Microsomal Detoxification Enzymes in Yam Bean [*Pachyrhizus erosus* (L.) Urban]

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Cytochrome P450s and glutathione-S-transferases (GSTs) constitute two of the largest groups of enzyme families that are responsible for detoxification of exogenous molecules in plants. Their activities differ from plant to plant with respect to metabolism and substrate specificity which is one of the reasons for herbicide selectivity. In the tuber forming yam bean, the legume *Pachyrhizus erosus*, their activities at the microsomal level were investigated to determine the detoxification status of the plant. The breakdown of the herbicide isoproturon (IPU) to two distinct metabolites, 1-OH-IPU and monodesmethyl-IPU, was demonstrated. GST activity was determined with model substrates, but also by the catalysed formation of the fluorescent glutathione bimane conjugate. This study demonstrates for the first time microsomal detoxification activity in *Pachyrhizus* and the fluorescence image description of microsomal GST catalysed reaction in a legume.

Key words: Fluorescent Conjugate, P450 Monooxygenase, Glutathione S-Transferase

Introduction

Cultivated plants are constantly exposed to attacks from pests, diseases and pollutants and crop failure can result in enormous agricultural and economic losses. With increasing use of agrochemicals and impact of pollution more detailed information is needed to understand plant response, pesticide selectivity and detoxification capacity (Schröder, 1997).

Plant membranes are the first compartments of living cells, which have to respond to foreign chemicals attack. Numerous lipophilic substances have been shown to accumulate in membranes, and their fate might be decisive for plant survival. Hence, the process of xenobiotic metabolism in membrane bound organelles is an essential step in the three-phase detoxification process of plants (Coupland 1991; Sandermann, 1994; Coleman *et al*., 1997).

The ability of plants to detoxify xenobiotics in cellular membranes depends on the presence of certain detoxification enzymes such as cytochrome P450 monooxygenases and glutathione S-transferases. However, up to now, the characterization of membrane defense systems is in its infancy, mostly due to difficulties encountered during the isolation of the proper microsomes.

Cytochrome P450 monooxygenases (CYPs, EC 1.14.14.1) constitute one of the major classes of phase one reaction enzymes responsible for detoxification of exogenous molecules in both animals and plants (Durst *et al*., 1997; Werck-Reichhart *et al*., 2000). In plants they are usually found bound to the endoplasmic reticulum or inner mitochondrial membranes. Besides representing potentially significant metabolic sinks for environmental contaminants (Sandermann, 1994), they are involved in the biosynthesis of hormones, lipids, and secondary compounds (Schuler, 1996). CYPs are present in a multitude of isoforms in plants and can be used for controlling herbicide tolerance and selectivity (Werck-Reichhart, 1995). CYPs activity in herbicide detoxification in cereals has been extensively studied, in barley (Bundock *et al*., 2003), in maize (Barrett, 1995; Persans *et al*., 2001), in rice (Zhong *et al*., 2002), in sorghum (Kahn *et al*., 1999) and in wheat (Frear, 1995; Forthoffer *et al*., 2001). By contrast, the study is far from equal in legumes. Only in mung bean, *Vigna radiata* (Mizutani *et al*., 1993), in alfalfa, *Medicago sativa* (Fahrendorf and Dixon, 1993) and in soybean (Siminszky *et al*., 1999) has any major investigation been done and to a lesser extent in *Vicia sativa* (Pinot *et al*., 1993). Complete genome sequences have been reported for CYPs from Jerusalem artichoke *(Helianthus tuberosus*; Teutsch *et al*., 1993), mung bean *(Vigna radiata*; Mizutani

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et al., 1993), and alfalfa *(Medicago sativa*; Fahrendorf and Dixon, 1993) and recently in *Arabidopsis* where a total of 224 cytochrome P450 genes was identified. However, the function of most of these genes is still unknown.

Glutathione-S-transferases (GSTs, EC 2.5.1.18) are like CYPs a diverse group of enzymes involved in detoxifying electrophilic xenobiotics by catalysing their conjugation with the tripeptide glutathione (GSH) subsequent to or without activation by CYPs. In plants they play a major role in detoxifying herbicides (Schröder, 1997). Hence, 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).

