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Research Article

ROLE OF MANNITOL ON *ABELMOSCHUS ESCULENTUS*L. (OKRA) AGAINST *ALTERNARIA* LEAF SPOT DISEASE

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ABSTRACT
In the present investigation, an attempt has been made to manage the different concentration of Mannitol, to increase plant growth and induce systemic disease resistance. Mannitol and other related inducers are known to affect various physiological and biochemical activities of plants and may play a key role in regulating their growth and productivity and also involved in local and endemic disease resistance in plants in response to various pathogenic attacks. The present investigation was conducted to know the effect of mannitol at different concentration and at different time intervals in *Abelmoschus esculentus* against *Alternaria* leaf spot disease. The mannitol treated okra seeds showed enhanced seed germination and seedling vigor. Among the different concentration of seeds treated with 1.5Mm concentration of mannitol at 3hr showed maximum seed germination and seedling vigor. Here, the protection of disease was through induction of systemic resistance. 10 days old seedings were challenge inoculated with the pathogen, and further the biochemical activities. PAL activity was highest in 6hr (3.2) and POX was recorded highest in 12hr (37.4) at the concentration of 1.5mM when compared to the control.

Keywords: *Abelmoschus Esculentus, Aalternaria Alternata, Mannitol, POL, POX*

INTRODUCTION
Abelmoschus esculentus (okra) is a commercial vegetables crop with considerable area under cultivation in Africa and Asia belongs to the family Malvaceae. It probably originates in Ethiopia and is widely spread all over tropical and sub tropical and warm temperature regions of the world. Okra plays an important role in the human diet by supplying fats, proteins, carbohydrates minerals and vitamins. Moreover, mucilage is suitable for certain medicinal and industrial applications. The optimum yield of okra is approximately 6.6 tons/ hectare. Okra requires warm temperature in the range of 20-30°C, with minimum temperature of 18°C and maximum temperature of 35°C. There are eight *Abelmoschus* sp. are cultivated in India currently. India is the first producer of okra in the world. Okra suffers from many disease, leaf spot is one of the serious disease in okra caused by *Alternaria alternata*. Plants posses a range of defense that can be actively expressed in response to pathogens and parasites of various scales, ranging from microscopic viruses to insect herbivorous. Induced resistance can be expressed locally at the site of infection, as well as systematically.

Mannitol plays a central role in the signal transduction pathways that results in systemic acquired resistance in plants. Seed soaking with mannitol gave the highest protection and helped to improve the induced systemic resistance in seedling stage (Monaim, 2012). Mannitol showed the lowest effect in all aspects. It could be suggested that mannitol used as seed soaking agent for controlling wilt disease of tomato plants, since they are safe, low cost and effective against the disease as stated above. It is found in a wide variety of natural products, including almost all plants. Initiation of a resistance response requires perception signal molecules either synthesized by the invading organisms or released from plant cell wall. These signal molecules have collectively been termed elicitors only a few have been defined at the molecular level as oligosaccharides and glycol-peptides (Ebel and Cosio, 1994). The term elicitor was used for molecule capable of inducing the production of phytoalexins stimulating any type of plant defense response. Concomitant with the induction of resistance, a number of enzyme activities have been documented to increase in host tissues. The hydrolase, glucanases, chitinase, and peroxidase have been documented to be involved in the defense reaction of plants against pathogens. Phenylalanine ammonia

lyase (PAL) is one of the biochemical markers of induced resistance. It is considered to be the principal enzyme of the phenylpropanoid pathway, which is the prime intermediary in the biosynthesis of phenolics and flavonoids (Hahlbrock and Scheel, 1989; Dixon and Lamb, 1990). It catalyses the conversion of L-phenylalanine to trans-cinnamic acid in the first step of the phenylpropanoid pathway and regulates the production of precursor for lignin biosynthesis along with other phenolic protectants in plant cell (Nicholson and Hammerschmidt, 1992). This pathway has been associated with the initiation of resistance in plants (Sticher *et al.,* 1997). The presence of phenolic compounds in plants and their synthesis in response to infection is associated with disease resistance. PAL is one of the most intensively studied enzymes in plant secondary metabolism because of its key role in phenylpropanoid biosynthesis (Whetten and Sederoff, 1995).

