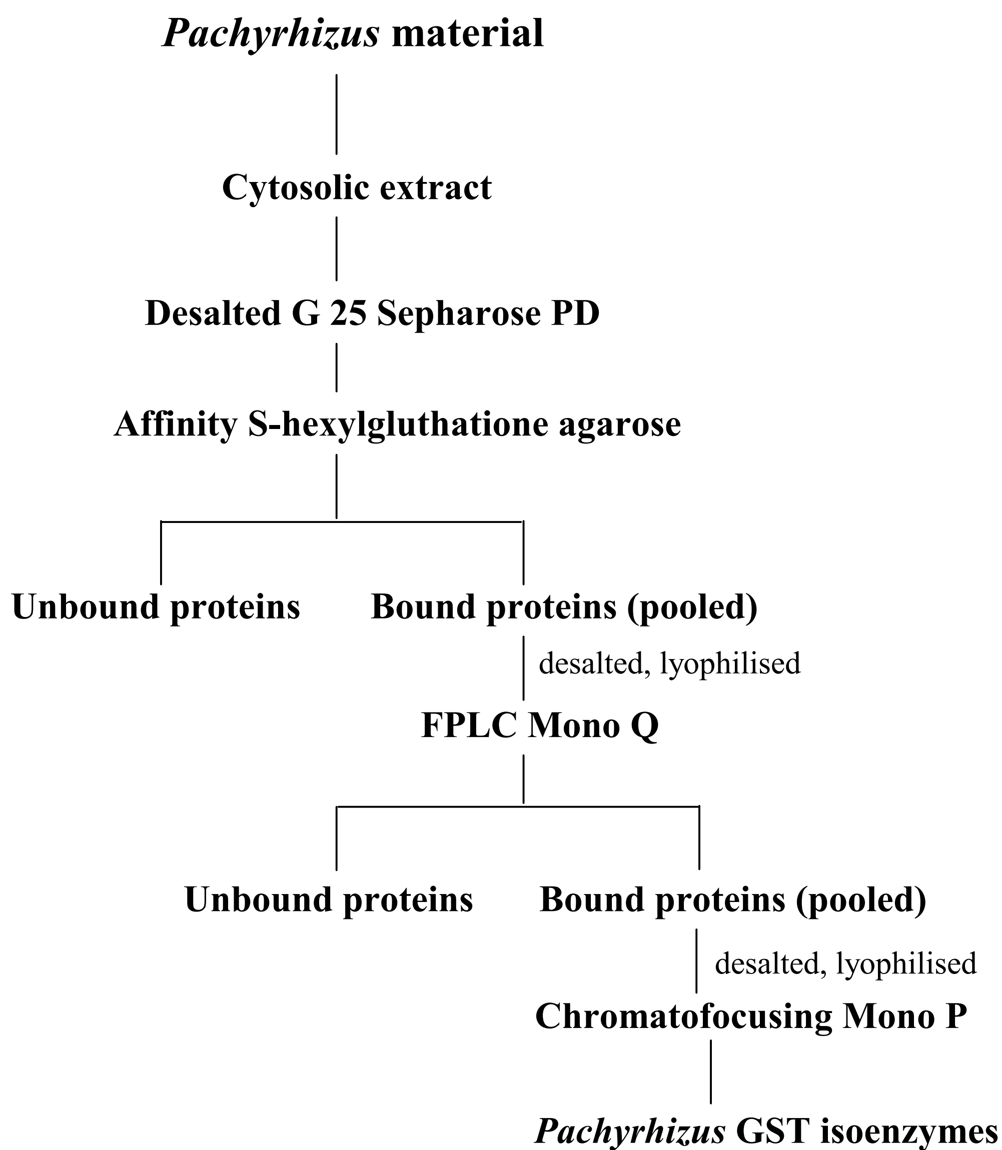


Figure 8. Schematic diagram for the isolation of GSTs isoenzymes from *Pachyrhizus,* Yam bean.

3. RESULTS

3.1. The Detoxification System

The general status of detoxification enzymes activity in the three-phase detoxification system was surveyed in *Pachyrhizus*. Purified protein extract from leaves and roots of *Pachyrhizus erosus* varieties EC 550 plants without any treatment were analysed for cytochrome P450 and peroxidase (POX) representative for detoxification phase 1 activity, glucosyltransferase (GT) and glutathione S-transferase (GST) for detoxification phase 2 activity and glucosidase for detoxification phase 3.

3.1.1. Detoxification Phase 1

Cytochrome P450 Activity

HPLC separation of radioactive labelled metabolites was applied to qualitatively confirm the metabolic activity of *Pachyrhizus* cytochrome P450 on ^{14}C isoproturon. Figures 9 compare HPLC chromatograms of non-enzymatic standard of ^{14}C isoproturon (taken as a reference) with that of *P. erosus* microsomal samples containing ^{14}C isoproturon - CYP activity. After 1 hour incubation of microsomal extracts with IPU, the reversed phase HPLC analysis resolved one major product peak and a second minor or trace peak in the enzyme assay (Figure 9).

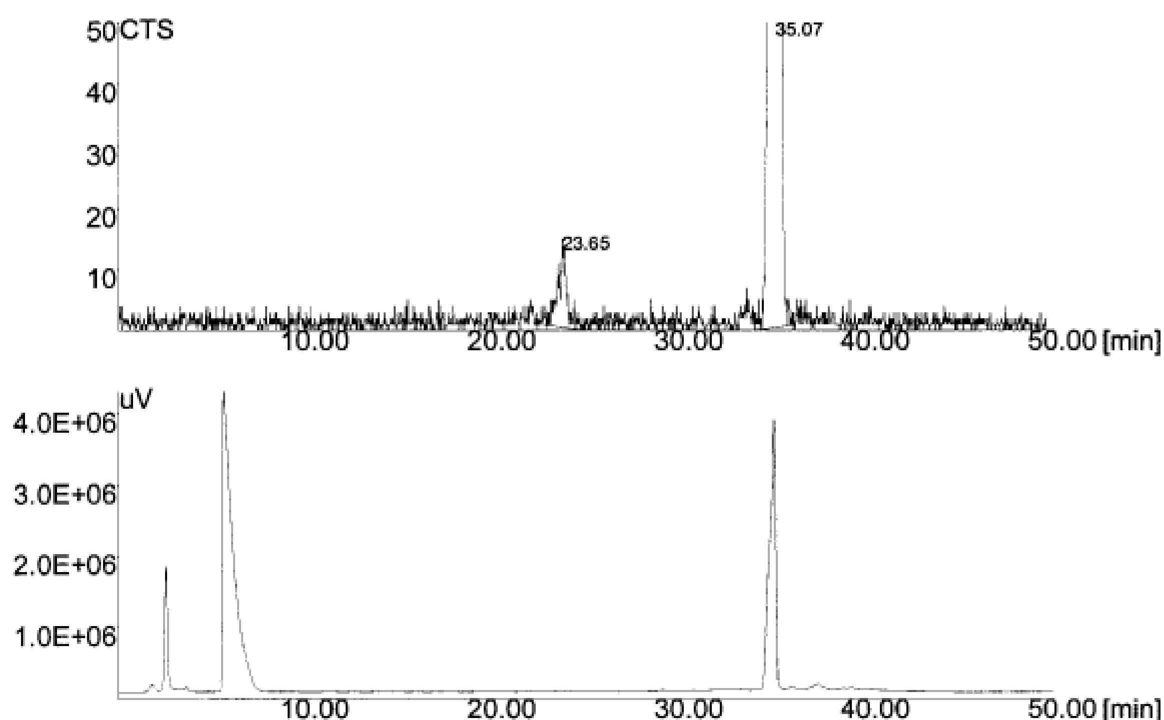


Figure 9. HPLC separation of ^{14}C Isoproturon metabolites from Cytochrome P450 metabolism

The major peak was identified to be 1-OH-isoproturon (4.1 %, 1OH-IPU) and the trace peak (1.4 %) was Monodesmethyl-isoproturon (Figure 10). The retention times (Rt) for the metabolites and substrate in the enzymatic radiolabel samples where comparable with that obtained for the standards. Enzymatic rates obtained for the conversion of IPU to the respective metabolites during the incubation with microsomal enzyme extract were found to be 9.13 ± 2 pkat mg $prot^{-1}$ for OH-IPU and 3.1 ± 1.3 pkat mg $prot^{-1}$ for Monodesmethyl-IPU.

The appearance of the second product peak, albeit as trace, suggests that either a second metabolite is produced by the same CYP or that more than one isoform of the enzyme is responsible for the detoxification process observed here.

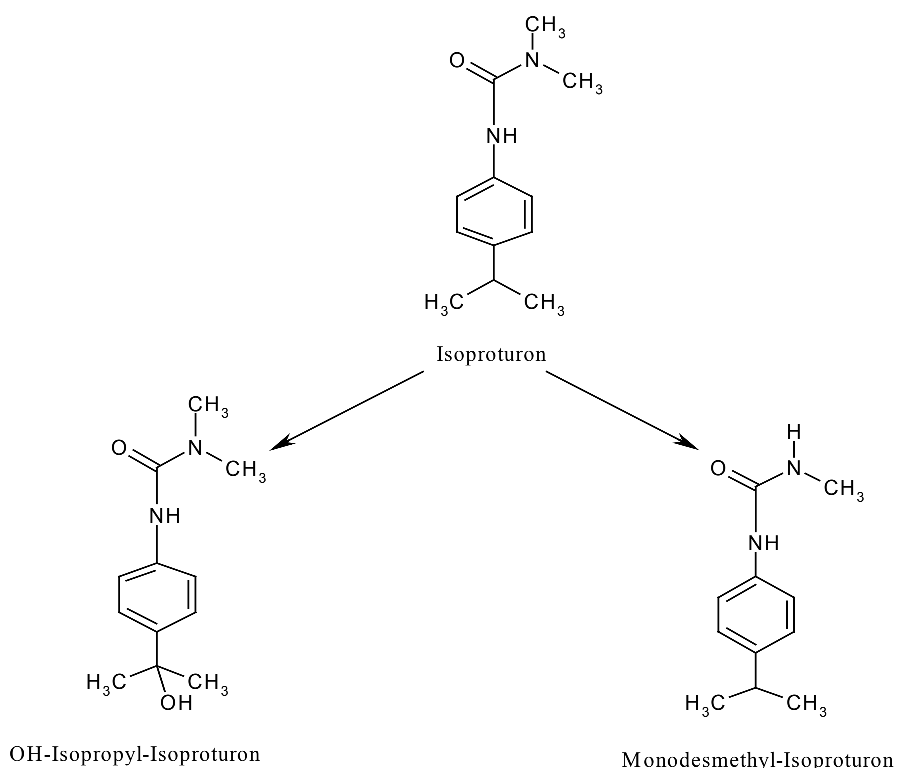


Figure 10. Molecular structures of the herbicide, Isoproturon, and its major metabolic products in Yam bean microsomal extracts after 1 hr of incubation.

Peroxidase Activities in *Pachyrhizus* Organs

Figure 11 represents activity of peroxidase activity in *Pachyrhizus erosus* tissues with guajacol as substrate. Activity ranges from 0.608 ± 0.094 µkat mg^{-1} in the seeds to 1.394 ± 0.047 µkat $mg-^{1}$ in roots. Peroxidase activity was 2-fold higher in the roots and tuber than in the leaves and seeds (Figure 11). Almost identical POX activity levels were observed between roots and tubers and between leaves and seeds.

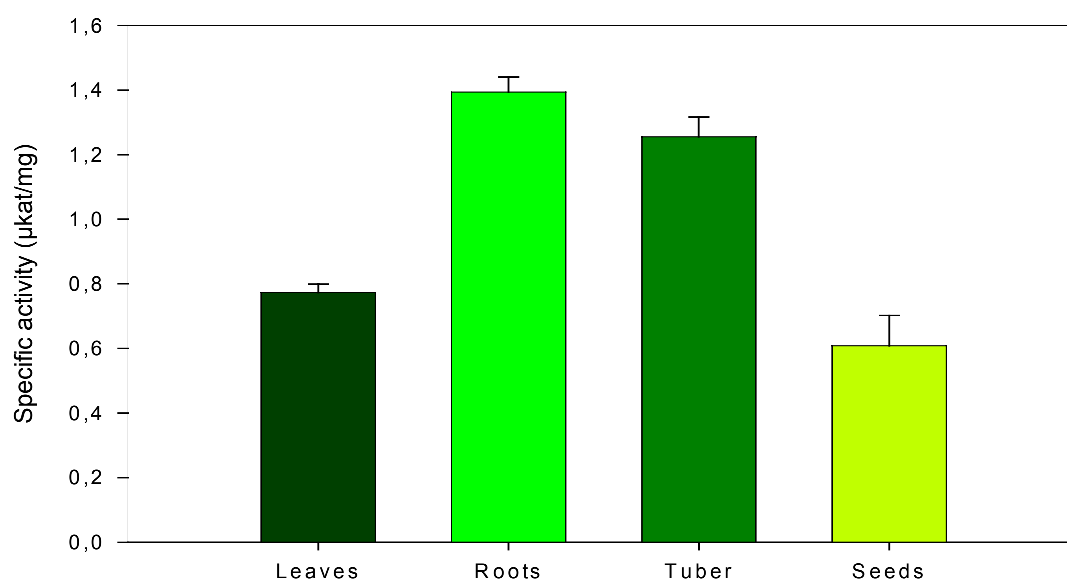


Figure 11. Activity of Peroxidase detoxification phase 1 enzymes in *Pachyrhizus* organs

3.1.2. Detoxification Phase 2

Glucosyltransferase Activities in *Pachyrhizus* Organs

Glucosyltransferase (GT) detoxification phase 2 enzyme activity for model substrate 2,4,5-trichlorophenol (TCP) conjugation was determined by HPLC separation of UDP-GT assays. Glucosyltransferase is an important detoxification step for substrates that have been hydroxylated or possess -OH, -NH or -SH functional groups.

Figure 12 compares HPLC chromatograms of non-enzymatic standard assay of TCP (taken as the reference) with that of samples containing TCP conjugation from UGT activity in tissues of *Pachyrhizus*. Samples used were *P. erosus* plant without any treatment. Whereas standard

control assay of only TCP (Figure 12e) exhibited one major peak, in contrast, addition of purified GT from samples resulted in the appearance of two peaks, parent and product peaks (Figures 12a, 12b, 12c and 12d).

The retention times (Rt) for the substrate in the samples where identical with that obtained for the reference, and product retention times similar in all enzymatic samples. The creation of the new product was also confirmed by the reduction in substrate peak area in the samples (Table. 17). The highest GT activity was obtained in the leaves at 0.58 nmol min^{-1} 12-fold higher than that in the roots. Activity was also high in the seeds as compared to the roots by 8-fold. There was no significant difference in GT activity between roots and tuber (Table. 17).

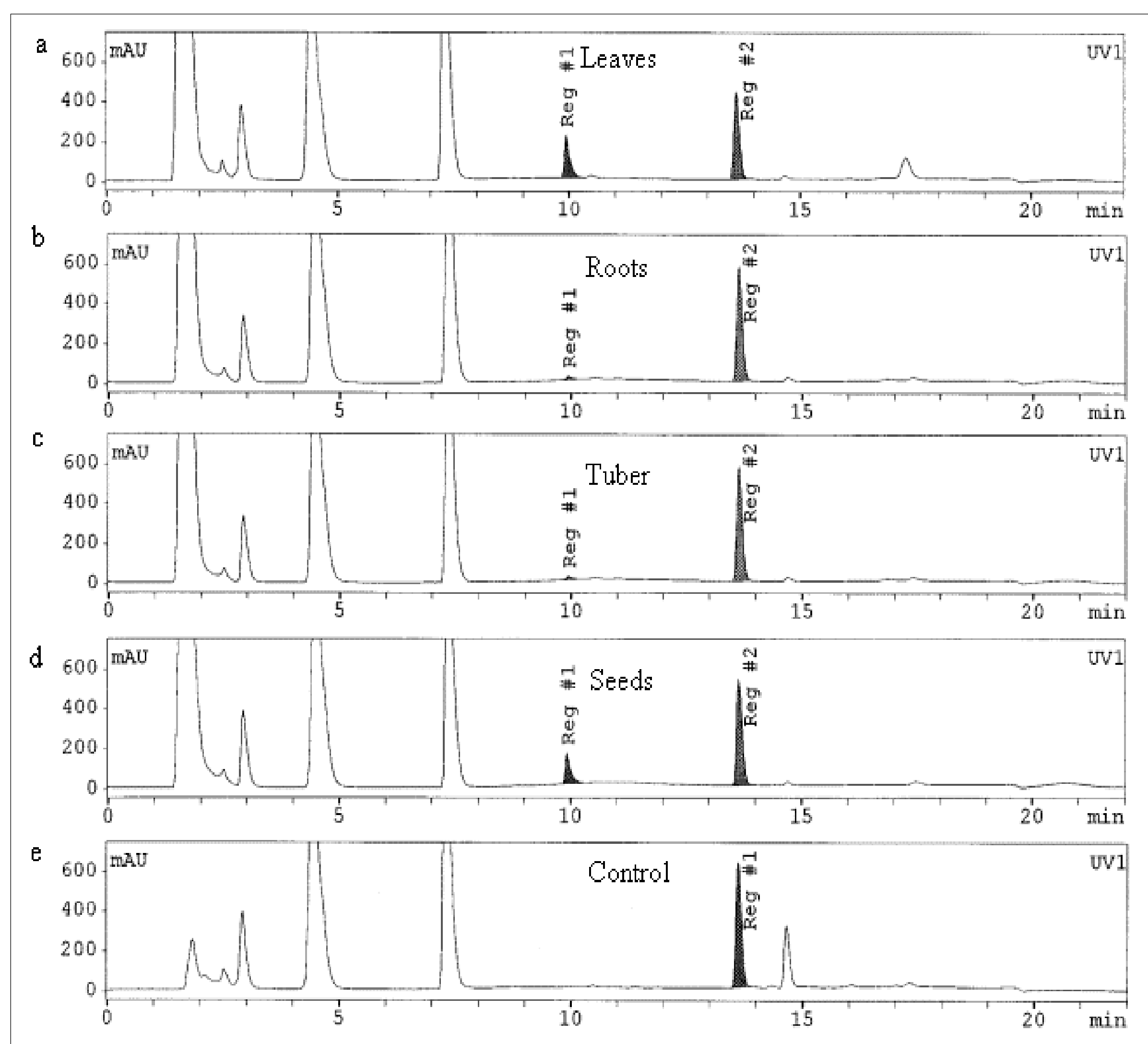


Figure 12. Expression patterns of HPLC separation of glucose conjugate from their aglycones in tissues of *Pachyrhizus* after GTs activity. (a) Leaves (b) roots (c) tuber (d) seeds and (e) control.

Table 17. Retention times and peak areas of HPLC separation of TCP conjugation in *P. erosus*

Fraction	Peak 1 = substrate		% reduction in	Peak 2 = product		Product
	R time	Peak area	peak area.	R time	Peak area	(nmol)
TCP	13.62	5046.77				
Leaves	13.62	3508.36	32.32	9.93	1675.72	0.58
Roots	13.63	4636.30	2.81	9.95	134.13	0.05
Tuber	13.65	4826.50	3.39	9.93	169.59	0.06
Seeds	13.65	4324.95	21.68	9.93	1197.40	0.39

3.1.3. Detoxification Phase 3

Glucosidase Activities in *Pachyrhizus* Organs

Activity of glucosidase, detoxification phase 3 enzyme, in *P. erosus* organs are represented in Figure 13. Glucosidase activity was significantly higher in the root at 27,26 ± 4,1 µkat $min^{-1}mg^{-1}$ than in leaves and seeds. There was no significant difference between leaves and seeds which have enzyme activity for the substrate at 19,42 ± 3,5 and 21,51 ± 2,83 µkat $min^{-1}mg^{-1}$ respectively.

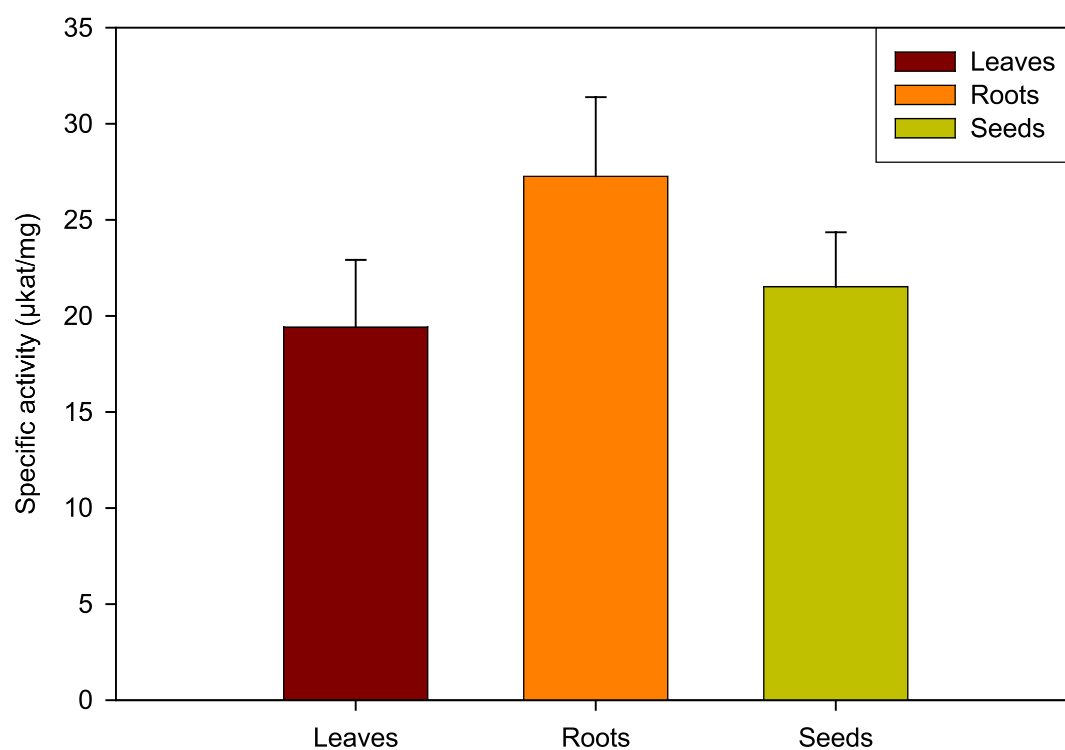


Figure 13. Activity of Glucosidase detoxification phase 3 enzymes in *Pachyrhizus* organs

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3.1.4. Glutathione Conjugating Activity and Thiol Content in *Pachyrhizus* Organs

Visualization of Microsomal Glutathione Conjugation

The formation of a fluorescent conjugate of monochlorobimane and GSH was followed microscopically in the microsomal leaf extract of *P. erosus* EC 550. Figure 14 shows the time course development of the GSH-MCB fluorescence conjugate. This is to our knowledge the first fluorescence image description of microsomal GST catalyzed reaction in a legume.

Fluorescence development begins immediately after incubation with just a tiny spot on a single microsome intensifying up to 16 minutes. At 20 minutes a shining fluorescence appeared in a large number of vesicles brightly expressed after 22 minutes.

