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Original Research Article

L-DOPA Inhibited Early Root Growth in Rice Involves Biochemical Alterations in Macromolecules and Associated Hydrolytic Enzymes.

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Abstract: The present study was conducted to explore the effect of L-DOPA (1-1000µM) on biochemical alterations during the early root growth under hydroponic conditions in the roots of *O. sativa* (rice). Seedling growth measured in terms of root length and shoot length exhibited a significant decline with increasing L-DOPA concentrations. It was observed that L-DOPA-exposure significantly enhanced the contents of water-soluble proteins and carbohydrates in roots of rice after 120h in a dose-response manner. Proteins and carbohydrates content increased by 7.7- and 2.3-folds, respectively at 1000 µM -exposure. On the other hand, the activities of hydrolyzing enzymes - proteases and amylases reduced apparently. At 1000 µM, activities of proteases, α-amylases, and β−amylases decreased by 1.91- , 1.90-, and 1.85- times, respectively. In addition, L-DOPA exposure significantly enhanced the activities of enzymes - peroxidases, polyphenol oxidases by 1.76- and 1.91-times, respectively, over control at 1000µM L-DOPA. An upregulation in the activities of these enzymes indicate their response to L-DOPA induced toxicity in rice seedlings and provide protection. The study concludes that L-DOPA-induced toxicity in hydroponically grown seedlings of rice involves the biochemical alterations in terms of macromolecules and the activities of associated hydrolytic enzymes to fight against the L-DOPA induced stress.

Key words: L-DOPA, proteases, α-amylases, and β−amylases

Introduction

Allelopathy can be defined as direct or indirect effect of any plant or microorganism on the survival, growth and reproduction of another plant mediated by the release of allelochemicals (Zeng, 2014). Farooq *et al.,* (2011) defined allelopathy as a phenomenon where different life forms such as fungi, viruses, microorganisms and plants synthesize secondary metabolites which influence biological and agricultural ecosystems either in a stimulatory or inhibitory manner. Allelochemicals are natural plant products exhibiting a wide array of chemical nature. These are synthesized within plants as secondary metabolites and are involved in various activities. Msafiri *et al.,* (2013) considered both positive and negative influences of allelochemicals by defining allelopathy as the ability of plants to inhibit or stimulate growth of other plants in the environment by excuding chemicals. Allelochemicals are plant secondary metabolites normally released into the environment (generally rhizosphere) through volatilization, leaching, root exudation, through cell death or through decomposition of plant residues in the soil (Khalaj *et al.,* 2013).

Velvet bean [*Mucuna pruriens* (L.) DC. Var. utilis)] of family Papilionaceae, is cultivated as smother or green manure crop for improving soil properties and selectively controlling weeds (Anaya, 1999; Tarawali *et al.,* 1999; Ayala *et al.,* 2000; Fujii 1999; Kubo *et al.,* 1995; Vadivel and Janardhanan, 2001). Based on *in vitro* bioassay and field studies, Fujii *et al.,* (1991) observed secondary metabolites released by roots and leaves of *M. pruriens* and the identified a non-protein amino acid−L-3,4-dihydroxyphenylalanine (hereafter referred to as L-DOPA). Further, Furubayashi *et al.,* (2005) observed that L-DOPA exuded from *Mucuna* roots accumulate into the soil and its bioactive concentrations (1 to 50 ppm) were enough to bring about the inhibitory effects (Nishihara, 2005; Fujii, 1999; Nishihara *et al.,* 2004). Later, L-DOPA was found to be an active principle of phytotoxic action (Tarawali *et al.,* 1999; Hachinohe *et al.,* 2004). L-DOPA is produced by the oxidation of tyrosine by tyrosinase- a copper containing enzyme. It is an essential precursor in the biosynthesis of several alkaloids, catecholamines, flavonoids, phenylpropanoids and melanin (Hahlbrock and Scheel, 1989), which are present in

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mammalian tissues, fruits and vegetables (Pattison *et al.,* 2002). Earlier it was observed that L- DOPA reduces root growth more than shoot growth (Fujii *et al.,* 1991; Nakajima *et al.,* 1999). L-DOPA has been found to be an antioxidant as it increases the melanin production and reduces the reactive oxygen species in the roots of plant species (Soares *et al.,* 2011). However, not many details are available regarding the mechanism of the phytotoxic action of L-DOPA towards radicle growth and physiological and biochemical aspects linked with variation in the macromolecular contents during hydroponic conditions in *Oryza sativa*. With this objective in mind a study was undertaken to explore the effect of L-DOPA on the content of macromolecules (proteins and carbohydrates) and the activities of hydrolyzing enzymes (α-amylases, β-amylases, proteases) and oxidoreductase enzymes (peroxidases and polyphenol oxidase) in test crop.

