International Journal of Agriculture Sciences
ISSN: 0975-3710&E-ISSN: 0975-9107, Volume 8, Issue 8, 2016, pp.-1080-1082.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000217

EFFECT OF PYRACLOSTROBIN ON INDUCTION OF PATHOGENESIS RELATED PROTEINS IN BRINJAL (*SOLANUM MELONGENA* L) AGAINST *PHOMOPSIS VEXANS*

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Received: January 30, 2016; Revised: February 13, 2016; Accepted: February 14, 2016

Abstract- In the present study, the effect of Pyraclostrobin treatment on induction of pathogenesis related proteins in a susceptible brinjal cultivar (*cv.* PPL) was studied upon challenge inoculation with *Phomopsis vexans*_MK2 isolate. Seeds treated with sterile distilled water served as controls. Seeds treated with 0.2% Pyraclostrobin along with respective control seeds (*cv.* PPL) were raised under nursery bed. Thirty days old seedlings were challenge inoculated with conidial suspension of *P. vexans* (1 x10^6 conidia/ml). Seedlings were harvested at regular intervals of 0, 3, 6, 12, 24, 48, 72 & 96 hours of post inoculation and analyzed for activity of pathogenesis related proteins (PRP's) namely β -1,3-glucanase, chitinase and lipoxygenase, colorimetrically. The results revealed that Pyraclostrobin treatment enhanced the activity of pathogenesis related proteins when compared to control seedlings (water inoculated, challenge inoculated with *P. vexans*) Significant differences (*P* - 0.001) in activity of β -1,3-glucanase was observed at 48hpi upon Pyraclostrobin treatment followed by challenge inoculation with *P. vexans*. An increased chitinase(*P*-0.03) activity was also recorded at 48-96 hpi upon Pyraclostrobin treatment and lipoxygenase activity was found maximum at 48 hpi. The results indicated thatPyraclostrobinhave a positive effect on the induction and activity of defense related proteins like β -1,3-glucanase, chitinase and lipoxygenase.

Keywords- *Solanum melongena*, *Phomopsis vexans*, Lipoxygenase, Chitinase, Glucanase

Citation: Mahadevakumar S. and Janardhana G.R. (2016) Effect of Pyraclostrobin on Induction o Pathogenesis Related Proteins In Brinjal (*Solanum Melongena* L) against *Phomopsis Vexans.* International Journal of Agriculture Sciences, ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 8, Issue 8, pp.-1080-1082.

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Introduction

Fruit rot and leaf blight disease caused by *Phomopsisvexans*(Sacc. & P. Syd.) Harter, 1914, is of major concern in brinjal(*Solanum melongena* L.) producing areas of India as it reduces yield and marketable value of the crop by 20−30%.In Karnataka, leaf blight and fruit rot disease is a major limiting factor for brinjal crop production in recent years [1]. Various management strategies have worked out to effectively manage the disease with fungicides, fertilizers and biocontrol agents [2-5]. In recent years, the control of fungal diseases with fungicides focused on the sole purpose of reducing inoculum. The concept of fungal disease control gained new perspectives with the introduction of Strobilurins and Pyraclostrobins with due advantages of positive physiological effects on plants [6,7]. Strobilurin group of fungicides (Pyraclostrobin) have been used to control various fungal diseases including *Phomopsis viticola* of grapes, cankers of peach caused by *P. amygdali* and *P. longicolla*, *Cercospora kukuchi*which offered better protection than other tested fungicides [8-10]. In the present study effect of Pyraclostrobin on induction of defense enzymes such as β - 1,3 glucanase, chitinase and lipoxygenase in susceptible brinjal cultivar upon challenge inoculation with *P. vexans* was studied.

Materials and Methods

Seed Samples: Brinjal seeds samples *viz., cv.* Purple Pusa long (PPL) was procured from the National Seed Corporation Ltd. and stored at 4°C until further use. Susceptible cultivar seeds without pyraclostrobin treatment and challenge inoculations were used as control. The increase in PR-Proteins could have been due to the genotype. It is normal for PR-Proteins to be up and down regulated during different stages of plant growth. The question here is whether the increase in PR-Proteins had any effect on *P. vexans*.