The induction of peroxidase (POX) activity in plants occurs in response to numerous biotic and abiotic stimuli, including exposure to pathogens or elicitor preparations, chemical oxidizing agents, red light, and mechanical stimuli (Hammerschmidt *et al*., 1982; De-Jaegher, *et al*., 1985; Casal, *et al.,* 1994). The generality of this response is probably related to the multiple forms and overlapping functions of POX in normal plant development and following induction by abiotic and biotic elicitors (Lagrimini and Rothstein, 1987). For example, POX is believed to play roles in auxin catabolism, the oxidation of phenolics to form lignin, the cross-linking of hydroxyproline-rich glycoproteins in plant cell walls, and the production and breakdown of hydrogen peroxide and other reactive oxygen species (Klotz and Lagrimini, 1996). The roles that POX can play in cell wall toughening and in the production of toxic secondary metabolites and its simultaneous oxidant and anti-oxidant capabilities can make it an important factor in the integrated defense response of plants to a variety of stresses (Hammerschmidt *et al.,* 1982; DeJaegher *et al.,* 1985; Felton *et al.,* 1989). The present study an attempt has been made to induce systemic resistance on okra against leaf spot disease by using abiotic inducers and further study is required to develop new strategies to control the disease.

MATERIALS AND METHODS

Source of Collection and Screening and Isolation of Causal Pathogen

Okra seeds, leaves and stems showing typical leaf spot symptoms were collected from different okra growing regions of Mysore district, T. Narasipura for the isolation of pathogen *Alternaria alternata.* The seed samples were collected from farmers, public seed agencies and Namdari seeds in Mysore. The causal organism *Alternaria alternata* was isolated followed by the method of (Narasimha Murthy *et al.,* 2012) from okra leaves showing typical leaf spot symptoms. The infected leaves were cut in to bits and 2-3 times washed with tap water and then surface sterilized using 1% Sodium hypochlorite for one minute and followed by washing with sterilized distilled water Single separated lesions on leaf materials were incubated in a humid chamber at 25^0C for 24 hr and then examined for conidial chains under Stereomicroscope. Conidia from separate conidiophores were picked using glass needles and plated on PDA agar medium. The plates were then incubated at 25^0 C and after 2 days the plates were thoroughly examined under binocular microscope, and identified pathogen based on standard procedures. The identified fungus was further sub cultured, maintained in petriplates on PDA media and used for further studies.

Preparation of Inoculum

Pure culture of *Alternaria alternata* was grown on potato dextrose agar medium in the dark at 18^0C for 14 days from the date of inoculation. From this pure culture conidia were harvested by flooding the surface of the petridish with sterile distilled water (10ml) and gently scraping the surface of the media with an L-shaped glass rod to dislodge the conidia. The conidial suspension was stirred with a magnetic stirred for 1 hr and strained through 4 layers of cheese cloth to remove the mycelial fragments. The concentration was then adjusted to 1×10^5 conidia per ml using haemocytometer (Safeeulla, 1976).

Pathogenicity Test

Detached leaf assay was done to prove pathogenicity of *Alternaria alternata* in okra plants. The spore suspension of pathogen was prepared in sterile distilled water from 10-days-old culture. The

concentration of spore suspension was adjusted to 1 X 10^5 conidia/ml using Haemocytometer. For detached leaf assay, healthy leaves from 25-30 days-old plant were collected and washed with running tap water and blot dried. These leaves were kept in a moist chamber prepared by placing moist blotter discs in Petri plates. The leaves were pin pricked at the centre with a sterile needle and 50 µl of spore suspension 1 X 10^5 conidia/ ml were injected onto the leaf. The setup was incubated in a moist chamber for 7 days and regular observations were made daily for appearance and symptoms around the pricked region.

Preparation of Mannitol Solution in Different Concentration for Inducer Treatment

18.217gm of Mannitol was dissolved in minimum quantity of distilled water until no granules were left over and the final volume was made up to 100 ml using standard flask to get 0.1M Mannitol stock solution. From this stock solution different concentration of 0.5mM, 1.0mM, 1.5mM and 2mM mannitol were prepared further studies.

