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Research article

Effects of glyphosate on metabolism of phenolic compounds (pal)

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ABSTRACT

The present study had as a goal to purify and characterize the enzyme Phenyl alanine ammonia lyase from the nodules and Rhizobia of cajanus cajan plant grown in NaCl and glyphosate conditions. N (grown in normal condition), Ng (grown in 4% ai glyphosate), H (grown in 300mM NaCl) & Hg (grown in 300mM and 6%ai glyphosate). In vitro and in vivo studies of enzyme PAL, isolation, purification, substrate specificity, optimum pH and temperature, activators and inhibitors, kinetic properties, molecular weight by SDS-PAGE and the action of glyphosate on PAL enzyme was checked.

Keywords: Phenyl alanine ammonialyase, Cajanus cajan,Rhizobium, SDS-PAGE, Glyphosate..

INTRODUCTION

Numerous experiments have been carried out of the possible impact of herbicide activity on soil microorganisms. Although, a steady increase in the application of herbicide on crop plants, it has become necessary to learn more about their impact on the symbioses of Rhizobiaceae- leguminous plants. There are evidences to indicate that, when applied in field cultivations of leguminous plants, herbicides can have some influence on nodulation, dinitrogen fixation and development of growing plants. It is believed that these compounds may also cause numerous side effects on growth and survival of soil microorganisms.

Pigeon pea (Cajanus cajan) a major food legume and important source of protein, vitamins and minerals, however production and availability is not enough to fulfill the requirements of human consumption because soil salinity is a major abiotic stress in plant agriculture had lead to the low productivity of crop. It has been speculated to be due to the uncontrolled and unplanned use of weedicide, which keeps accumulating in the soil, enhancing the salinity. This Salinity also affects the microbial world beneath the soil. Rhizobium is thus affected along with the plant in such stress condition affecting nitrogen fixation and thus the crop.

Glyphosate is a broad spectrum herbicide widely used to kill unwanted plants. However, herbicidal action probably arises from the inhibition of the biosynthesis of aromatic amino acids. This amino acid (phenylalanine, tyrosine and tryptophan) are used in the synthesis of protein and are essential or growth and survival of most plants. One particular enzyme important in aromatic amino acid synthesis called 5–enol pyruvyl shikimate-3phosphate and PAL synthase, is inhibited by glyphosate [1].

Glyphosate also may inhibit or repress two other enzyme, chlorismate mutase and prephrenate hydratase involved in other steps of the synthesis of same amino acids. These enzymes are all part of shikimic acid pathway present in higher plants and microorganisms but not in animal. Cremlyn demonstrated that herbicide N- (phosphono methyl)-glycine, commonly known as glyphosate, inhibits aromatic amino acid biosynthesis, resulting in, the slowed or halted growth associated with glyphosate treatment. Jaworski, however find that 3-deoxy –D-arabino– heptulosonate-7 phosphate synthetase was inhibited in vitro by 10mM glyphosate.

Induction of PAL activity has been shown to reduce aromatic amino acid pool levels to reduce growth rates. The molecular mode of

action of glyphosate on PAL enzyme of Rhizobium nodules in vitro and in vivo clarify the effects on microorganism and thus the nitrogen fixation, which ultimately reduces the productivity of crop. Davidson and Duke hypothesized that action of glyphosate on PAL activity might inhibit growth in three ways. a) Depletion of free pools of phenyl alanine and possibly tyrosine, thus inhibiting protein synthesis.

B) Production of toxic levels of ammonia, provided amination reactions do not keep pace with deaminations or c) increased levels of growth–inhibiting phenolic compounds. In this paper our study deals with in vitro and in vivo effect caused due to stress and glyphosate on the enzyme activity of aromatic amino acid biosynthesis of Rhizobuim. Secondarily, we have sought to add to the understanding of the relationship between PAL, its substrate and its activity [2].