Materials and Methods

Technical grade L-DOPA (L-3,4-dihydroxyphenylalanine) used in the present study was purchased from Loba Chemie, India. Healthy seeds of rice (*Oryza sativa*) were procured from the local market. These were surface sterilized with sodium hypochlorite (NaOCl, 0.1 %, *w/v*) and washed under running tap water followed by rinsing in distilled water. For biochemical estimations, all the reagents and chemicals used were of technical grade and procured from Sisco Research Laboratory Pvt. Ltd., India; Sigma Co., St. Louis, USA; Merck Ltd., India; Acros, Belgium; and Loba-Chemie Pvt. Ltd., India.

Studies under hydroponic conditions

Seeds of *O. sativa* imbibed for 6 h at room temperature (25 ºC) were germinated on a wet filter paper in enamel trays (32 cm × 23cm × 7 cm) lined with a moist cotton wad. Three day old seedlings were acclimatized in pure water for 24 h in glass beakers (500 ml capacity). After that they were exposed to different concentrations of L-DOPA *viz*. 0 (control), 1, 10, and 100, and 1000 µM for 120 h in a growth chamber. The experimental conditions for the experiment was set at day /night temperature of and 25/20 (±2) °C, relative humidity of 75±3%, and a photoperiod of 12 h (7.30 AM –19.30 PM) at a photosynthetic photon flux density (PPFD) of ~240 µmol photons m^{-2} s^{-1}. After 120 h, root and shoot length of the hydroponically raised seedlings was measured with the help of a centimeter ruler. Following this, *Oryza sativa* seedlings were harvested and roots were choped for biochemical estimations.

Estimation of protein and carbohydrate content

Nearly 200 mg of root tissue was homogenized in 10 ml of distilled water. After passing through a double layer of muslin cloth, the sample was centrifuged at 15,000 g for 15 min and the supernatant was collected for the estimations. Water soluble protein content was determined using Folin-Ciocalteu reagent against bovine serum albumin as a standard as per Lowry *et al.,* (1951). Estimation of carbohydrate content was done using anthrone reagent against a standard of glucose (Loewus, 1952).

Preparation of enzyme extract

Crude enzyme extracts were prepared by homogenizing nearly 100 mg of root tissue in a pre-chilled pestle and mortar with 5 ml of 0.1 M phosphate buffer (pH=7). Homogenates were centrifuged at 15,000 g for 25 min at 4°C rotor temperature in a Sigma Centrifuge. The fraction of supernatant thus obtained was used for determining the activities of proteases, α–amylases and β–amylases, peroxidases and polyphenol oxidases (PPO). The supernatant was stored at – 4°C before enzyme assays. An aliquot of the supernatant was used to determine protein content using bovine serum albumin standard as per Lowry *et al., (*1951).

Enzyme assays

Protease activity was estimated using Casein (1% in 0.1 M phosphate buffer, pH=6) as a substrate (Basha and Beevers, 1975). Activity of α-amylases was assessed as per Muentz (1977) using starch as a substrate; whereas β- amylases was estimated following the method of Bernfeld (1951) with modifications suggested by Dure (1960). Peroxidases were assayed using 0.2 M hydrogen peroxide as a substrate following the methology given by Malik and Singh (1980). The specific activity of polyphenol oxidases was determined using catechol (0.01 M in 0.1 M phosphate buffer, pH=6) as a

substrate as per Van Lelyveld and Pretorious (1973).

Data analysis

The experiments were performed in a randomized design with five replicates, each consisting of a single Petri dish with 20 seeds each. All the experiments were repeated and the data presented is of a single experiment since the differences between two experiments were less than 5%. The data were analyzed by one-way ANOVA and means were separated using post hoc Tukey's test at $P< 0.05$.