Fungal pathogen: *Phomopsis vexans*_MK2 isolate was obtained from a leaf blight affected brinjal and was used to challenge inoculate Pyraclostrobin treated susceptible brinjal seedlings. Please explain from which locality and how was *P. vexans* isolated? On what media did it grow, at what temperatures and for how long?

Seed treatment with Pyraclostrobin

10 g of brinjal seeds (*cv.* PPL) was selected and treated with 0.2% Pyraclostrobin. Treated seeds and untreated seeds(water treated) were raised in nursery beds. 30 day old seedlings were challenge inoculated with conidial suspension of *P. vexans*_MK2 (1x10^6 conidia/ml). Seedlings challenge inoculated with *P. vexans* without Pyraclostrobin treatment and sterile distilled water (SDW) served as controls.

Defence enzymes analysis

Sampling

Seeds treated with Pyraclostrobin with their respective control were sown in poly cups supported with Coconut coir peat and kept at 28±2ºC and 12 h/ 12 h light/ darkness photoperiod. The 30-day-old seedlings were challenge inoculated with *P. vexans*_MK2 (1x10^6 conidia/ ml). A total of 300 seedlings were challenge inoculated and at each time interval, 20 seedlings were harvested. The inoculated brinjal seedlings were harvested at 0, 3, 6, 12, 24, 36, 48, 72, and 96 hours of post inoculation (hpi), immediately frozen at -20°C until used for further biochemical enzyme analysis.

Protein estimation

One gram of each sample was ground to a fine powder with liquid nitrogen and protein was extracted with Tris HCl (50mM, pH 7.6) buffer. Protein content in the extract was estimated by the dye binding assay [11] using Bovine Serum Albumin

(Sigma) as standard (describe the standard curve used) and expressed as microgram of protein per gram of plant tissue. More information needed on the method and replications.

β -1,3-glucanase assay

β -1,3-glucanase assay was carried by the Laminaria-dinitrosalicylate method described by Pan *et al*. [12]. One gram each of Brinjal seedlings collected at different time intervals were extracted with 5 ml of Sodium Acetate buffer (SAB; 0.05M, pH 5.2). The extract was centrifuged at 12000 rpm for 15 min at 4ºC. The supernatant was used as crude enzyme extract to quantify the β -1,3-glucanase activity. The reaction mixture containing 62.5 µl of crude enzyme extract was added to Laminarin (4%, 62.5 µl) and were incubated at 40ºC. The reaction was stopped by adding 375 µl of dinitro-salicylic acid reagent and heated for 5 min in boiling water bath. The resulting coloured solution was diluted and made up to 5 ml (4.5 ml SDW), vortexed and the absorbance was read at 500 nm Colorimetrically How did you set up a standard curve for quantification.. For blank, the crude enzyme extract was mixed with the substrate (4% Laminarin) at 0 h incubation. The experiment was repeated thrice taking three replicates each time.

Chitinase assay

One gram each of harvested brinjal seedlings collected at different time intervals were extracted in 5 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4ºC. The homogenate was centrifuged at 12000 rpm for 25 min at 4ºC in a refrigerated high speed centrifuge. The supernatant was then used as crude enzyme source to estimate the Chitinase colorimetrically. Chitinase was assayed using N-acetyl glucosamine (Sigma) as standard. The assay mixture was consisted of 10 µl sodium citrate buffer (1 M, pH 4.2), 0.4 ml of enzyme extract solution and 0.1 ml of colloidal chitin (1 mg/ ml). The contents were incubated for 2 h at 37ºC and the reaction was stopped by centrifugation. Monomers of N-acetyl glucosamine released after incubation were estimated using dimethyl amino benzaldehyde reagent (DMAB) at 595 nm [13]. The enzyme activity was expressed in terms of nmol of N-acetyl glucosamine released/ min/ mg protein. The experiment was repeated thrice taking three replicates each time.