MATERIAL AND METHOD

Collection of samples The Rhizobium was isolated aseptically from the root nodules of Cajanus cajan (L.) Millsp. Plant of AKPH 2022 variety. The strains were identified and isolated according to Shende & Patil, 200.

Rhizobium inoculums

Fresh nodules were collected from Cajanus cajan plants. N plant grown in normal condition, NG grown in presence of 4% glyphosate, H grown in 300mM NaCl, HG grown in 300mM NaCl and 6% glyphosate. Fresh nodules from four different plants were collected, crushed and inoculated in YM medium aseptically.

Enzyme Extraction and assay

Preparation of cell free extract: rhizobial strains-Rhizobium strains were cultured in 250ml flask containing 100ml of autoclaved yeast extract mannitol broth (YEM). The cultures were grown in automatic shaker for 3 days at 32oC.

Rhizobium cells were harvested by centrifugation at 6000g for 20 min, at 5oC in a refrigerated centrifuge, washed thrice with distilled water and the cell mass was ground in pre-chilled motor and pestle placed on ice bath. Centrifuge at 4100g for 20min. The supernatant was used as a source of crude enzyme.

Nodules

One gm nodules from respective plants N, Ng, H and Hg were frozen chilled for 10min with 2 gm Al203 and extracted with 5ml of 0.05 M sodium phosphate buffer pH 8. Centrifuge at 4100g for 20min. The supernatant was used as a source of crude enzyme.

Assay of PAL and protein

Assay of PAL was carried according. The enzyme unit used is that of Havir and Hanson (10). 1 unit (U) = 1, umol cinnamic acid formed per min at 30 C.Protein Assays. Soluble protein was extracted and assayed as before [3].

Purification of PAL

The crude supernatant is treated with 2ml of 2% protamine sulphate solution, stirred for 15 min and then centrifuged, precipitate is discarded. The supernatant was fractionated with (NH4)2SO4 (90% saturation). The mixture is stirred for 15min, centrifuged and the precipitate is collected by centrifugation and dissolved in 2ml of 0.01 sodium phosphate buffer pH 7 and dialyzed overnight against distilled water. Column was prepared using DEAE cellulose and fresh phosphate buffer pH7 was used for elution, fractions were collected by adding buffer gradients (0.05M NaCl-0.5mM NaCl).

Electrophoresis

Samples containing 20% (w/v) sucrose, 3% SDS, 7% (w/v) 2- mercaptoethanol and 0.002% (w/v) bromophenol blue were heated at 90oC for 5min before loading. SDS-PAGE was performed according to the method of Laemmli Following electrophoresis proteins were stained with Coomassie brilliant blue R-250. Markers were used for molecular weight determination.

Substrate specificity, temperature and pH were checked

Inhibitor studies-PAL was assayed spectrophotometrically in the presence of a range of different phenolic compounds with different concentration of inhibitor and substrate. Phenolics were dissolved in EtOH, the presence of which (up to 10% v/v) in the assay mixture did not inhibit PAL activity.

Effects of glyphosate on Plants

Growth- Documentation of the sensitivity of Cajanus cajan plant to the herbicidal action of glyphosate. Rhizobium strains were grown in presence of 1mM glyphosate in YM medium with control. O.D was noted [5].

Enzyme Sensitivity to Glyphosate

Glyphosate at 2 mM concentration was tested as a potential inhibitor of the PAL enzymes isolated from N, Ng, H & Hg plants.

