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RESEARCH ARTICLE

EFFECT OF BIOTIC AND ABIOTIC INDUCERS ON INDUCTION OF DEFENSE ENZYMES IN SUNFLOWER

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ABSTRACT

The present study explores the ability of biotic and abiotic agents as inducers to stimulate the plant immunity assessed through bio chemical estimations and to control the spread of fungal pathogen Alternariaster helianthi (Hansf.) E.G. Simmons in infected plants. Three chemical inducers α-Amino Butyric Acid (AABA), ɣ-Amino Butyric Acid (GABA) and Salycilic Acid (SA) and two biological agents Trichoderma harizanum and Pseudomonas fluorescens as biological inducers on eight sunflower genotypes were studied. Bio chemical estimations of known plant defense enzymes viz., total phenols, peroxidases, total sugars, Phenyl Annine Lyase (PAL) and catalase were done spectro photo metrically at different intervals to understand the efficacy of inducers in developing resistance in plants. Among all the inducers tested GABA has shown effective response in systemic induction of resistance, followed by T. harizanum. All treated plants has shown high release of components at 18hours after inducer application. Observed defensive response of sunflower plants against the pathogen is different in inducer treated plant from untreated plant. In conclusion, chemical inducer GABA should be considered excellent and the results indicate that a comprehensive evaluation of inducers is advisable as they combine both direct antifungal activity against the targeted pathogen and the ability to develop prime plant immunity against various infections.

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INTRODUCTION

Alternariaster blight, is one among the most devastating diseases of sunflower (*Helianthus annuus* L.) due to its challenging management. Although use of chemicals for containing crop diseases is followed frequently, emergence of pathogen strains resistant to the chemicals has become a serious problem to overcome therefore, development of cultivars with induced systemic resistance (ISR) to microbial pathogens is considered as the most plausible disease management. Strategy depending on the induction of natural disease resistant mechanisms by employing inducers of resistance has been shown to be a practical possibility in certain crops (Narayanasamy 2008). Various synthetic and biological agents have been proposed in inducing resistance in plants and making them capable to fight against various diseases. These agents are considered as inducers basing on their capability of induction of resistance in treated plants. Use of induced systemic resistance in crop protection against different diseases is a modern concept for plant disease management.

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Alteration in the oxidative enzyme systems like peroxidase and polyphenol oxidase on induction by necrotizing pathogens and plant acquisition of resistance to further pathogenic attack have led many investigators to accept that the plant pathogensis related proteins are involved in the acquired resistance phenomenon (Bowles., 1990; Tomiyama., 1963). The biochemical basis of resistance to infection by *A.helianthi* is poorly elucidated therefore, the present investigation will shed light on induced systemic acquired resistance in resistant, moderately resistant and susceptible genotypes under the influence of both biotic and abiotic inducers and help to investigate the biochemical basis of resistance against *A.helianthi,* an incitant of Alternaria leaf blight, one of the most wide spread disease in tropical regions of the world.

MATERIALS AND METHODS

Pathogen and plants

Fungal pathogen, *A.helianthi* was obtained by the collection of leaf samples from naturally diseased sunflower plants showing typical symptoms of Alternariaster blight at sunflower farm, Indian Institute of Oilseeds Research, Hyderabad.

Infected leaf spots were excised, thoroughly washed with tap water and surface sterilized for 45 seconds with 0.2% sodiumhypocholorite solution, then rinsed three times in sterilized distilled water and dried between folds of sterile whatman filter paper. The surface sterilized samples were plated onto sunflower leaf extract medium. *Alternariaster helianthi* isolate was identified morphologically by bright field microscopy and pure culture was maintained on Sunflower Leaf Extract Medium (SLEM) (Sujatha *et al.,* 1997).