Results and Discussion

The seedling growth of *O. sativa* (measured in terms of root and shoot length) was adversely affected upon exposure to L-DOPA (Table 1). The inhibitory effect was dose dependent and growth declined with increasing concentrations of L-DOPA. Root length decreased by ~14%, 28%, 40% and 53% in response to 1, 10, 100 and 1000 µM L-DOPA, in comparison to control. Similarly, shoot length was also found to decrease, it was reduced by ~13, 18, 22 and 31% upon exposure to 1, 10, 100 and 1000 µM L-DOPA, respectively. The reduction in shoot length was comparatively lesser and the inhibitory effect was more pronounced on root length. Thus, the roots were selected for the further studies. In continuation, the present study revealed that upon L-DOPA treatment water soluble protein and carbohydrate content increased significantly ($P<0.05$) in the roots of *Oryza sativa* (Table 2). The effect was evident in a concentration dependent manner. The protein content exhibited an increase of 52.6%, 63.2%, 200.0% and 668.4% at 1, 10,100 and 1000 µM L-DOPA treatment, respectively, as compared to control (Table 2). These observations are in agreement with earlier observations of Terzi *et al.,* (2003) who suggested that increase in the protein content is mainly due to an increase in the enzymatic proteins in cucumber seedlings treated with juglone but Sultan and Fatma (1999) and Wu *et al.,* (2007) opined that it may be the defence mechanism adopted by the plants to minimize the effect of allelochemicals. Further, the activity of protein-hydrolyzing enzyme, proteases, decreased with the increasing L-DOPA concentration. Protease activity decreased by ~59%, 71%, 82% and 91% over control upon treatment with 1, 10, 100 and 1000 µM L-DOPA (Fig. 1). The increase in protein content could be associated with the reduced protease activity.This decline in the activity of proteases indicated that the plant was not able to hydrolyze proteins under L-DOPA stress. The results in the present study corroborated with the previous observations reported by Batish *et al.,* (2006).

Table 1: Effect of different concentrations of L-DOPA on root length (cm) and shoot length in *O sativa*.

Conc.(µM)	Root length (cm)	Shoot length (cm)
0	11.8±0.08a	8.7±0.04a
1	10.1±0.06b	7.6±0.02b
10	8.5±0.05c	7.1±0.03c
100	7.2±0.04d	6.8±0.06c
1000	5.6±0.06e	6.0±0.03d

Different alphabets along a column represent significant difference applying Tukey's test ($P<0.05$).

Table 2: Effect of L-DOPA on the content of water-soluble proteins and carbohydrates in rice roots.

Conc.(µM)	Proteins (mg g^{-1}f.wt.)	Carbohydrates (mg g^{-1} f.wt.)
0	1.90 ± 0.07a	3.68 ± 0.0[2a]
1	2.80 ± 0.07b	5.62 ± 0.04b
10	3.10 ± 0.20b	6.43 ± 0.03c
100	5.70 ± 0.01c	6.68 ± 0.03 d
1000	14.60 ± 0.12d	8.53 ± 0.03e

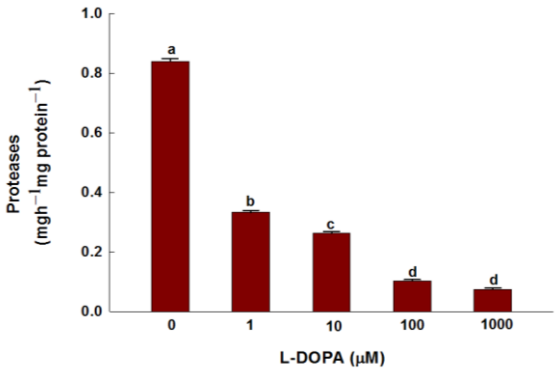
Data represented as mean±S.E of five independent replicates; Different alphabets within a column at a particular treatment represent significant difference from control at $P< 0.05$ applying post hoc Tukey's test.

Al-Watban and Salama (2012) tested the allelopathic potential of aqueous extracts of aerial parts of *Artemisia monosperma* and reported an increase in the protein content with declining activity of proteases in *Phaseolus vulgaris*. Similarly, Chum *et al.,* (2012), also, observed a significant enhancement in the protein content of *Brassica oleracea* var. capitata and a decrease in the activity of proteases upon exposure to Benzoxazolinone, a well-known allelochemical. On the contrary, El- Khatib and Hegazy (1999) and El- Khawas and Shehata (2005) observed a decline in the protein content in leguminous crops in response to extracts of *Acacia nilotica*.

