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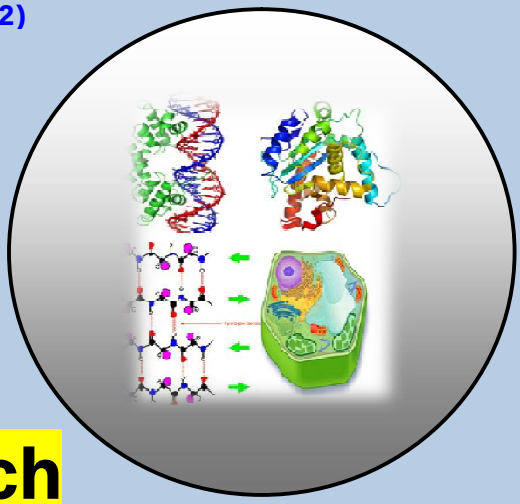
ISSN 0970-4973 (Print)
ISSN 2319-3077 (Online/Electronic)

Index Copernicus International Value
IC Value of Journal 4.21 (Poland, Europe) (2012)
Global Impact factor of Journal: 0.587 (2012)
Scientific Journals Impact Factor: 2.597

J. Biol. Chem. Research
Volume 31 (2) 2014 Pages No. 929-941



Journal of Biological and Chemical Research
(An International Journal of Life Sciences and Chemistry)

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 31, No. 2: 929-941 (2014)
An International Journal of Life Sciences and Chemistry

ISSN 0970-4973 Print
ISSN 2319-3077 Online/Electronic

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RESEARCH PAPER
Received: 11/07/2014 Revised: 22/07/2014 Accepted: 23/07/2014

Enhanced Defence Enzyme Activity by Seed Treatment with Partially Purified ns-LTPs in Pearl Millet against Downy Mildew Disease

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ABSTRACT

Downy mildew disease of pearl millet caused by Sclerospora graminicola is a major threat in pearl millet growing areas. In the present study, partially purified ns-LTP extracts isolated from different seed materials (maize, rice and wheat) and evaluated for their efficacy of hypersensitive response and in inducing defence related enzymes against downy mildew pathogen. Hypersensitive reaction responses (HR) were observed in all the crude ns-LTP treated pearl millet seedlings followed by challenge inoculation and there was also an increase in the activity of defense enzymes. The maximum PAL, POX and LOX activities of 252.3, 77.8 and 59.4 U, respectively was observed in rice ns-LTP treated seedlings followed by maize and wheat seedlings. This combined action of these defence enzymes and HR may account for increased downy mildew disease resistance in pearl millet.

Key words: ns-LTPs, Defence Related Enzymes, Pearl Millet, Sclerospora graminicola, HR and Downy Mildew Disease Resistance.

INTRODUCTION

Pearl millet (*Pennisetum glaucum* L. R. Br.) is the most widely grown type of millets. It is widely grown in semi-arid regions of the world for food and fodder and is the staple diet for farm households in the world's poorest countries and among the poorest people. The world area cropped to pearl millet is about 26 million hectares and in India, the crop is grown in about 9.8 million hectares with a total grain production of 7 million tonnes (Khairwal et al. 2008). One of the major biotic yield-reducing factors of pearl millet is the downy mildew disease caused by an oomycete biotrophic fungus *Sclerospora graminicola* [(Sacc.) Schroeter] resulting in 40-60% crop loss (Thakur et al. 2003).