Effect of Okra Seeds Priming with Mannitol on Seed Germination and Seedling Vigor

Seeds of okra susceptible variety (*Arka anamica*) were taken; surface sterilized in 1% sodium hypochlorite solution for 1 min and soaked in different concentrations of Mannitol (100 ml) for 3h and 6h respectively. Distilled water treated seeds served as control. The experiment was conducted with 100 seeds in four replicates and repeated thrice. The treated seeds (one set inoculated with the conidia of *A. alternata* at 1x 10^5 concentration and another set without inoculation) were placed on wetted paper towels (ISTA, 2005) at equal distance and the same was incubated for 10 days at 25±2^0 C. Germination percentage was analyzed by the method of Abdul Baki and Anderson (1953). The percentage of germination, root length and shoot length was recorded and vigor index was calculated as mentioned below;

$$\text{Percentage of germination} = \frac{\text{No. of seed germinated}}{\text{Total No. of seed plated}} \times 100$$

Vigor index= seed germination % × (mean root length+ mean shoot length).

The concentration of the Mannitol, which showed effective seed germination and vigor Index, were used for future studies.

Biochemical Studies:

Sampling of seedlings for Biochemical Studies

The activity of defense enzymes like peroxidase and phenylalanine ammonia-lyase activity during the host pathogen interaction in okra was studied. Okra seeds treated with different concentrations of 0.5mM, 1.0mM, 1.5mM, and 2mM of Mannitol at different time intervals of 3h and 6h. 10 days old seedlings were inoculated with a spore suspension 1× 10^5 spores/ml of the pathogen by root dip method and incubated at 25±2^0C. Seedlings were harvested at different times intervals of 0, 6, 12, 18 and 24 hr of post inoculation of the pathogen. The treated Okra seedlings were carefully uprooted without causing any damage to root and leaf tissues, blot dried and stored at 4^0 C until subsequent use for PAL and POX assays respectively. The seedlings treated with distilled water served as control.

Estimation of PAL and POX

PAL Enzyme Extraction

Seedlings (1 gm) were homogenized in 1 ml of ice cold 25 mM Tris buffer (pH 8.8) in a pre-chilled mortar and pestle on ice and the homogenate was centrifuged at 10,000 rpm for 30 min at 4° C. The supernatant was collected and used as enzyme source.

PAL Enzyme Assay

PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson *et al.,* (1984). The reaction mixture (3 ml) consisted of 0.3 ml of enzyme extract, 1.5 ml of substrate buffer (50mM of L-phenylalanine) and 1.2 ml of 25 mM Tris-HCl, pH-8.8. The reaction mixture was mixed well and incubated at 40°C for 2h. 0.18 ml of 5N HCl was added to the reaction mixture and vortexed and optical density (OD) was read at 290 nm. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient at 9630 m^{-1} (Dickerson *et al.,* 1984). Blank containing 1.5 ml of substrate buffer + 1.2 ml of 25 mM of Tris-HCl (pH-8.8) and 0.3 ml of enzyme were added after the addition of 0.180 ml of 5N HCl. The total activity and specific activity was

calculated using the following formula, and the enzyme activity was expressed in terms of µ mol of TCA/ mg protein/hr. Experiments were conducted in three replicates and repeated thrice.

Total activity = OD x 100 (slope value of TCA) X µ mol of TCA

$$\text{Specific Activity} = \frac{\text{Total activity}}{\text{mg/protein}} \times 2$$

Estimation of Peroxidase (POX) Activity

One gram of seedlings was homogenized in one ml of 10mM phosphate buffer (pH 6.0) in a pre-chilled mortar and pestle on ice. The homogenate was centrifuged at 12,000 rpm for 20 min at 4º C and the supernatant served as enzyme source.

Peroxidase enzyme assay was carried out as described by Hammerschmidt *et al*., (1982) with minor modifications. The reaction mixture (3 ml) consisted of 0.25% (v/v) Guaiacol in 10 mM Potassium phosphate buffer (pH 6.0) containing 10 mM hydrogen peroxide. Addition of 100 µl of crude enzyme extract initiated the reaction, which was measured Spectrophotometrically at 470 nm for 1 min. One unit of POX enzyme activity is defined as change in absorbance min^{-1} mg^{-1} protein. Experiments were conducted in three replicates and repeated thrice.

Protein Estimation - Lowry's Method

The protein content in the crude extract was estimated by following the standard procedure of Lowry's method (1951), using BSA (Sigma) as the standard. The reaction mixture consists of 50 µl of sample and the volume was made up to 1 ml using distilled water. To the same solution 5 ml of reagent C was added (100ml of 2% Na_2CO_3 in 0.1 N NaOH + 1 ml of 1% $CuSO_4$ + 1ml of 2% sodium potassium tartarate) and mixed well, the same was incubated at room temperature for 10 min. After that 0.5 ml of Folin's reagent was added to each tube and mixed thoroughly. The reaction mixture in the test tubes was again incubated at room temperature for 30 min and OD (Optical Density) was read at 660 nm. The blank solution contains 100 µl of distilled water + Reagent C + Reagent D (Folin's reagent). In another set of tubes containing BSA solution the volume was made up to 1ml with 0.1N NaOH and to that reagent C and reagent D were added and incubated.

