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Screening of NADPH Cytochrome P450 Reductase and Alkaline Protease Activities in Germinating Cajanus Cajan L. Seedlings In The Presence of Herbicides and Protein Synthesis Inhibitors

KEYWORDS: NADPH-Cytochrome p450 reductase, Cajanus cajan, germination, xenobiotics.

§Shankar Mular	Rajyalakshmi Amancherla	§Zia H Khan
§Department of Biochemistry, Shri Shivaji College of Arts, Commerce And Science. Akola (M.S.) Maharashtra State Highway 204, Ramdaspeth, Akola, Maharashtra 444003	§Department of Biochemistry, Shri Shivaji College of Arts, Commerce And Science. Akola (M.S.) Maharashtra State Highway 204, Ramdaspeth, Akola, Maharashtra 444003	§Department of Biochemistry, Shri Shivaji College of Arts, Commerce And Science. Akola (M.S.) Maharashtra State Highway 204, Ramdaspeth, Akola, Maharashtra 444003

ABSTRACT *The detoxifying enzyme NADPH-Cytochrome p450 reductase (Cyt p 450) which plays a key role in metabolism of xenobiotics in plants assumes great significance during seed germination particularly when the germinating seedlings are exposed to chemicals such as protein synthesis inhibitors and herbicides.*

Our study is an attempt to get insights into how the Cyt p 450 is varying with protein reserve mobilization when Cajanus cajan seedlings are exposed to cycloheximide, diclofop and sulfonyl urea. The alkaline protease and Cyt p 450 activities were determined in the germinating seedlings of two varieties of Cajanus cajan namely ICP 7203-1 and Yashoda for a period of 8 days post germination. The results showed that the Cyt p 450 activities were gradually rising during the seedling growth and assumed a peak on the 6th day. The physical injury and the chemical challenge to the germinating seedlings resulted in a 3-4 fold increase in Cyt p 450 activity. The sulfonyl urea has elicited more Cyt p450 activity than diclofop whereas the cycloheximide exposure has resulted in the lowest activity of the NADPH Cytochrome p450 reductase in the seedlings.

INTRODUCTION

Plants accumulate protein reserves in developing seeds. The major amount of these reserves consists of specific storage proteins (Shewry & Casey, 1999), like globulins, which predominate in dicotyledonous seeds, or prolamins, which are the major storage proteins of most cereals and pulses. Storage proteins are not only accumulated and mobilized in specific storage tissues but also in axis organs like the radical and the embryonic shoot (Tiedemann, etal 2000). Protein mobilization in storage tissues does not start in all regions simultaneously (Smith, D.L, 1981). During germination small amounts of storage proteins are broken down in limited regions. This degradation might be mediated by stored proteinases. It is not known whether storage protein mobilization in embryonic axes is mediated by de novo formed or by stored proteinases, neither is it known to what extent early protein biosynthesis during germination in axis and storage tissue (Bewley, 1982&1997) depends on free amino acids released by protein mobilization.

Expression of NADPH-cytochrome P450 reductase (Cyt P_{450}), one of the predominant detox enzymes, is regulated both developmentally prior to seed maturation and during germination, and differentially in the cotyledons, radicle and seedling tissues (Timothy J.T, etal, 2000). In order to explore the how protein reserve mobilization is varying with NADPH-cytochrome P450 reductase levels we have measured total protein content, protease activity and free amino acids released by protein mobilization along with Cyt P_{450} activity developed in germinating seedlings of 2 varieties of Cajanus cajan namely ICP-1 (ICP 7203-1) and Yashoda for a period of 8 days post germination.

The Cyt P_{450} activity is found to increase when the plants are challenged not only with physical injuries but with exposure to harmful chemicals as well (Morant. M, etal,2003). Since Cajanus is an important pulse crop cultivated widely all over the world it is important to get insights into the levels of detox enzyme Cyt P_{450} which participates in metabolism of xenobiotics. That is why in this study NADPH- cytochrome P450 reductase activity was also measured in the context of physical injury and in the presence of 2mM concentration of herbicides sufonyl urea and diclofop. The variations in the development of NADPH-cytochrome P450 reductase were also investigated in the presence of 2Mm Cycloheximide, is also an inhibitor of protein biosynthesis.

Materials and Methods

The seeds of ICP-1 and Yashoda were germinated in the dark and the seedlings were allowed to grow for 8 days.2 mM solutions of diclofop, sulfonyl urea and cycloheximide were prepared and used instead of distilled water for seed germination and growth. The protein reserve mobilization study was done in the cotyledons of control seeds germinated with distilled water and in the cotyledons of the seedlings germinated in the presence of diclofop, sulfonyl urea and cycloheximide. NADPH-cytochrome c reductase activity was determined in the excised hypocotyls of the seedlings grown in the presence of herbicides and cycloheximide to study their effect on the development of the reductase. To study the effect of physical injury on the development of NADPH-cytochrome c reductase activity the enzyme assay was also done in the hypocotyls cut into pieces and incubated on moist filter papers. This was done from day 2 because the hypocotyls were too short to be excised on day 1 after germination.