Numerous isoforms of plant GSTs have been found to occur in the cytosol (Marrs, 1996) and microsomal forms have been reported in spruce (Schröder and Belford, 1996), onion (Schröder and Stampfl, 1999) and other plant species (Pflugmacher *et al*., 2000). Though GSTs activity can be determined in all parts of plants, studies on activities of specific GST in individual cell types or tissues are few (Jepson *et al*., 1998; Edwards *et al*., 2000). Also, like CYPs, GST has been more widely studied in cereals than in legumes. The growing interest in plant GSTs is attributed to their agronomic value, since it has been demonstrated that glutathione conjugation is the major reason for tolerance, diversification and selectivity of crops for a variety of xenobiotics.

The present research is focused on the tolerance of the tuber-bearing legume, yam bean, *Pachyrhizus* DC (Vietmeyer, 1986) to xenobiotics with view of its possible establishment, utilisation and large-scale production in developing countries. The plant has been described as a sustainable crop, high yielding vegetable and is today cultivated in numerous countries in the southern hemisphere (Sørensen, 1988, 1996). Especially lacking are data on the performance of the plant after application of agrochemicals, *i.e.* herbicides, fungicides and insecticides. In this report the status of the main detoxification enzyme systems in membranes (microsomes), cytochrome P450 and glutathione-S-transferase, involved in pesticides metabolism in *Pachyrhizus* are evaluated for the first time.

Materials and Methods

Cultivation

Yam bean, *Pachyrhizus erosus* (accession EC 550) was grown under greenhouse conditions in pots at the GSF – National Research Centre for Environment and Health, Neuherberg, Germany. The pots were 24 cm high and 26 cm wide, containing 7 kg of soil in a mixture of 46% loam, 31% sand and 23% gravel. The mean temperature was 27 °C during the 9 h daylight and 18 °C during the dark period. The light intensity was 4.0 klux. The relative humidity was maintained at 70%. Five seeds were sown in each pot at a depth not below 2 cm. The pots were watered regularly to ensure seed germination and seedling development. Leaves were harvested separately from plants at vegetative phase 3 before the onset of tuber formation (Sørensen *et al*., 1993), four weeks after sowing. The samples were immediately immersed in liquid nitrogen and stored at −80 °C.

Recovery of plant microsomal extracts

Frozen samples of leaves weighing between 3 to 10 g, were crushed and ground in liquid nitrogen in a chilled mortar with a pestle to a smooth paste. 10% insoluble polyvinylpyrrolidone (PVP 80) were added to the fine powder followed by five volumes (v/w) of extraction buffer 0.1 M Tris[Amino(hydroxymethyl)aminomethane]-HCl buffer (pH 7.8; buffer A) containing 2 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, and freshly prepared 0.25 M saccharose, 5 mM dithioerythritol (DTE) and 1 mM phenylmethylsulfonylfluoride (PMSF). The mixture was homogenized at 13,000 rpm with an Ultra Turrax (T8, Kika Werke, Stauffen) for 2 × 90 s. The homogenate was pressed through a double layer of miracloth (Calbiochem) and centrifuged at 10,000 × *g* at 4 °C for 5 min. The supernatant was ultra-centrifuged at 105,000 × *g* at 4 °C for 1 h. The resulting pellet was the microsomal fraction and the supernatant (designated as crude extract) was the cytosolic fraction.

Production of microsomal enzymes

The pellet containing microsomal fraction was re-suspended 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 at 105,000 × *g* at 4 °C for 1 h. 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 served for the measurements of both cytochrome P450 and microsomal GST activity.

Isolation and partial purification of cytosolic GST

Glutathione S-transferase in the cytosol was isolated for determination of activity against that of the microsomes. Solid ammonium sulphate was added to the supernatant (designated as crude extract) obtained after first ultra-centrifugation to give a saturation of 40%. After stirring for 30 min on ice, precipitated proteins were removed by centrifugation at 39,200 × *g* at 4 °C 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. 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, Freiburg), which had been 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 aliquots of 0.5 ml and stored at −80 °C until use. This fraction served only for determination of cGST activity.