Statistical Analysis

All error bars in figures are one standard error. In tables, significant differences were determined by overlap of two standard errors. All values represent means of from two to four separate determinations**.**

RESULT AND DISCUSSION

The PAL enzymes were purified in vitro and in vivo from N, NG, H and HG nodules of Cajanus cajan plants grown in different stress conditions. PAL was isolated maximum in HG condition from nodules and rhizobia as compared to N, NG & H plants. The enzyme is widely distributed in higher plant and mainly involved in defense mechanism. In microorganisms it has a catabolic role, allowing them to use L–phenyl alanine as a sole source of carbon and nitrogen. PAL was isolated from Ustilago maydis also from Medicago. Sativa L. (alfa alfa) in stress condition. PAL catalyses the biotransformation of L-phenylalanine to trans-cinnamic acid and ammonia. Its catabolic role in microorganisms and its importance in the phenyl propanoid pathway of plants, PAL have gained considerable significance in several clinical, industrial and biotechnological applications. The enzymes natural ability to break down L- phenyl alanine makes PAL a reliable treatment for the genetic condition phenyl ketonuria. The enzyme in Vivo & in

Vitro had sufficient recovery yield (Table 1-8).

The enzyme appeared as a single protein band on SDS-PAGE gel, with a molecular mass, approximately 45 & 64 kDa in N, NG, H and HG (Figure 1), 64KD and 45KD in nodules (in vivo) & (in vitro) 64 kDa Rhizobium band of Cajanus Cajan plant. PAL isolated from Rhodosporidium torvloides contain molecular mass of 76.88 KDa. The molecular weight didn't showed much difference in PAL assay, though the HG band was more prominent as compared to others, this might be due to the maximum protein content found in H and Hg. The protein is increased in plants grown in different stress condition of 300 mM NaCl (H) And 6% glyphosate & 300mM NaCl (Hg). Although, as compared to normal (N) and normal glyphosate (NG) condition. They concluded that the protein content was increased in stress due to enhance accumulation of osmolytes to cope with the stresses. Jesus Jorrin et al reported the rise in protein content isolated from alfa alfa in stress condition, however large significant difference in PAL activity was not seen. In Hg PAL activity was increased might be due to the plant isolated and characterized were resistant to saline and glyphosate stress. The isolated PAL enzyme when treated with NaCl 300mM the enzyme activity was inhibited completely in vitro and in vivo (Figure 2 & Figure 3) as well in H and Hg some enhance activity could be seen as they might had adapted to NaCl stress and glyphosate at 5µM the activity could be seen till 20 µm both in vitro and in vivo and suddenly the activity decreased showing the inhibitory effect on enzyme, same in case of glyphosate acted on PAL the activity showed a slight increase in NG and HG & its decline showing the inhibitory effect in vivo and in vitro (Figure 4 & 5). The Haderlie et al. reported that the glyphosate inhibits the aromatic amino acid biosynthesis, such a primary effect would slow or halt protein synthesis, resulting in decrease activity. Hoagland Re reported the induction of phenyl alanine ammonia lyase (PAL) activity has been shown to reduce aromatic amino acid pool levels sufficiently to reduce growth and effect nitrogen fixation. In previous work with roots of maize seedlings, showed 50 % reduction in the free pool of phenyl alanine6. Our data, in the context of that of others indicate that glyphosate could inhibit PAL activity, since many stress conditions reduce the activity. Duke So et. al. has shown that glyphosate alters membrane properties within 1 h after treatment suggesting a primary effect. Glyphosate may act through reduction of PAL with resulting aromatic amino acid pool depletion and decreased protein synthesis.

However, the herbicidal action probably arises from the inhibition of the biosynthesis of aromatic amino acids. Glyphosate also may inhibit or repress other enzymes in shikimic acid pathway. They do play the same role in the soil microorganism Rhizobium by inhibiting the PAL enzyme in amino acid pool and thus affecting the biological nitrogen fixation and ultimately the crop. The Hg and Ng plants grown in resistant conditions to stress NaCl and glyphosate still showed inhibitory effect of glyphosate on PAL enzyme and protein synthesis. Robert E. Hoogland indicated that glyphosate significantly interferes with non-aromatic as well as aromatic amino acid synthesis. Also, Roisch and lingens found in vitro effect of glyphosate on the enzymes chorismate mutase and prephenate dehydrogenase involved in aromatic amino acid synthesis in E. coli.