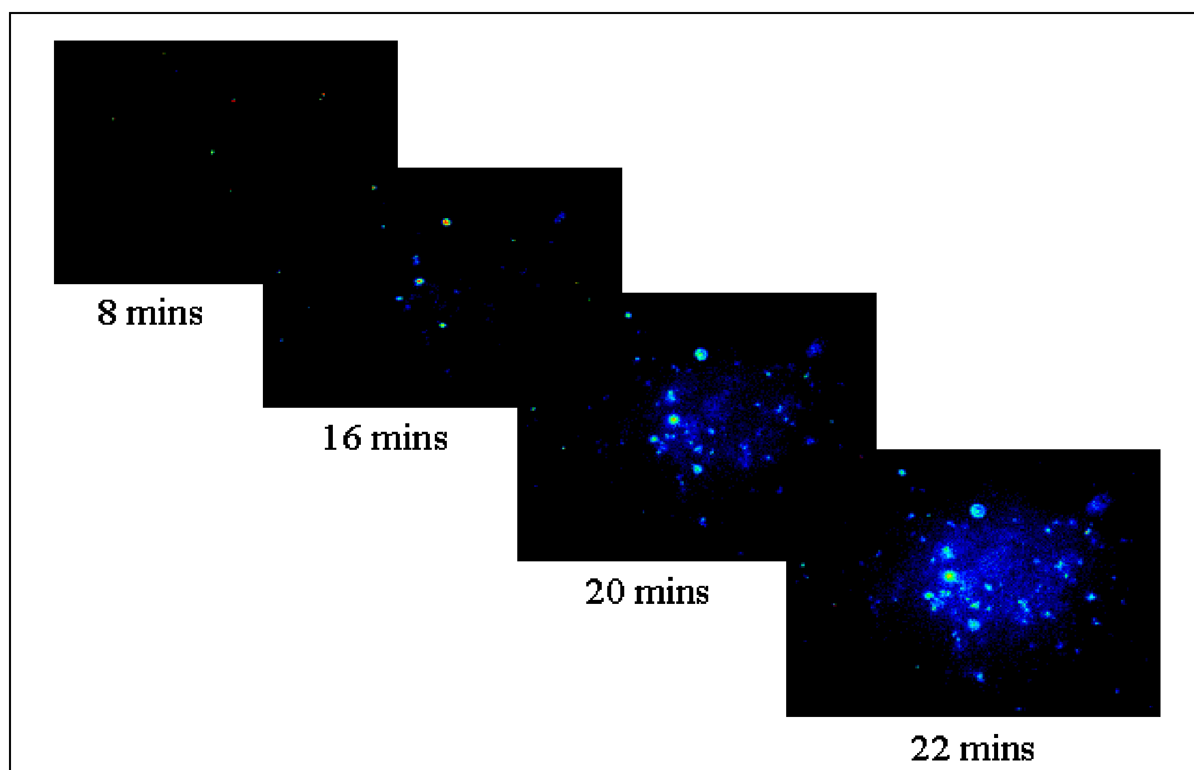

Figure 14. Novel finding: First fluorescence description of microsomal GST in a legume. Time course of fluorescence development of GSH-MCB conjugate as monitored microscopically in microsomes of yam bean, *Pachyrhizus erosus* EC 550.

Levels of Low Molecular Weight Thiols in *Pachyrhizus*

The content of low molecular weight thiols, glutathione GSH and homoglutathione hGSH were measured in *P. erosus* EC 550 control leaves and roots to confirm which was the dominant thiol for xenobiotic conjugation. Figure 15 shows a HPLC profile of eluting thiol and retention time. Pachyrhizus leaves contained on average 13-fold more GSH than hGSH and 2-fold more GSH than in the roots (Table 18). The level of GSH in the roots was also higher than of hGSH by 10.5-fold.

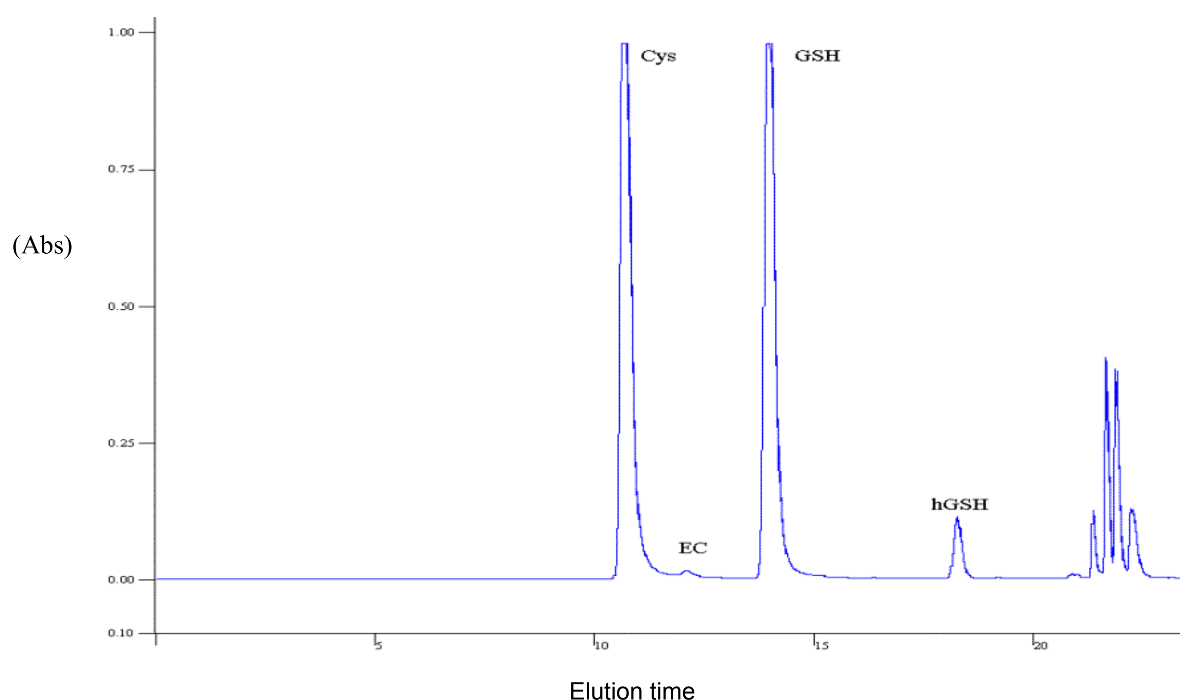

Figure 15. Representative HPLC profile of Bromobimane labelled low molecular weight thiols in cytosolic extracts of *Pachyrhizus*. Retention time were Cys: cysteine 10.6; EC: γ glutamylcysteine 12.1; GSH glutathione 13.9 and hGSH: homoglutathione 18.3 min. GSH is the major thiol content

Table 18. Content of low molecular weight thiol in *Pachyrhizus* leaves and roots

	mmol g^{-1} FW	
	GSH	**hGSH**
Leaves	0,39 ± 0,11	0,03 ± 0,005
Roots	0,21 ± 0,05	0,02 ± 0,003

3.2. Treatment of Plants under Environmental Stress Conditions

3.2.1. Analysis of Water Stress Measurements and Yield

Leaf Relative Water Content

The progression of drought was monitored by leaf relative water content (LRWC). Figure 16 shows changes in LRWC in stressed and control plants during the 21 days water stress period. LRWC varies between 61-75% in stress plants and between 72-78% in their controls. Significant differences in the LRWC were observed on the 6th day with a decrease of 15% in AC 102, 19% in EC 550 and 14% in TC 361 when compared with their controls. Differences between treatment and control were maintained until harvest.

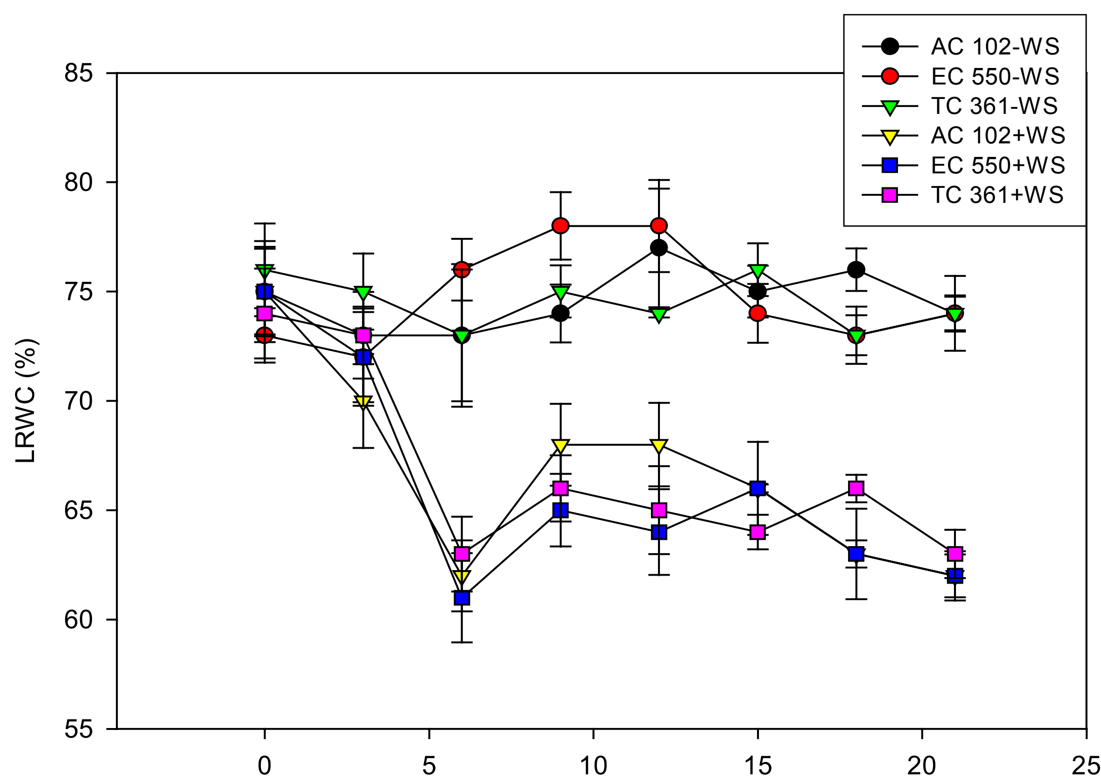

Figure 16. Changes in leaf relative water content during 21 days water stress period in *Pachyrhizus*. Significant difference was observed from 6th day until harvest. -WS = minus water stress (control plants) and +WS = plus water stress (treated/stressed plants).

Soil Moisture Content

For all three varieties significant differences were also observed in soil moisture content (SMC) in the pots of stressed plants (Figure 17). Significant differences were observed from the 6th day with a decrease of 15% in AC 102, 19% in EC 550 and 14% in TC 361 when compared with their controls. Final figures for SMC in stressed plants levelled out at 5.0, 5.8 and 6.8 % in AC 102, EC 550 and TC 361 respectively. Resulting decrease from their controls was 64% in AC 102, 55 % in EC 550 and 58% in TC 361.

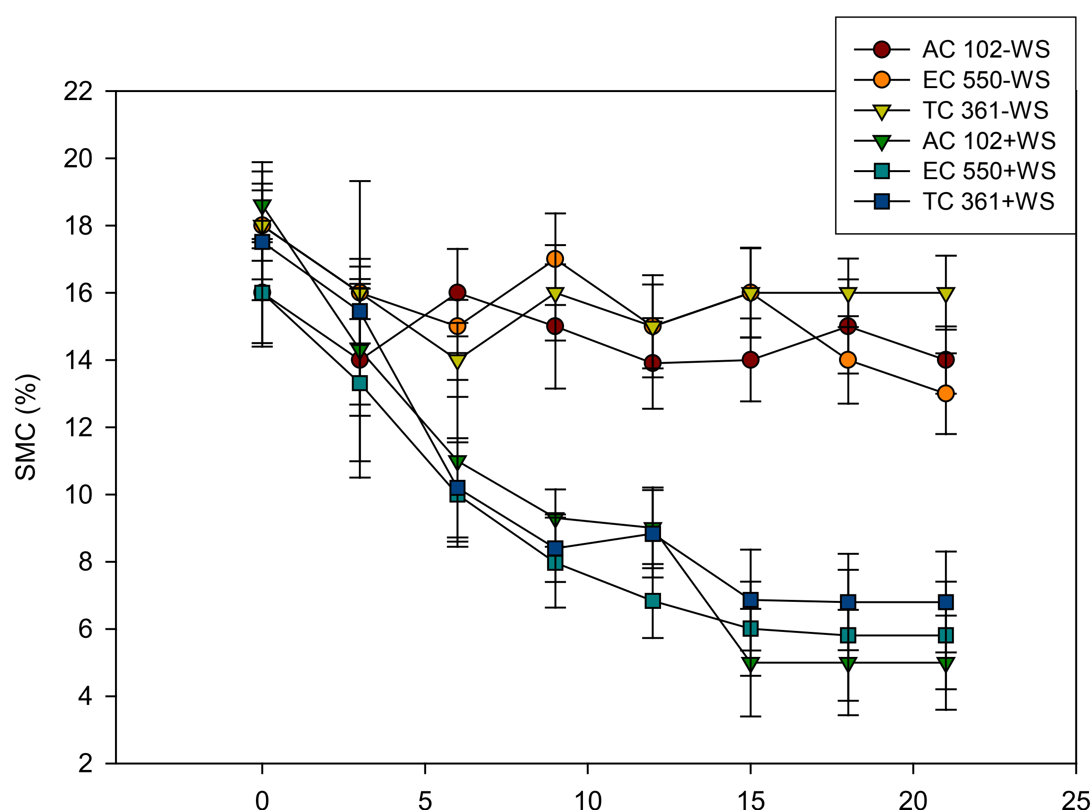

Figure 17. Changes in soil moisture content during 21 days water stress period in *Pachyrhizus*. Significant difference was observed from 6th day until harvest. -WS = minus water stress (control plants) and +WS = plus water stress (treated/stressed plants).

Tuber Yield

When tuber yields performance (tuber fresh weight) TFW of the three varieties *P. ahipa*, *P. erosus*, and *P. tuberosus* under environmental stress conditions were evaluated significant variations between the varieties were observed (Figure 18).

Fresh tuber yield ranged from 136 g/plant recorded for O_3 treated *P. tuberous* TC 361 to 455 g/plant for CO_2 treated *P. erosus* EC 550. Elevated level of CO_2 increase tuber yield in all three varieties whilst O_3 exposure caused significant reduction by 36, 33 and 31 % in *P. ahipa* AC 102, *P.erosus* EC 550 and *P. tuberous* TC 361 respectively. Under water stress conditions there was no significant difference in tuber yield in TC 361 as compared to the control. However, in EC 550 and AC 102 tuber yield was reduced by 8 and 22 % respectively.

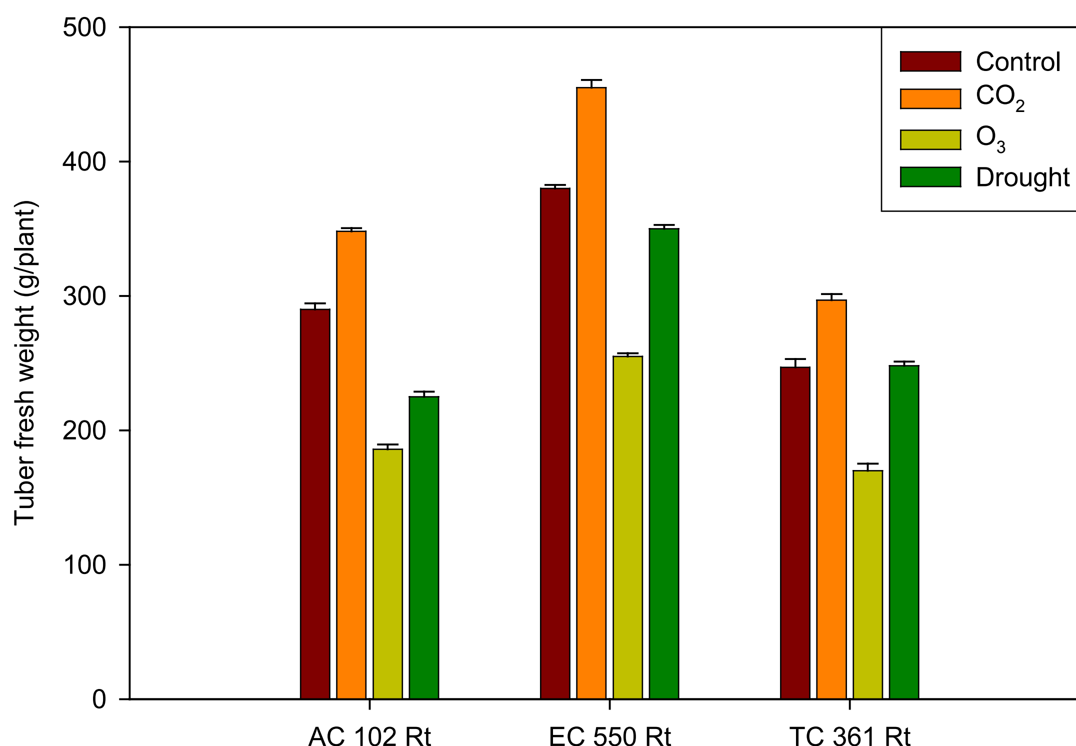

Figure 18. Effect of CO_2, O_3, and water stress on tuber yield in three varieties of *Pachyrhizus*

Germination

Percentage germination and survival rate of seeds from all three accessions of *Pachyrhizus* was satisfactory. Over 90% was achieved under environmental conditions whilst seeds, which were treated with pesticides, achieved over 98% percentage germination. The treatment of the seeds might have been a contributing factor in increasing germination potential and maintaining survival rate.

Morphological Appearance of Plants

Environmental stress conditions of elevated CO_2 (770 ppm) and O_3 (200 ppb) applied on *Pachyrhizus* plants for the isolation and purification of GST enzyme induced changes in morphology and yield over the period of exposure.

Visual estimate of the effects of CO_2 on Pachyrhizus leaves demonstrated that 700 ppm CO_2 caused bleaching after prolonged exposure (8 weeks) leaving them with a yellow appearance (Figure 20a and 20b). This could be attributed to the breakdown of the chlorophyll. Ambient air control plants did not show any damage or alterations (Figure 20e and 20f).

Stress symptoms of ozone exposure were apparent in older leaves. Foliar injuries of red dry lesions were visible on both the abaxial and adaxial surfaces in matured leaves within the second week of the experimental treatment (Figure 20c and 20d). Besides injuries of red dry lesions, symptoms of premature senescence were observed in oldest leaves. Ambient air control plants did not show lesion development or symptoms of premature senescence (Figure 20e and 20f).

Concentration of Leaf Chlorophyll Pigments

To assess the big differences in leaf appearance between treatments associated with chlorophyll depletion, the concentrations of chlorophyll pigments in the leaves were determined. Table 18 shows the concentration of *P. erosus* leaf pigments under environmental stress conditions of CO_2, O_3 and water stress. Results obtained revealed that environmental stress affects chlorophyll contents.

The concentration of chlorophyll *a*, chlorophyll *b*, and total chlorophyll were reduced approximately by 64, 32 and 49% under elevated levels of CO_2, O_3 and water stress conditions respectively. As observed visually the effect was more severe under elevated CO_2 where the concentration of both chlorophyll *a* and chlorophyll *b* were three times reduced. This difference was reflected in the absorption spectra obtained for the pigments (Figure 19).

Leaves from CO_2 treatment produce a chlorophyll *a*/*b* ratio of 3.34, which is within the *a*/*b* ratio range of 3.2 to 4, for highlight chloroplasts and sun-exposed plants (Lichtenthaler, 1987). The chlorophyll ratio for O_3 treated leaves was at neutral level of 3. Chlorophyll ratios

for control and water stress leaves were 2.69 and 2.54, respectively and well in the ratio range of 2.5 to 2.9 for low-light chloroplasts and shade plants.

The strongest effect of environmental stress on total carotenoids concentrations was also observed in CO_2 treated leaves with 60% reduction. The loss of carotenoids was more moderate in drought leaves at 36% and low in O_3 treated leaves at only 10%.

Table 19. Concentration of *P. erosus* leaf pigments (mg/ml) under environmental stress conditions

Pigment	Control	CO_2	Drought	Ozone
Chlorophyll a	11,6	4,4	5,8	8,2
Chlorophyll *b*	4,3	1,3	2,3	2,7
Total Chlorophyll	15,9	5,7	8,1	10,9
Carotenoids	800,6	322,5	510,6	720,3
Chlorophyll *a*/*b*	2,7	3,3	2,5	2,9

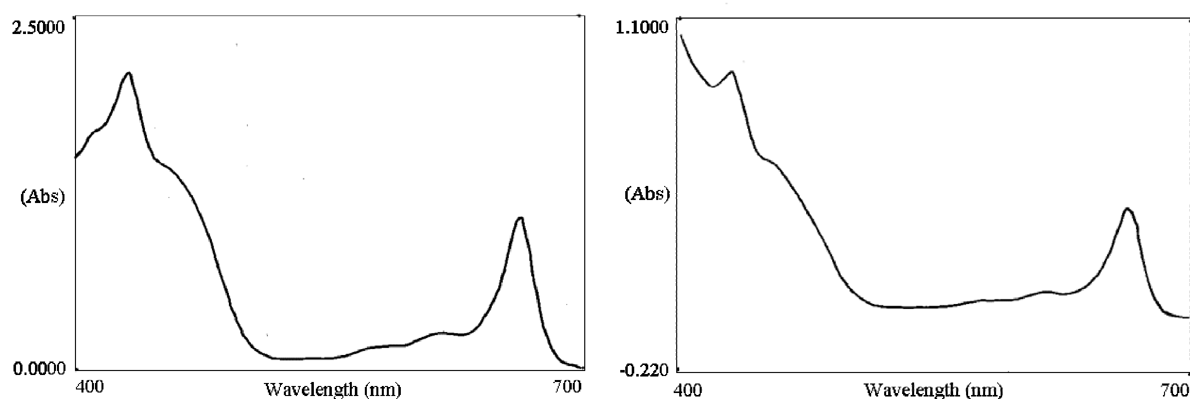

Figure 19. Absorption spectrum for freshly isolated chlorophyll pigments from *Pachyrhizus* plants in (80%) aqueous acetone (a) control plants under ambient air (b) plants exposed to elevated level (700ppm) of CO_2

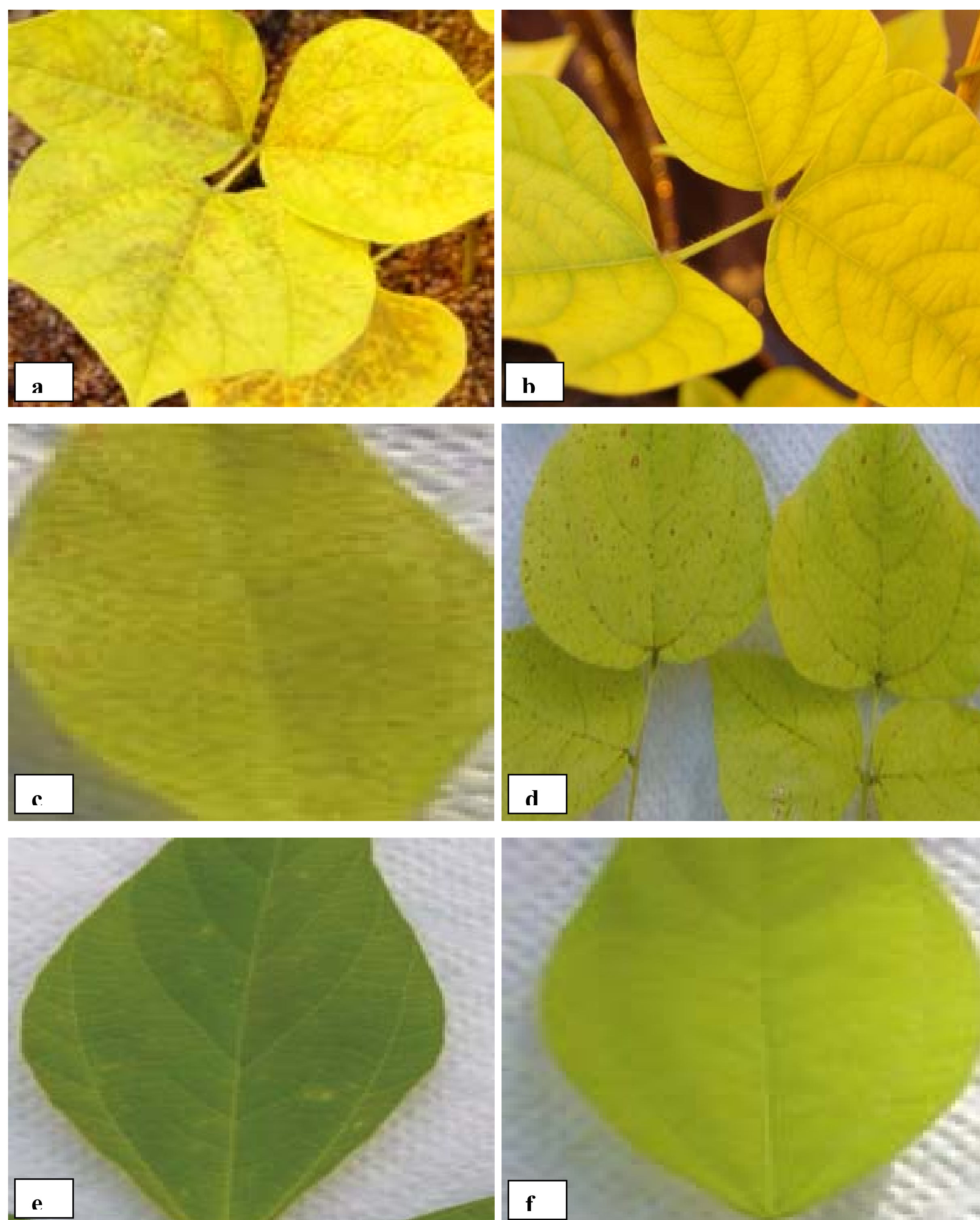

Figure 20. Visual symptoms of leaf injury in *Pachyrhizus* plants under environmental stress conditions of elevated levels of CO_2 (770 ppm) and Ozone (200 ppb). Fig. (a and b) Bleaching effect of CO_2: yellowing of leaves Fig. (c and d) Ozone induced injury: red lesions on leaves. Fig. (e and f) Leaves from control plants maintained in ambient air.