Standard curve was drawn against µg of BSA. From the standard curve the amount of protein in the sample was determined and protein/ gram of sample were calculated. To calculate the specific activities of the enzymes, protein content in Mannitol treated seedlings were estimated by Lowry's methods (Lowry *et al.,* 1951) using BSA (Sigma) as a standard. Experiments were conducted in two replicates and repeated twice.

RESULTS AND DISCUSSION

Results

Screening, Isolation and Identification of the Pathogen

The causal organism *Alternaria alternata* was isolated from okra leaves showing typical leaf spot symptoms. Single separated lesions on leaf material were incubated in a humid chamber at 25ºC for 24 hrs and then examined for sporulation under stereo-binocular microscope. The pathogen was identified based on the morphological, conidial, fruiting bodies and culture characters. The identified pathogen conidia were picked using sterile needle and inoculated onto PDA slants/plates for further studies.

Pathogenicity Test

Preparation of Inoculums and detached leaf assay

The spore suspension of *Alternaria alternata* was prepared using sterile distilled water from 10 – 15 days old pure culture. The concentration of spore was adjusted to 1×10^5conidia / ml using Haemocytometer. The detached leaves inoculated with 1×10^5 conidia / ml of *Alternaria alternata* was found to be virulent and effective.

Pin prick inoculated part of the leaf expressed severe infection and spots were noticed on the leaves after 7 days of inoculation from humidity conditions.

In contrast to the control leaf which is pin prick inoculated with distilled water, which is completely free from the pathogen infection.

Effect of Okra Seeds Priming with Mannitol on Seed Germination and Seedling Vigor

Okra seed (susceptible variety Arkaanamica) priming with different concentration of Mannitol was tested for their efficacy on seed germination and seedling vigor test was conducted. The experiment was done with four replicates in 3h and 6h duration of treatments one set is inoculated with pathogen with Mannitol and another set is uninoculated with Mannitol and water treated seeds served as control in all replicates. After 10 days incubation the maximum germination (78%) and seedling vigor 1021 was recorded in 1.5mM at 3h of seed treatment.

Followed by other concentration where the percentage of germination and seedling vigor reduced sequentially and distilled water treated seeds showed less germination and seedling vigor then compare to other concentration

Defense- Related Enzyme Assay

Okra seeds were primed with different treatments as detailed above. After seed treatment seedlings were harvested at 0h, 6h, 12h, 18h, and 24h pathogen after inoculation and incubation at -30^{0}C until enzyme extraction. These seedlings samples were extracted and used for PAL and POX activity studies.

PAL and POX Activity in Mannitol Treated Seeds

Ten days old okra seedlings treated with different concentrations of (0.5mM, 1.0mM, 1.5mM and 2.0mM) of Mannitol after inoculation with *Alternaria alternata* was assayed for time course study of PAL and POX activity as defense related enzymes at 0, 6, 12, 18, 24hr per inoculation. 1.5mM Mannitol seedlings showed a maximum PAL activity at 3h seed treatment which was followed by other concentration with PAL activity which remained higher when compared to the control.

The other concentration of Mannitol treated seedlings for 3h also followed the same pattern as that of 6h treated seedlings where the activity of after reaching a peak at 6hpi declined sequentially at 12h and 18hpi. PAL activity was determined by the calculating the conversion of L-Phenylalanine to t-cinnamic acid through PAL assay procedure and PAL activity expressed in µ mol of t-cinnamic acid/ mg protein/hr. In the present study an increase in PAL activity was positively correlated with increase in disease resistance.

The Mannitol treated seedlings at different concentration were studied for POX activity also. The specific activity of POX also followed the same pattern as that of PAL activity but maximum activity of POX was observed in 12hpi in 1.5mM concentration when treated for 3h. The activity was declined at 24hpi in both duration of treatment.

Seedlings treated for 6h also showed maximum activity at 12hpi which was lower than that of 3h treated. Control seedlings recorded less activity when compared to other concentration. POX activity was expressed as units/ mg/protein/min.