Enzyme assays

Alkaline Protease assay

Protease activity was determined according to the modified Anson's method. 1.0 ml of the cotyledonary extract was taken in a test tube and 1.0 ml of 0.1 M Tris HCl buffer pH 9.4 added to it. One ml of the substrate (2% Hammersten's casein pH 7.0) was added to the buffer - enzyme solution and incubated at 37°C for 10 minutes in a water bath.

At the end of 10 minutes, 10.0 ml of 5N TCA (trichloroacetic acid) was added to stop the reaction. The precipitated casein was then filtered off and 5.0 ml of the filtrate were taken in a test tube. To this 10.0 ml of 0.5N NaOH solution and then 3.0 ml of the folin ciocalteu reagent (one ml diluted with 2 ml of distilled water) were added. Final readings were taken in a spectrophotometer at 750 nm. Blanks of the samples were prepared by adding the TCA before the addition of substrate.

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NADPH Cytochrome P450 assay
NADPH Cytochrome P450 assay was done as described by Manjunath Seth (1993). Briefly NADPH- cytochrome c reductase activity was determined spectrophotometrically at 550 nm as described using a reaction mixture containing 40uM cytochrome c, 50 mM Tris HCl (pH 7.4), 10 mM MgCl2, 150 mM KCl, and 2 mM NaN3. Cytochrome P450 content was determined spectrophotometrically using a ELICO Junior Spectrophotometer Model CL-27.

Determination of free amino acids and protein contents
Protein concentration was estimated by the Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard and free amino acids was determined by ninhydrin method.

Estimation of Amino Acids
The free amino acids were estimated in the protein-free supernatant by the Ninhydrin method (Raghuramulu et al., 1983).

A suitable aliquot of the cotyledonary extract was made up to 3ml with water and mixed with 2ml of 20% TCA. The precipitated protein was filtered. A suitably diluted sample of the filterate in 0.2ml was mixed with 1.8ml of citrate buffer (0.1 M; P^H 5.0)and 1ml of ninhydrin reagent (2g of ninhydrin and 0.3 g hydrindantin dissolved in 75ml of methyl cellosolve +25ml of 4M acetate buffer, P^H 5.5).The contents were heated in a boiling water bath for 15 min, cooled and 5ml diluents solution (equal volumes of water and isopropanol) was added immediately and mixed. The extinction was read at 570 nm against reagent in ELICO Junior Spectrophotometer Model CL-27. Standards containing known amounts of leucine (5-40µg) were run simultaneously under identical conditions and the free amino acid content is expressed as mg per g wet tissue.

The SDS PAGE was carried out by the method of Lammeli, (1970).

Statistical Analysis
Each value presented in figures represents the arithmetic mean of three the independent determinations unless otherwise stated. The level of significance was calculated by Student "t" test.

Results and discussion
The results showed that the protein contents in germinating seedlings have decreased from day 1 to day 8 it was also observed that there was a concomitant increase in the proteolytic activities. The free amino acid content has increased from day 1, reached a peak and then declined on the 8th day. This shows that both the selected varieties of Cajanus cajan followed that normal trends in the case of protein reserve mobilization and development of proteolytic enzyme. Activity of NADPH cyt p450 reductase from ICP-1 and Yashoda was measured over a period of 8 days post germination and the results were analyzed.

The analyses showed that NADPH cyt p450 reductase activity in healthy growing normal cotyledons started to increase from 0.036±0.001 & 0.038±0.001 units on the day 0 in ICP-1 and Yashoda respectively and reached a maximum of 0.102±0.002 & 0.112±0.002 units on the 6th day of seedling growth. It has declined on the 8th day to 0.053±0.001 & 0.081±0.002 units in ICP-1 and Yashoda respectively.

This prompted us to explore further into the variations and trends in the development of NADPH-cytochrome c reductase activity in different conditions of physical injury and exposure to chemical inhibitors and herbicides.