3.2.2. Analysis of *Pachyrhizus* Cytosolic and Microsomal GST Activities under Environmental Stress Conditions

Results obtained from each environmental stress condition applied to *Pachyrhizus* plants are shown in Tables 19 and 20. GST catalyzed activities toward model substrate CDNB was analysed for glutathione conjugate in both cytosolic and mirosomal crude protein extracts of all three varieties of *Pachyrhizus*; *P. ahipa*, *P. erosus* and *P. tuberous* investigated.

Activity was highly variable ranging from 0.3 ± 0,04 nkat/g in microsomal root extract of AC 102 to 1.5 ± 0,04 µkat/g in cytosolic extract of O_3 treated leaves in all three varieties (Tables 19). The high variability was associated to the large difference in cytosol GST (cGST) activity and that of the microsomes, sub cellular or membrane bound organelles (microsomal GST, mGST).

Table 20. Cytosolic and microsomal GST activity (µkat/g) foc CDNB in leaves (Lf) and roots (Rt) of control and stressed *Pachyrhizus* plants. (AC 102 = *P. ahipa*, EC 550 = *P. erosus*, TC 361 = *P. tuberous*). Results are expressed as means ± SD. nd = not detectable.

Variety	Organ	Control	CO_2	O_3	Drought
AC 102	Cytosol Leaves	0,468 ± 0,03	0,974 ± 0,01	1,529 ± 0,00	0,115 ± 0,08
	Microsomal Lf	0,037 ± 0,02	0,062 ± 0,00	0,013 ± 0,05	nd
	Cytosol Roots	0,045 ± 0,01	0,471 ± 0,01	0,166 ± 0,01	0,029 ± 0,00
	Microsomal Rt	0,003 ± 0,04	0,039 ± 0,01	0,038 ± 0,03	nd
EC 550	Cytosol Leaves	0,953 ± 0,01	1,140 ± 0,01	1,498 ± 0,00	0,359 ± 0,00
	Microsomal Lf	0,015 ± 0,05	0,019 ± 0,14	0,058 ± 0,01	nd
	Cytosol Roots	0,419 ± 0,00	0,275 ± 0,01	0,142 ± 0,01	0,057 ± 0,00
	Microsomal Rt	0,013 ± 0,01	nd	0,020 ± 0,03	nd
TC 361	Cytosol Leaves	0,791 ± 0,00	0,876 ± 0,02	1,530 ± 0,04	1,183 ± 0,01
	Microsomal Lf	0,006 ± 0,00	0,065 ± 0,02	0,708 ± 0,09	nd
	Cytosol Roots	0,197 ± 0,00	0,399 ± 0,01	0,626 ± 0,11	0,293 ± 0,01
	Microsomal Rt	0,014 ± 0,03	nd	0,006 ± 0,05	0,020 ± 0,03

Generally, GST activity for the conjugation of CDNB in the cytosol was far greater than that in the microsomal extract (Figure 21). Under control conditions, activities in the leaf cytosol were 13, 64 and 132 fold higher than in the leaves microsomal, where as in the roots cytosol they were 15, 32 and 14 fold higher than in the roots microsomal in AC 102, EC 550 and TC 361 respectively. Similarly large differences between cytosol and bound membranes in GST activities were also observed under stress conditions (Table 20).

Table 21. Cytosolic and microsomal GST activity (µkat/g) for CDNB in leaves (Lf) and roots (Rt) of stressed *Pachyrhizus* plants expressed as number of times greater (Fold increase) and as percentage of the activity present in control plants (=100%). (AC 102 = *P. ahipa*, EC 550 = *P. erosus*, TC 361 = *P. tuberous*).

Variety	Organ	CO_2		O_3		Drought	
		Fold increase	% Increase	Fold increase	% Increase	Fold increase	% Increase
AC 102	Cytosol Leaves	2,1	208	3,3	327	0,2	25
	Microsomal Lf	1,7	166	0,4	36	nd	nd
	Cytosol Roots	10,5	1047	3,7	369	0,6	64
	Microsomal Rt	13,2	1323	12,6	1257	nd	nd
EC 550	Cytosol Leaves	1,2	120	1,6	157	0,4	38
	Microsomal Lf	1,3	125	3,9	387	nd	nd
	Cytosol Roots	0,7	66	0,3	34	0,1	14
	Microsomal Rt	nd	nd	1,6	155	nd	nd
TC 361	Cytosol Leaves	1,1	111	1,9	193	1,5	150
	Microsomal Lf	10,8	1080	118	11797	nd	nd
	Cytosol Roots	2	202	3,2	318	1,5	150
	Microsomal Rt	nd	nd	0,4	40	1,4	139

nd = not detectable
Note that (µkat/g) refers to total activity of GST with CDNB, -fold refers to -fold purification of the enzyme, and % is an abbreviation for percent yield.

In the cytosol, compared between plant organs, leaves and roots, total GST activity is greater in the leaves than in roots. Leaf cytosolic activities were 10, 2 and 4 fold higher than those in the roots under control conditions in AC 102, EC 550 and TC 361 respectively. In contrast activities at the microsomal level between leaves and roots were mostly equal (Figure 21).

Elevated levels of CO_2 and O_3 enhanced GST activity in both leaves and roots cytosol developing a linear increase in the leaves from control to ozone treatment (Figure 21). However, this pattern was changed in *P. erosus* EC 550 cytosol roots, which shows a general linear decline with stress treatment (Figure 21b).

Under CO_2 exposure leaves cytosolic activities increase by 208%, 120% and 111% in AC 102, EC 550 and TC 361 respectively. In the roots cytosol it was enhanced 11 fold in AC 102 and 2 fold TC 361 (Table 20). In EC 550 the effect was somehow concealed at 66%. At the microsomal level mGST activity after CO_2 exposure was strongly enhanced in the leaves of all three varieties and roots of *P. ahipa* AC 102. However, in the roots of EC 550 and TC 361 it was not detectable (Figure 21b).

The highest activities under environmental stress conditions were achieved in cytosolic extracts of 200 ppb O_3 treated leaves at 1.5µkat/g, 2-3 fold higher than the control. Although activities were generally low in sub cellular organelles the highest mGST activity from this extract was also ozone induced in *P. tuberous* TC 361 at 0.71µkat/g, 118 fold higher than the control.

In contrast, to the inductive rates of GST activities obtained with CO_2 and O_3 exposures, prolonged dehydration, induced drought generally reduced GST activity in the cytosol except in *P. tuberous* TC 361. In the leaves and roots cytosolic crude extracts of *P. ahipa* AC 102 and *P. erosus* EC 550 activity of cGST was reduced substantially under drought conditions to 75% and 62% in leaves, and at 36% and 86% in roots respectively, of the corresponding activity in well-watered plants (Table 20).

Under drought condition membrane bound activity (mGST) was consistently not detectable in roots except in TC 361 microsomal roots. As shown Table 19, TC 361 exhibited cGST activities toward CDNB 150% (1.5-fold) higher in both cytosolic leaves and roots as compared to control plants. Drought induced TC 361 mGST activity in bound membranes was 139% (1.4 fold) higher than that of the control.

Between varieties and at the cytosolic level GST activity was higher in EC 550 leaves and roots under control conditions as compared to the other varieties (Figure 22a). Varietal differences in GST activities under CO_2 exposure were negligible or equal (Figure 22b). Similarly, cGST leaves activities for all three varieties exposed to 200 ppb ozone were equal whilst in the leaves microsomes and roots cytosol they were greater in TC 361 than those of AC 102 and EC 550 (Figure 22c). Under drought conditions cGST activities in *P. tuberous* TC 361 were 10 and 3 fold higher in leaves cytosol whilst in the roots cytosol they are 10 and 5 fold higher than in AC 102 and EC 550 respectively (Figure 22c).

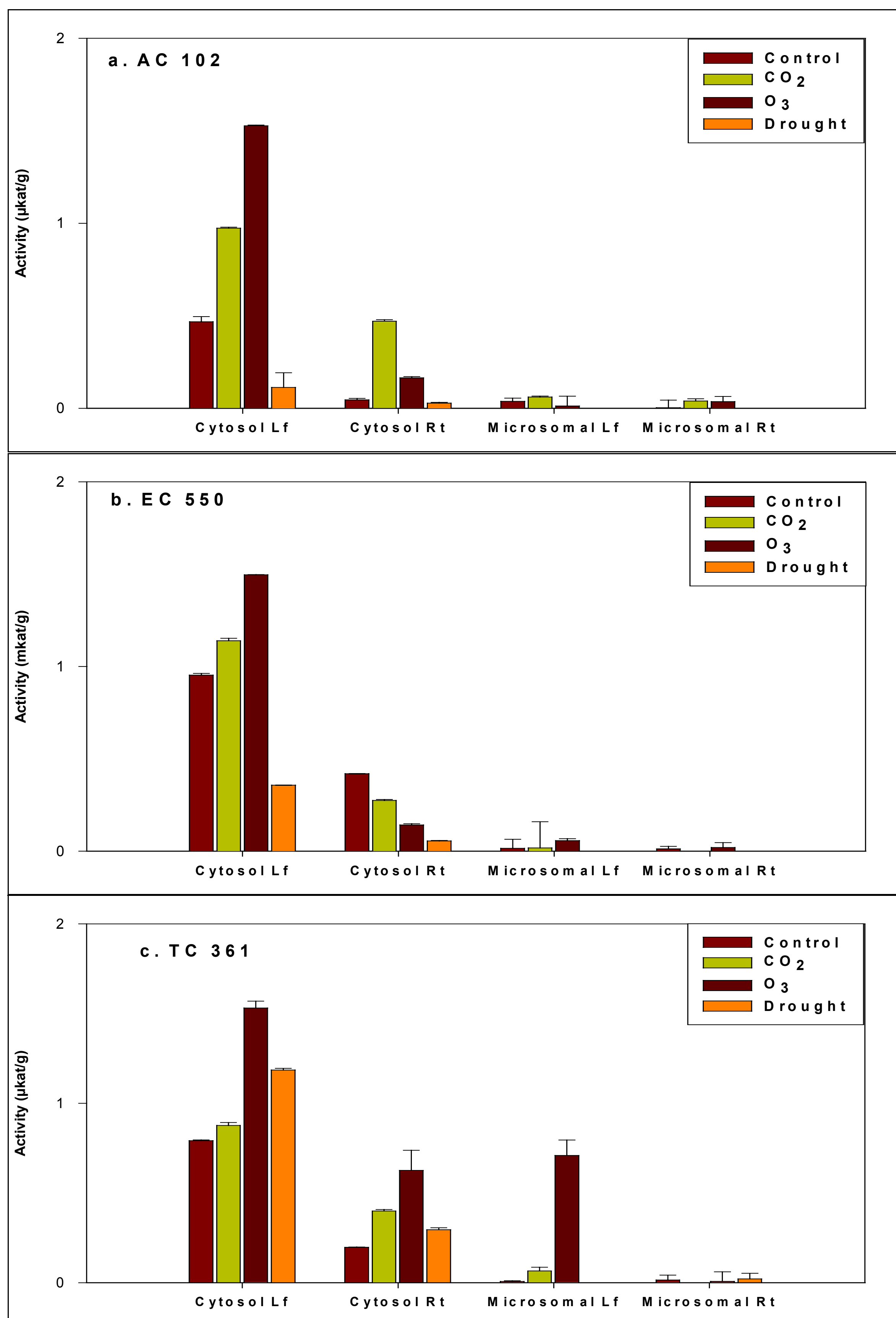

Figure 21. Cytosolic and microsomal GST activity (µkat/g) in control and stressed plants of *Pachyrhizus*. (a) *P. ahipa* AC 102 (b) *P. erosus* EC 550 (c) *P. tuberous* TC 361.

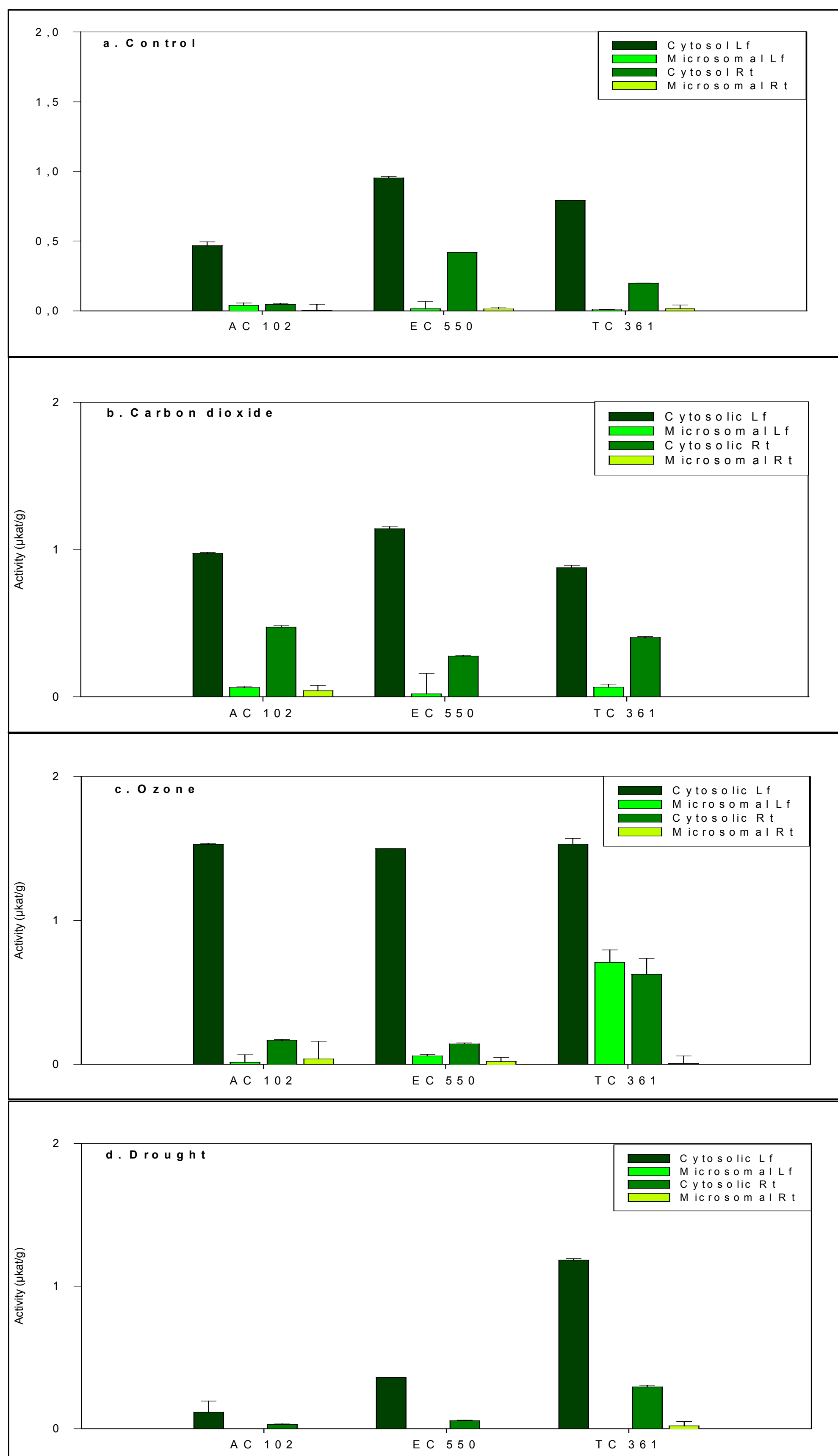

Figure 22. Cytosolic and microsomal GST activity (µkat/g) compared between *Pachyrhizus* varieties in (a) Control (b) CO_2 (c) O3 (d) Drought treated plants.

Derived equations showing the relationship between *Pachyrhizus* varieties on the level of total GST activity induced as a result of environmental stress conditions is summarized in Table 22.

Table 22. Level of GST activity (µkat/g) as induced by environmental stress conditions in three varieties of *Pachyrhizus*; *P. erosus*, EC 550, *P. ahipa* AC 102 and *P. tuberous* TC 361.

Control leaves cytosol	**Control roots cytosol**
EC 550 > TC 361 > AC 102	EC 550 > TC 361 > AC 102
Control leaves microsomal	**Control roots microsomal**
AC 102 ≥ EC 550 > TC 361	EC 550 = TC 361 > AC 102
CO_2 leaves cytosol	**CO_2 roots cytosol**
EC 550 > AC 102 ≥ TC 361	AC 102 > EC 550 ≥ TC 361
CO_2 leaves microsomal	**CO_2 roots microsomal**
AC 102 ≥ EC 550 ≥ TC 361	AC 102 > EC 550 = TC 361
O_3 leaves cytosol	**O_3 roots cytosol**
EC 550 = TC 361 = AC 102	TC 361> AC 102 ≥ EC 550
O_3 leaves microsomal	**O_3 roots microsomal**
TC 361> EC 550 > AC 102	AC 102 > EC 550 > TC 361
Drought leaves cytosol	**Drought roots cytosol**
TC 361> EC 550 > AC 102	TC 361> EC 550 > AC 102
Drought leaves microsomal	**Drought roots microsomal**
TC 361= EC 550 = AC 102	TC 361> EC 550 = AC 102

From the equations derived above a pattern of varietal selectively for GST activity seem to evolve. It is observed that in control plants and generally under conditions of elevated CO_2 EC 550 has the dominant GST activity. AC 102 is next followed by TC 361. Under conditions of elevated O_3 and drought the pattern is completely changed. TC 361 has the dominant GST activity followed by EC 550, whereas AC 102 tends to have lowest activities in all investigated cases.

3.2.3. Analysis of *Pachyrhizus* Cytosolic GST Activities after Pesticide Treatment

Cytosolic GST Activities

Pachyrhizus is as a rule propagated by its seeds. Therefore the most serious threat for its survival and continued cultivation are those related to pest attack i.e. fungi and insects on the seeds. Treatment of *Pachyrhizus* seed with pesticides; Captan (fungicide), Fenoxaprop (herbicide) and Imidacloprid (insecticide) did not affect germination or result in any visual stress symptoms during incubation or growth.

Analysis for the induction of GST activity towards CDNB by three selected pesticides has been examined in the three cultivated *Pachyrhizus* varieties; *P. ahipa*, *P. erosus* and *P. tuberous* (Table 22, Figure 23).

Table 23. Cytosolic GST activity (µkat/g) in leaves (Lf) and roots (Rt) of control and pesticide treated *Pachyrhizus* plants (AC 102 =*P. ahipa*, EC 550 = *P. erosus*, TC 361 = *P. tuberous*). Results are expressed as means ± SD.

Variety	Organ	Control	Captan	Fenoxaprop	Imidacloprid
AC 102	Cytosol Lf	1,824 ± 0,007	1,195 ± 0,013	1,505 ± 0,005	1,222 ± 0,008
AC 102	Cytosol Rt	0,184 ± 0,013	1,033 ± 0,006	0,585 ± 0,002	0,642 ± 0,008
EC 550	Cytosol Lf	2,278 ± 0,008	1,518 ± 0,014	2,181 ± 0,017	1,787 ± 0,014
EC 550	Cytosol Rt	0,367 ± 0,017	3,831 ± 0,007	0,415 ± 0,010	1,158 ± 0,004
TC 361	Cytosol Lf	1,206 ± 0,019	1,637 ± 0,017	1,401 ± 0,006	1,938 ± 0,010
TC 361	Cytosol Rt	0,143 ± 0,020	0,534 ± 0,001	0,032 ± 0,089	0,802 ± 0,008

Cytosolic GST activity was present in all varieties and organs. Activity varied from 0.03µkat/g to 3.8µkat/g and was generally greater in leaves than in roots except in EC 550 captan treated roots were the highest activity was achieved at 3.8µkat/g, reaching a 10-fold increase compared to control plants (Table 22).

There were marked varietal differences in GST activities in the leaves and roots (Figure 23). In *P. ahipa* AC 102 and *P. erosus* EC 550 leaves GST activity was reduced or unchanged for all pesticide treatments applied (Figure 23a and 23b). In contrast to the effects in TC 361 GST activity were enhanced by 136%, 116% and 161% for Captan, Fenoxaprop and Imidacloprid respectively (Table 23, Figure 23c).

The situation in the roots was significantly different to that of the leaves in that though GST activity was generally enhanced by all treatments however, with fenoxaprop treatment activity in EC 550 was unchanged and in TC 361 substantially reduced by 88% (Table 24).

The significant increase of GST activity in EC 550 captan treated roots specifically stands out (Figure 24) and is also 2.5-fold higher as compared to the highest activity obtained under environmental stress conditions.

Table 24. Cytosolic and microsomal GST activity in leaves (Lf) and roots (Rt) of stressed *Pachyrhizus* plants expressed as number of times greater (Fold increase) and as percentage of the activity present in control plants (=100%). (AC 102 = *P. ahipa*, EC 550 = *P. erosus*, TC 361 = *P. tuberous*).