Table 1: Seed Germination and Seedling Vigor Treated with Different Concentration of Mannitol at 3hrs (Inoculated with Pathogen)

Concentration of Mannitol	%Germination	MSL (cm)	MRL(cm)	Seedling Vigor
0.5 mM	68±0.21^{cd}	7.1±0.25^{ab}	4.9±1.24^{ab}	816±0.24^{bc}
1 mM	72±1.23^{b}	7.2±0.45^{ab}	4.5±1.26^{c}	842±0.59^{b}
1.5 mM	78±1.25^{a}	7.6±0.36^{a}	5.5±1.56^{ab}	1021±0.47^{a}
2 mM	61±1.24^{cd}	6.8±0.56^{bc}	4.9±1.86^{bc}	782±1.26^{d}
Control	60±0.45^{d}	6.2±0.45^{cd}	4.1±1.24^{d}	703±1.28^{de}

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

Table 2: Seed Germination and Seedling Vigor Treated with Different Concentration of Mannitol at 6hrs (Inoculated with Pathogen)

Concentration of Mannitol	% Germination	MSL(cm)	MRL(cm)	Seedling Vigor
0.5mM	49±0.23^{c}	6.8±0.98^{bc}	4.9±1.87^{c}	573±0.47^{bc}
1 mM	46±0.56^{cd}	7.1±0.25^{b}	5.0±0.59^{b}	556±0.45^{d}
1.5 mM	59±0.48^{ab}	8.2±0.54^{a}	6.2±0.48^{a}	751±0.26^{a}
2 mM	50±1.45^{b}	6.6±0.26^{d}	3.5±1.98^{cd}	598±0.21^{b}
Control	40±0.25^{e}	5.0±0.25^{de}	2.7±0.48^{de}	548±0.58^{de}

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

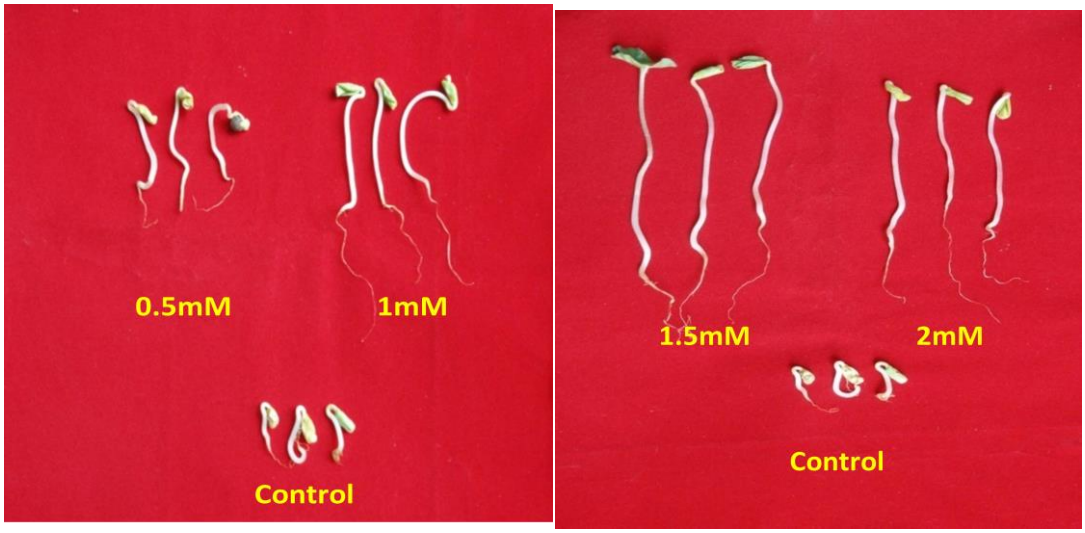

Figure 1: Seed Germination and Seedling Vigor of Okra Seeds Treated with Different Concentration of Mannitol at 3hrs (Inoculated with Pathogen)