Induction of resistance in the host plants is highly versatile and elastic, even the susceptible plants can be protected from disease by developing systems to induce and enhance the plant's own defense mechanisms (Garcia-Brugger et al. 2006). During ISR in plants, several well-characterized defense reactions such as hypersensitive reaction (HR) (Zhang et al. 2004), oxidative burst (Yaeno et al. 2004), reinforcement of cell wall structures through lignification or callose deposition (Zhao et al. 2005; Soylu, 2006), accumulation of antimicrobial phytoalexins (Soylu, 2006) and induction of defense-related proteins with antifungal properties (Andreu et al. 2006) have been extensively reported in many plant species. Pre-treatment of plants with biotic or abiotic inducers can enhance resistance to subsequent attack not only at the site of treatment, but also in tissues distant from the initial infection sites (Soylu et al. 2003). Non-specific Lipid Transfer Proteins (ns-LTPs) are typically encoded by a small multi gene family present in diverse plant species and are often designated as ns-LTPs due to their affinity for multiple substrates (Kader, 1996). The structural, biochemical and physiological data have confirmed that ns-LTPs are involved in plant defense resistance to biotic and abiotic stresses and in the assembly of protective extra cellular hydrophobic polymers such as cutin and suberin (Kader, 1996). The expression of ns-LTPs can be induced by environmental stress factors such as pathogen invasion (Kader, 1996), high or low temperature (Hughes et al., 1992 and Hincha, 2002), drought and chemical treatments (Trevino, 1998; Dani et al. 2005). Studies from several research groups showed that ns-LTPs take a certain effect in plant defense mechanisms (Molina et al. 1993; Carvalho et al. 2001; Gomes et al. 2003; Manjula et al. 2014) and further evidences on ns-LTPs have proved their role in plant defense signalling (Maldonado et al. 2002). It has been reported by many investigators that during induction of resistance, activation of defense systems are necessary such as hypersensitive reaction (HR) and production of defense related enzymes. Hence, in the present study, an attempt was made to analyse the effect of seed treatment of crude ns-LTP extracts which gave protection to downy mildew disease in pearl millet for induction of defense related enzymes during host pathogen interaction.

MATERIAL AND METHODS

Seed materials

Seeds of *Zea mays* (maize- NAH-1137) and *Oryza sativa* (rice- BR-2655) were obtained from Zonal Agricultural Research Station, V.C. Farm Mandya. *Triticum aestivum* (Wheat- DWR-162) seed samples were obtained from Dharwad Agricultural University, Dharwad and same were used to extract crude ns-LTPs.

Host plant

Seeds of pearl millet that are highly susceptible (7042S) and highly resistant (IP18296) to downy mildew disease were obtained from the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patencheru, India. The seeds were obtained under a Material Transfer Agreement and used throughout the study.

Source of pathogen and inoculum preparation

S. graminicola was isolated from pearl millet cv. 7042S grown in a heavily infested field. The pathogen was maintained on its susceptible host until use.

Leaves of pearl millet showing profuse sporulation of *S. graminicola* on the abaxial side were collected in the evening from the plants maintained in field conditions were thoroughly washed under running tap water to remove sporangia. The leaves were then blot-dried, cut into small pieces and maintained in a moist chamber for sporulation. The following morning, fresh sporangia were washed into distilled water. For use as inoculum, the zoospores concentration was adjusted to $4x10^4$/ ml using Haemocytometer (Safeeulla et al. 1976) and used as source of inoculum for further studies.

Extraction, partial purification and seed treatment with of ns-LTPs

The ns-LTPs from different seed samples (maize, rice and wheat) were isolated using the modified procedure of (Gorjanovic et al. 2005). Highly susceptible (7042S) pearl millet seeds were treated separately with ns-LTP extracts from maize, rice and wheat at 100 µg/ ml concentrations (w/ v) (which offered maximum protection against downy mildew disease under greenhouse conditions in our previous studies) (Manjula et al, 2014) by soaking method and were kept at 25±2° C in a rotary shaker for 6 h at 100 rpm/ min to facilitate the penetration of the protein into the seeds. Seeds soaked in sterile distilled water (SDW) for some time served as negative control and resistant seeds (IP18296) served as positive control.