Variety	Organ	Captan		Fenoxaprop		Imidacloprid	
		Fold increase	% Increase	Fold increase	% Increase	Fold increase	% Increase
AC 102	Cytosol Lf	0,7	66	0,8	83	0,7	67
AC 102	Cytosol Rt	5,6	560	3,2	317	3,5	348
EC 550	Cytosol Lf	0,7	67	1,0	96	0,8	78
EC 550	Cytosol Rt	10,4	1045	1,1	113	3,2	316
TC 361	Cytosol Lf	1,4	136	1,2	116	1,6	161
TC 361	Cytosol Rt	3,7	373	0,2	22	5,6	561

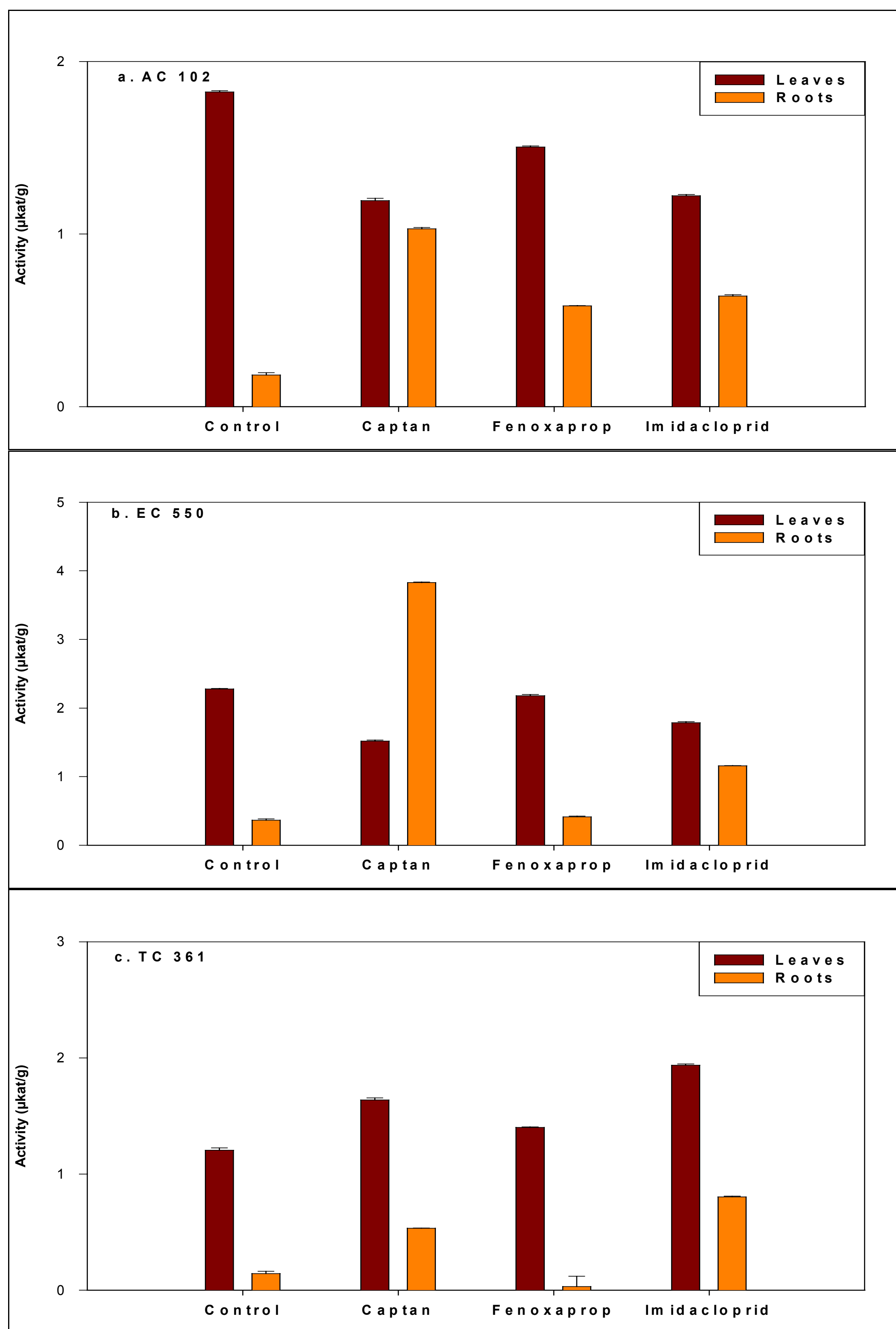

Figure 23. Cytosolic GST activity (µkat/g) in control and pesticide treated plants of *Pachyrhizus*. (a) *P. ahipa* AC 102 (b) *P. erosus* EC 550 (c) *P. tuberous* TC 361.

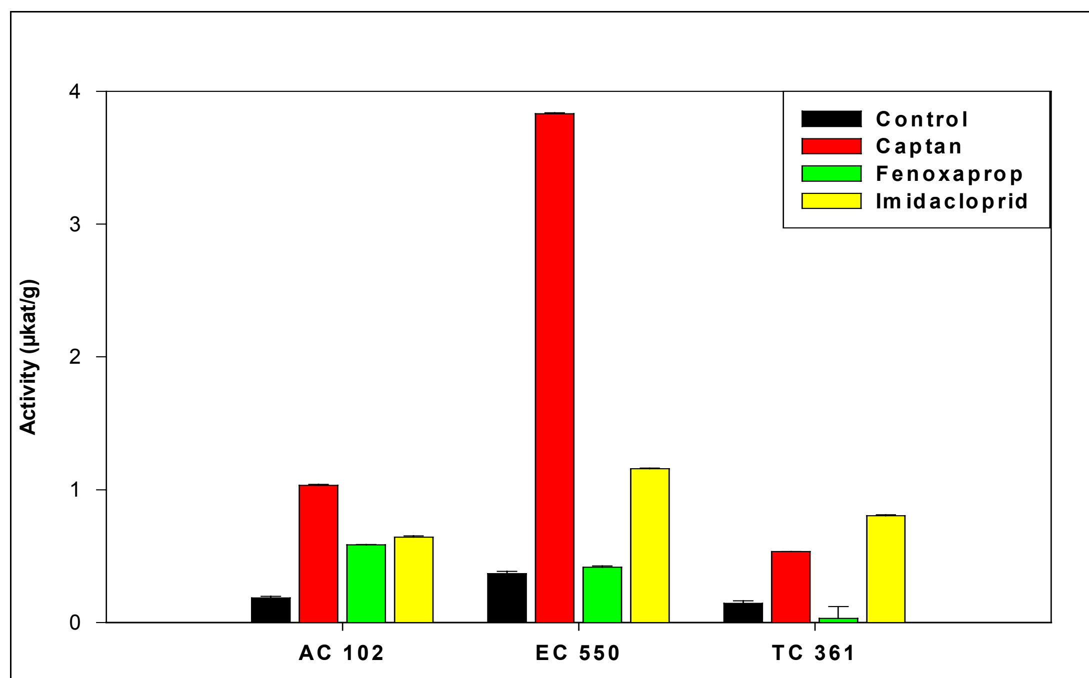

Figure 24. Cytosolic root GST activity (µkat/g) compared between *Pachyrhizus* varieties in control and pesticide treated plants.

The most prominent effect is seen with Captan treatment. Strongest increase of GST activity is again found in variety EC550. With a view to a possible denaturation of GST, the observed low residual activity in TC 361 after treatment with Fenoxaprop is also of high importance.

Derived equations showing the relationship between *Pachyrhizus* varieties on the level of total GST activity induced as a result of pesticide treatments is summarized in Table 25.

Table 25. Level of Cytosolic GST activity (µkat/g) as induced by pesticide treatment in three varieties of *Pachyrhizus*; *P. erosus*, EC 550, *P. ahipa* AC 102 and *P. tuberous* TC 361.

Control leaves	**Control roots**
EC 550 > AC 102 > TC 361	EC 550 > AC 102 > TC 361
Captan leaves	**Captan roots**
TC 361> EC 550 > AC 102	EC 550 > AC 102 > TC 361
Fenoxaprop leaves	**Fenoxaprop roots**
EC 550 > AC 102 > TC 361	AC 102 > EC 550 > TC 361
Imidacloprid leaves	**Imidacloprid roots**
TC 361> EC 550 > AC 102	EC 550 > TC 361 > AC 102

Similarly as with data from environmental stress the equations derived from pesticide treatment leads to a developing pattern of varietal selectively for GST activity. Generally, EC 550 has the dominant activity for pesticide treatment. It is followed by AC 102, which is followed by TC 361. This latter finding is different from the result obtained with the environmental stressors.

3.3. Further Purification

Choice of Matrices for Affinity Chromatography

The three different affinity chromatographic bed materials GSH-NH_2-Agarose, GSH-S-Agarose and S-Hexylgluthatione used specifically for GST affinity purification were evaluated in a preliminary trial for choice of affinity matrix. The elution performance was investigated with respect to protein recovery, binding capacity and GST activity as shown in Table 26.

Table 26. *Pachyrhizus* protein content, enzyme activity and percentage recovery from preliminary trials of three different GSH affinity chromatographic matrices.

Substrate	Total Protein (mg/ml)	Total Activity (µkat/ml)	Specific Activity (µkat/mg)	% Recovery	Purification fold
Crude extract	10.7	77.8	7.2	100	1
S-Hexylgluthatione	0.11	54.0	490.9	66	68
GSH-NH_2-agarose	0.08	14.8	185	19	27
GSH-S-agarose	0.07	2.3	32.9	3	5

Significant differences in binding capacity, recovery and GST activity were observed. From S-Hexylgluthatione agarose column the highest percentage of protein recovery at 66% and binding capacity for the isolation of required proteins was obtained (Table 26). The maximal activity of GST obtained from S-Hexylgluthatione agarose was 2.6-fold and 14.5-fold higher than that for GSH-NH_2-agarose and GSH-S-agarose respectively.

The protein has a higher affinity with S-Hexylgluthatione agarose obtaining 68% purification compared to 27 and 5% in GSH-NH_2-agarose and GSH-S-agarose respectively. All further purification through affinity chromatography for of all *Pachyrhizus* samples were subsequently carried out on S-Hexylgluthatione agarose columns.

The elution protocol of the preliminary trials was adjusted slightly to maximize the degree of affinity binding within the GSH gradient. The linear salt gradient that goes parallel with the GSH gradient was increased up from 0.3 to 0.5M.

3.3.1. Purification of *Pachyrhizus* GSTs

Several methods exist for the purification of enzymes from plant materials amongst which are affinity chromatography and ion exchange chromatography, Mono Q.
Cytosolic crude extracts from both (environmental stress conditions and pesticides treatments) and from all three varieties were further purified by affinity chromatography and ion exchange chromatography, Mono Q (Fast protein liquid chromatography, FPLC), for more detailed analysis of *Pachyrhizus* GSTs.

3.3.2. Affinity Chromatography

GSH affinity chromatography elution profiles of selected samples used for further purification of GSTs from *Pachyrhizus* are shown in Figures 25 to 29. Generally, the affinity profile patterns of samples were similarly. Non-bound proteins without affinity for the glutathione resin but with GST activities eluted within the first 8 fractions well separated from the GST fraction tightly bound to affinity matrix. GSTs were eluted within the GSH gradient fractions 11 to 20.

However, there were differences between samples in binding capacity and percentage recovery of GST bound protein. In leaves of EC 550 from control plants and CO_2 treatment, and AC 102 from O_3 treatment nearly 80% of the total proteins were eluted as non-bound proteins (Figures 25a, 26a and 27a) reflecting a low leaf GST binding capacity for these proteins under the selected conditions. In contrast, well over 80% of the total proteins in roots from all treatments (Figures 25b to 29b), in leaves of TC 361 from drought treatment (Figure 25a), and EC 550 leaves from captan treatment (Figure 26a) were eluted as bound proteins. In these samples GST activity was eluted with increasing salt concentration up to 0.5 M NaCl reflecting a high GST binding capacity with broad GST specific activities. Most of the protein showing enzymatic activity was eluted in the broad peak.

EC 550 Control

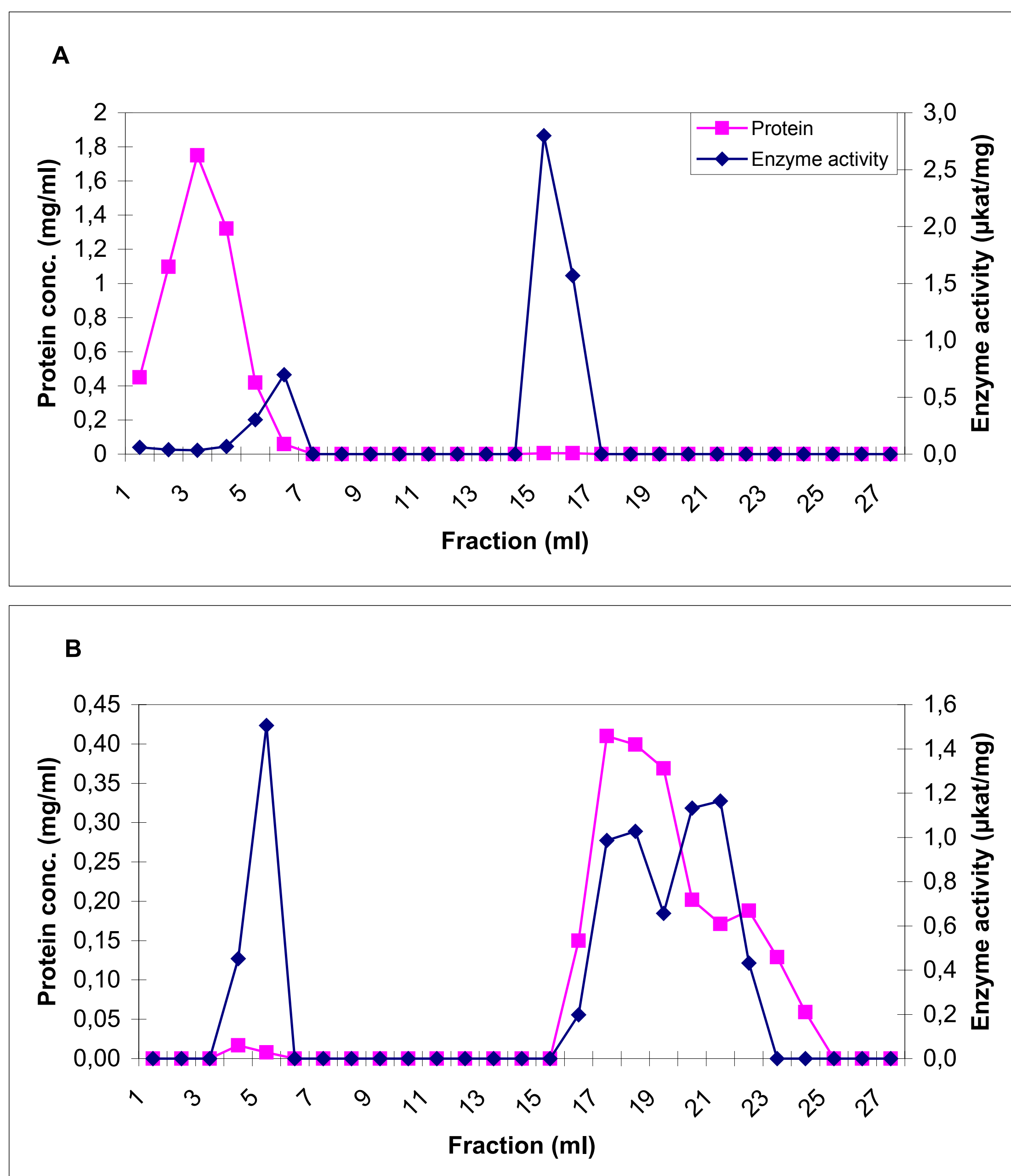

Figure 25. Elution profile of proteins from S-Hexyl GSH affinity column in control plants of *P. erosus* EC 550 in terms of GST activity as indicated by change in absorbance at 340 nm and protein contents as indicated by absorbance at 280 nm. (A) Leaves (B) Roots. Wash buffer (25 mM Tris–HCl pH 7.8) and elution buffer (25 mM Tris–HCl pH 7.8, 1-16 mM GSH and 0.1-1M NaCl). Active fractions were pooled, preconcentrated and chromatographed on a Mono-Q column.

Tables 27 to 32 summarize the results of purification of GSTs from *Pachyrhizus* crude extracts through affinity chromatography to ion exchange chromatography (Mono Q) using CDNB as substrate. Percentage recoveries of S-Hexylgluthatione affinity purified total GST varied greatly from 8.5% as in EC 550 O3 leaves to 92.3% as in TC 361 captan treated roots (Table 25 to 30). Generally percentage recoveries and GST specific activities were observed in the roots than in the leaves.

In some of the samples specific conjugating activity of affinity purified GSTs was approximately equivalent to that of the crude extract. For other samples activity increased ranging from 2 to 11-fold (Table 27 to 32).

EC 550 Carbon dioxide

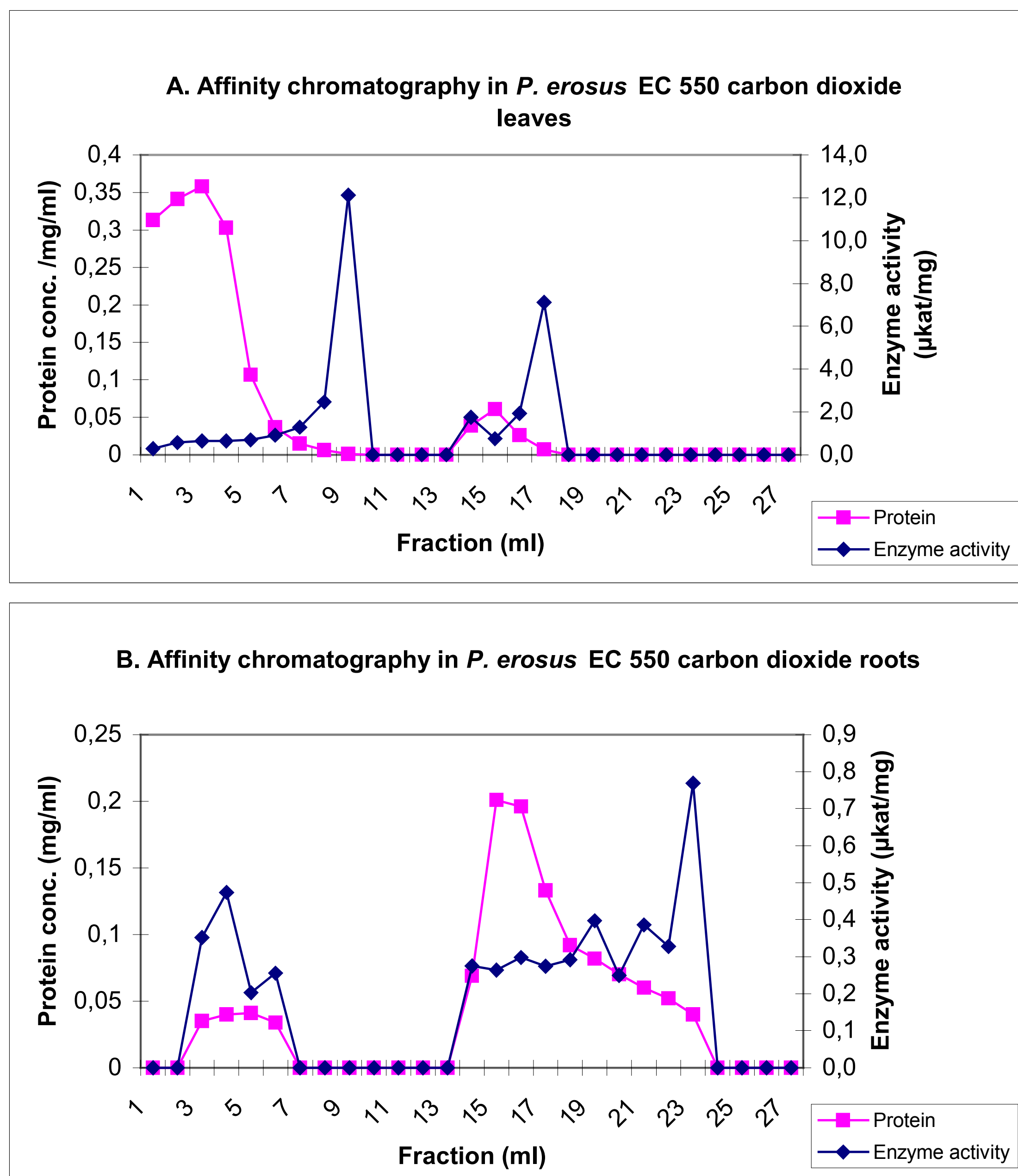

Figure 26. Elution profile of proteins from S-Hexyl GSH affinity column in CO_2 plants of *P. erosus* EC 550 in terms of GST activity as indicated by change in absorbance at 340 nm and protein contents as indicated by absorbance at 280 nm. (A) Leaves (B) Roots. Wash buffer (25 mM Tris–HCl pH 7.8) and elution buffer (25 mM Tris–HCl pH 7.8, 1-16 mM GSH and 0.1-1M NaCl). Active fractions were pooled, preconcentrated and chromatographed on a Mono-Q column.

AC 102 Ozone

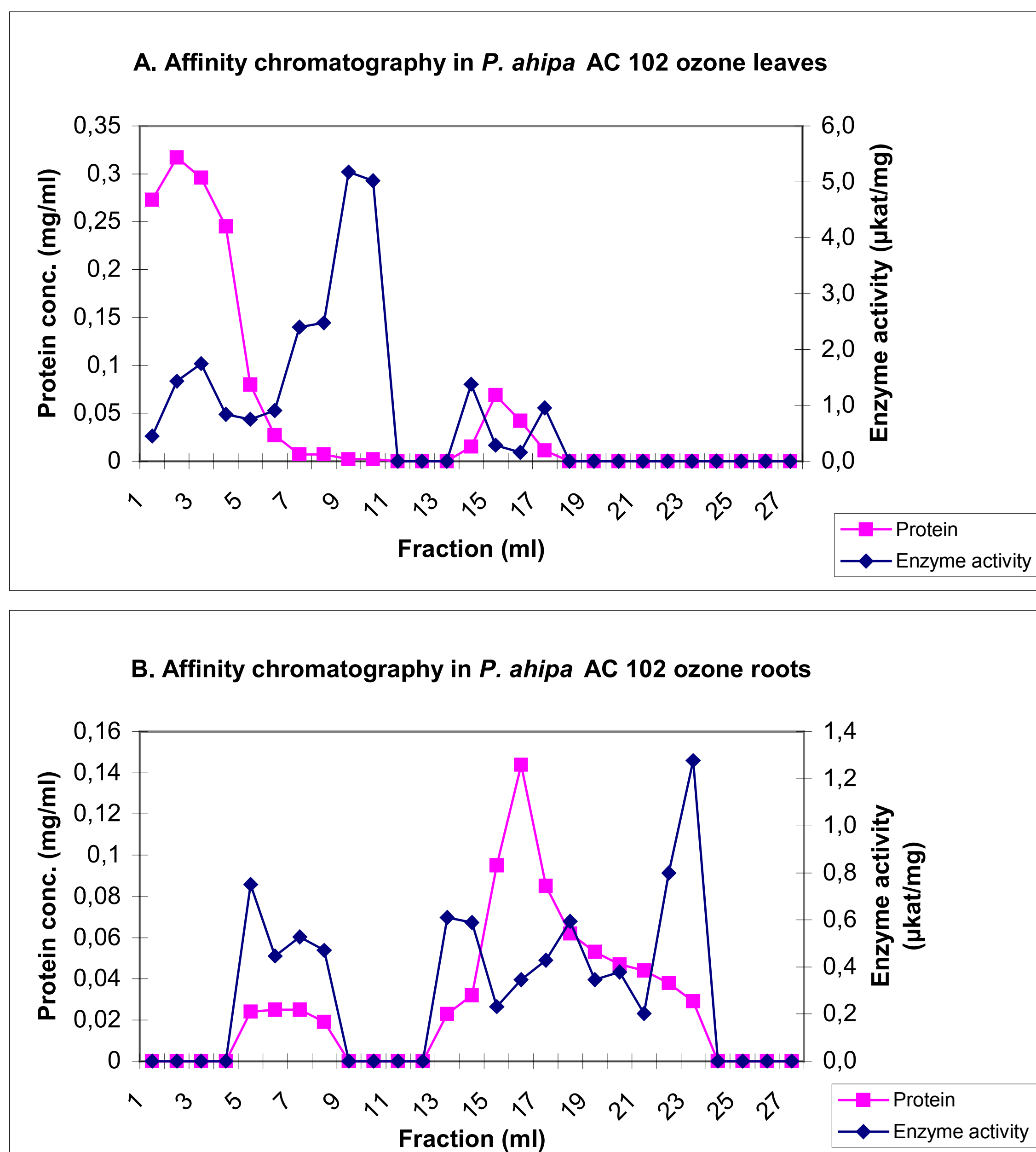

Figure 27. Elution profile of proteins from S-Hexyl GSH affinity column in O_3 plants of *P. ahipa* AC 102 in terms of GST activity as indicated by change in absorbance at 340 nm and protein contents as indicated by absorbance at 280 nm. (A) Leaves (B) Roots. Wash buffer (25 mM Tris–HCl pH 7.8) and elution buffer (25 mM Tris–HCl pH 7.8, 1-16 mM GSH and 0.1-1M NaCl). Active fractions were pooled, preconcentrated and chromatographed on a Mono-Q column.