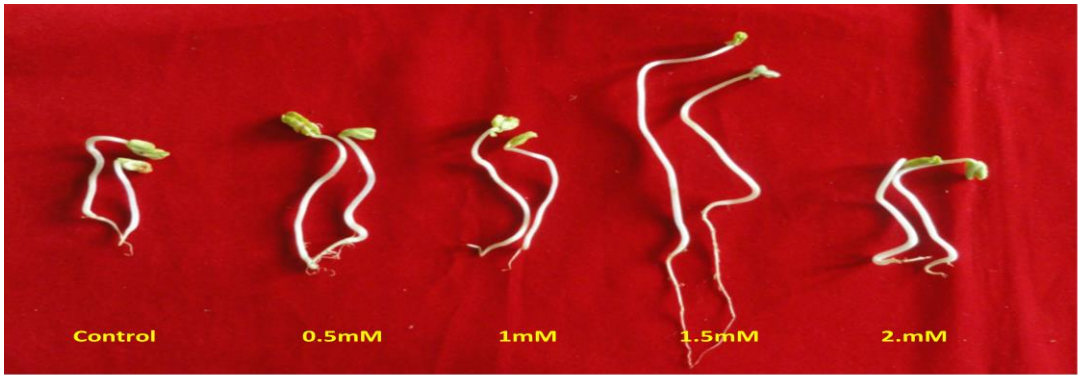

Figure 2: Seed Germination and Seedling Vigor of Okra Seeds Treated with Different Concentration of Mannitol at 6hrs (Inoculated with Pathogen)