Sampling of seedlings

The crude ns-LTPs treated pearl millet seeds along with controls were placed on Petri plate lined with moistened blotter discs and incubated at 25±2º C. Two-day-old seedlings were carefully removed without damaging the roots and dipped in *S. graminicola* spore suspension at 4 x 10^4 zoospores/ ml. Both the inoculated and uninoculated pearl millet seedlings (susceptible, susceptible crude ns- LTPs treated and resistant) were harvested at , 3, 6, 9, 12, 24, 48 and 72 hours after post inoculation (hpi) and for hypersensitive response pathogen inoculated samples (susceptible, susceptible crude ns- LTPs treated and resistant) were harvested at 0, 3, 6, 9, 12, 24 hpi

Morphological Study

Time-course analysis for hypersensitive reaction (HR)

HR studies were carried out in pearl millet seedlings following the method of Kumudini et al. (2001). The crude ns-LTPs treated pearl millet seeds along with controls were placed on Petri plate lined with moistened blotter discs and incubated at 25±2° C. Two-day-old seedlings were carefully removed without damaging the roots and dipped in *S. graminicola* spore suspension at 4 x 10^4 zoospores/ ml. The inoculated and un-inoculated pearl millet seedlings were observed at hourly intervals for the external appearance of necrotic spots or streaks on the coleoptile region of tested seedlings. The experiment consisted of four replicates of 25 seedlings each and repeated three times.

Per cent HR = No. of seedlings with necrotic spots X 100
Total no. of seedlings taken

Biochemical studies

Phenylalanine Ammonia Lyase (PAL) activity

One gram fresh weight of seedlings harvested at above mentioned time intervals were homogenized in 1 ml of ice cold 25 mM Tris buffer of pH 8.8, containing 32 mM of 2-mercaptoethanol in a pre-chilled mortar and pestle.

The extract was centrifuged at 10,000 rpm for 25 min at 4° C and the supernatant was used as enzyme source. Reaction mixture containing 0.5 ml of enzyme extract was incubated with 1 ml of 25 mM Tris-HCl buffer of pH 8.8 and 1.5 ml of 10 mM L-phenylalanine in the same buffer for 2 h at 40° C. The activity was stopped using 5 N HCl (Geetha et al. 2005). PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Enzyme activity was expressed as μmol of trans-cinnamic acid/ mg protein/ h. The experiment was repeated thrice taking three replicates each time.

Peroxidase (POX) activity

Two-day-old seedlings which were inoculated and harvested along with un-inoculated samples were used for enzyme extraction. One gram fresh weight of seedlings harvested at above mentioned time intervals were macerated with 0.2 M sodium phosphate buffer (pH 6.5) in a pre-chilled mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 15 min at 4° C to get the supernatant and used as enzyme source. Peroxidase (POX) activity was determined following the method of Hammerschmidt et al. (1982). The reaction mixture of 3ml consisted of 0.25% (v/v) guaiacol in 10 mM potassium phosphate buffer (pH 6.9) containing 10 mM hydrogen peroxide. Five micro litres of crude extract was added to initiate the reaction, which was followed calorimetrically at 470 nm. POX activity was expressed as the increase in absorbance at 470 nm/ mg protein/ min. The experiment was repeated thrice taking three replicates each time.

Lipoxygenase (LOX) activity

Two-day-old seedlings which were inoculated and harvested along with un-inoculated samples were used for enzyme extraction. One gram fresh weight of the seedlings were homogenized in 0.2 M sodium phosphate buffer (pH 6.5) containing 1% poly vinyl pyrrol idone (PVP), 0.1% Triton X-100 and 0.04% sodium meta-bisulfite. The homogenate was centrifuged at 9000 *g* for 20 min at 4° C and the supernatant was used as the enzyme source. Enzyme activity was measured by monitoring the appearance of the conjugated dienehydroperoxide at 234 nm. Linoleic acid was used as substrate, which was prepared according to the standard method (Axelrod et al. 1981). Activity was recorded for 3 min using a spectrophotometer. The enzyme activity was expresses in terms of m mol quinone formed/ mg protein/ min. The experiment was repeated thrice taking three replicates each time.

Protein estimation

Protein content in extracts was estimated by the dye binding method (Bradford, 1976) using bovine serum albumin (Sigma) as a standard.

Statistical analysis

Each experimental data was subjected to analysis of variance (ANOVA) using SPSS Inc16.0. Significant effects of treatments were determined by the magnitude of the F value ($P \leq 0.05$). Treatment means were separated by Tukey's HSD test.