Kinetic properties of the enzyme PAL shows specificity for substrate (Figure 6 & 7and Table 9). (Figure -8 &9) showed maximum activity at pH 8 and temperature 40oC for all strains (Figure 10 & 11), same was found in the isolation of PAL from Medicago Sativa L. by Jesus jorrin Fluctuations observed in the kinetic constants of PAL enzymes have been explained based on association/dissociation of PAL subunits, the occurrence of PAL isoforms with differing substrate affinity or the presence of two substrate binding sites differing in their substrate affinity and interaction with each other It has also been suggested that during purification or storage, modifications of structure, which could be caused by proteolytic enzymes as well as inactivation or modification of one or more active sites, could lead to variations in kinetic behaviour [6].

The influence of certain metal ions and ethylenediaminetetraacetic acid (EDTA) on PAL activity was investigated (Table-10 & 11). Among all tested metal ions, only Ag+, Hg+2, Zn+2 completely inhibited enzyme activity, while Cu+2, Fe+2, Mg+2 and reduced enzyme activity (Table 3). On the other hand, K+ and Mn+2 ions slightly stimulated PAL activity, indicating the potential roles of both metal ions as cofactors for enzyme- substrate reactions, in addition to a stabilizing effect on various other enzymes. PAL activity was strongly inhibited by Hg+2, a strong inhibitor of most PAL, including those produced by Thermomyces lanuginosus (20), Aspergillus caespitosus and Penicillium citrinum EDTA, a metal chelator, decreased PAL activity, indicating that metal ions are required for the activity of purified enzymes. Phenyl hydrazine, Trans-Cinnamic acid, catechol were also inhibited, all PAL preparations were inhibited by trans cinnamic acid, The end product of the reaction. (In case of reversal reaction, concentrations of t-CA greater than 135 mmol/L were enough to cause substrate inhibition (23). Steinrucken et. al. (24) claim that salinity and herbicides may affect the legume – Rhizobium relationship by 1) affecting the host plant (i.e. there may be a reduction in root biomass that leads to fewer infection sites or by affecting the carbohydrate supply to existing nodules. 2) affecting Rhizobial survival or growth that leads to a decreased potential for rhizobial infection on root hairs. 3) inhibiting or inactivating the biochemical signaling that plants require to initiate nodules development – this inhibition could affect rhizobia or plants and 4) inhibiting nodule development by reducing the capacity for cell division. Thus glyphosate & NaCl may

directly affect plant growth and thus indirectly affect rhizobia and N2 fixation; it also may have an impact on rhizobial survival and growth. The application of glyphosate in agricultural systems may exert side effects on the soil microflora, including a possible shift in microorganism community structure. This may be particularly true that it interfere with amino acid biosynthesis and therefore also my affect microbial metabolism.

This research has focused on negative effects of herbicidal interactions with Rhizobia and N2 fixation in Cajanus cajan plant. As nitrogen fixation associated with the legume – Rhizobium relationship offers benefits not only to the legume themselves, but potentially to subsequent crops by increasing the amount of soil N, this in turn, decreases fertilizer costs. Thus herbicides that are suitable for agricultural use should have very little or no effect on crop. This research shows the impact of herbicide on legume – Rhizobium relationship at the enzymatic and molecular level. Further studies examining the relationship between herbicides and N2 fixation are warranted.

CONCLUSION

From the above results, it is concluded that *Lageneria siceraria* used traditionally and inbackward areas to treat ringworm infection, showed significant antifungal activity. The experimental evidence obtained in the laboratory model could provide a rationale for the traditional use of this plant as antifungal. The activity was almost reached about to the standard. The plant may be further explored for its phytochemical profile to recognize the active constituent accountable for antifungal activity. Thus the present experiments scientifically proved its traditional claim for the beneficial effect in the ringworm infection from aqueous extractand better from ethanolic root extract of *Lageneria siceraria*.

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