Lipoxygenase assay

Lipoxygenase (LOX) activity was measured following the method described by Axelrold et al. [14]. One gram each of harvested brinjal seedlings collected at different time intervals were homogenized with 3 ml of ice cold 0.2 M sodium phosphate buffer [pH 6.0, 0.5% (m/v) PVP, 0.1% TritonX-100 and 0.02% sodium meta-bisulphite]. The homogenate was centrifuged at 12000rpm for 15 min at 4ºC and the supernatant was used as the enzyme source. Enzyme activity was measured by monitoring the appearance of the conjugated dienehydroperoxide at 234 nm after 3 minutes colorimetrically. Linoleic acid was used as a source of substrate. The experiment was repeated thrice taking three replicates each time.

Statistical Analysis: Data on the temporal pattern accumulation of pathogenesis related proteins estimated at different intervals of time were subjected to analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects were determined by the magnitude of the F value ($P \leq 0.05$). Means were separated by Tukey's HSD test.

Results

Quantification of defence related enzymes (β -1, 3-glucanase; chitinase; and lipoxygenase) in Pyraclostrobin treated seedlings upon challenge inoculation is presented in [Table-1].

Table-1 *Temporal pattern activity of pathogenesis related proteins (β-1,3-Glucanase; Chitinase; &Lipoxygenase) in brinjal seedlings upon seed treatment with biotic and abiotic inducers.*

Treatments	Hours of Post Inoculation							
	0	3	6	12	24	48	72	96
β-1,3-Glucanase –GLU(µ mol/ min/ mg protein)								
Pyra + *P. vexans**	1.44±0.02^{a}	2.01±0.06^{a}	3.40±0.14^{a}	3.95±0.06^{a}	4.78±0.11^{a}	5.78±0.14^{a}	6.75±0.13^{a}	5.54±0.23^{a}
*P. vexans***	1.46±0.02^{a}	1.68±0.06^{b}	2.75±0.12^{b}	3.65±0.22^{b}	4.38±0.40^{b}	4.73±0.13^{b}	5.68±0.14^{b}	5.30±0.08^{b}
Control****	1.39±0.02^{a}	1.50±0.02^{b}	2.52±0.09^{b}	3.40±0.06^{b}	3.47±0.03^{c}	4.78±0.04^{b}	5.51±0.24^{b}	4.47±0.27^{c}
Chitinase - CHI (nmol of N-acetyl glucosamine released/ min/ mg protein)								
Pyra + *P. vexans*	0.23±0.03^{a}	0.65±0.05^{a}	0.81±0.08^{a}	0.95±0.06^{a}	1.79±0.11^{a}	2.73±0.13^{a}	2.92±0.27^{a}	2.88±0.13^{a}
P. vexans	0.20±0.02^{a}	0.54±0.01^{ab}	0.81±0.08^{a}	0.96±0.13^{a}	1.72±0.12^{a}	1.88±0.19^{b}	2.46±0.02^{b}	2.07±0.06^{b}
Control	0.18±0.03^{a}	0.30±0.03^{b}	0.57±0.03^{b}	0.40±0.07^{b}	0.46±0.03^{b}	0.84±0.01^{c}	1.40±0.04^{c}	1.07±0.32^{c}
Lipoxygenase - LOX (Units at 234 nm mg protein/ min)								
Pyra + *P. vexans*	0.24±0.02^{a}	0.35±0.02^{a}	0.77±0.03^{a}	1.18±0.07^{a}	1.41±0.01^{a}	1.77±0.04^{a}	1.67±0.04^{a}	1.28±0.02^{a}
P. vexans	0.15±0.00^{b}	0.30±0.01^{ab}	0.40±0.01^{b}	0.80±0.04^{b}	0.74±0.02^{b}	0.86±0.02^{b}	0.74±0.03^{b}	0.71±0.01^{b}
Control	0.14±0.02^{b}	0.26±0.02^{b}	0.47±0.03^{b}	0.51±0.03^{c}	0.53±0.01^{c}	0.62±0.02^{c}	0.38±0.01^{c}	0.30±0.04^{c}

Note: *Pyra + *P. vexans*-Pyraclostrobin seed treatment and challenge inoculation after 30 days of seedlings growth; **P. *vexans*-seeds without pyraclostrobin treatment but challenge inoculated after 30 days of sowing; ***Control-Seedlings without pyraclostrobin and challenge inoculation; and *Values are the means (means ± SE) of three independent replicates and same letter(s) between the treatments in the column are not significant different according to Tukey's HSD (P≤0.05).