RESULTS

Effect of partially purified ns-LTP extracts on time-course analysis of HR

All the partially purified ns-LTPs treated seedlings along with resistant seedling offered significant HR reaction when challenge inoculated with the pathogen.

HR reaction was observed as early as 3 hpi in inducer treated and resistant seedlings and gradually increased and reached maximum at 24 hpi. A maximum of 82% HR response was observed in rice ns-LTP treated seedlings followed by maize and wheat ns-LTP treated seedlings. The resistant seedlings offered a maximum of 88% HR response, while the susceptible inoculated seedlings offered 22% HR at 24 hpi. There was an increase in percent of HR cells with time gap (Table 1).

Biochemical studies

Phenylalanine Ammonia Lyase (PAL) activity

Significant differences in PAL enzyme activity was observed in inducer treated and control seedlings at different time intervals. PAL activity in all the samples increased with increase in time interval. A gradual increase of PAL activity from 0 hpi (146.4 U) to 48 hpi (281.2 U) was observed in resistant inoculated samples and decreased thereafter. Similar trend of PAL activity was observed in rice ns-LTP treated inoculated seedlings with a maximum of 252.3 U at 48 hpi, while the maize and wheat ns-LTP treated inoculated seedlings offered maximum PAL activity at 24 hpi and it was maintained thereafter. The susceptible inoculated seedlings offered a maximum of 166.6 U of PAL activities at 48 hpi (Fig. 1). The uninoculated pearl millet samples showed similar trend of PAL activity of that of inoculated samples but the activity was lower when compared to inoculated samples.

Peroxidase (POX) activity

All the inducer treated and control seedlings showed significant difference in POX enzyme activity with time interval between challenge inoculations. POX enzyme activities were higher in crude ns-LTP treated and pathogen inoculated seedlings when compared with distilled water treated uninoculated ones. In all the samples tested there was a steady increase in enzyme activity up to 48 hpi, except in wheat ns-LTP treated seedlings where maximum activity was observed after 72 hpi (Fig. 2). Maximum POX activity was observed in rice ns-LTP treated seedlings followed by maize and wheat inducer treated seedlings. The highest POX activity of 82.40 U was observed at 48 hpi in resistant inoculated seedlings followed by rice and maize crude ns-LTP treated susceptible inoculated seedlings, which showed 77.80 and 69.20 U of POX activity, respectively. In time-course measurements of POX activity (from 0 to 72 hpi), rice crude ns-LTP treated seedlings showed more POX enzyme activity in both inoculated and un-inoculated susceptible seedlings over other treatments except resistant controls. There was up to 1.9 fold increases in POX activity in rice crude ns-LTP treated inoculated and 2.1 fold POX activities in resistant inoculated samples over the SDW treated uninoculated controls.

Time course analysis of Lipoxygenase (LOX) activity

Lipoxygenase activity was estimated in both inoculated and uninoculated seedlings raised from resistant, inducer treated and susceptible cultivars. The LOX activity at 0, 3, 6, 9, 12, 24, 48 and 72 hpi is depicted in Fig. 4. The pearl millet seedlings raised from rice ns-LTPs treated and challenge inoculated seeds showed higher level of LOX activity of 54.8 U at 24 hpi compared to susceptible seedling which offered 30.4 U at same time interval. LOX activity in inducer treated and inoculated seedlings was noticed as early as 3 hpi and reached maximum at 24 hpi and decreased or maintained thereafter (Fig. 3).

There was no significant increase in LOX activity in SDW treated pearl millet seedlings upon inoculation. LOX activity in resistant seedlings was higher compared to induced resistant seedlings. However, LOX activity in control seedlings remained lesser than the induced and resistant seedlings.