Similar to protein content, a gradual increase was observed in carbohydrate content in response to L-DOPA in concentration dependent manner. It was

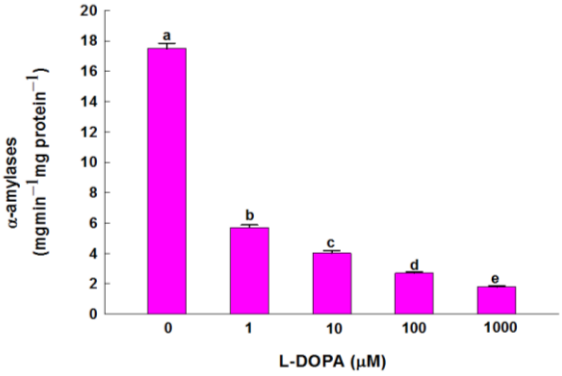
observed that carbohydrate content increased by ~1.8 folds at 100 µM L-DOPA and it enhanced further to ~2.3 times over control at 1000 µM (Table 2). This observation parallels the previous studies which indicate that allelochemicals from other plants affect the macromolecular content (Cameron and Julian, 1980; Rice, 1984; Mersie and Singh, 1993; Singh *et al.,* 2002; Terzi *et al.,* 2003; El-Khawas and Shehata, 2005). The increase in carbohydrate content in the present study could be attributed to the decreasing activity of amylases upon exposure to L-DOPA treatment. It was observed that L-DOPA - exposure significantly decreased the activity of amylases (both α and β). The activity of α-amylases decreased in the range 67.4% to 89.7% over that of control (Fig. 2). Likewise, the activity of β- amylases declined in between 54.6%-84.8% over control in response to 1-1000 µM L-DOPA (Fig. 3). The decreased activity of amylases clearly indicated the incapability of the plant to fulfill increased energy demands of the tissue upon exposure with L-DOPA treatment. Al-Watban and Salama (2012) observed an inhibition in the amylase activity as well as sugars in *Phaseolus vulgaris* upon treatment with the extracts of *Artemisia monosperma*. Abdulghadar *et al.,* (2008) opined that increased content of carbohydrates is suggestive of the fact that plant is under stress and is gathering up its energy reserves to meet the conditions of adversity. Our results are in line with the increased sugars in maize in response to leaf extracts of Acacia and Eucalyptus (Sahar *et al.,* 2005).

Fig.1: Effect of L-DOPA on the specific activity of proteases in the root tissue of *O. sativa*.



Different alphabets at a particular treatment represent significant difference from control at *P*< 0.05 applying post hoc Tukey's test.

Fig.2: Effect of L-DOPA on the specific activity of α−amylases in the root tissue of *O. sativa*.

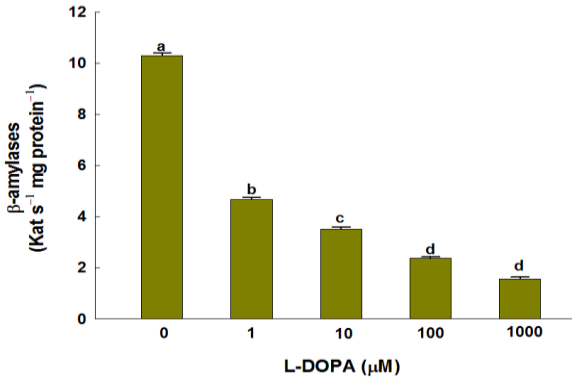


Different alphabets at a particular treatment represent significant difference from control at *P*< 0.05 applying post hoc Tukey's test.