Cytochrome P450 enzyme assay

The cytochrome P450 activity from microsomal extract was measured according to Salaün *et al.* (1989), Mougin *et al.* (1991) and Haas *et al.* (1997). The reaction was started by the addition of buffered microsomal enzyme (25 mM Tris-HCl buffer, pH 7.8, containing 2 mM EDTA and 20% glycerol) to the substrate ^{14}C-ring labelled isoproturon [IPU, 3-(4-isopropylphenyl)-1,1 dimethylurea, specific radioactivity = 507 Bq μg^{-1}, radiochemical purity > 98%], pre-incubated for 5 min. The hydrolysis of IPU occurred in the presence of 2 mM NADPH plus a regenerating system containing 6.7 mM glucose-6-phosphate and 0.4 U glucose-6-phosphate dehydrogenase. The reaction mixture with a final volume of 250 μl was further incubated at 25 °C for 1 h in a shaker. The reaction was stopped by the addition of 100 μl cold acetone and the resulting mixture was centrifuged for 10 min at 13,000 x *g*.

HPLC determination of cytochrome P450 activity

Aliquots of reaction mixtures obtained from the organic phase of the supernatant were analysed by high-pressure liquid chromatography (HPLC) using a reversed-phase C-18 column (LiChrospher 100, 5 μm, 250 × 4 mm; Merck, Darmstadt). The HPLC system was equipped with a UV-VIS detector, 240 nm (Merck, Darmstadt), and a radioactivity detector (Berthold, Wildbad). The separation of metabolites proceeds in a linear gradient of acetonitrile in water from 5 to 60% in 35 min at a flow rate of 0.80 ml min^{-1}. Parent compound and metabolites were identified by comparing the retention times with reference substances.

GST enzyme assays and protein determination

GST activity was determined spectrophotometrically using CDNB (1-chloro-2,4-dinitrobenzene) as a model substrate according to Habig *et al.* (1974) and with DCNB (1,2-dichloro-4-nitrobenzene) following the assay method of Schröder *et al.* (1990). The reaction mixture for CDNB assay was performed at 340 nm ($\varepsilon_{340\,nm}$ = 9.6 mM cm^{-1}) and contained 0.1 M potassium phosphate buffer, pH 6.4, 60 mM GSH and 30 mM CDNB dissolved in ethanol. The assay with DCNB as substrate was monitored at 345 nm ($\varepsilon_{345\,nm}$ = 8.5 mM cm^{-1}), with a reacting mixture containing 0.1 M potassium phosphate buffer, pH 7.5, 60 mM GSH and 30 mM DCNB dissolved in ethanol. All measurements were corrected for non-enzymatic conjugation rates. Enzyme activity is expressed as μkat mg^{-1}. Protein contents were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Fluorescence microscopy

The conjugation of xenobiotics to 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). MCB reacts with GSH to form a fluorescent bimane conjugate thus making it possible to trace the GSH 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-GSH adducts in the microsomal fraction was examined by fluorescence microscopy.

Enzyme assay and microscopic measurement

The fluorescence of the conjugate was determined using an inverted microscope (Zeiss Axiovert 100), a monochromator (TILL) for excitation and TILL imaging software for data acquisition and data analysis (Schröder and Stampfl, 1999). Aliquots of 30 μl from freshly prepared microsomal fraction of leaves of *P. erosus* EC 550 were transferred to clean microscopic cover slides and suspended in 50 μl 1% Phytagel (Sigma, Dreieich). 10 μl of 20 mM GSH was added followed by 10 μl of 50 mM MCB. The assay was allowed to incubate for 5 min at room temperature. The slide covered with a cover slip was mounted in a special measuring chamber fixed on the stage of an inverse fluorescence microscopy (Axiovert 100, Objective: Flat Neofluar 20 × 0.50, Zeiss, Jena). The GSH-MCB adduct formation was monitored with excitation at 380 nm and emission was measured at 510 nm. The fluorescence measurements were made over a period from 40 min. Three to five attempts were repeated.