TC 361 Drought

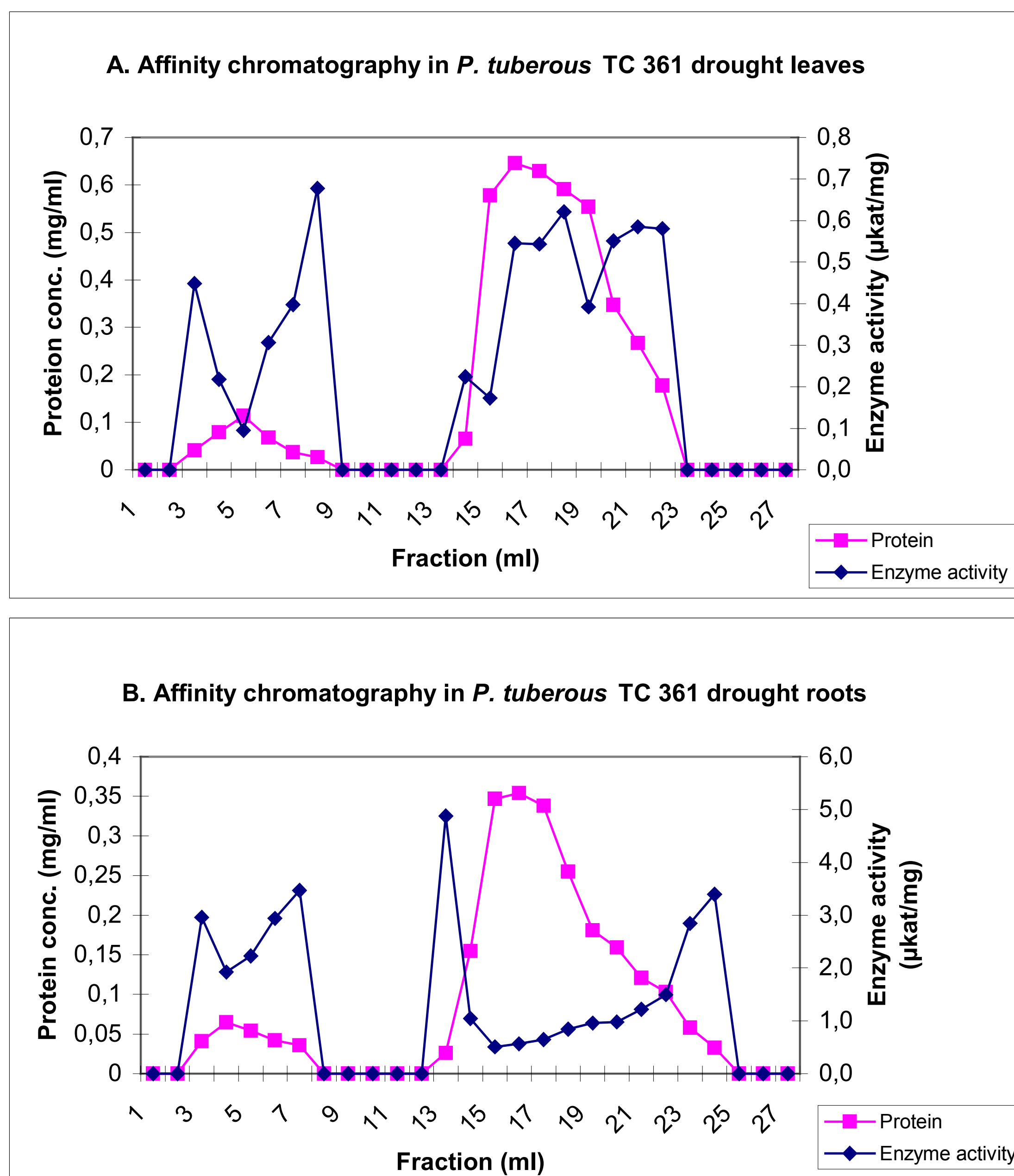

Figure 28. Elution profile of proteins from S-Hexyl GSH affinity column in water stress plants of *P. tuberous* TC 361 in terms of GST activity as indicated by change in absorbance at 340 nm and protein contents as indicated by absorbance at 280 nm. (A) Leaves (B) Roots. Wash buffer (25 mM Tris–HCl pH 7.8) and elution buffer (25 mM Tris–HCl pH 7.8, 1-16 mM GSH and 0.1-1M NaCl). Active fractions were pooled, preconcentrated and chromatographed on a Mono-Q column.

EC 550 Captan

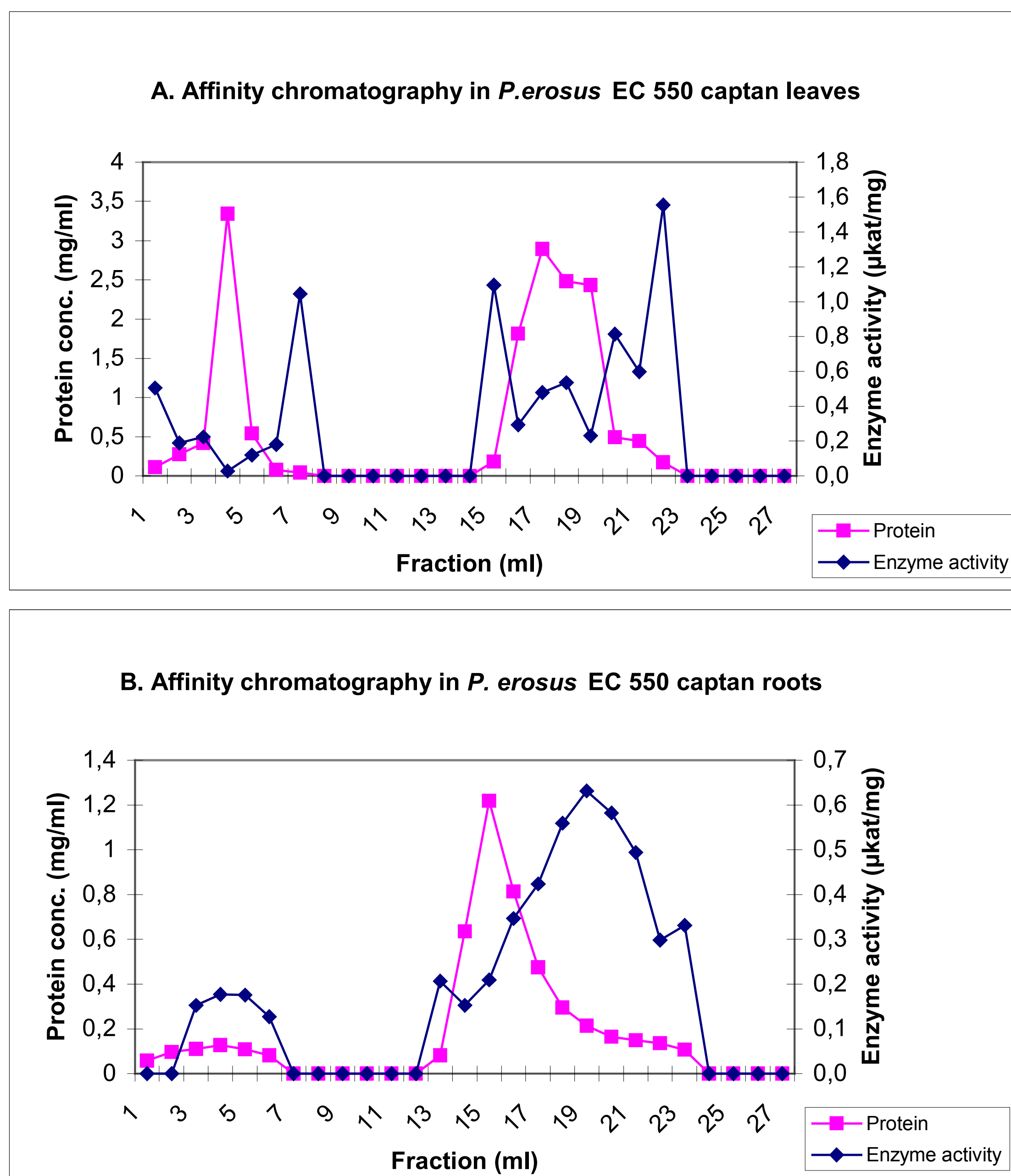

Figure 29. Elution profile of proteins from S-Hexyl GSH affinity column in fungicide (Captan) treated plants of *P. erosus* EC 550 in terms of GST activity as indicated by change in absorbance at 340 nm and protein contents as indicated by absorbance at 280 nm. (A) Leaves (B) Roots. Wash buffer (25 mM Tris–HCl pH 7.8) and elution buffer (25 mM Tris–HCl pH 7.8, 1-16 mM GSH and 0.1-1M NaCl). Active fractions were pooled, preconcentrated and chromatographed on a Mono-Q column.

3.4. Fast Protein Liquid Chromatography FPLC

Mono Q

Pooled affinity-bound GSTs fractions were subjected to Fast Protein Liquid Chromatography (FPLC), on a Mono Q system for further GST purification and separation on the basis of their ionic charge.

Results obtained from this second purification step are shown in elution profiles Figures 27 to 31 and Table 25 to 30.
From the elution profile FPLC purified GSTs activity revealed the presence of two *Pachyrhizus* GST fractions as shown in control plants (Figure 27). The first group of GSTs can be described as the low salt eluting fraction, eluting between 0.2 to 0.4 M NaCl gradient. Whilst the second group can be identified as the high salt eluting fraction, eluting with salt gradient 0.6 to 0.8M (Figure 27).

Similar patterns of salt eluting GSTs fractions were observed in treated samples but forming a continuous activity band between 0.25 and 0.8 M of the salt gradient (Figure 28 to 31). GST activity eluted in several joint peaks linked together.

GST specify activity increased with FPLC purification and as with affinity purified fractions FPLC purified GST specific activity was generally higher in roots than in the leaves. This could be attributed to the low protein concentration in roots than in leaves. The highest GST specific activity under environmental conditions was obtained in TC 361 roots after drought treatment at 4.87 µkat/mg with 17-fold purification on FPLC (Table 27), whilst under agrochemical treatment TC 361 fenoxaprop treated roots had the highest GST specific activity at 5.78 µkat/mg with 5-fold purification.

FPLC elution profiles indicate that their might be a number of overlapping GST isoenzymes with similar ionic strength in *Pachyrhizus*. In order to get a more detailed separation active fractions were pooled, desalted, lyophilised and reconstituted for further purification.

Table 27. Summary of three purification steps of GST enzyme from leaves and roots of *P. ahipa* AC 102 plants under environmental stress conditions.

	Treatment	AC 102 Leaves			AC 102 Roots		
		Crude extract	Affinity	Mono Q	Crude extract	Affinity	Mono Q
Volume		3,5	1	2	3,5	1	2
Total Protein	**Control**	12,63	1,31	0,77	0,41	0,21	0,07
	CO_2	12,01	1,17	0,21	1,18	0,19	0,07
	O_3	10,30	1,01	0,31	3,17	0,2	0,05
	Drought	8,40	0,88	0,08	1,28	0,69	0,08
Total Activity	**Control**	1,34	0,23	0,22	0,2	0,11	0,14
	CO_2	1,66	0,42	0,12	0,36	0,09	0,09
	O_3	2,37	0,32	0,26	0,38	0,11	0,11
	Drought	0,17	0,12	0,10	1,07	0,15	0,16
Specific Activity	**Control**	0,11	0,18	0,28	0,5	0,52	1,98
	CO_2	0,14	0,36	0,55	0,31	0,49	1,33
	O_3	0,23	0,32	0,84	0,12	0,55	2,27
	Drought	0,02	0,13	1,20	0,1	0,22	2,06
Recovery %	**Control**	100	17,5	16,3	100	52,6	68,1
	CO_2	100	25,6	7,0	100	25,7	25,2
	O_3	100	13,5	10,9	100	28,3	29,1
	Drought	100	68,2	57,2	100	14,2	14,9
Purification	**Control**	1	1,7	2,7	1	1	4
	CO_2	1	2,6	4,0	1	1,6	4,3
	O_3	1	1,4	3,7	1	4,6	18,8
	Drought	1	6,5	59,2	1	2,1	19,9

Table 28. Summary of three purification steps of GST enzyme from leaves and roots of *P. erosus* EC 550 plants under environmental stress conditions.

	Treatment	EC 550 Leaves			EC 550 Roots		
		Crude extract	Affinity	Mono Q	Crude extract	Affinity	Mono Q
Volume		3,5	1	2	3,5	1	2
Total Protein	**Control**	12,34	1,21	0,24	1,26	0,94	0,07
	CO_2	10,25	0,94	0,09	2,21	0,6	0,06
	O_3	6,34	1,03	0,54	1,2	0,25	0,05
	Drought	8,78	0,80	0,06	1,17	0,26	0,05
Total Activity	**Control**	1,57	0,15	0,19	0,91	0,7	0,06
	CO_2	1,30	0,28	0,08	0,57	0,19	0,17
	O_3	3,03	0,26	0,18	0,31	0,1	0,17
	Drought	0,52	0,13	0,09	0,45	0,13	0,17
Specific Activity	**Control**	0,13	0,12	0,78	0,72	0,75	0,87
	CO_2	0,13	0,30	0,88	0,26	0,32	2,85
	O_3	0,23	0,25	0,33	0,26	0,39	3,45
	Drought	0,06	0,16	1,51	0,38	0,51	3,16
Recovery %	**Control**	100	9,5	11,9	100	77,7	7
	CO_2	100	21,5	6,1	100	33,7	28,9
	O_3	100	8,5	5,9	100	31,6	54,8
	Drought	100	24,2	17,3	100	29,4	37,9
Purification	**Control**	1	1,0	6,1	1	1	1,2
	CO_2	1	2,3	6,7	1	1,2	11,0
	O_3	1	1,1	1,5	1	1,5	13,4
	Drought	1	2,7	25,3	1	1,3	8,2

Table 29. Summary of three purification steps of GST enzyme from leaves and roots of *P. tuberous* TC 361 plants under environmental stress conditions.

	Treatment	TC 361 Leaves			TC 361 Roots		
		Crude extract	Affinity	Mono Q	Crude extract	Affinity	Mono Q
Volume		3,5	1	2	3,5	1	2
Total Protein	**Control**	11,38	1,28	0,57	3,86	0,82	0,05
	CO_2	10,28	1,05	0,15	3,24	0,7	0,06
	O_3	13,62	1,35	0,14	2,22	0,61	0,05
	Drought	11,89	1,12	0,08	2,53	0,76	0,05
Total Activity	**Control**	2,26	0,30	0,25	0,56	0,21	0,05
	CO_2	1,27	0,14	0,13	0,68	0,17	0,12
	O_3	2,55	0,34	0,11	0,39	0,17	0,19
	Drought	1,85	0,33	0,09	0,71	0,23	0,26
Specific Activity	**Control**	0,20	0,23	0,44	0,15	0,26	1,01
	CO_2	0,12	0,14	0,82	0,21	0,24	2,04
	O_3	0,19	0,25	0,74	0,17	0,27	4,12
	Drought	0,16	0,29	1,05	0,28	0,31	4,87
Recovery %	**Control**	100	13,3	11,1	100	37,7	9,6
	CO_2	100	11,3	10,0	100	24,9	17,6
	O_3	100	13,3	4,2	100	43,2	48,2
	Drought	100	17,9	4,6	100	32,6	36,4
Purification	**Control**	1	1,2	2,2	1	1,8	7
	CO_2	1	1,1	6,7	1	1,1	9,7
	O_3	1	1,3	3,9	1	1,6	23,8
	Drought	1	1,9	6,8	1	1,1	17,3

µkat/mg refers to specific activity of GST with CDNB, -fold refers to -fold purification of the enzyme, and % recovery is for percent yield. Percent yield are expressed as percentage of total activity of crude extract.

Table 30. Summary of three purification steps of GST enzyme from leaves and roots of *P. ahipa* AC 102 plants under agrochemical conditions.

	Treatment	AC 102 Leaves			AC102 Roots		
		Crude extract	Affinity	Mono Q	Crude extract	Affinity	Mono Q
Volume		3,5	1	2	3,5	1	2
Total Protein	Control	10,90	0,81	0,08	0,10	0,06	0,04
	Captan	13,52	0,96	0,10	1,88	0,28	0,10
	Fenoxaprop	20,69	1,35	0,06	0,93	0,27	0,09
	Imidacloprid	18,00	1,12	0,09	0,61	0,15	0,04
Total Activity	Control	1,63	0,32	0,14	0,18	0,06	0,16
	Captan	1,71	0,20	0,22	0,72	0,12	0,16
	Fenoxaprop	2,16	0,48	0,21	0,37	0,16	0,17
	Imidacloprid	1,80	0,23	0,26	0,28	0,08	0,17
Specific Activity	Control	0,15	0,40	1,76	0,77	1,03	4,34
	Captan	0,13	0,21	2,31	0,38	0,42	1,57
	Fenoxaprop	0,10	0,35	3,51	0,40	0,59	1,91
	Imidacloprid	0,10	0,21	3,00	0,45	0,54	4,11
Recovery %	Control	100	19,8	8,5	100	33,2	88,8
	Captan	100	11,6	13,0	100	16,4	22,6
	Fenoxaprop	100	22,2	9,9	100	42,6	46,1
	Imidacloprid	100	12,9	14,5	100	29,7	60,7
Purification	Control	1	2,7	11,8	1	1,3	5,7
	Captan	1	1,6	18,3	1	1,1	4,1
	Fenoxaprop	1	3,4	33,7	1	1,5	4,8
	Imidacloprid	1	2,1	30,0	1	1,2	9,0

Table 31. Summary of three purification steps of GST enzyme from leaves and roots of *P. erosus* EC 550 plants under agrochemical conditions.

	Treatment	EC 550 Leaves			EC 550 Roots		
		Crude extract	Affinity	Mono Q	Crude extract	Affinity	Mono Q
Volume		3,5	1	2	3,5	1	2
Total Protein	Control	35,06	1,04	0,14	0,48	0,12	0,04
	Captan	22,65	1,11	0,15	5,03	1,07	0,38
	Fenoxaprop	19,68	1,14	0,09	0,39	0,17	0,06
	Imidacloprid	35,70	1,27	0,09	4,18	0,69	0,16
Total Activity	Control	3,27	1,11	0,16	0,21	0,06	0,18
	Captan	2,21	0,52	0,31	1,07	0,31	0,21
	Fenoxaprop	2,82	1,46	0,18	0,23	0,11	0,20
	Imidacloprid	2,56	0,49	0,25	1,06	0,21	0,22
Specific Activity	Control	0,09	1,06	1,09	0,44	0,51	4,11
	Captan	0,10	0,47	2,01	0,21	0,29	0,56
	Fenoxaprop	0,14	1,29	2,05	0,58	0,67	3,17
	Imidacloprid	0,07	0,39	2,71	0,25	0,31	1,33
Recovery %	Control	100	33,9	4,8	100	29,5	83,0
	Captan	100	23,6	14,0	100	28,9	19,6
	Fenoxaprop	100	51,8	6,5	100	48,6	88,0
	Imidacloprid	100	19,3	9,6	100	20,2	20,5
Purification	Control	1	11,4	11,7	1	1,2	9,3
	Captan	1	4,8	20,6	1	1,4	2,6
	Fenoxaprop	1	9,0	14,3	1	1,1	5,4
	Imidacloprid	1	5,4	37,7	1	1,2	5,3

Table 32. Summary of three purification steps of GST enzyme from leaves and roots of *P. tuberous* TC 361 plants under agrochemical conditions.

	Treatment	TC 361 Leaves			TC361 Roots		
		Crude extract	Affinity	Mono Q	Crude extract	Affinity	Mono Q
Volume		**3,5**	**1**	**2**	**3,5**	**1**	**2**
Total Protein	**Control**	5,07	0,45	0,05	0,30	0,05	0,03
	Captan	12,82	0,98	0,06	0,19	0,15	0,05
	Fenoxaprop	9,71	0,93	0,04	0,20	0,05	0,04
	Imidacloprid	13,69	1,23	0,09	0,23	0,16	0,05
Total Activity	**Control**	0,69	0,18	0,17	0,22	0,18	0,17
	Captan	1,87	0,24	0,30	0,20	0,19	0,15
	Fenoxaprop	1,02	0,31	0,20	0,23	0,17	0,15
	Imidacloprid	1,96	0,25	0,22	0,28	0,23	0,22
Specific Activity	**Control**	0,14	0,40	3,49	0,73	3,30	4,89
	Captan	0,15	0,25	5,10	1,09	1,26	2,84
	Fenoxaprop	0,11	0,34	5,11	1,17	3,17	5,78
	Imidacloprid	0,14	0,21	2,33	1,26	1,45	4,33
Recovery %	**Control**	100	26,0	25,4	100	79,5	74,4
	Captan	100	13,0	16,1	100	92,3	75,1
	Fenoxaprop	100	30,7	20,1	100	74,0	68,1
	Imidacloprid	100	12,9	11,2	100	81,6	77,7
Purification	**Control**	1	2,9	25,7	1	4,5	6,7
	Captan	1	1,7	35,0	1	1,2	2,6
	Fenoxaprop	1	3,2	48,7	1	2,7	5,0
	Imidacloprid	1	1,4	16,3	1	1,2	3,4

EC 550 Control

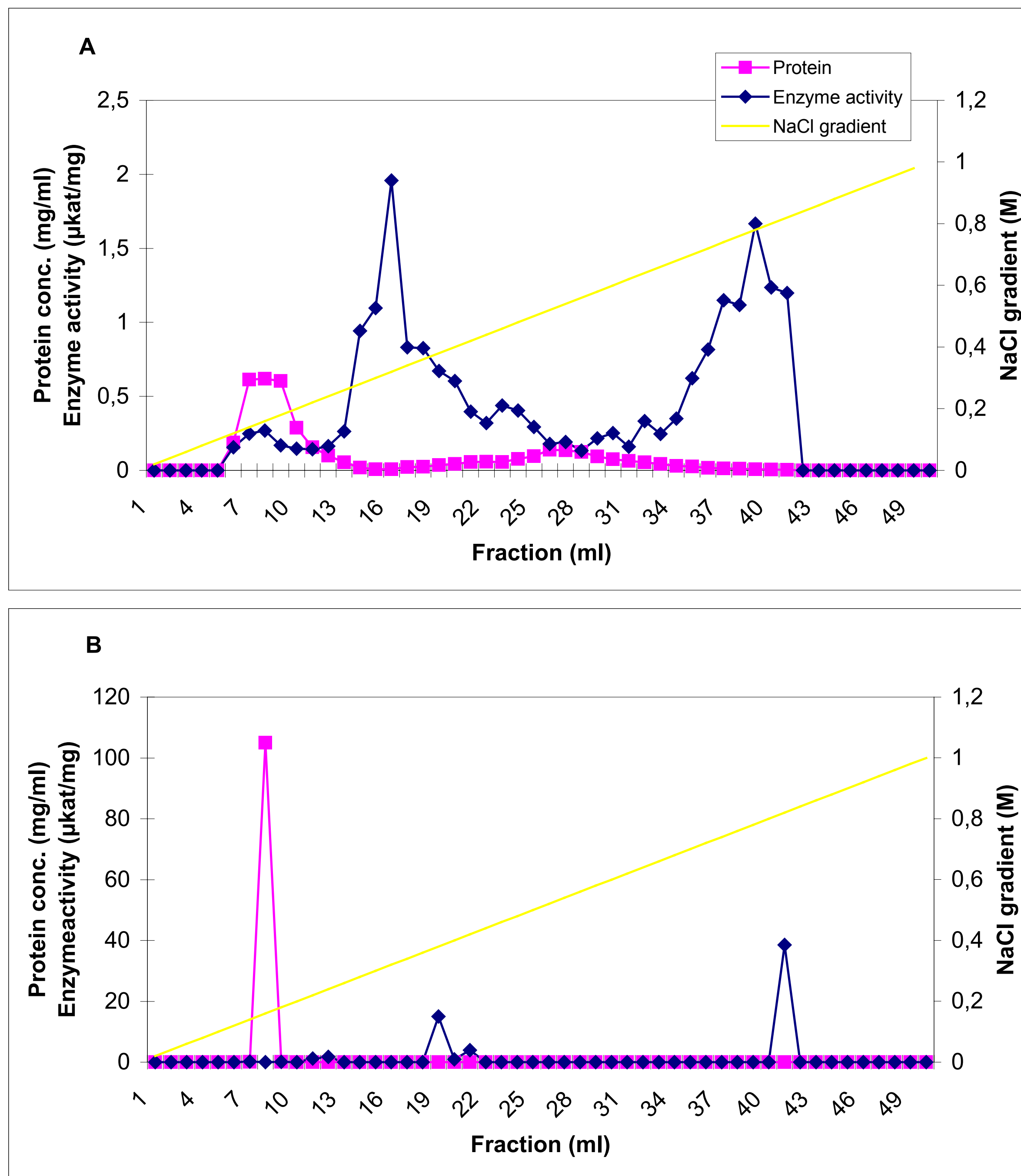

Figure 30. Elution profile of active protein fractions from affinity, chromatographed on Mono-Q column in control plants of *P. erosus* EC 550. GST activity is indicated by change in absorbance at 340 nm and protein contents by absorbance at 280 nm. (A) Leaves (B) Roots. Mobile phase A buffer (25 mM Tris–HCl pH 7.5) and mobile phase B buffer (25 mM Tris–HCl pH 7.5, and 1M NaCl). Active fractions were pooled, lyophilised and chromatographed on a Mono-P column.