Earlier, Chrispeels and Varner (1967) observed that decreased activity of the amylases can be due to its limited production which could be the consequence of the inhibition of gibberllins stimulated α-amylase synthesis and its secretion. Kaplan and Guy (2004) reported an increase in specific activity of the β-amylases in response to allelochemical stress which is contradictory to our studies where the activity of this enzyme decreased with increasing concentration of L-DOPA. In the present study, the activity of PPOs increased by ~31.5%, 58.6%, 71.2% and 90.9% at 1, 10, 100 and 1000 µM L-DOPA treatment respectively compared to control (Fig. 4). Likewise studies of Hachinohe and Matsumoto (2007) exhibited higher activity of polyphenoloxidases in lettuce which may be ascribed to active melanin synthesis or greater generation of ROS. Kim *et al.,* (2005) reported an apparent increase in the activity of polyphenol oxidases in *Cassia mimosoides* L.var.nomame with the treatment of extracts of *Phytolacca americana* L. containing phenolic compounds. A significant increase was noticed in the activity of peroxidases (PODs) (Fig. 5) suggestive of L-DOPA induced stress in rice roots. PODs are a group of enzymes that are also responsible for the plant seedling growth and development. Soluble POD catalyzes the oxidation of structurally diverse phenolic substrates to protect the cells from toxic influence of oxygen radicals (Santos *et al.,* 2004). Many workers have reported that the activity of these enzymes is enhanced in response to allelochemical induced stress (Devi and Prasad, 1996; Politycka, 1996;

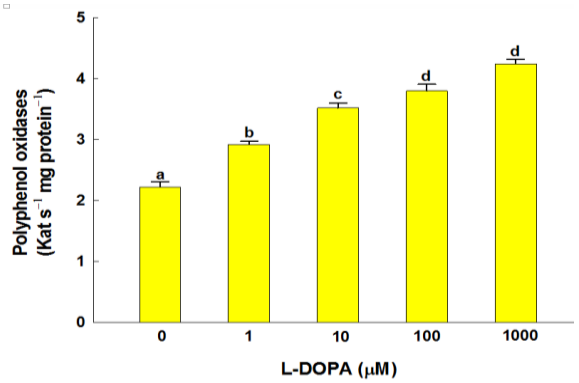
Zeng *et al.,* 2001; Moradshahi *et al.,* 2003; Ponce, 2004). Soares *et al.,* (2007) reported that the activity of peroxidases, a key enzyme in the lignification of cell wall, increase in response to L-DOPA possibly due to melanin formation. Polyphenol Oxidases play a vital role in plant defense in response to a variety of toxins/ allelochemicals. Chowhan *et al.,* (2011) reported a significant increase in the activities of peroxidases (POX) and polyphenol oxidases (PPO) in rice roots in response to β-pinene (an oxygenated monoterpene) in a dose- and time-dependent manner. The current investigations are concordant with the findings of Chowhan *et al*., (2011).

Fig.3: Effect of L-DOPA (1-1000 µM) on the specific activity of β−amylases in the root tissue of *O. sativa* after 120 h.



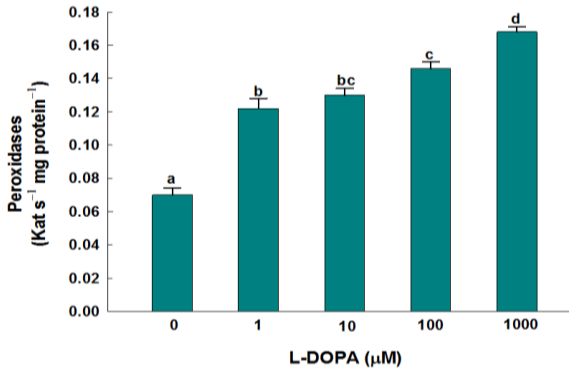
Different alphabets at a particular treatment represent significant difference from control at *P*< 0.05 applying post hoc Tukey's test.

Fig. 4: Effect of L-DOPA (1-1000 µM) on the specific activity of Polyphenol oxidases in the root tissue of *O. sativa* after 120 h.



Different alphabets at a particular treatment represent significant difference from control at *P*< 0.05 applying post hoc Tukey's test.

Fig.5: Effect of L-DOPA (1-1000 µM) on the specific activity of peroxidases in the root tissue of *O. sativa* after 120 h.



Different alphabets at a particular treatment represent significant difference from control at *P*< 0.05 applying post hoc Tukey's test.

Conclusions

In summary, the present study concludes that L-DOPA induced toxicity in the roots of *O. sativa* involve the biochemical alterations in terms of macromolecules and the activities of related enzymes to cope with the phytotoxic action of L-DOPA.

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