Results and Discussion

Cytochrome P450 activity

HPLC separation of radioactive labelled metabolites was applied to confirm qualitatively the metabolic activity of *P. erosus* cytochrome P450 on ^{14}C-isoproturon. Fig. 1A and 1B compare HPLC chromatograms of reference substances of ^{14}C-isoproturon and metabolites with that of *P. erosus* microsomal sample containing ^{14}C-isoproturon-CYP activity. After 1 h incubation of microsomal extracts with IPU, the reversed phase HPLC analysis resolved one major metabolite peak and a second minor or trace metabolite peak in the enzyme assay (Fig. 1B). The major metabolite peak was identified to be 1-OH-isoproturon (3-[4-(1-hydroxyisopropylphenyl)]-1,1-dimethyl urea, 4.1%) and the trace metabolite peak (1.4%) was monodesmethyl-isoproturon, [3-(4-isopropylphenyl)-1-methyl urea, Fig. 1B]. The retention times (RT) for the metabolites and substrate in the enzymatic radiolabelled samples were 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 1-OH-IPU and 3.1 ± 1.3 pkat mg prot^{-1} for monodesmethyl-IPU. They are within the range of rates described for microsomal CYP from other plant species (Didierjean *et al.*, 2002). The formation of the metabolites confirmed that isoproturon induces CYP activity and the enzyme's ability to hydroxylate and demethylate IPU (Table I). 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. Earlier reports indicated that several phenylurea derivatives might be substrates of the same P450 enzyme, and that the same P450 might be able to carry out the double dealkylation of the molecules (Frear, 1995). In Jerusalem artichoke (*Helianthus tuberosus*) Robineau *et al.* (1998) showed that CYP76B1 catalyses the mono- and di-*N*-demethylation of both chlortoluron and isoproturon and indicate that plant tolerance to phenylurea and selectivity of these herbicides thus relies on the presence and relative expression of at least one CYP. Further studies would be required to determine how many P450 isoenzymes are involved in herbicide metabolism in yam bean and how their levels are regulated. Evidence points to the fact that as there is a number of herbicides presently known to be metabolised by plant CYP (Werck-Reichhart *et al.*, 2000). The metabolism of isoproturon in *Pachyrhizus* suggests that other herbicides of the class of phenylureas could be substrates.

Table I. Retention times, relative peak areas of HPLC separation and enzymatic conversion rates of isoproturon metabolites from cytochrome P450 metabolism in *Pachyrhizus erosus*. Values represent means from 3 independent measurements.

Fraction	Peak 1 Metabolite 1	Peak 2 Metabolite 2	Peak 3 = substrate ^{14}C Isoproturon
Retention time	23.50	33.87	35.52
Peak area (%)	4.12 ± 0.9	1.38 ± 0.6	94.82 ± 0.9
Amount per sample [μg]	4.9 ± 1.1	1.66 ± 0.7	113.87 ± 1.1
Enzyme activity [pkat mg prot^{-1}]	9.13 ± 2.05	3.09 ± 1.3	