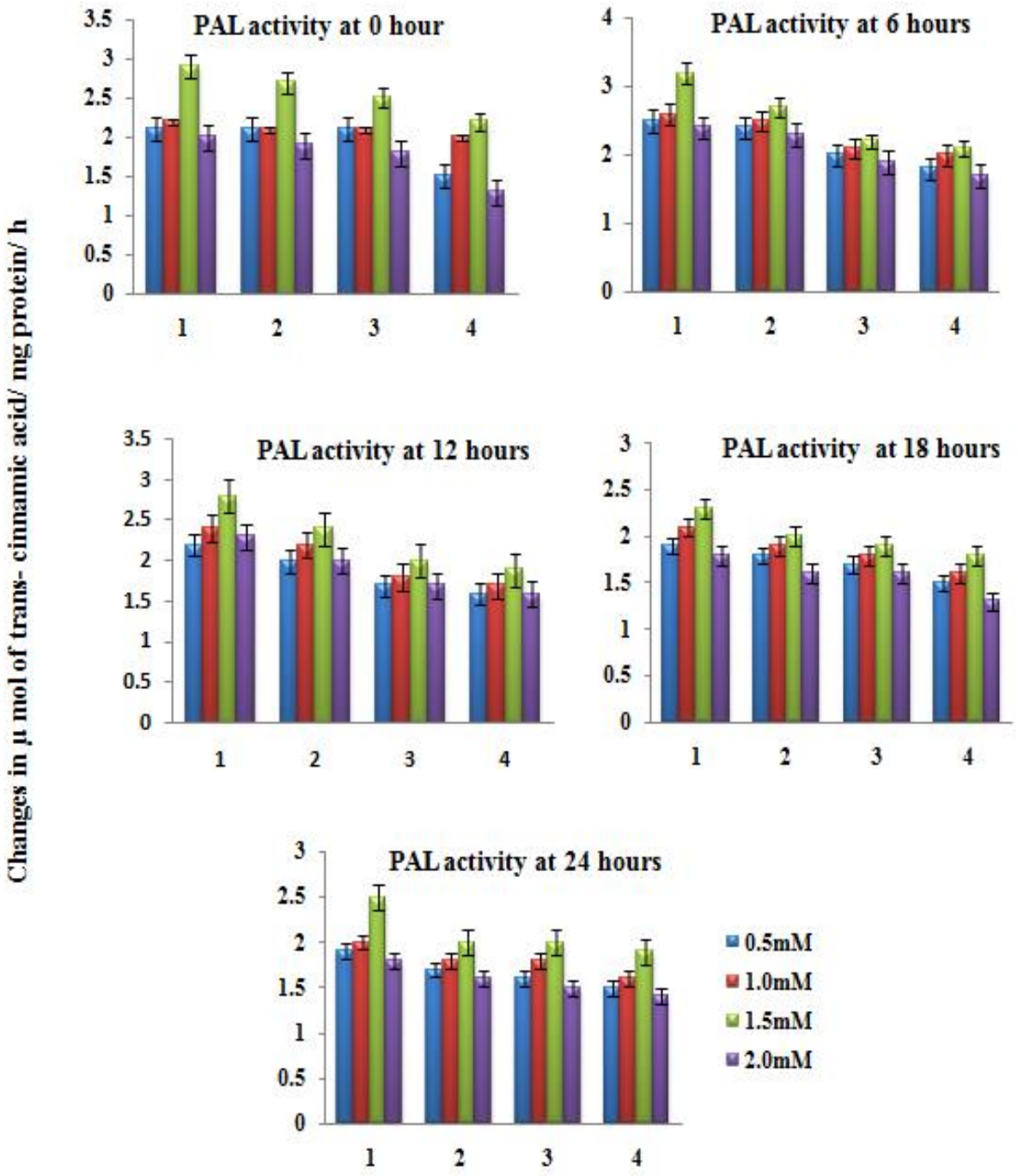

Figure 3: Temporal Pattern of Accumulation of PAL Enzyme in Okra Seedlings after Seed Treatment with Different Concentrations of Mannitol (0.5, 1.0, 1.5 and 2.0mM) at Different Time Intervals; Lines on the Bars Indicate Standard Error
1- Mannitol Treated Inoculated;
2- Mannitol Treated Uninoculated;
3- Distilled Water Treated Inoculated;
4- Distilled Water Treated Uninoculated