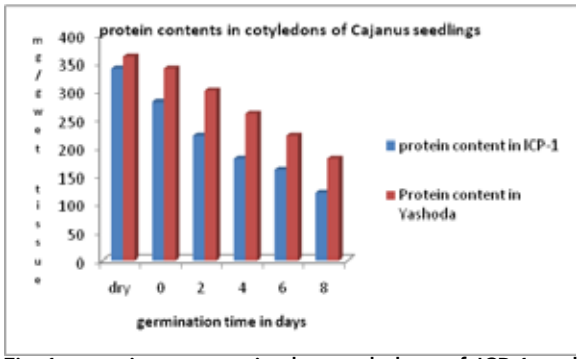

Fig 1: protein contents in the cotyledons of ICP-1 and Yashoda Cajanus seedlings

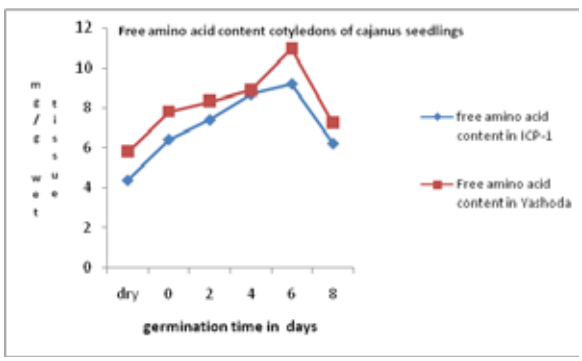

Fig 2: Free amino acid contents in the cotyledons of ICP-1 and Yashoda Cajanus seedlings

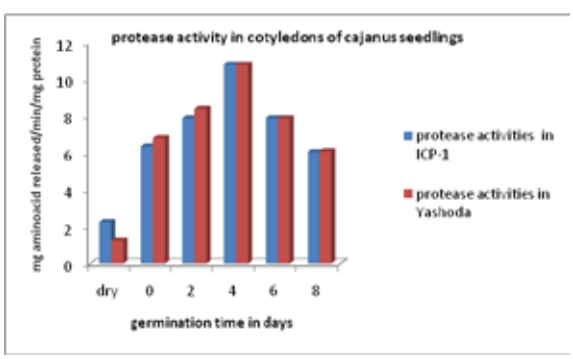

Fig 3: Alkaline protease activity in the cotyledons of ICP-1 and Yashoda Cajanus seedlings

Injury of the hypocotyledons from 2 to 8 day old seedlings by cutting them into 1-mm fragments, followed by incubation in a moist atmosphere for 10 h, results in a 3 fold time-dependent increase in NADPH-cytochrome c reductase activity and spectrally detectable P450.The trends in the development of NADPH-cytochrome p450 reductase activity were studied in excised hypocotyledons. The study of Activity of cyt p450 reductase in the cotyledons of germinating seedlings in ICP-1 and Yashoda varieties over a period of 8 days the has showed that the highest activity in ICP-1 and Yashoda was about 0.102±0.002 & 0.112±0.002 units whereas in excised hypocotyledons it was 0.304±0.002 & 0.312±0.002 units clearly indicating that there is more than 3 fold increase in the activity of cyt p450 reductase if there is a physical injury to hypocotyledons.

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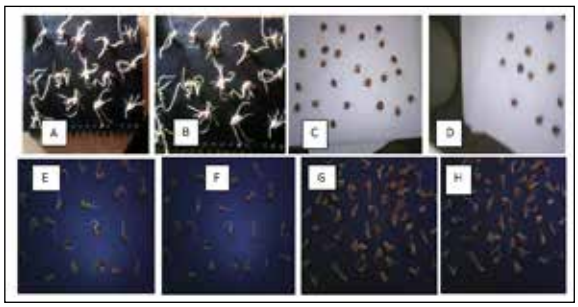

Fig 4 A&B: Normal 6 day old sprouted seedlings of Cajanus cajan varieties ICP-1 and Yashoda; C&D exposed to Cycloheximide, E&F exposed to Diclofop, G&H exposed to Sulfonyl urea

To assess the extent of rise or fall in the Activity of cyt p450 reductase in the presence of chemical challenges like the protein synthesis inhibitors and herbicides the enzyme assay is done in the excised hypocotyledons after germinating the seeds in the presence cycloheximide, diclofop and sulfonyl urea respectively. The results have showed that the maximum activity which was less than the controls was observed in the six day old hypocotyls exposed to sulfonyl urea and diclofop. The sulfonyl urea exposed hypocotyls of ICP-1 and Yashoda varieties exhibited maximum activity of 0.182±0.002 and 0.282± 0.002 units whereas diclofop exposed hypocotyls of ICP-1 and Yashoda varieties exhibited maximum activity of 0.092±0.002 and 0.102± 0.002 units.The activity has absolutely declined in the hypocotyls of cycloheximide exposed seeds and only 0.003 ±0.001 units and of 0.002± 0.001units were observed on the six day old hypocotyls of ICP-1 AND Yashoda varieties respectively.