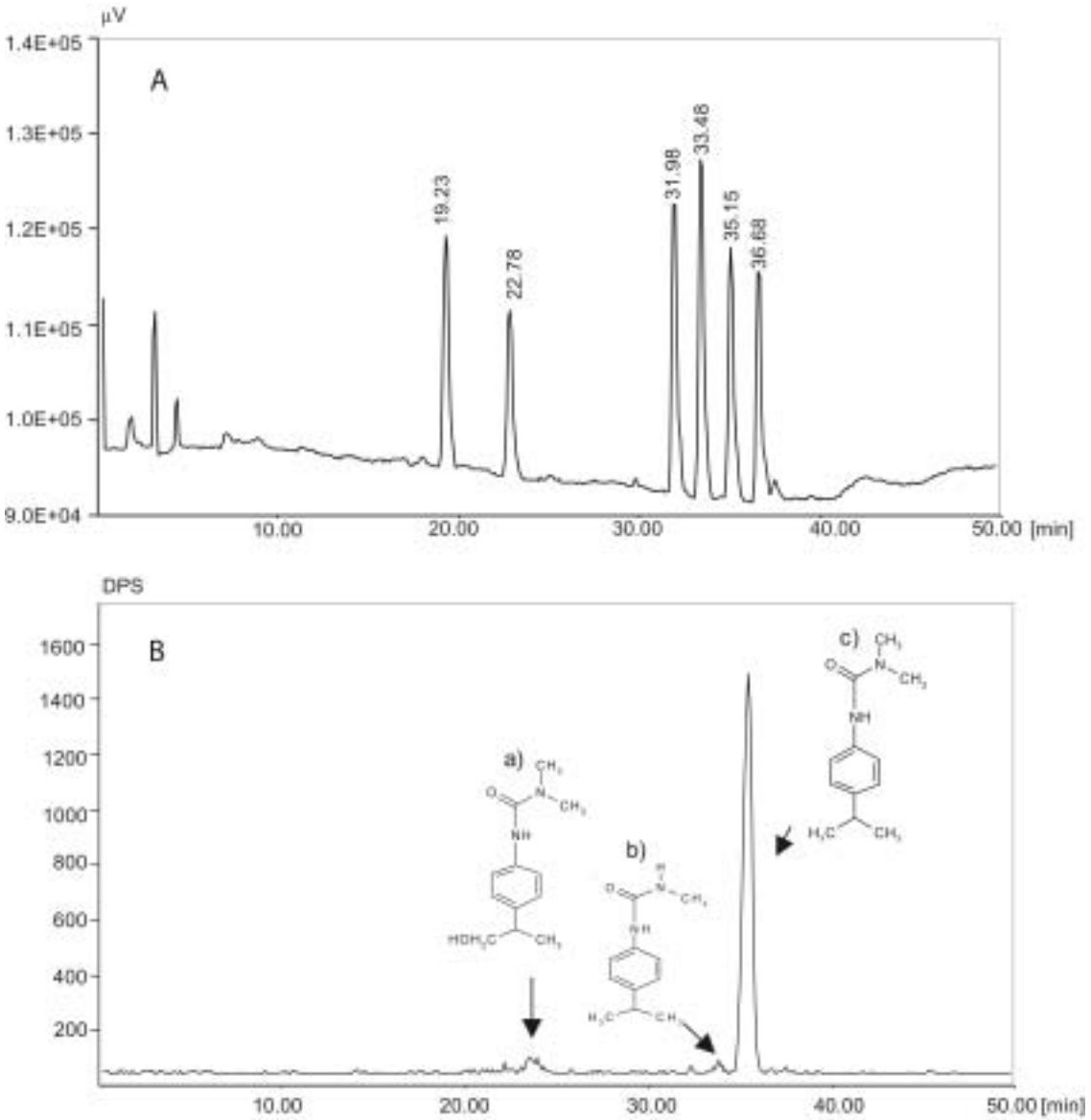

Fig. 1. Metabolism of isoproturon (IPU) in yam bean. (A) Standard reference peaks of isoproturon metabolites (19.23 min: 2-OH-monodesmethyl-IPU; 22.78 min: 1-OH-isoproturon; 31.98 min: didesmethyl-IPU; 33.48 min: monodesmethyl-IPU; 35.15 min: isoproturon; 36.68 min: 4-isopropylanilin). (B) HPLC separation of ^{14}C-isoproturon metabolites from yam bean cytochrome P450 metabolism in microsomal extracts after 1 h of incubation.

Glutathione S-transferase activity

Three protein extracts (crude, cytosolic and microsomal) from leaves of *Pachyrhizus erosus* (EC 550) contained GST activity for the conjugation of the model xenobiotics CDNB and DCNB (Table II). Although not herbicides, these model substrates have been applied as to characterize GST activity for xenobiotics in many plants (Lamoureux and Rusness, 1989; Schröder, 1997). Earlier studies (Schröder and Wolf, 1996; Schröder and Götzberger, 1997) have revealed that CDNB and DCNB may be conjugated by several GST isoforms with overlapping substrate specificity and

Table II. Activity of glutathione S-transferase (μkat mg^{-1}) in leaves of *Pachyrhizus erosus* EC 201 with CDNB and DCNB as model substrates. Measurements were done in triplicate and corrected for non-enzymatic values.

Substrate	CDNB [μkat mg^{-1}]	DCNB [μkat mg^{-1}]
Crude extract	124.71 ± 1.30	3.73 ± 0.37
Cytosolic extract [80% $(NH_4)_2SO_4$ saturation]	209.00 ± 1.21	5.25 ± 1.17
Microsomal extract	76.90 ± 5.44	63.20 ± 4.61

the presence or absence of certain isoforms determines the overall defence capacity of a given plant.

In *Pachyrhizus*, CDNB conjugation occurred at the highest rates and proved to be the better substrate for GST in all three extracts used. In crude extracts, CDNB was converted to the respective glutathione conjugate at rates of 124 μkat mg^{-1}. GST activity for the conjugation of CDNB increased two-fold in the cytosolic fraction (209 μkat mg^{-1}) with increased purification after 80% ammonium sulphate saturation. Activity with DCNB was only 3.7 μkat mg^{-1} in crude extracts and increased slightly to 5.25 μkat mg^{-1} after ammonium sulphate precipitation. In the microsomal fraction though, there is virtually no significant difference between activity for the substrates CDNB and DCNB (76 μkat mg^{-1} and 63 μkat mg^{-1}, respectively).