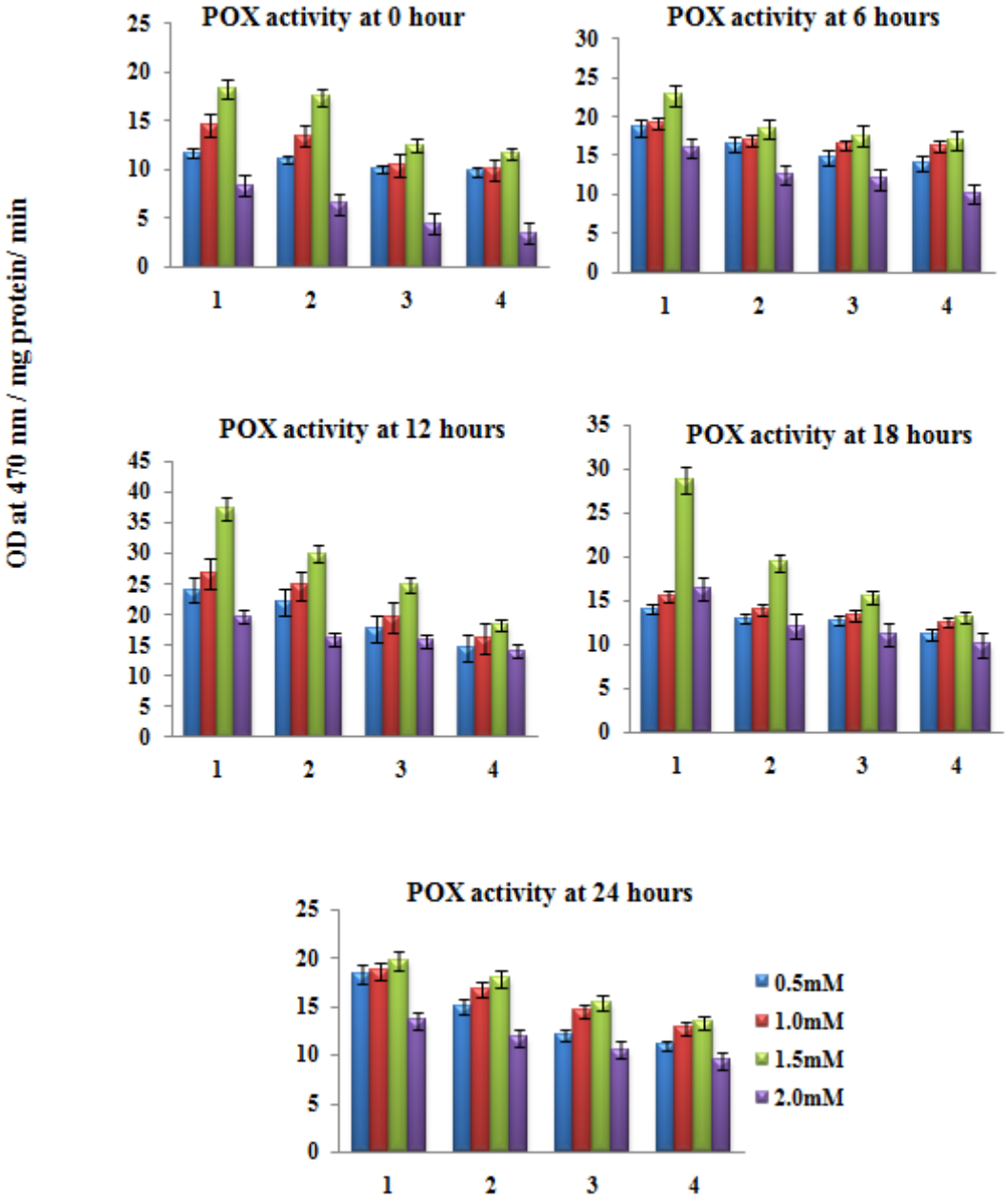

Figure 4: Temporal Pattern of Accumulation of POX Enzyme in Okra Sedlings after seed Treatment with Different Concentrations of Mannitol (0.5, 1.0, 1.5 and 2.0mM) at Different Time Intervals; Lines on the Bars Indicate Standard Error
1- Mannitol Treated Inoculated;
2- Mannitol Treated Uninoculated;
3- Distilled Water Treated Inoculated;
4- Distilled Water Treated Uninoculated

Discussion

The use of abiotic inducer is to reduce disease incidence caused by plant pathogens is continuously being developed and is being used in a variety of vegetable crops. The natural resistance of plants to pathogens

is based on constitutive and inducible barriers. There are many reports on application of chemicals triggers plants latent defense mechanism in response to infection by pathogen. Mannitol plays central role in the signal transduction pathways that results in systemic acquired resistance in plants. Seed primed with Mannitol gave more yields when compare to non primed seeds. Several growth promoting response was generated in tomato seedlings sprayed with Mannitol observed a significant increasing the percentage of wilt disease controlling of tomato plants (Monaim, 2012). In the present study Mannitol significantly enhanced seed quality parameter at 1.5mM concentration in a minimum period of 3hr and it helps to inducing resistance in host. Presoaking okra seeds in 1.5mM for 3hr could be effective in combating the adverse effects of the pathogen. The results demonstrate that there was a significant difference between the treatments.

In the present study, effort has been made to know the effect of exogenous application of Mannitol by seed treatment in induction of resistance in okra against *Alternaria alternata.* Induction of resistance was achieved by treating the seeds for 3h in 1.5mM Mannitol and it helps in developing a resistance in plants. Mannitol application induces accumulation of PR proteins (Ryals *et al.,* 1997). Many of the PR proteins have antimicrobial activity in *in-vitro* and they serve as biochemical markers for the onset of the defense response. The importance of phenylalanine ammonia lyase (PAL) enzyme in plant defense mechanism has been reported on many plant species against invading pathogens. The results of the present study substantiate this in okra leaf spot pathogen interaction in PAL and POX enzyme. This strengthens the hypothesis that Mannitol activates the signal transduction pathway, thus, leading to expression of SAR. In the investigation also, activity of the defense enzyme PAL was observed to increase sharply in response to seed treatment with Mannitol in the presence of the pathogen. Maximum PAL activity was observed in 1.5mM concentration in 3hr seed treatment. Increase in the concentration of Mannitol feeding to the seedlings PAL activity decreased with respect to time intervals after inoculation at both 3hr after seed treatments, but remained higher than the control which showed lesser activity in both treatments. The induction response of POX was similar to that of PAL. The induction of POX appears to be an early event in plant microbe interaction. The correlation between increased POX activity during incompatible interactions and re enforcement of cell wall with phenol compounds has been reported (Cordeler *et al.,* 2003). Our results show that peroxidase show constitutive presence in both treated and untreated seedlings that peroxidase perform a variety of physiological roles in plants and carryout wide range of diverse activity including hypersensitive reaction, lignifications, cross linkage of phenolics and glycoproteinse and phytoalaxin production (Doubrava *et al.,* 1988). Mannitol significantly improved the germination and seedling vigor performance including the elicitation of defense related enzymes of okra at a concentration of 1.5mM for minimum period of 3hr seed treatment and it helps to eliminate the adverse effect of the pathogen.

Conclusion

From the present investigations it can be concluded that mannitol plays an important role in inducing disease resistance and promoting plant growth in okra against leaf spot disease under controlled conditions. Okra seeds treated with mannitol for 3hr duration showed improved seed germination, seedling vigor, uniform plant growth and reduced disease incidence compared to control okra plants under controlled conditions.

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