Biochemical constituents *viz.,* sugars, catalase, peroxidase, PAL and polyphenol oxidase are known to impart resistance against diseases. In this context these factors were estimated in resistant, moderately resistant and susceptible genotypes before and after inoculation of the pathogen to know the relative change in host. DRSH-1, KBSH-1, KBSH-44, Morden, SCG-99, White Pollen, TX16R AND EC- 53792 were raised from the seeds in earthen pots containing red soil, sand, FYM in the ratio of 3:2:1 in green house conditions (28$\pm$ 2^0c temperature). Plants at the age of 25 days with three to four pairs of leaves were used for all the experiments. Fifteen days old well grown culture of *A.helianthi* has been scrapped and mixed with sterile distilled water to make a spore suspension of $1X10^6$ spores per/ml. Whole plants were inoculated by spraying (up to 15 ml/plant, depending on size) with spore suspension containing 0.05% Tween-20.

ISR compounds and their Application

AABA, GABA and Salycilic Acid were purchased from Sigma. Isolation and identification of fungal antagonists from rhizosphere soil was done as described by Rifai (1969). To Select the effective isolate, potentiality of different strains of *Trichoderma viride*, *Trichoderma harzianum*, *Bacillus Megaterium, Bacillus Circulans* and *Psuedomonas fluorescens* were screened under greenhouse conditions directly as *A.helianthi* is a slow growing fungal pathogen, it is difficult to evaluate in *invitro* conditions by dual plate technique. Selected isolates *T.harizanum* and *P.fluorescens* were found effective after primary screening conducted at Indian Institute of Oilseeds Research, Hyderabad. Application of the compounds on plants was done before 3 days of pathogen inoculation by spraying of each chemical inducer at 100 µg/ml and biocontrol agents at 0.2% concentration (talc formulation), foliar spray of 5ml/ plant using fine atomizer (hand sprayer) onto the upper leaf surfaces.

Sample collection and analysis

Leaf samples were collected from main stem above the ground level in labeled envelopes from inducer treated plants and untreated plants that serves as control at different time intervals (0hrs, 6hrs, 12hrs, 18 hrs, 24hrs, 48hrs and 96hrs). Extraction and estimation of peroxidases, polyphenol oxidase, catalase and Phenyl alanine ammonia lyase were estimated using standard procedures according to biochemical methods by sadashivam and manickam (2005). Peroxidase enzyme is estimated extracted by homogenizing the material in ice-cold 0.1 M phosphate buffer of pH 6.0 in a chilled pestle and mortar. Guaiacol is used as a substrate. H_20_2 is as donor. The absorbance was read at 436 nm.

Specific activity of the enzyme was expressed as per mg protein per min. Estimation of Polyphenol oxidase was done by using catechol oxidase as substrate for the reaction. Rate of increase in absorbance at 412 nm against the blank at every 30 seconds up to 3 min was noted. Change in absorbance was calculated and enzyme activity was expressed as micro moles per mg of protein per min. For the estimation of catalase, hydrogen peroxidase is substrate degraded by catalase enzyme. Rate of absorbance was recorded at 240nm at 30 sec interval. Enzyme activity was expressed as micro moles per mg protein/ min. For estimation of PAL activity spectrophotometrically absorbance were measured at 290nm against blank. Supernatant of homogenised leaf sample in borate buffer was used as enzyme source. Enzyme activity was expressed as micro moles per mg protein per minute. Total sugars were extracted from untreated (control) and leaves treated with each inducer and on each genotype (0, 6, 12, 18, 24, 48 and 96 hours after inoculation) and estimated according to Dubois *et al*. (1956). Total sugars were expressed as mg g-1 fresh weight of tissue.

RESULTS

To understand the host pathogen interaction under the influence of different biotic and abiotic inducers, an integrated study of changes in peroxidases, total sugars, poly phenol oxidase , PAL and catalases was done. Better understanding of the role of defense enzymes under the influence of inducers reveals their response in host to pathogen. Observation of biochemical estimations of different compounds in inducer treated and untreated sunflower plants of eight genotypes revealed the difference in release of biological compounds when induced with five inducers was different at 0 hrs, 6 hrs, 12 hrs, 18hrs, 24 hrs, 48 hrs and 96 hrs. Results at 18 hrs are presented here, data at different time intervals is available with author.