With respect to DCNB, GST activity in the microsomes (63 μkat mg^{-1}) is 12-fold higher compared to that in the cytosol (5.25 μkat mg^{-1}). Schröder and Stampfl (1999) had previously observed a similar ratio between CDNB and DCNB activities in microsomes isolated from onion epidermal tissue. With the relatively high GST-DCNB activity observed in the microsomes in comparison to the cytosol it can be concluded that the isoforms of GST are either more substrate specific in the cytosol than in the microsomes, or that the distribution of certain GST isoforms is strictly organelle specific. Moreover, it may be assumed that detoxification capacity for other compounds of interest may also be differently distributed between cytosol and the membrane fraction. Further studies will include the separation and identification of GSTs from cytosolic and microsomal sources to elucidate the effects described here.

Visualization of microsomal glutathione conjugation

The formation of a fluorescent glutathione conjugate was followed microscopically in the microsomal leaf extract of *P. erosus* (EC 550). Fig. 2 shows the time course of development of GSH-

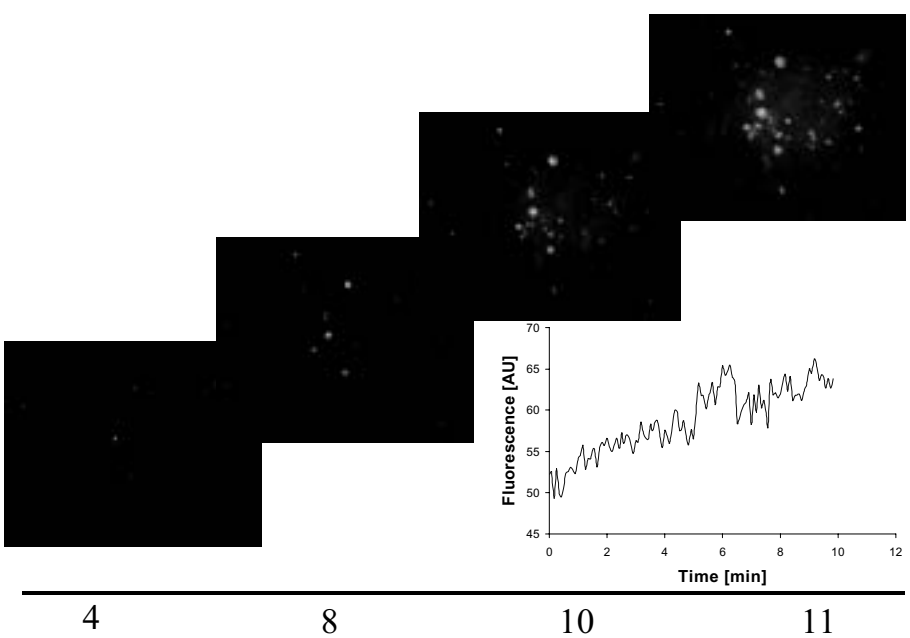

Fig. 2. Fluorescence description of microsomal GST in a legume. Time course of fluorescence development of GSH-MCB conjugate as monitored microscopically in microsomes of yam bean, *Pachyrhizus erosus* EC 550. Insert: Development of fluorescence across the microscopic slide during the incubation [AU].

MCB fluorescent conjugate. To our knowledge this image provides the first fluorescence description of microsomal GST catalysed reaction in a legume. Fluorescence development begins immediately after incubation with just a tiny spot on single microsomes intensifying up to 16 min. At 20 min a shining fluorescence appeared and brightly expressed after 22 min. The increased appearance of this fluorescence conjugate provides visual evidence of the catalytic conversion of monochlorobimane and confirms production of its nonphytotoxic glutathione conjugate.

In this study we have demonstrated that in the legume *Pachyrhizus* (yam bean), both enzyme classes are present, metabolising a herbicide as well as model substrates. We have also to our knowledge for the first time presented evidence of xenobiotic glutathione conjugation in the microsomes of a legume produced by the fluorescence of the conjugate. The identification of these enzymes involved in defence metabolism represents an important step towards a better understanding of *Pachyrhizus* reaction to environmental stress and the possible use of various pesticides, which will allow for a better justification of pesticide use in this plant.

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