DISCUSSION

Plants defend themselves from insect or pathogen attack through a wide variety of mechanisms which are stimulated by many different biotic/ abiotic inducers (Schneider and Ullrich, 1994; Murali et al. 2013 Thakur and Sohal, 2013). Local defense is commonly associated with the HR and programmed cell death in plants (Heath, 2000). HR is visually observed as brown necrotic spots or streaks representing localized cell death and as resistance response in interaction between plants in general and to downy mildew oomycetes in particular (Dangl et al., 1996; Kamoun et al., 1999).

Table 1. Time course study of hypersensitive reaction (HR) in pearl millet seedlings treated with ns-LTPs upon pathogen inoculation.

Treatments	Hours post inoculation (hpi)						
	0	3	6	9	12	18	24
Maize ns-LTP	12.00±1.00^{bc}	14.50±0.50^{c}	21.00±0.00^{b}	32.50±0.50^{a}	45.00±1.00^{b}	60.50±0.50^{b}	72.00±0.00^{c}
Rice ns-LTP	13.75±0.47^{b}	16.50±0.28^{b}	22.50±0.50^{b}	36.75±0.47^{a}	46.00±0.40^{b}	68.00±0.40^{a}	78.50±0.28^{b}
Wheat ns-LTP	11.25±0.25^{c}	13.75±0.47^{c}	20.75±0.47^{b}	30.75±0.47^{a}	50.75±0.47^{a}	58.75±0.47^{b}	69.500±0.28^{d}
Susceptible	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{c}	9.00±0.40^{b}	14.75±0.47^{c}	17.75±0.47^{c}	22.00±0.40^{e}
Resistant	15.75±0.47^{a}	18.75±0.47^{a}	24.75±0.47^{a}	34.25±2.46^{a}	48.75±0.47^{a}	66.50±0.28^{a}	83.75±0.25^{a}

Values are means of four independent replicates. Means followed by the same letter(s) within the same column are not significantly different according to Tukey's HSD.

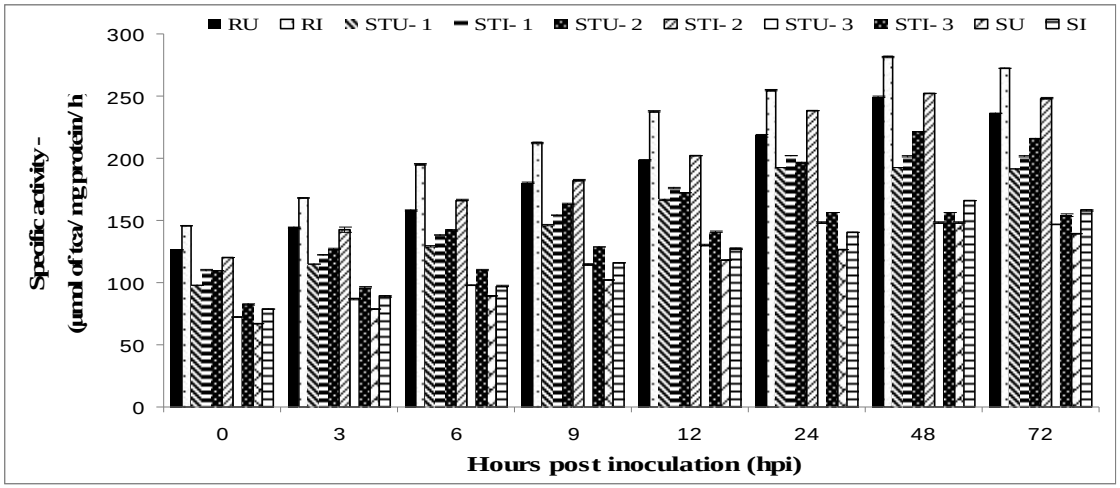

Fig. 1. Time course study of Phenyl ammonia lyase (PAL) activity in pearl millet seedling upon seed treatment with partially purified ns-LTPs.