Peroxidases are ubiquitous enzymes found in virtually all green plants, they often increases as a response to stress and role is cellular protection. Peroxidase activity was tested, optical density at 420 nm and expressed in micro moles/mg of protein/ min. Among the leaf samples, highest peroxidase activity was seen in GABA induced DRSH-1 cultivar at 18 hours after inoculation followed by *T. harizanum*, AABA, SA and *P.flourescens* (1.855, 1.785, 1.720 and 1.620 *respectively*). Each genotype have reacted to each inducer differently showing a range of increase of peroxidase release. Release of peroxidase at 18hrs post inoculation DRSH-1 has shown highest enzyme activity of 1.990 and in KBSH-1 enzyme activity recorded was 1.960 followed by SCG-99 (1.680), TX16R(1.455), EC-53792 (1.315l), white pollen (1.200), KBSH- 44 (1.005) and morden (0.750) in the same given order. Second effective inducer that increased the release of peroxidase enzyme in all the genotypes was *T.harizanum* which is known for its anti fungal potency. Enzyme activity has varied from 1.855 to 0.735, highest observed in DRSH-1 and least in cultivar morden. However, noted to be least among the tested, observed an enhanced enzyme activity when compared to untreated control at 0.050. Effectivity of AABA is followed by *T.harizanum* ranging its effect on enzyme activity release from 1.785 (DRSH-1) to 0.705 (Morden). Salycilic

acid was next to AABA in its effect on release of enzymes ranging from 1.720 (DRSH-1) to 0.675 (morden). Least in performance among the studied inducers was *P.flourescens* with the release of 1.620 in DRSH-1 to 0.600 in morden. Estimations were given in Table 1. Poly phenol oxidases are wide spread enzymes which oxidize plant phenolic compounds, known increased release in response to infections. Spectrophotometrical estimations of polyphenol oxidase release at 450 nm proved the activity of inducers in promoting enzymes release. Its activity was expressed in micro moles/ mg of protein/ min. Maximum poly phenol oxidase activity was recorded in GABA treated DRSH-1 with 3.380 and on par activity in KBSH-1 and SCG-99 with 2.940 and 2.906 respectively and minimum effect on morden with 1.190 against 0.445 in same cultivar, untreated control. *T.harizanum* followed by AABA also has shown potential influence on the enzyme activity at 3.370 and 3.210 respectively. *P.fluorescens* effect on enzyme activity was 2.740 and 2.560 in DRSH-1 and SCG-99 respectively. Influence on enzyme activity in morden was 0.933. Observations were given in Table 2.

Incontrast to the trend in other biochemical estimations total sugars release was less in Hybrid varieties (6.660 DRSH-1, 10.380 – KBSH-1) and more release was observed in susceptible variety morden with 33.320 against 45.670 release in untreated control in the same variety. Same inverted trend is followed in efficiency of inducers too with highest release in least effective inducer *P.fluorescens* 10.280 in DRSH-1 and 43.440 in Morden. Total sugars release observed was estimated in mg/ g of fresh tissue. Table 3 represents the values of estimation of total sugars.

Catalase activity expressed in mg/protein/min, considered as another defensive enzyme followed the regular pattern of release as observed in the other inducers, in GABA treated plants with highest release of 3.610 in DRSH-1 followed by 3.526 mg/protein/min in KBSH-1 and recorded least in morden with the release of 1.663. Catalase release was least effected by *P.fluorescens* with 3.193mg/ml and 2.966 in DRSH-1 and KBSH -1 respectively. Under the same treated inducer morden has shown the release of catalase at 1.260 mg/protein/ min. values presented in Table 4.