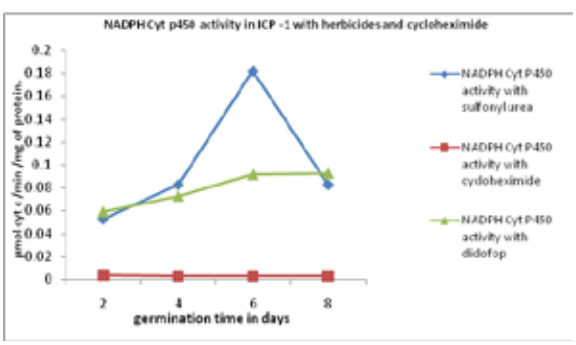

Fig 5: NADPH Cyt p450 activity in ICP-1 hypocotyledons with herbicides and cycloheximide

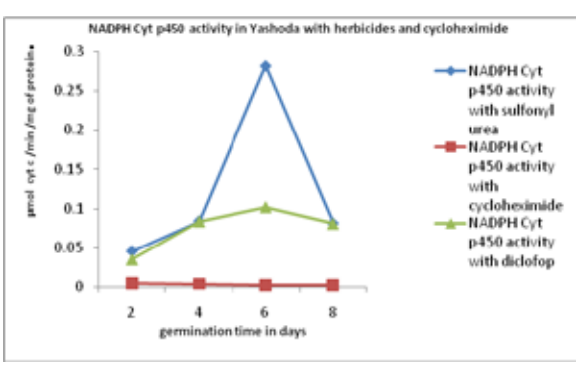

Fig 6: NADPH Cyt p450 activity in Yashoda hypocotyledons with herbicides and cycloheximide

Diclofop is found to suppress the NADPH Cytochrome Reductase activity more than sulfonyl urea in the cajanus varieties ICP-1 and Yashoda. The physical injury of the seedlings of both varieties has caused the 3-4 fold increase of the NADPH Cytochrome Reductase activities in the hypocotyls whereas herbicide challenge has also elicited 3-4 fold rise of the enzyme activity in both the varieties. In order to investigate the effect of herbicides and cycloheximide on the microsomal proteins SDS PAGE of the microsomal fractions of the hypocotyls was carried out and the results are shown as below.

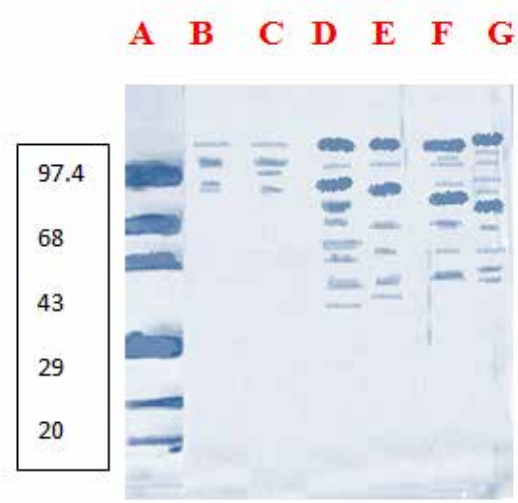

Fig 7: SDS PAGE of the microsomal proteins from 2 varieties of Cajanus cajan ICP-1 and Yashoda

Lanes A TO G

A: medium molecular weight markers from Bangalore genie, 97.4 to 20 K Da Starch phosphorylase,97.4; Bovine Serum albumin ,68 ; Ovalbumin, 43 ; Carbonic anhydrase,29; Soyabean Trypsin inhibitor, 20;Lysozyme, 14.3.

B: microsomal proteins from ICP-1 with 2 Mm cycloheximide

C: microsomal proteins from Yashoda with 2 Mm cycloheximide

D: microsomal proteins from ICP-1 with 2 Mm sulfonyl urea

E: microsomal proteins from Yashoda 2 Mm sulfonyl urea

F: microsomal proteins from ICP-1 2 Mm diclofop

G: microsomal proteins from Yashoda 2 Mm diclofop

The SDS PAGE analysis of microsomal preparations from the excised hypocotyls of the seedlings from ICP-1 and Yashoda varieties challenged with herbicides and cyclheximide has lead to the better understanding of the phenomena happening to the detoxifying enzyme, the NADPH cytochrome p450 reductase.

CONCLUSIONS

The protein synthesis inhibitor, cycloheximide has considerably inhibited the de novo biosynthesis of microsomal proteins (lanes B&C; Fig 7) and that is why the development of Cyt P_{450} enzyme was also inhibited to the minimum levels. Our study shows that Sulfonyl urea has increased the activity of Cyt P_{450} enzyme more than diclofop. It might be due to higher metabolic activity elicited in response to sulfonyl urea that focuses more on cyt p 450 systems which however needs to be verified by further research into the metabolism of sulfonyl urea in the developing seedlings of Cajanus cajan.

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