EC 550 Carbon dioxide

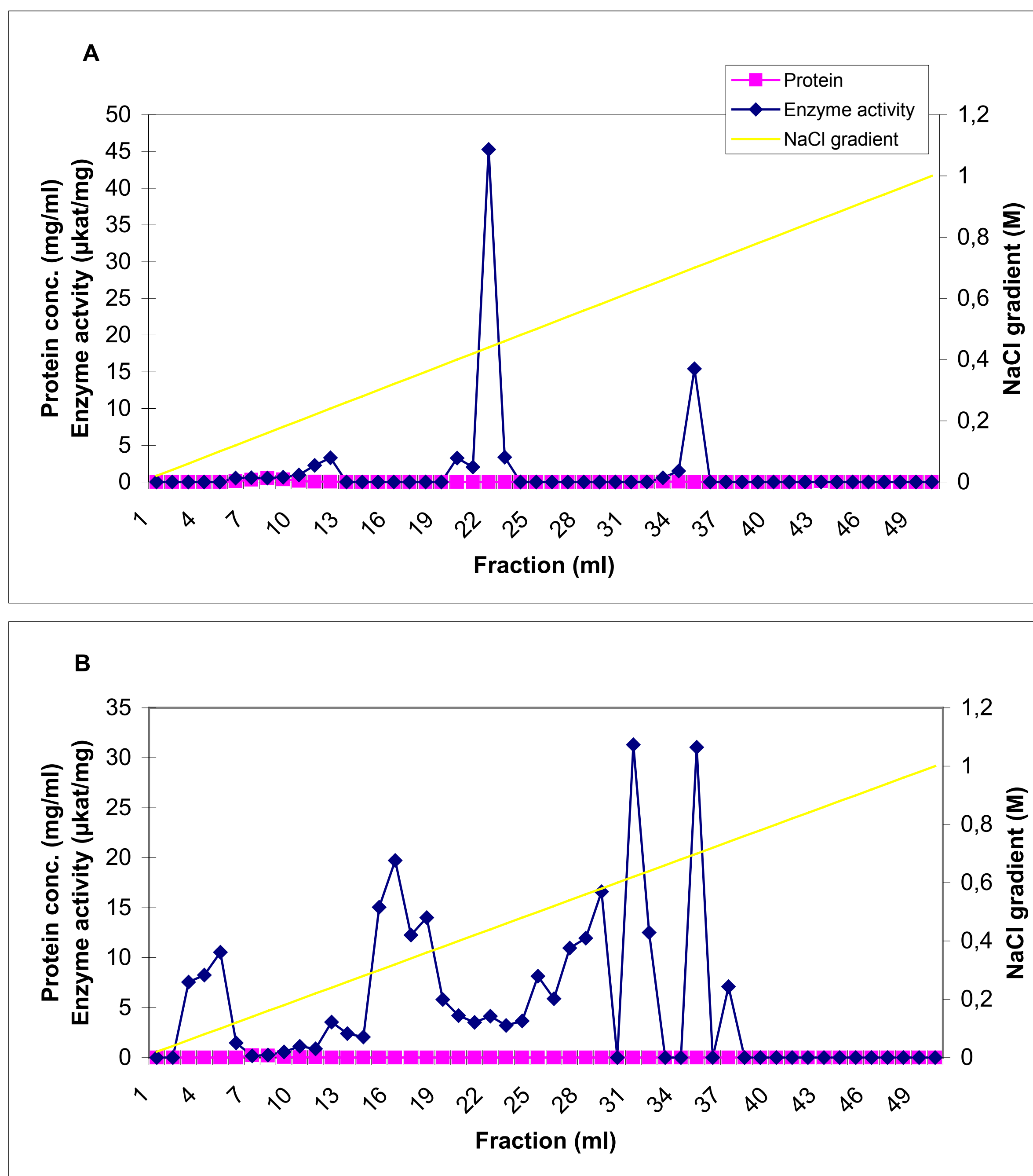

Figure 31. Elution profile of active protein fractions from affinity, chromatographed on Mono-Q column in CO_2 plants of *P. erosus* EC 550. GST activity is indicated by change in absorbance at 340 nm and protein contents by absorbance at 280 nm. (A) Leaves (B) Roots. Mobile phase A buffer (25 mM Tris–HCl pH 7.5) and mobile phase B buffer (25 mM Tris–HCl pH 7.5, and 1M NaCl). Active fractions were pooled, lyophilised and chromatographed on a Mono-P column.

AC 102 Ozone

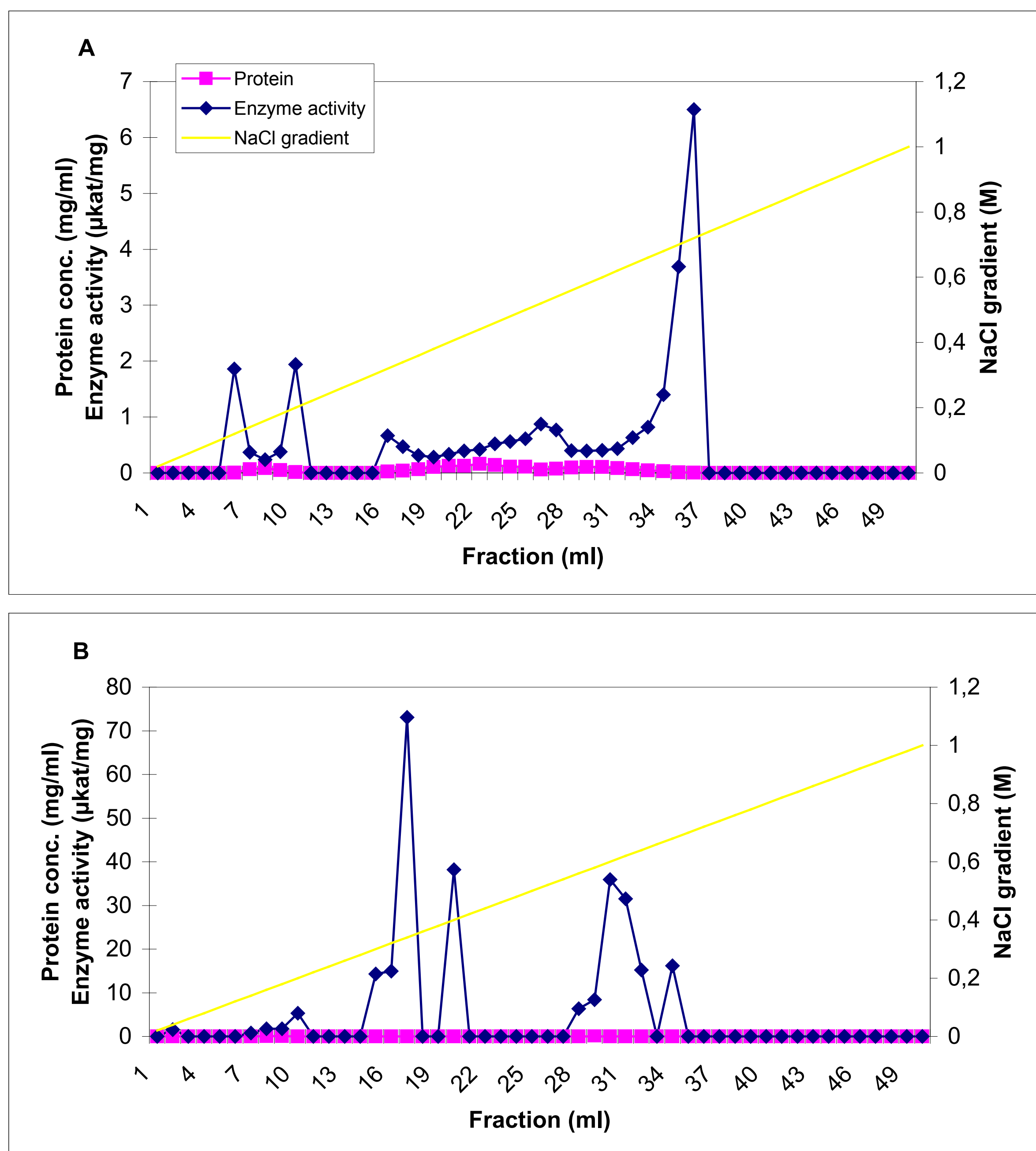

Figure 32. Elution profile of active protein fractions from affinity, chromatographed on Mono-Q column in O_3 plants of *P. ahipa* AC 102. GST activity is indicated by change in absorbance at 340 nm and protein contents by absorbance at 280 nm. (A) Leaves (B) Roots. Mobile phase A buffer (25 mM Tris–HCl pH 7.5) and mobile phase B buffer (25 mM Tris–HCl pH 7.5, and 1M NaCl). Active fractions were pooled, lyophilised and chromatographed on a Mono-P column.

TC 361 Drought

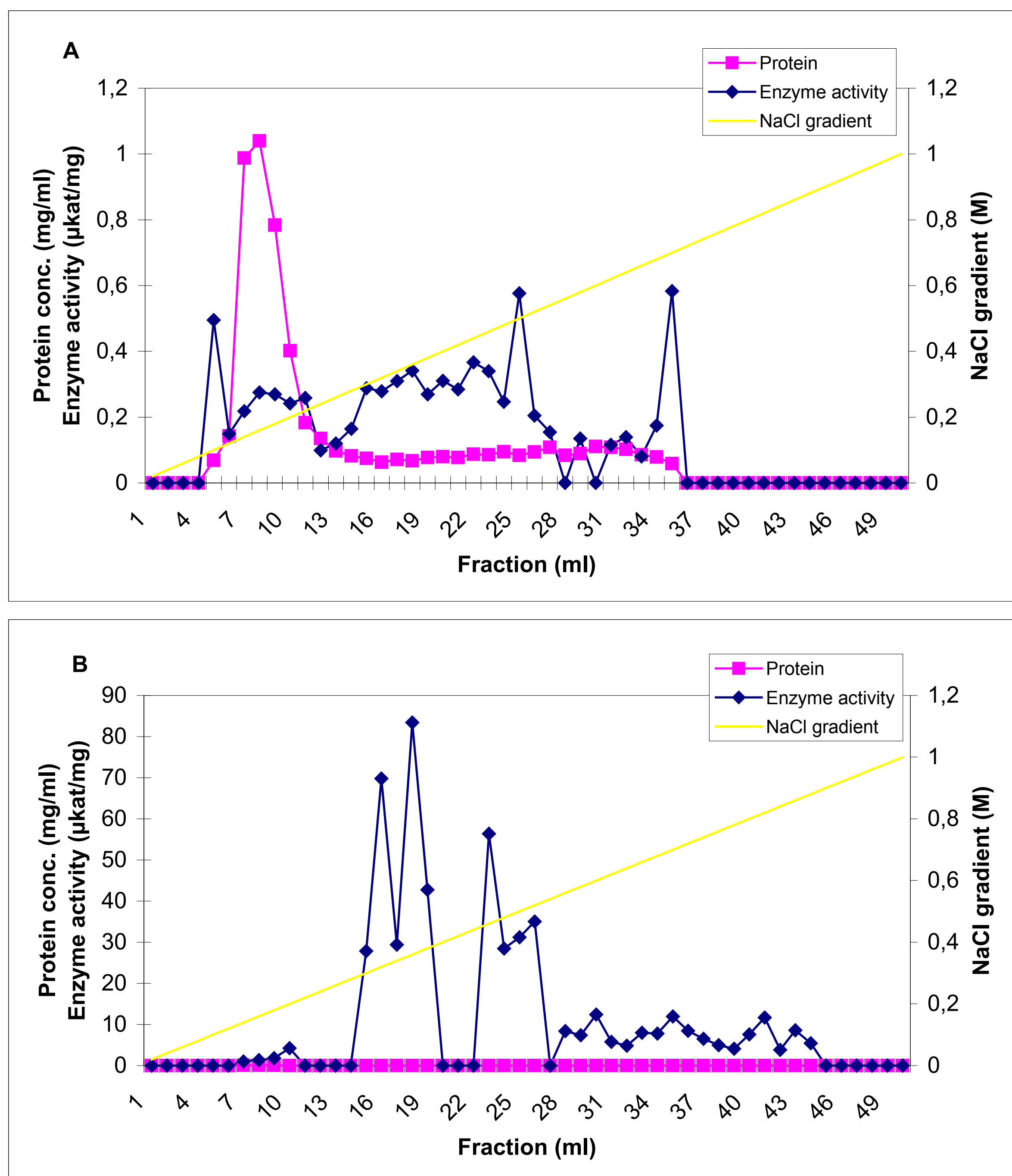

Figure 33. Elution profile of active protein fractions from affinity chromatographed on Mono-Q column in water stress plants of *P. tuberous* TC 361. GST activity is indicated by change in absorbance at 340 nm and protein contents by absorbance at 280 nm. (A) Leaves (B) Roots. Mobile phase A buffer (25 mM Tris–HCl pH 7.5) and mobile phase B buffer (25 mM Tris–HCl pH 7.5, and 1M NaCl). Active fractions were pooled, lyophilised and chromatographed on a Mono-P column.

EC 550 Captan

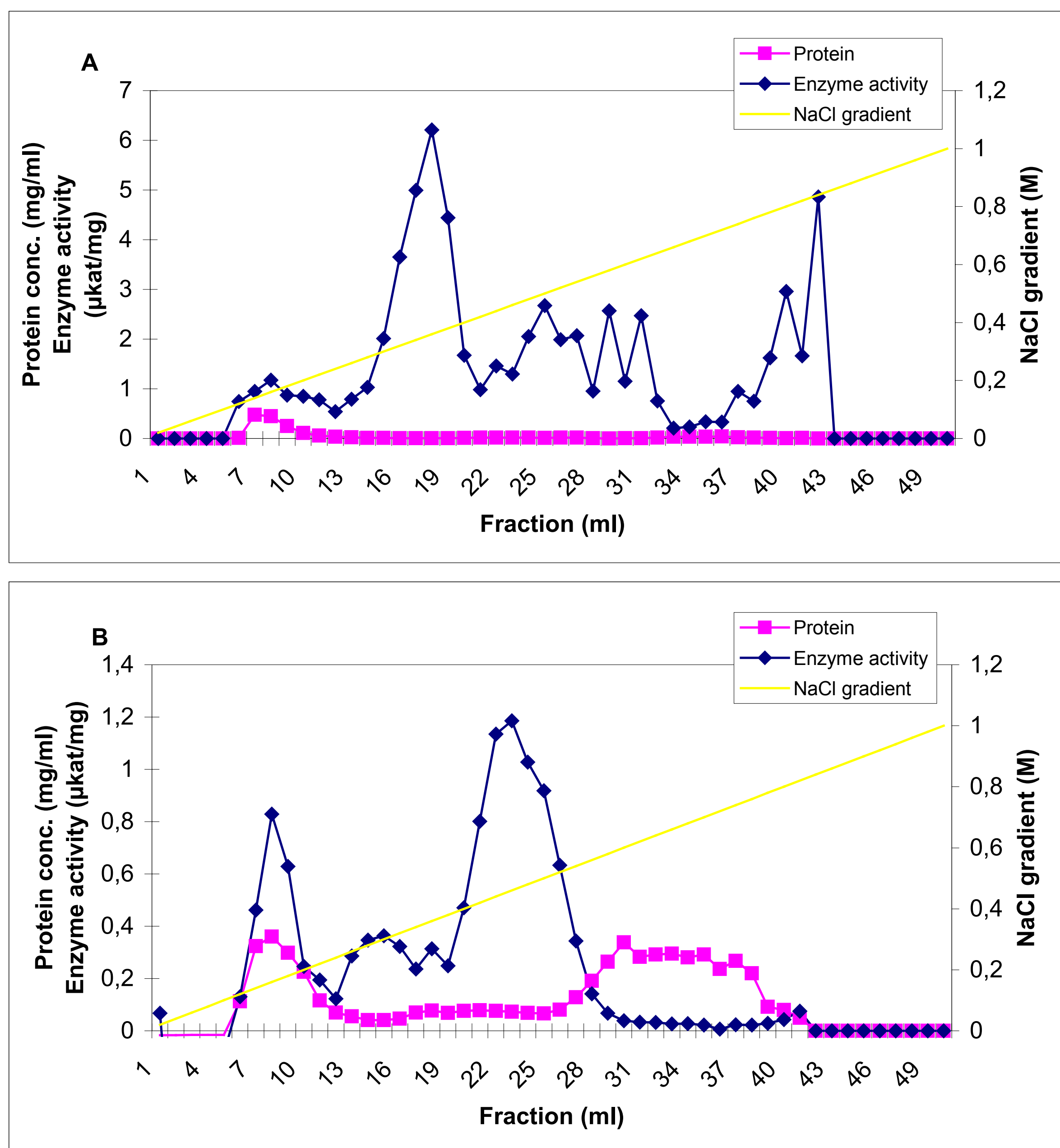

Figure 34. Elution profile of active protein fractions from affinity, chromatographed on Mono-Q column in fungicide (Captan) treated plants of *P. erosus* EC 550. GST activity is indicated by change in absorbance at 340 nm and protein contents by absorbance at 280 nm. (A) Leaves (B) Roots. Mobile phase A buffer (25 mM Tris–HCl pH 7.5) and mobile phase B buffer (25 mM Tris–HCl pH 7.5, and 1M NaCl). Active fractions were pooled, lyophilised and chromatographed on a Mono-P column.

3.4.1. Chromatofocusing: Separation of Subunits of GSTs

Mono P

Anion exchange chromatography had resulted in the resolution of several distinct GST peaks on a salt gradient. To obtain more detailed information on these GST peaks under different treatments, samples including a control (with both leaves and roots) were selected for further purification and separation of isoenzymes units by chromatofocusing, for the determination of isoelectric points (pI) (Table 33). Selection was made on bases of high GST activity induced by applied treatments and across cultivars.

Lyophilised reconstituted fractions of selected samples from Mono Q purification were applied on a Mono P column. The elution profile obtained from all samples produced a general pattern of a main peak followed by minor peaks (Figures 35 to 39). From each of the fractions a major GST isoenzyme can be distinguished. In some cases up to three forms can be identified as in the case of O_3 treated *P. ahipa* AC 102, water stress *P. tuberous* TC 361 and captan treated *P. erosus* EC 550 leaves (Figures 37, 38, 39) (Table 35). From control leaves and roots of *P. erosus* EC 550, and captan treated roots two isoforms were distinguished. Whilst in CO_2 treated *P. erosus* EC 550 leaves and roots only the major isoforms were clearly distinguished.

The isoforms separated were temporarily named according to the variety, treatment applied, the type of plant organ and order or position of elution as shown in (Table 35).
The pIs for the major isoforms or GST 1 from CO_2 treated leaves and roots, O_3 treated leaves, water stress leaves and root and captan treated leaves were found to be identical in a pI region of 6.75 to 7.07 (Table 34). Differences in GST 1 pIs were observed between the above group and those of control plants pI (5.80 to 6.16), O_3 treated roots pI (5.60 to 5.93) and captan treated roots pI (6.45).

In drought treated TC 361 the GST 1 peak in leaves (*Pt*-wsGST L1) and roots (*Pt*-wsGST R1) eluted with the same pI at 6.9. The second isoforms also eluted at a similar pI of 6.7. The elution pIs for the third isoforms were almost identical (Table 34). The leaves (*Pt*-wsGST L3) eluted at pI region of 4.5-4.4 whilst that for roots (*Pt*-wsGST R3) was 4.10. Identical pIs for leaves and roots with the same treatment were also observed for GST 3 in O_3 treated *P. ahipa* AC 102.

In captan treated *P. erosus* EC 550 the three isoforms identified in the leaves have significantly different pIs from the two identified in the roots. Significant differences were also observed between leaves and roots pIs for GST 1 in O_3 treated *P. ahipa* AC 102, and for GST 2 and GST 3 pIs between treatments of O_3, water stress and captan (Table 34).

GST specific activity for CDNB conjugation in all samples increased several fold after separation of the isoforms when compared to the crude homogenate (purification Table 33). The highest specific activities were obtained with the major isoform GST 1. The most active GST isoform was expressed in captan treated *P. erosus* EC 550 corresponding to pI values 6.85 to 7.07 and found to be 678.8-fold more active than the crude homogenate but recovery was low at 6%. In general, specific activity and recovery for GST 2 and GST 3 were very low when compared to GST 1 (Table 36).

Isoelectric chromatofocusing indicated *Pachyrhizus* GST isoenzymes are found within the acidic pI range of 7 to 4 and that almost two-thirds have pI in the weak acidic region 6 to 7. Physiochemical analysis and characterization was continued only on the major peaks GST 1 of each samples.

Table 33. Samples selected for further purification and separation of GST isoforms

Sample	Total Protein (mg/ml)	Total Activity (µkat/ml)	Specific Activity (µkat/mg)
EC 550 Control			
Leaves	1,26	0,91	0,72
Roots	12,34	1,57	0,13
EC 550 CO_2			
Leaves	10,25	1,30	0,13
Roots	2,21	0,57	0,26
AC 102 O_3			
Leaves	10,30	2,37	0,23
Roots	3,17	0,38	0,12
TC 361 Drought			
Leaves	11,89	1,85	0,16
Roots	2,53	0,71	0,28
EC 550 Captan			
Leaves	22,65	2,21	0,10
Roots	5,03	1,07	0,21

EC 550 Control

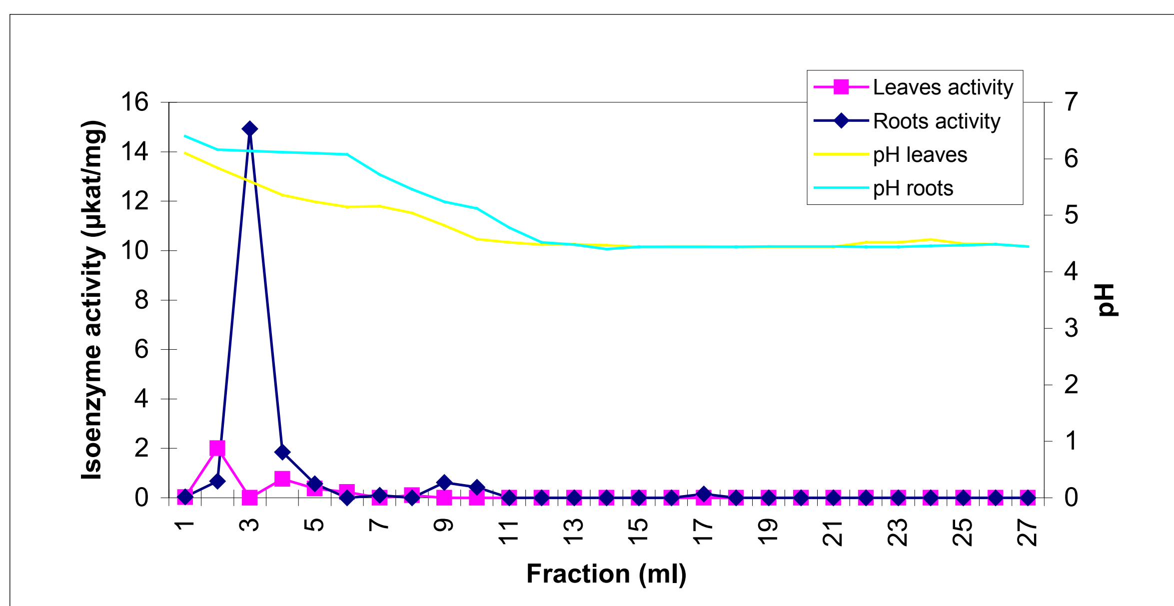

Figure 35. Elution profile of active protein fractions from Mono-Q column chromatographed on Mono-P column in control plants of *P. erosus* EC 550. (A) Leaves (B) Roots. GST activity of isoenzymes is indicated by change in absorbance at 340 nm. Mobile phase A buffer (25 mM Bis-Tris–HCl pH 7.4) and mobile phase B (Polybuffer74/HCl pH 4.0).