Table 1. Effect of different inducers on peroxidase activity in different genotypes of sunflower at 18 hours after pathogen inoculation

Genotype	Perioxidases (µ moles /mg protein/min)					
	GABA*	T.harizanum	AABA*	Salycilic Acid	P.fluorescens	Control
DRSH-1	1.990	1.855	1.785	1.720	1.620	0.125
KBSH-1	1.960	1.850	1.755	1.500	1.250	0.115
KBSH-44	1.005	0.885	0.835	0.800	0.770	0.045
Morden	0.750	0.735	0.705	0.675	0.600	0.050
SCG-99	1.680	1.585	1.495	1.330	1.300	0.093
White pollen	1.200	1.095	1.045	1.030	0.985	0.044
TX16R	1.455	1.415	1.300	1.200	1.190	0.085
EC-53792	1.315	1.295	1.245	1.195	1.045	0.100

α-Amino Butyric Acid (AABA) and ɣ-Amino Butyric Acid (GABA)*

Table 2. Effect of different inducers on Polyphenols Oxidase activity in different genotypes of sunflower at 18hours after pathogen inoculation

Genotype	Polyphenols Oxidase (µ moles /mg protein/min)					
	GABA*	T.harizanum	AABA*	Salycilic Acid	P.fluorescens	Control
DRSH-1	3.380	3.310	3.270	2.980	2.740	0.976
KBSH-1	2.940	2.660	2.430	1.580	1.330	0.908
KBSH-44	2.100	2.003	1.943	1.833	1.783	0.481
Morden	1.190	1.153	1.100	1.033	0.933	0.445
SCG-99	2.906	2.746	2.703	2.660	2.560	0.676
White pollen	2.266	2.243	2.146	2.090	2.043	0.557
TX16R	2.806	2.740	2.666	2.520	2.383	0.645
EC-53792	2.543	2.486	2.403	2.276	2.266	0.567

α-Amino Butyric Acid (AABA) and ɣ-Amino Butyric Acid (GABA)*

Table 3. Effect of different inducers on total sugars activity in different genotypes of sunflower at 18hours after pathogen inoculation

Genotype	Total Sugars (mg/g fresh weight)					
	GABA*	T.harizanum	AABA*	Salycilic Acid	P.fluorescens	Control
DRSH-1	6.660	7.680	8.520	9.460	10.280	11.520
KBSH-1	10.380	11.660	13.020	13.980	14.820	18.840
KBSH-44	23.240	24.820	26.280	28.440	29.520	32.860
Morden	33.320	36.300	39.440	41.480	43.440	45.670
SCG-99	13.320	13.980	15.480	16.280	16.660	22.300
White pollen	13.020	14.020	16.220	16.980	19.840	23.420
TX16R	14.660	16.380	16.520	18.320	19.140	21.080
EC-53792	12.520	12.980	14.580	15.460	19.280	20.120

α-Amino Butyric Acid (AABA) and ɣ-Amino Butyric Acid (GABA)*

Table 4. Effect of different inducers on catalase activity in different genotypes of sunflower at 18hours after pathogen inoculation

Genotype	Catalase (µ moles /mg protein/min)					
	GABA*	T.harizanum	AABA*	Salycilic Acid	P.fluorescens	Control
DRSH-1	3.610	3.533	3.416	3.306	3.193	1.124
KBSH-1	3.526	3.406	3.330	3.120	2.966	1.012
KBSH-44	2.446	2.310	2.220	2.116	2.050	0.545
Morden	1.663	1.516	1.453	1.406	1.260	0.432
SCG-99	3.413	3.283	3.176	3.070	2.950	1.060
White pollen	2.850	2.766	2.680	2.603	2.500	0.627
TX16R	3.483	3.266	3.150	3.053	2.900	0.745
EC-53792	3.156	3.020	2.916	2.800	2.716	0.713

α-Amino Butyric Acid (AABA) and Ɣ-Amino Butyric Acid (GABA)*

Table 5. Effect of different inducers on phenylannine ammonia lyase activity in different genotypes of sunflower at 18hours after pathogen inoculation