[RU- Resistant Uninoculated; RI- Resistant Inoculated; STU-1: Susceptible Treated Uninoculated (Maize ns-LTPs); STI-1: Susceptible Treated Inoculated (Maize ns-LTPs); STU-2: Susceptible Treated Uninoculated (Rice ns-LTPs); STI-2: Susceptible Treated Inoculated (Rice ns-LTPs); STU-3: Susceptible Treated Uninoculated (Wheat ns-LTPs); STI-3: Susceptible Treated Inoculated (Wheat ns-LTPs); SU- Susceptible Uninoculated, SI- Susceptible Inoculated. Data of enzyme activity are means of three different experiments and bars indicate ± SE. **]**

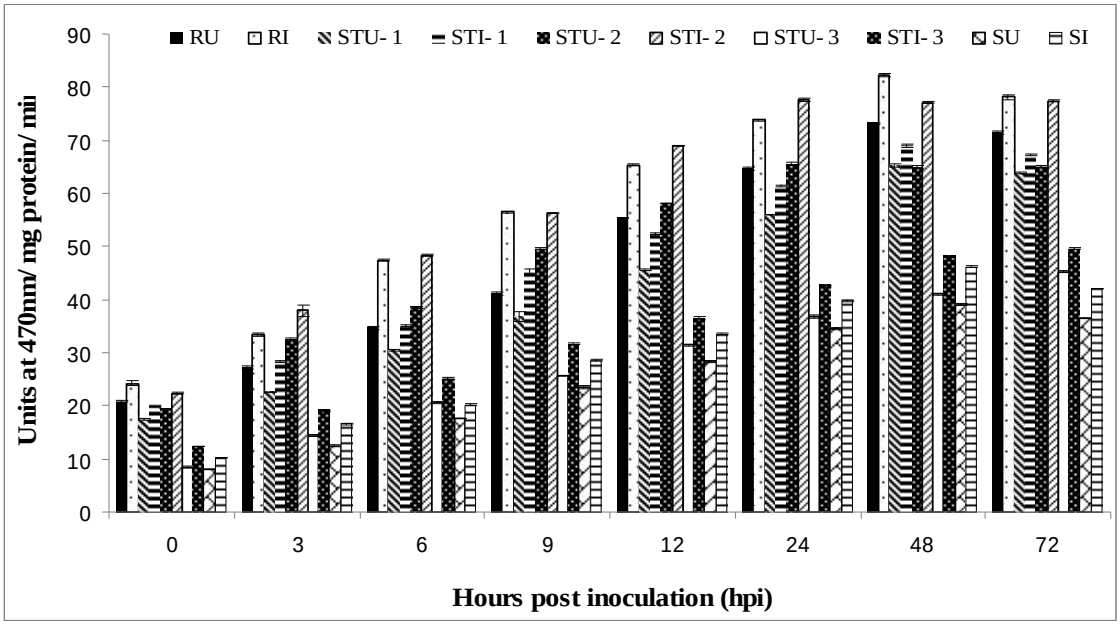

Fig. 2.Time course study of Peroxidase (POX) activity in pearl millet seedling upon seed treatment with partially purified ns-LTPs.

[RU- Resistant Uninoculated; RI- Resistant Inoculated; STU-1: Susceptible Treated Uninoculated (Maize ns-LTPs); STI-1: Susceptible Treated Inoculated (Maize ns-LTPs); STU-2: Susceptible Treated Uninoculated (Rice ns-LTPs); STI-2: Susceptible Treated Inoculated (Rice ns-LTPs); STU-3: Susceptible Treated Uninoculated (Wheat ns-LTPs); STI-3: Susceptible Treated Inoculated (Wheat ns-LTPs); SU- Susceptible Uninoculated, SI- Susceptible Inoculated. Data of enzyme activity are means of three different experiments and bars indicate ± SE. **]**

The present study revealed a rapid expression of HR in the resistant seedlings while the susceptible seedlings showed HR at later hours after inoculation. The early HR response in partially purified ns-LTP treated seedlings, similar to that of resistant reflects the rapid response to the pathogen infection sensitized by the ns-LTPs treated. The difference in rapidity and degree of HR as a response to inoculation with *S. graminicola* observed could be due to difference in expression of associated resistance genes. Such variation depending on the interacting pathogen and plant genotype has been documented by (Kamoun et al. 1999).