EC 550 Carbon dioxide

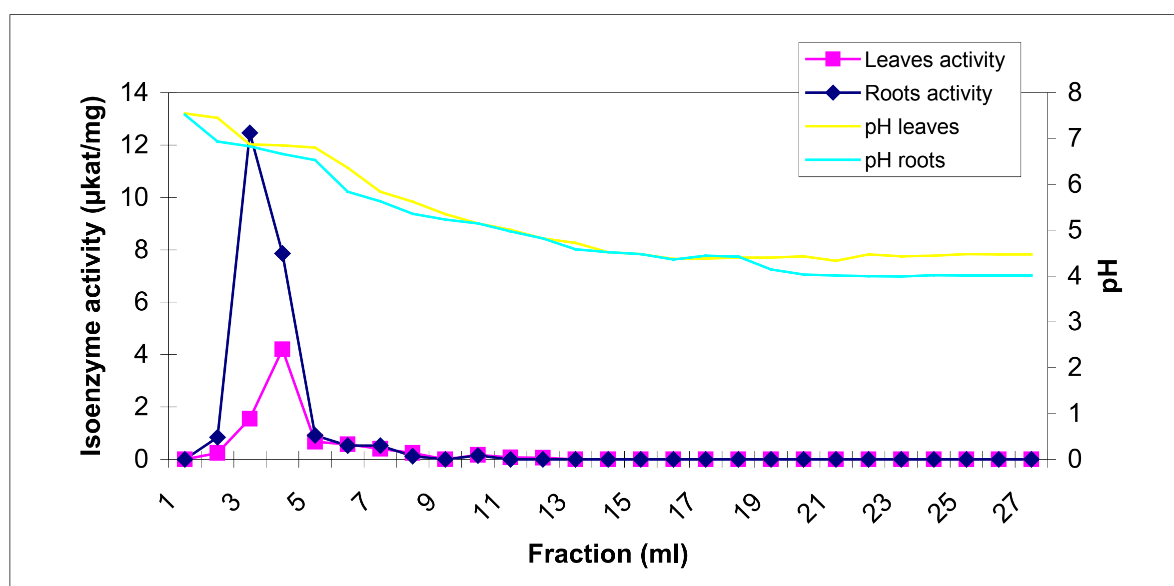

Figure 36. Elution profile of active protein fractions from Mono-Q column chromatographed on Mono-P column in CO_2 treated plants of *P. erosus* EC 550. (A) Leaves (B) Roots. GST activity of isoenzymes is indicated by change in absorbance at 340 nm. Mobile phase A buffer (25 mM Bis-Tris–HCl pH 7.4) and mobile phase B (Polybuffer 74/HCl pH 4.0).

AC 102 Ozone

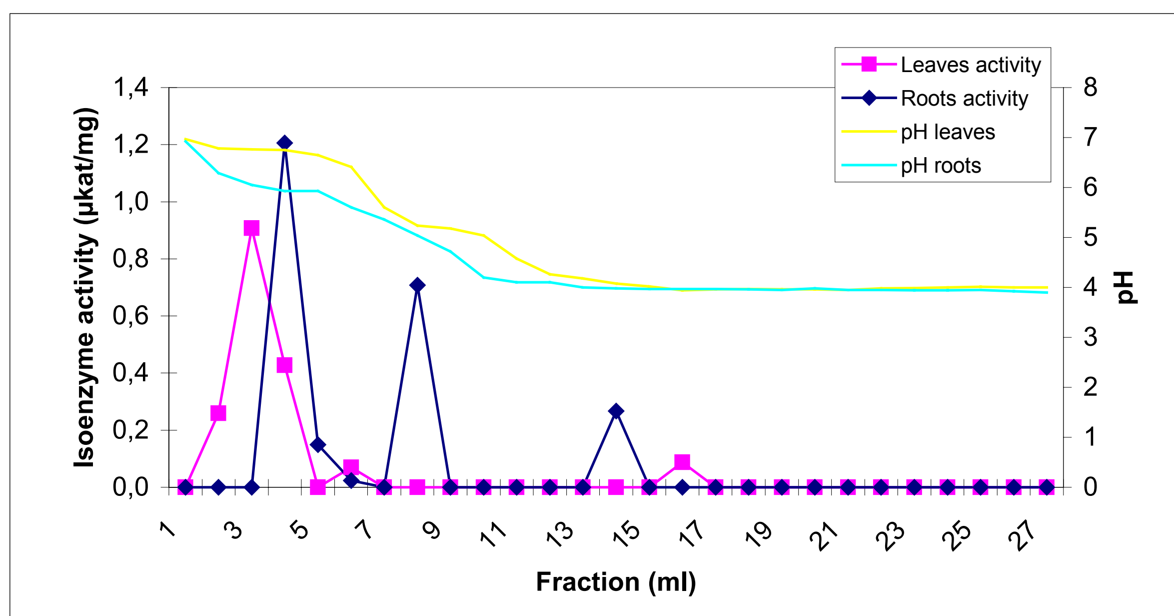

Figure 37. Elution profile of active protein fractions from Mono-Q column chromatographed on Mono-P column in O_3 treated plants of *P.* ahipa AC 102. (A) Leaves (B) Roots. GST activity of isoenzymes is indicated by change in absorbance at 340 nm. Mobile phase A buffer (25 mM Bis-Tris–HCl pH 7.4) and mobile phase B (Polybuffer 74/HCl pH 4.0).

TC 361 Drought

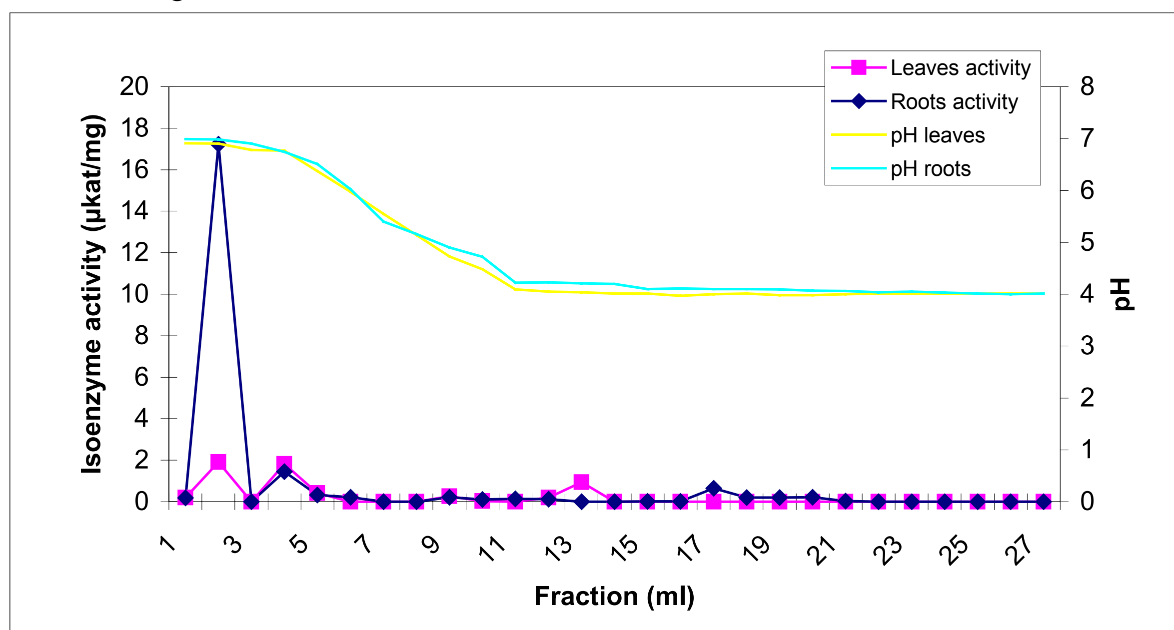

Figure 38. Elution profile of active protein fractions from Mono-Q column chromatographed on Mono-P column in water stressed plants of *P.* tuberous TC 361. (A) Leaves (B) Roots. GST activity of isoenzymes is indicated by change in absorbance at 340 nm. Mobile phase A buffer (25 mM Bis-Tris–HCl pH 7.4) and mobile phase B (Polybuffer 74/HCl pH 4.0).

When EC 550 had been treated with Captan, the main GST peak from mono P was found to contain two distinct GST peaks in both roots and leaf. The roots GST peaks were between 5 and 2µkat/mg in their capability to conjugate CDNB, whereas the activity in leaves was more than 10-fold higher for the same proteins (Figure 39).

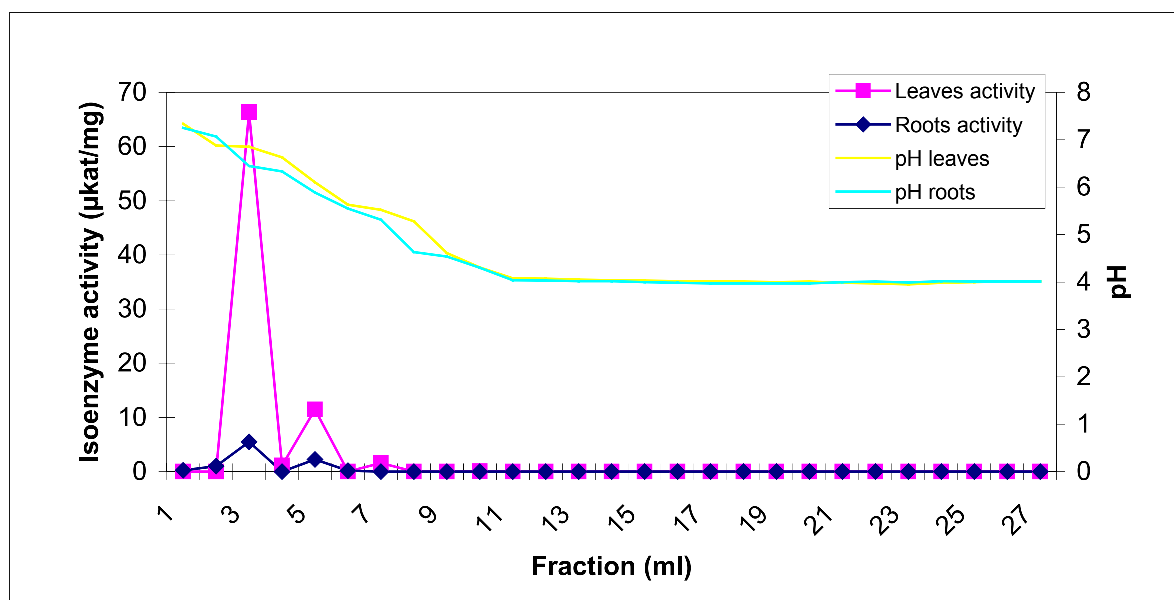

Figure 39. Elution profile of active protein fractions from Mono-Q column chromatographed on Mono-P column in captan (fungicide) treated plants of *P.* erosus EC 550. (A) Leaves (B) Roots. GST activity of isoenzymes is indicated by change in absorbance at 340 nm. Mobile phase A buffer (25 mM Bis-Tris–HCl pH 7.4) and mobile phase B (Polybuffer 74/HCl pH 4.0).

Table 34. Isoelectric points (pI) of *Pachyrhizus* isoforms separated after chromatofocusing. GSTs within the same group or column e.g. GST 1 was separated into 1a and 1b to clearly show the differences and similarity between the pI range.

Accession	Treatment	GST 1a	GST 1b	GST 2a	GST 2b	GST 3
EC 550	Control Leaves	5,80 – 6,10		5,10 – 5,36		
	Control Roots	6,10 – 6,16		5,72		
EC 550	CO_2 Leaves		6,80 – 6,87			
	CO_2 Roots		6,66 – 6,85			
AC 102	Ozone Leaves		6,75 – 6,78		6,41	3,94
	Ozone Roots	5,60 – 5,93		5,04		3,98
TC 361	Drought Leaves		6,90		6,77	4,05 – 4,04
	Drought Roots		6,90		6,74	4,10
EC 550	Captan Leaves		6,85 – 7,07	6,10		5,52
	Captan Roots	6,45		5,89		

Table 35. Nomenclature of *Pachyrhizus* isoforms separated after chromatofocusing on a Mono P system

Accession	Treatment	GST 1	GST 2	GST 3
EC 550	Control Leaves	*Pe*GST L1	*Pe*GST L2	
	Control Roots	*Pe*GST R1	*Pe*GST R2	
EC 550	CO_2 Leaves	*Pe*-co$_2$GST L1		
	CO_2 Roots	*Pe*-co$_2$GST R1		
AC 102	Ozone Leaves	*Pa*-o$_3$GST L1	*Pa*-o$_z$GST L2	*Pa*-o$_z$GST L3
	Ozone Roots	*Pa*-o$_z$GST R1	*Pa*-o$_z$GST R2	*Pa*-o$_z$GST R3
TC 361	Drought Leaves	*Pt*-wsGST L1	*Pt* -wsGST L2	*Pt*-wsGST L3
	Drought Roots	*Pt*-wsGST R1	*Pt*-wsGST R2	*Pt*-wsGST R3
EC 550	Captan Leaves	*Pe*-capGST L1	*Pe*-capGST L2	*Pe*-capGST L3
	Captan Roots	*Pe*-capGST R1	*Pe*-capGST R2	

Table 36. Further purification of *Pachyrhizus* GST isoforms by chromatofocusing

Isoenzyme	Crude extract Specific activity (µkat/mg)	Mono P Specific activity (µkat/mg)	Recovery %	Purification factor
EC 550 Control				
*Pe*GST L1	0,13	2,01	3,4	15,7
*Pe*GST L2		0,76	0,6	6,0
*Pe*GST R1	0,72	14,93	3,3	20,8
*Pe*GST R2		0,61	1,4	0,9
EC 550 CO_2				
Pe-co$_2$GST L1	0,13	4,20	4,2	31,9
Pe-co$_2$GST R1	0,26	12,46	24,0	48,3
AC 102 Ozone				
Pa-o$_3$GST L1	0,23	0,91	3,50	3,9
Pa-o$_z$GST L2		0,07	0,10	0,3
Pa-o$_z$GST L3		0,09	0,13	0,4
Pa-o$_z$GST R1	0,12	1,21	4,70	10,0
Pa-o$_z$GST R2		0,71	5,90	5,8
Pa-o$_z$GST R3		0,27	2,30	2,2
TC 361 Drought				
Pt-wsGST L1	0,16	1,91	2,70	12,3
Pt-wsGST L2		1,82	0,80	11,7
Pt-wsGST L3		0,26	0,44	1,7
Pt-wsGST R1	0,28	17,22	14,50	61,3
Pt-wsGST R2		1,45	2,40	5,2
Pt-wsGST R3		0,22	1,04	0,8
EC 550 Captan				
Pe-capGST L1	0,10	66,32	6,00	678,8
Pe-capGST L2		11,47	5,70	117,4
Pe-capGST L3		0 ,08	0,06	0,8
Pe-capGST R1	0,21	1,02	7,40	4,8
Pe-capGST R2		2,25	2,70	10,6

3.4.2. Physicochemical Properties

pH Optima

The pH optima of *Pachyrhizus* GST 1 isoenzymes activity for the formation of GSH-CDNB conjugates at the active site of the isoenzymes was determined between pH range 3 to 9.

Table 37 shows result of physiochemical analysis of *Pachyrhizus* isoenzymes GST 1 including their pH optima. Values obtained revealed that the samples exhibit similarities for optimal pH range. All samples optimum points were within one interval range 6 and 7 as represented in elution profile of captan treated *P. erosus* EC 550 (Figure 40).

The sensitivity of the isoenzymes was tested when different buffering systems were used for separate pH points (see Table 38). While potassium phosphate and Tris-HCl buffers provide suitable environment, in which the enzymes operates GST isoenzyme activities in MES at pH 6 and HEPES at pH 7 were complete lost. As a result each MES and HEPES buffers were replaced with Tris-HCl at pH 6 and 7 and activity was obtained.
The pH range within which isoenzymes activity was maintained varied from pH 4 to 7.5. GST isoenzymes activity for the

Table 37. Physiochemical parameters of GST Isoenzymes from *Pachyrhizus* Leaves and roots

Variety	Treatment	Organ	pI	pH	Temp. ^{o}K	Activation energy ($KJmol^{-1}$)
EC 550	Control	leaves	5.80 – 6.10	6.5	304	26.62 ± 1.71
EC 550	Control	roots	6.10 – 6.16	6.4	303.5	28.15 ± 6.81
EC 550	CO_2	leaves	6.80 – 6.87	6.6	303	25.66 ± 6,13
EC 550	CO_2	roots	6.66 – 6.85	6.2	303	31.59 ± 7.92
AC 102	O_3	leaves	6.75 – 6.78	6.3	304	26.23 ± 4.88
AC 102	O_3	roots	5.60 – 5.93	6.4	303.5	25.47 ± 6.00
TC 361	Drought	leaves	6.90	6.6	303	25.85 ± 6.34
TC 361	Drought	roots	6.90	7	307.5	26.23 ± 7.88
EC 550	Captan	leaves	6.85 – 7.07	6.8	304.5	42.70 ± 1.13
EC 550	Captan	roots	6.45	6.5	303	25.28 ± 6.16

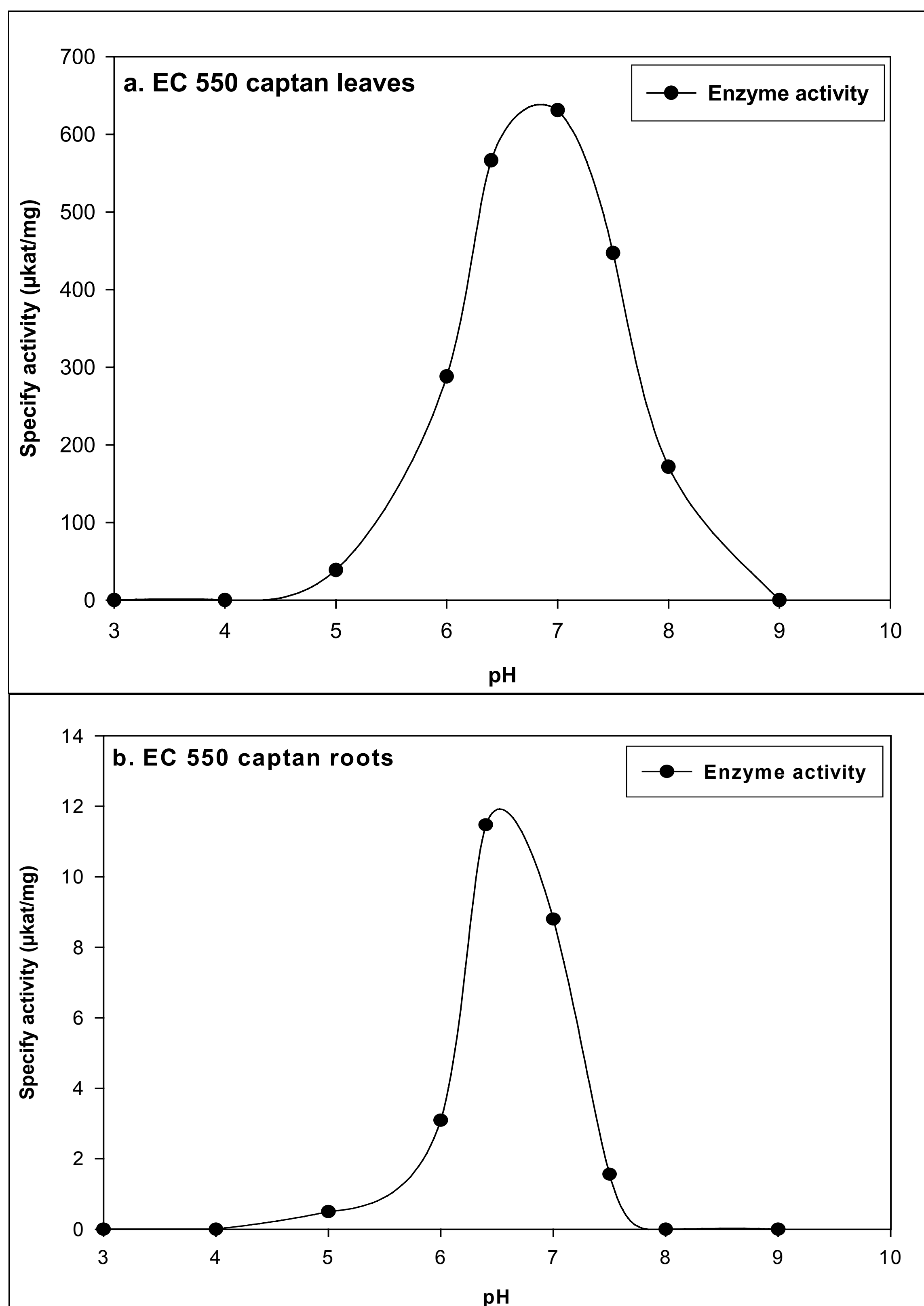

Figure 40. Representation of pH dependence of GST(CDNB) activity from *P.* erosus EC 550 captan treated plants. (A) Leaves isoenzyme *Pe*-capGST L1 (B) Roots isoenzyme *Pe*-capGST R1. GST activity of isoenzymes is indicated by change in absorbance at 340 nm obtained at different pH values. Buffer 0.1M. Citric pH4, KH_2PO_4 pH 5, Tris/HCl pH 6, K_2HPO_4 pH 6,4, Tris/HCl pH 7 and 8, Ches pH 9. Each experimental point is the mean of three replicates ± SE.

Table 38. pH of selected active buffers suitable for *Pachyrhizus* GST stability over the active pH range (0.1M, 25°C)

Buffer	pH
Citric	4
KH_2PO_4	5
Tris/HCl	6
K_2HPO_4	6.4
Tris/HCl	7
Tris/HCl	7.5
Tris/HCl	8
Ches	9

3.4.3. Temperature Optima and Activation Energy

The temperature optima of *Pachyrhizus* GST 1 isoenzymes activities with CDNB for different stress treatments, determined over a temperature range of 10 to 70 ^oC (283 to 343 ^oK) are shown in Table 37. The optimum range was 30 to 35 ^oC (303 to 308 ^oK). Isoenzymes of *P. erosus* EC 550 control plants and O_3 treated *P. ahipa* AC 102 exhibit identical optima activity at 31 ^oC. Identical temperature optima were also observed for isoenymes from CO_2 treated *P. erosus* EC 550, water stress TC 361 leaves and captan treated *P. erosus* EC 550 roots at 30 ^oC. Optimum temperature for isoenzyme from captan treated leaves was one degree above the control at 32^oC whilst that for water stress TC 361 roots were four degrees higher than EC 550 control plants at 35 ^oC.

Generally, temperature profiles of the selected isoenzymes were similar. Figure 41 shows a representative profile of temperature dependence activity for water stress *P. tuberous* TC 361 GST isoenzyme. Above 10 ^oC and up to 30 ^oC isoenzyme activity increases steady approximately doubling the rate for every 10-degree rise in temperature. Above optimum temperature range 30 to 35 ^oC isoenzyme activity begins to decline with increasing temperature and at 60 ^oC activity was lost due to enzyme denaturation. At the lower end of the temperature range below 10 ^oC there was also no enzyme activity.