Genotype	Phenylannine ammonia lyase (µ moles /mg protein/min)					
	GABA*	T.harizanum	AABA*	Salycilic Acid	P.fluorescens	Control
DRSH-1	3.620	2.843	2.585	2.509	2.280	1.020
KBSH-1	3.089	2.784	2.535	2.143	2.118	0.980
KBSH-44	1.525	1.447	1.404	1.368	1.035	0.850
Morden	1.212	1.196	1.155	1.099	0.913	0.650
SCG-99	3.050	2.690	2.198	2.043	1.995	0.970
White pollen	2.134	2.126	2.007	1.436	1.067	0.950
TX16R	2.860	2.609	2.128	1.491	1.346	0.960
EC-53792	2.602	2.432	2.089	1.679	1.076	0.950

α-Amino Butyric Acid (AABA) and Ɣ-Amino Butyric Acid (GABA)*

PAL estimations in GABA induced leaf samples were higher when compared to other inducers. PAL, as the first enzyme in the general pathway of phenyl propanoid metabolism, is directly involved in the synthesis of active metabolites, including phenols that are associated with the localised resistance processes (Cao et al., 2008). At 18 hrs GABA effectivity on DRSH-1 was 3.620, 3.089 in KBSH-1 and lowest was 1.212 in morden. *P.fluorescens* has shown less effectivity when compared to other inducers however, there is a significant increase in release against untreated control. Effectivity recorded was 2.280 and 2.118 in DRSH-1 and KBSH-1 respectively. least among the release was seen in susceptible variety morden - 0.913. PAL activity was calculated in micro moles/mg of protein/ min. Table 5 represents the values.

DISCUSSION

It is well known that the mechanisms of induced resistance appear to involve the priming of plant defences that display a more rapid response following a challenge inoculation with a virulent strain of a pathogen (Hammerschmidt, 2009). The importance of biochemical characterization of the defence in the physiology of disease resistance is widely accepted as different mechanisms of resistance operate in different host plants (Tomiyama, 1963). An increase in polyphenol oxidase activity has been observed in infected plant tissue and was credited with blocking infection through the action of its oxidation products (Maxwell and Bateman, 1967). Certain chemicals like salicylic acid, jasmonic acid and organics like cow urine and bioagents like *Pseudomonas* sp. are known to induce systemic resistance when applied exogenously in small quantities (Klessig and Malamy, 1994). The *in vivo* role of the PR-proteins like chitinase and ß-1,3 glucanase is to protect the host from invasion by fungal pathogen and they are integral components of a general disease resistance mechanism (Verburg and Huynh, 1997). Ratnam *et al.* (2001) reported that with the treatment of salycilic acid (5 mM) observed an increased amount of phenol content in plant and less severity of disease the plants and developed induced systemic resistance. *Trichoderma* species, necro trophic mycoparasites easily isolated from the soil, are efficient in controlling plant pathogens, especially those with resistance structures, because they act through several antagonism mechanisms such as antibiosis, antibiotic production, competition, and induction of resistance in addition to growth promotion of some plants (Howell, 2003). Chitra (2004) has documented Bio control agents inoculated groundnut seeds has developed induced systemic resistance against *A. alternata.* Savitha (2004) observed induced systemic resistance against Alternaria blight in sesame, by seed treatment of salicylic acid and *Psuedomonas fluorescens*. Karthikeyan *et al.* (2005) reported that isolate of *Psuedomonas fluorescens* was found to inhibit the growth of the pathogen *Alternaria palandui* leaf blight in onion with the foliar spray of talc based formulation. Thus, showing treated plants showed significant increase in levels of defense enzymes in compared to untreated plants. Chen yu *et al*. (2014) reported that the activity of defense related enzymes enhanced drastically including peroxidases, polyphenol oxidases and Phenylalnine ammonia lyase and induces resistance against *Pencillium expansum* when treated with GABA and its affectivity was high when GABA at 100 µg/ ml inoculated 24 hrs before pathogen inoculation. The above mentioned supportive findings to the present investigation done it can be concluded that in management of Alternariaster blight of sunflower induced systemic resistance is one of the best methods to enhance the production of defense enzymes and increase resistance towards the infection.

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