HR as a defensive response and as a marker of resistance has been established earlier in pearl millet downy mildew interaction with various inducers like BTH (Geetha and Shetty, 2002), unsaturated fatty acids (Amruthesh et al. 2005), PGPF (Murali et al. 2013) and *Lactuca sativa* (Mythrashree et al. 2013).

Pathogenesis related (PR) proteins are induced in pathological situations in plants, (Bowles, 1990) produced via salicylic-dependent pathway and considered a part of the multiple defense systems of plants (Kombrink and Somssich, 1997). Different kinds of proteins are found to play certain roles in the plant defense mechanism and the resistance to plant pathogens (Belkhadir et al. 2004). In the present study, partially purified ns-LTPs from maize, rice and wheat induces the defense related enzyme activities like PAL.POX and LOX activities which inturn gave protection against *S. graminicola* causing downy mildew disease in pearl millet. The activities of PAL, POX and LOX were induced distinctly and increased significantly in partially purified ns-LTPs treated and inoculated seedling over the SDW treated uninoculated controls. PAL has been demonstrated in metabolic activity of many higher plants and in synthesis of several defense-related secondary compounds like phenols and lignin (Hemm et al. 2004).

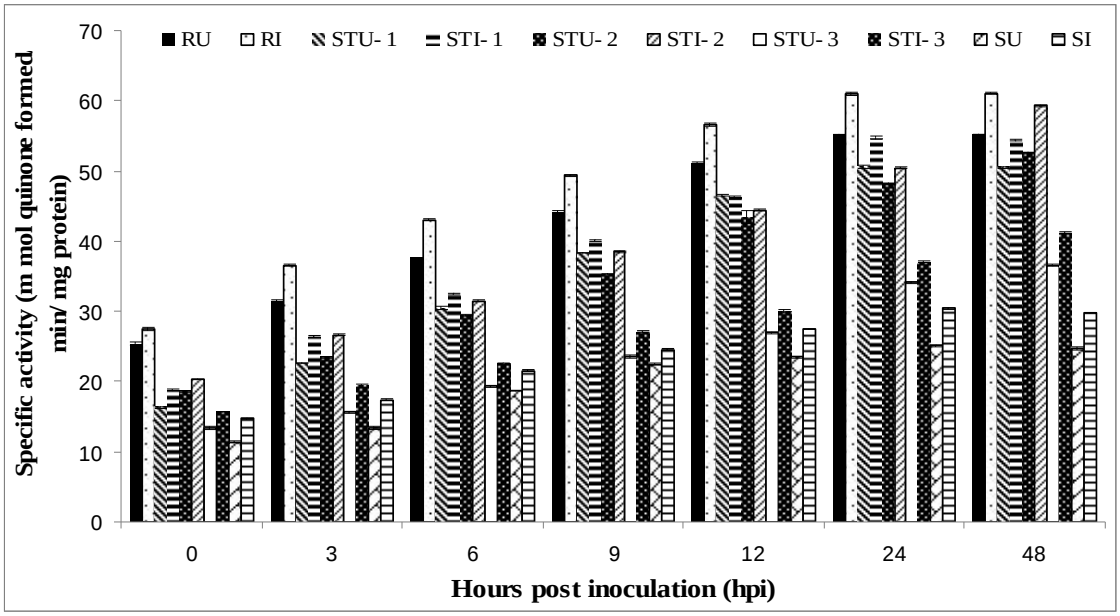

Fig. 3.Time course study of Lipoxygenase (LOX) activity in pearl millet seedling upon seed treatment with partially purified ns-LTPs.