Activation energies (E_a) for GST isoenzymes catalysed net conjugation of CDNB with GSH were determined from temperature dependency profiles (Table 37). Arrhenius plots of GST

activity with respect to temperature were linear over the temperature range of 10 to 30 °C (283 to 303 °K). Activation energy for captan treated *P. erosus* EC 550 leaves isoenzymes was significantly different from the others. It required a net energy of 42.7 $KJmol^{-1}$ whilst the others were in a range of 25 to 31 $KJmol^{-1}$. Results are means ± S.E.

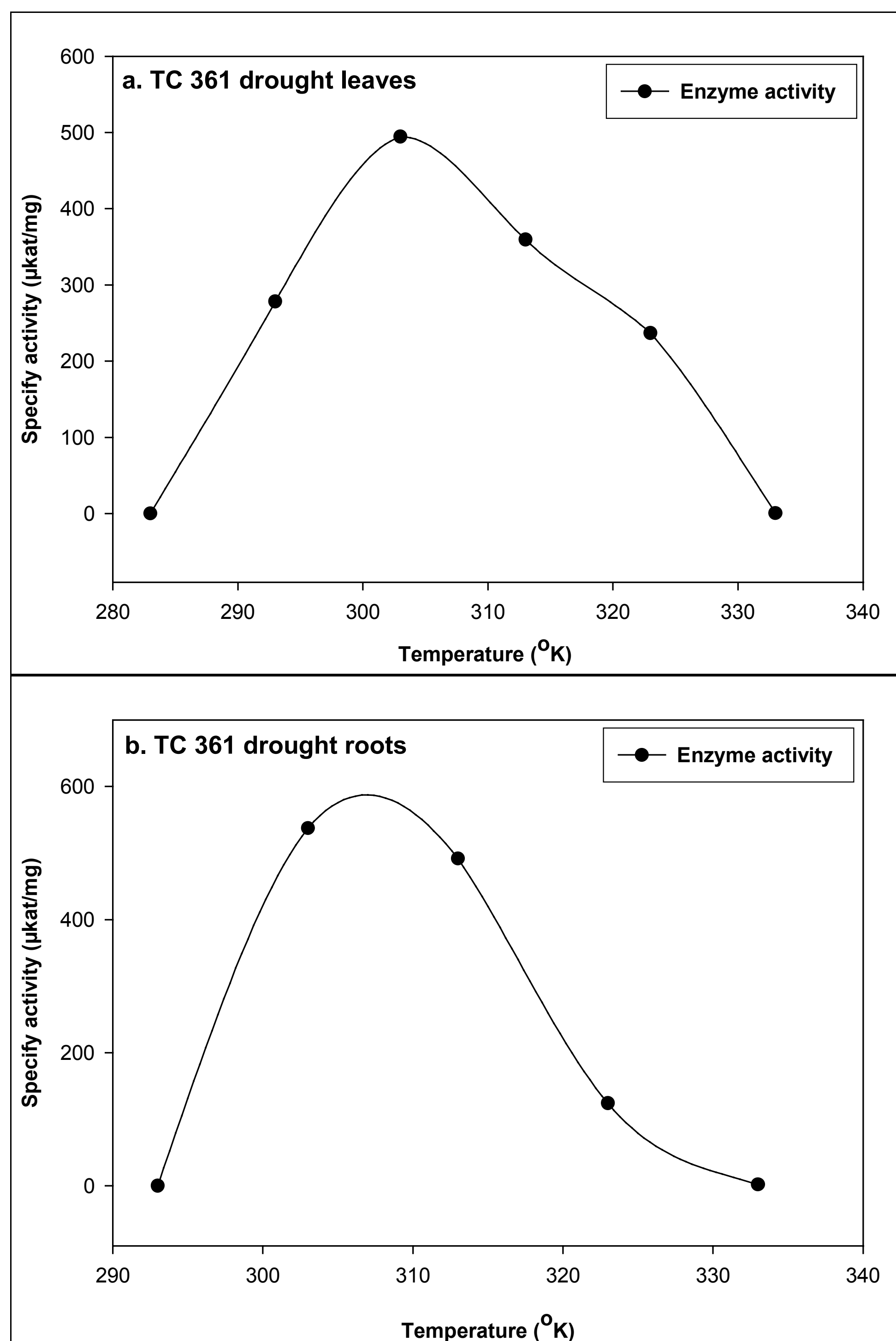

Figure 41. Representation of Temperature optima profile of GST(CDNB) activity from *P.* tuberous TC 361 water stressed plants. (A) Leaves isoenzyme *Pt*-wsGST L1 (B) Roots isoenzyme *Pt*-wsGST R1. GST activity of isoenzymes is indicated by change in absorbance at 340 nm obtained at different temperature (^{o}K). Each experimental point is the mean of three replicates ± SE. Molecular weight of isoenzymes were determined by SDS-PAGE

3.4.4. Catalytic Properties of *Pachyrhizus* GST Isoenzymes

Analysis of K_m and V_{max}

Kinetic parameters, Michaelis Menten constants K_m and maximum velocity V_{max} were determined from Lineweaver-Burk plots for selected purified *Pachyrhizus* GST isoenzymes after chromatofocusing. The correlation coefficients for the fits were all above 0.9 (Table 39).

The K_m values vary in the range of 0.98 to 4.98mM and show that there is a higher affinity of the enzyme towards its substrate, CDNB in the leaves (for both control and treated plants) than in the roots (Table 39).

The apparent K_m for captan (fungicide) treated *P. erosus* EC 550 isoenzymes in both leaves and roots were lower and significantly different from those of control and other treated plants (Table 35). Similar K_m was observed for isoenzymes in the leaves from control (2,35 ± 0,28 mM), CO_2 (2,69 ± 0,87 mM), O_3 (2,39 ± 0,28 mM) and water stress (2,29 ± 1,01 mM) plants. In contrast captan treated leaves isoenzyme demonstrate a high affinity for CDNB with a k_m value of (0,98 ± 0,18 mM).

The maximum velocity (V_{max}) clearly separate four different groups of isoenzymes (Table 39) for which references can also be drawn from other physiochemical parameters analysed.
V_{max} obtained for isoenzyme from captan treated leaves reveal that it has the fastest reaction rate significantly different from the others at 718,76 ± 63,24 µkat/mg. Next isoenzyme group which could be separated is that of CO_2 treated roots which had a V_{max} of 168,58 ± 46,27 µkat/mg. This is followed by the isoenzyme from water stress roots with a V_{max} of 71,75 ± 15,35 µkat/mg. The fourth group serves for the rest of the isoenzymes, with V_{max} below 10 µkat/mg.

Table 39. Kinetic Parameters of Pachyrhizus GST Isoenzyme separated after chromatofocusing

Variety	Treatment	Organ	K_m	R²	V_{max}
EC 550	Control	leaves	2,35 ± 0,28	0,998	6,59 ± 0,49
EC 550	Control	roots	4,98 ± 2,82	0,987	7,96 ± 3,44
EC 550	CO_2	leaves	2,69 ± 0,87	0,99	9,42 ± 1,99
EC 550	CO_2	roots	3,65 ± 1,41	0,99	168,58 ± 46,27
AC 102	O_3	leaves	2,39 ± 0,28	0,998	8,62 ± 0,63
AC 102	O_3	roots	3,33 ± 1,38	0,987	4,34 ± 1,25
TC 361	Drought	leaves	2,29 ± 1,01	0,978	8,29 ± 2,29
TC 361	Drought	roots	2,37 ± 0,80	0,987	71,75 ± 15,35
EC 550	Captan	leaves	0,98 ± 0,18	0,991	718,76 ± 63,24
EC 550	Captan	roots	1,43 ± 0,56	0,969	9,87 ± 2,11

4. DISCUSSION

4.1. *Pachyrhizus* Detoxification Status

The status of detoxification enzymes in the Yam Bean, *Pachyrhizus,* has been investigated and the findings show that the plant possess enzymes for the metabolism of xenobiotics in all three detoxification phases without any inducement. Furthermore, activity of P450 monooxygenase, peroxidase, glucosyltransferase and glutathione S-transferase could be determined in all investigated parts of the plant varieties under consideration.
Activities obtained are comparable to other cultivated crops like maize (Mozer *et al*., 1983; Fuerst *et al.*, 1994), wheat (Thom *et al*., 2002) and soybean (Skipsey *et al*., 1997).

For a root tuber crop, *Pachyrhizus* shows a high peroxidase activity in the roots compared to the leaves and seeds. As a tuber crop such level of expression in the roots will enhance root growth (Briggs and Fry, 1987). Previous reports (Debus *et al*., 1989; Schröder *et al*., 1992; Roy *et al*., 1992 and Levine *et al*., 1994) have suggested that elevated detoxification enzyme activities might serve as indicators of environmental stresses in plants. It would be an interesting subject of further studies to elucidate the peroxidase pattern in this plant, as it might be exposed to many stressors in its natural environment.

The P450 monoxygenase system of *Pachyrhizus* has been investigated using Isoproturon (IPU) as a xenobiotic substrate. Metabolic breakdown of Isoproturon to hydroxy-IPU and Monodesmethyl-IPU has been shown in a wheat cell culture (Haas, 1995). The rates obtained with the Yam Bean microsomal extracts were 9 and 3 pkat per mg protein for these metabolites. It is obvious from this result, that *Pachyrhizus* converts IPU to the same initial metabolites that are formed in wheat. In the frame of this work it was not possible to clarify whether the formation of both metabolites is catalysed by one or several P450 isoenzymes. However it is likely that more than one P450 isoform exists in the Yam bean.

The expression of glucosyltransferase, a typical phase II detoxification enzyme activity, was also determined in different *Pachyrhizus* organs. This survey demonstrated the potential of *Pachyrhizus* GTs to conjugate different xenobiotic compounds with glucose and indicated a differential expression in leaves, roots, tuber and seeds. Similar differences were found when analysing enzyme preparations from different plant species with xenobiotic phenols (Pflugmacher and Sandermann, 1998; Harvey *et al*., 2002) and may reflect differences in the

presence, in the number or in the activity of GT isoenzymes capable of glucosylating these xenobiotics.

Glucosidases are typical detoxification phase III enzymes, and antagonistic to the conjugating GT activities. The glucosidase activity in Yam Bean was expressed to a higher extent in the roots than in the leaves and seeds. It is however not known, whether the observed high level of Glucosidase is constitutive or inducible. Inducibility would be a relatively new finding as the enzyme is generally thought to be a housekeeping enzyme with steady expression in plant tissues.

The major focus of this thesis was on the characterization of the phase II enzyme, glutathione S-transferase (GST), in the *Pachyrhizus* plant, as it was speculated that this enzyme would have a central role in the detoxification of typical pesticides used also for other legumes. Hence, it was the aim to demonstrate GST activity for selected substrates and in plants under typical stress. Another question arising from the beginning of the project was the availability of glutathione or homoglutathione (hGSH) in Yam Bean, as a necessary prerequisite for the conjugation of xenobiotics in vivo.

The predominant low molecular weight thiol was found to be glutathione rather than homoglutathione. The contents of GSH was determined to be 13-fold higher than the level of hGSH. With approximately 0.4 mmoles per g FWt in the leaves, the glutathione content in leaves is twice as high as in the roots. Contrary to this, hGSH values have been found to be almost equal (0.03 and 0.02 mmoles per g, respectively) in leaves and roots. The levels of GSH concentration obtained in *Pachyrhizus* are comparable to GSH contents in other plants for which similar results have been reported (Klapheck *et al*., 1991; Foyer *et al*., 2000).

The enzyme catalysed formation of a fluorescent conjugate of monochlorobimane provides evidence of cell type-specific distribution of GST and GSH. Bimanes have been introduced some decades ago as diagnostic markers for free thiols and protein SH-groups. Whereas the monobromobimane reacts spontaneously with sulfhydrylgroups, monochlorobimane needs catalytic support of GSTs to form a fluorescent conjugate. The formation of the monochloro-bimane-glutathione conjugate was observed to be enzymatically catalyzed as seen from its higher rate of conjugate formation in the presence of cell homogenate.

It was attempted to depict this conjugation reaction microscopically in vivo. Thus, to our knowledge we are able to show for the first time a series of fluorescence images demonstrating a microsomal GST catalysed reaction in a legume. Reports on mGST especially that on plants are few and scanty (Schröder and Belford, 1996, Pflugmacher *et al*., 2000). The reason for the low level of information on microsomal enzyme activities is however not the result of lacking interest, but of the laborious experimental procedure entailed in its extraction. In this case, the amount of starting material that should be used to obtain a sound level of activity was very high.

4.2. GST Expression in *Pachyrhizus* under Environmental Stress Conditions

The activity of the major detoxification phase II enzyme, GST, under different environmental stressors has been determined and purified in several varieties of Yam Bean. The purification protocol applied followed published procedures and yielded similar purification factors (Schröder and Götzberger, 1997).

Purification and characterization of GSTs is well documented for cereals but is less well known for legumes, especially lacking in tuberous legumes. In cereals, some differences in GST responses were observed depending on the species. To compliment data on leguminous plants more knowledge about the possible novel GST isoenzymes in tuberous legumes is needed and meaningful. In the present research the purification and characterization of GST isoenzymes by a combination of glutathione (GSH)-affinity chromatography, Fast protein liquid chromatography using both, anion exchange (Mono Q) and isoelectric focusing (Mono P) from *Pachyrhizus* is described. Such an attempt using multiple purification steps and including GSH-agarose as an affinity matrix has previously been published for GST from spruce (Schröder and Berkau, 1994) and maize (Nicolaus *et al*., 1996).

The general level of GST activity towards the model compound CDNB in the plants cytosol was higher than that of the microsomal extract. This finding corroborates earlier reports of over 10-fold higher cGST activity as compared to mGST. The difference between treatments in *Pachyrhizus* GST induction level was immediately realized in the partially purified crude extract activities.

Under environmental stress GST activity for the conjugation of CDNB achieved its highest level with 200 ppb elevated O_3 in the leaves. Ozone induced stress on plants has been

frequently reported to be connected to visible injury and GST response to exposure to ozone has been reported with conflicting results (Foyer *et al*., 2000).

The enhanced response of *Pachyrhizus* GST to elevated O_3 levels exhibited a high potential of the plant's tolerance to acclimatize to such stress. It does also raise the open question whether GSTs are contributing to the detoxification of reactive oxygen species within the plant. So far, no respective conjugate of any biological metabolite has been reported in plants. Despite the presence of GST ant their induction, the impact of ozone was detrimental to the plant. Leaves developed typical ozone lesions, and tuber production was reduced by 30% as compared to the untreated control plants.

Similar to the ozone treatment, *Pachyrhizus* GST was also enhanced under elevated levels of CO_2. During the CO_2 treatment the leaves of the plants were yellowing strongly, an effect that has not been observed with other species subjected to the same treatment in the chambers. The yellowing was due to a strong decrease in all pigments in the leaves, especially chlorophyll b which was reduced to 25 % of controls. The chlorophyll a/b ratio of 3,3, however, was similar to plants under high light condition. One explanation for their observed negative effects might be that, although elevated carbon dioxide can cause some plants to grow more rapidly, increased carbon dioxide can also worsen the adverse effects of high light intensity and exert stress to the plant. It has to be mentioned, that tuber production was maintained during this treatment in all varieties.

Temporary drought will be a severe problem for any plant that is expected to grow in Africa. Water stress reduced the level of GST activity in the plants except for *P. tuberous* TC 361, which shows significant increase in GST activity in both leaves and roots. Studies carried out on response to drought in *Pachyrhizus* demonstrate that *P. erosus* is a good drought avoider (Annerose and Diouf, 1994; Belford *et al*., 2001) whilst *P. ahipa* is said to be a drought tolerance species (Annerose and Diouf, 1994). A detailed state of tolerance in *P. tuberous* has not yet been fully determined. With the significant GST induction it might be that *P. tuberous* is much more sensitive to water stress.

The levels of GST activity demonstrated by the varieties under the different treatments provide a means of selectivity of those plant varieties that can favorably response to the environmental stress condition. The difference in enzyme activity may be physiologically

relevant as it also indicates the differences existing between the varieties in response and adaptation to environmental stress.

4.3. GST Activity under Pesticide Stress

The basis for pesticide selectivity, besides target specific properties, might rely on the fact that plants are able to detoxify some pesticides fast enough to avoid accumulation to phytotoxic levels. To investigate these effects, *Pachyrhizus* plant seeds were treated with pesticide Captan, Fenoxaprop and Imidacloprid and the seedlings were later analyzed for GST activity.

Highest induction of GST root activity was observed in EC 550 for all treatment varieties. Taking samples over a time series, it was possible to determine that GST expression can be controlled spatially or temporary (Cole *et al*., 1994). With the pesticide treatment at the time of plant analysis GST activity was only dominant in the roots four weeks after sowing. On the other hand, significant GST loss was observed in TC 361 after Captan fungicide treatment. *Pachyrhizus* seeds are normally treated with fungicides for the control of post and pre harvest fungal infections. The information collected here provides the justification for continued use of these fungicides except in TC 361. Here the fungicide might competitively inhibit GSTs which has been speculated in a paper suggesting the use of GST as a biosensor detector for Captan conjugation (Choi *et al*., 2003).

In order to obtain the most important isoenzyme, which makes a difference in *Pachyrhizus* detoxification systems, selected samples from different treatments of elevated level of carbon dioxide CO_2, Ozone O_3, water deprivation drought and fungicide Captan were further purified. Purification of distinct GST isoenzymes was achieved using affinity chromatography, ion exchange chromatography and isoelectric focusing.

4.4. Purification

GSH affinity chromatography may be used routinely for the purification of GST from plants, were a single step purification procedure with simple elution by GSH yields an almost homogeneous preparation of GST in one single peak. However isoenzyme separation requires more complicated methods and was most successful with isoelectric focusing.

Chromatofocusing has been described as a suitable separation for numerous GST isoenzymes in animals (Bogaards *et al.*, 1992) and plants (Schröder and Berkau, 1993; Schröder and Götzberger, 1997). After isoelectric focusing up to four different groups of isoenzymes can be distinguished from the samples on basis of their pI range. The four pI ranges can be distinguished as 6.75 – 7.07, 5.80 – 6.16, 5.04 – 6.10 and 3.94 – 5.52. Plant GST pIs are generally found to range from 4 – 7 whereas animal GST pIs are in the basic range.

The control plants exhibited only two GST peaks with distinct difference between leaves and roots. The CO_2-treated leaves were found to have only one GST family eluting at pIs between 6.8 and 6.9, which was not found in the controls. This GST peak, however, was found in each of the extracts of the treated plants that were further investigated. It represented the main GST in ozone treated plant leaves, whereas the root enzymes had a significantly lower pI. Ozone exposed plants were found to have the most acidic GST form of all treatments. Under drought, the isoforms in roots and leaves seemed to have identical IEPs, very closely related or identical to the ozone treatment.

Captan treated plants had three GST groups in the leaves, but, contrary to the other treatments, only two GST peaks in the roots.
The major isoenzyme peaks from each sample were used for enzyme characterization. The pH optima of the isoenzymes varied slightly and corresponded to the pIs found during chromatofocusing. Temperature optima four different groups of isoenzyme can be differentiated and in combination with their activation E_a clear distinctions between isoforms were possible.

Catalytic constants K_m for CDNB in the leaves were low compared to the roots, which demonstrates that the leaves are better suited to detoxify certain xenobiotics. Changes observed in the K_m are a significant indication of alteration in the isoenzyme pattern between leaves and roots and in developmental stages (Schröder and Götzberger, 1997).

The flexibility of the GST system to react to environmental changes with a change in isoenzyme patterns becomes again visible in this study. As has been demonstrated before, (Schröder and Wolf, 1997), stressors might be able to induce but also reduce the expression of certain GSTs to a large extent. This makes the GST system a valuable bioindicator, but it does not explain the damage patterns observed under some treatments.

Pachyrhizus seems well equipped with detoxification enzymes at every stage of the previously mentioned detoxification cascade. The activity is well comparable with the detoxification capacity in other crops, and it has become clear from this study that it will be possible to improve Yam bean yields by controlling pests and weeds in plantations of this valuable plant.

Further studies should include the sequencing of the purified GSTs to understand the flexibility of the detoxification system and to elucidate the regulatory aspects of this enzyme family. It was only possible in the frame of this work to demonstrate the presence of the detoxification enzymes. It will of course now be required to demonstrate true detoxification of agrochemicals and to uncover the metabolic pathways of their breakdown, in order to facilitate a risk assessment for consumers of this plant and its products.

5. SUMMARY

- The general status of the detoxification enzymes system of *Pachyrhizus* was surveyed to determine the level of activity of the respective enzymes in the detoxification metabolic system. From phase 1 (cytochrome P450 and peroxidase), Phase 2 (glucosyltransferase and glutathione-S-transferase) and from Phase 3 (glucosidase). Results reveal that *Pachyrhizus* is well equipped with typical detoxification enzymes and that detoxification can be achieved with a wide range of xenobiotic substrates and agrochemicals.

- The conjugation of GSH to MCB was followed in the microsomes where the first fluorescence image description of microsomal GST catalyzed reaction in a legume was demonstrated. Microsomal GST activity was found to be in the range of 0.3 ± 0,04 nkat/g to 0.7 ± 0,09 µkat/g.

- The relative abundance of glutathione (GSH) to homoglutathione (hGSH) as the main conjugating substrate was determined by HPLC. GSH was found to be dominant major free thiol for xenobiotic conjugation.

- Under induced conditions of elevated levels of CO_2 and O_3, water stress and from plants treated with the fungicide Captan, GST was purified and partially characterized. GST activity was determined in both microsomes and cytosol. *Pachyrhizus* GST showed high variation, differing between treatments as well as between varieties (*P. erosus*, *P. ahipa* and *P. tuberous*).

- Purification of isoforms was achieved by NH_3SO_4 precipitation and chromatographic techniques, namely: affinity chromatography, FLPC of Mono Q (anion exchange) and Mono P (Isoelectric focusing). Determination of isoelectric points, K_m and V_{max}, temperature and activation energy were used to partially characterized and separate four different groups of GST isoenzymes.

- The study indicates that GST expression may serve as an excellent model system for the investigation of stress-induced response in *Pachyrhizus* and the plant's ability to response to and withstand stress.

6. ZUSAMMENFASSUNG

Die Entgiftung von Fremdstoffen und Agrochemikalien ist ein essenzieller Prozess im Leben von Pflanzen, da sie als sessile Organismen widrigen Umwelteinflüssen und der Aufnahme solcher Substanzen hilflos gegenüber stehen. In der vorliegenden Arbeit wurde die Aktivität einiger der geläufigsten pflanzlichen Entgiftungsenzyme, unter anderem Cytochrom P450 Monooxygenasen, Peroxidase, Glucosyltransferase, Glutathion S-Transferase und Glucosidase aus Pachyrhizus-Proteinextrakten bestimmt, um einen Überblick über die Toleranz der Pflanze gegenüber Agrochemikalien zu erhalten.

Die Studien belegten, dass Pachyrhizus die entsprechenden Enzyme enthält und in der Lage ist, Modellsubstrate und Pestizide, unter ihnen die herbizide Isoproturon und Fenoxaprop-ethyl sowie das Fungizid Captan und das Insektizid Imidacloprid zu entgiften.

Mit dem Focus auf Glutathion S-Transferasen, einer Klasse herausragender Entgiftungsenzyme, wurde der Einfluss von Trockenheit, Ozon und erhöhtem CO_2 auf den Entgiftungsstoffwechsel studiert. Erneut zeigten die Entgiftungsenzyme hohe Variabilität und starke Induktion unter dem Einfluss des Fungizids. Die weitere Reinigung der Isoformen von GST aus Pachyrhizus belegte, dass Umwelteinflüsse das Isoenzymmuster der GST verändern. Diese Veränderungen können als Biomarker für Stress herangezogen werden. Weitere Studien werden folgen, in deren Rahmen molekularbiologische Untersuchungen durchgeführt werden sollen, um die Regulation der Entgiftungskapazität zu verstehen.

7. REFERENCES

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