[RU- Resistant Uninoculated; RI- Resistant Inoculated; STU-1: Susceptible Treated Uninoculated (Maize ns-LTPs); STI-1: Susceptible Treated Inoculated (Maize ns-LTPs); STU-2: Susceptible Treated Uninoculated (Rice ns-LTPs); STI-2: Susceptible Treated Inoculated (Rice ns-LTPs); STU-3: Susceptible Treated Uninoculated (Wheat ns-LTPs); STI-3: Susceptible Treated Inoculated (Wheat ns-LTPs); SU- Susceptible Uninoculated, SI- Susceptible Inoculated. Data of enzyme activity are means of three different experiments and bars indicate ± SE. **]**

The spatio-temporal time gap studies of PAL activities in resistant, induced resistant and susceptible pearl millet cultivars showed significant induction after pathogen infection and at 48 hpi the enzyme activity reached peak. The increase in PAL activity in all the test seedlings after pathogen inoculation and higher activity in resistant compared to induced resistant and susceptible cultivars indicates a possible role for PAL enzyme during pathogen infection and host resistance. PAL activity in rice ns-LTP treated seedlings was higher when compared to other ns-LTP treated and control seedlings. Similar results were obtained in pearl millet by Sudisha *et al*. (2011), wherein seed treatment with raw cow milk and amino acids enhanced PAL activity when compared to control. Likewise, there was an early increase in PAL activity in rice upon infection with *P. oryzae* (Wang et al. 2004) and in barley in response to fungal pathogens and elicitor treatments (Kervinen et al. 1998).

Involvement of protein components and peroxidase activity in plant diseases resistance has been documented in several plant patho-systems (Martin et al. 2003; Carvalho et al. 2006). Induction of peroxidases due to pathogen interaction has been studied in a number of patho-systems, where novel peroxidases appeared and higher activity was recorded in resistant cultivars compared to susceptible ones (Ramanathan et al. 2001). Plant peroxidises exists as isoenzymes with diverse expression profiles ranging from hypersensitive reaction, lignification, cross-linking of phenolics, glycoproteins, and suberisation to phytoalexin production (Baysal et al, 2003). In the present study, inoculation with *S. graminicola* resulted in more pronounced POX activity in the resistant cultivar particularly 48 hpi. High POX activity has been associated with resistance in pepper to *P. capsici* (Alcazar et al. 1995) and in tomato against *F. oxysporum* f.sp. *lycopersici* and *A. alternata* (Hameed et al. 2010).

LOXs are widely known to vitally contribute to plant defenses against pathogenic microorganisms. LOX enzyme activity has also been shown to be induced rapidly during a disease-resistance response and more slowly in a susceptible interaction. This association of increased LOX activity and an effective defense response has been observed for several plant pathosystems (Burow et al. 2000; Gobel et al. 2001). In the current study, LOX activity in challenge inoculated resistant was evident at 3 hpi and reached maximum at 24 hpi in resistant and susceptible seedlings, while in partially purified ns-LTP treated seedlings (rice and wheat) maximum LOX activity was observed at 48 hpi. Increased LOX activity was also noticed in untreated inoculated samples. Further, the distilled water treated control of both resistant and susceptible pearl millet seedlings exhibited a constant increase in LOX activity, even without elicitor-treatment or pathogen inoculation indicating the constitutive level of the enzyme. An increase in LOX activity in response to infection has been reported in many host-pathogen systems and correlated with plant resistance against pathogens (Kolomiets et al. 2001, Porta et al, 2008). Similarly, Sandhu et al. (2007) reported that the lipoxygenase activity was always higher in Brazilian rice cultivars resistant to leaf blast disease than in susceptible cultivars. The present study highlights the efficiency of the tested biotic agent in inducing defense related enzymes upon challenge inoculation with the pathogen in pearl millet after seed treatment. The results of the study confirm that inducers of crude ns-LTPs from plant source can be used as a source of inducer in pearl millet against downy mildew disease and the findings have also evidenced the interconnection of ISR and defense responses.

ACKNOWLEDGEMENTS

The authors are grateful to the University Grants Commission (UGC) for awarding Major Research Project to corresponding author for financial assistance. Authors are also thankful to Indian Council of Agricultural Research (ICAR), All India Co ordinated Pearl Millet Improvement Project (AICPMIP), Government of India, Mysore Center, Institution of Excellence (IOE) Project Authorities, University of Mysore, Mysore, India and Department of Botany DST-FIST for research facilities.

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