Significantly higher activity of β-1,3glucanase activity was observed in seedlings treated with Pyraclostrobin upon challenge inoculation with *P. vexans* when compared to control seedlings [Table-1]. No significant change in enzyme activity among seedlings challenge inoculated with *P.vexans* and water was observed. The β-1,3 glucanase activity was maximum at 72 hpi in Pyraclostrobin treated seedlings and decreased thereafter.

Chitinase enzyme activity was observed at 48-96 hpi in Pyraclostrobin treated followed by challenge inoculated *(P. vexans)* seedlings compared to controls [Table-1]. Highest chitinase activity (2.92U) was observed at 72 hpi in Pyraclostrobin treated seeds followed by challenge inoculation with *P. vexans*. No significant change in chitinase activity was observed among other two treatments.

The Pyraclostrobin treated seedlings showed an elevated LOX activity at 96 hpi. Highest LOX activity of 1.77U was recorded at 48 hpi in seedlings treated with Pyraclostrobin upon challenge inoculation with *P. vexans* and the enzyme activity decreased thereafter.

Discussion

Plant diseases are mainly controlled by fungicide treatments as the resistant varieties and crop rotation merely contribute to successful disease control. The strobilurin class of fungicides comprises a variety of synthetic plant protecting compounds, which are having a broad-spectrum antifungal activity [15]. There are evidences in support of direct influences of strobilurins on plant physiology [16]. These physiological effects include the so-called "greening," that is, even in the absence of challenge by pathogen attack; plants treated with strobilurins are

intense green and look healthier than plants that have not been treated with strobilurin fungicides [16]. In addition to their fungicidal activity, strobilurins enhance the capability of plants to ward off pathogens. Several well-characterized defense reactions including induction of defense-related proteins with antifungal properties have been reported in many plant species during disease development in plants [17,18].

Phomopsis vexans is knownto cause leaf blight, fruit rot, stem blight and calyx blight on brinjal [1-5,19-21]. Brinjal being one of the economically important vegetable crops seriously suffers from various diseases and pests. Chemical as well as bio-agent based studies have been used to manage the disease [2,3]. In the present study, effect of Pyraclostrobin on induction of pathogenesis related proteins during seed germination was analysed by Colorimetric assays in the susceptible brinjal cultivar *cv*. PPL followed by challenge inoculation with *P. vexans*.

The study revealed that, the Pyraclostrobin treatments enhanced the activity of defense related enzymes in early seed germination and seedling development. Similar results were also reported from Strobilurin group of fungicides which offered maximum disease protection and enhanced defense enzyme activity among virus inoculated tobacco and bean plants [22,23]. Several of the QoI fungicides currently registered in the United States are considered by the Environmental Protection Agency to be reduced-risk pesticides [23], due to their reduced risk on human health and environment [22, 24]. Pyraclostrobin fungicides are known to influence physiology through the interaction with electron transfer in plant mitochondria [6,25-27] and also helps in increased photo-assimilation, delayed senescence with enhanced concentrations of nitrogen, chlorophyll and protein and changes in hormone status [28-30]. In the present study the Pyraclostrobin seed treatment inducing early defence reaction was witnessed in the form of enhanced activity of three defence enzymes such as β-1,3-glucanse, chitinase and lipoxygenase. The results suggest that Pyraclostrobin known to have physiological effect during seed germination and early seedling development. This study is not complete. It should have determined if elevated PR-Proteins actually reduced fungal growth at seedling stage.

Acknowledgements:

First author (Mahadevakumar, S.) would like to thank Department of Science and Technology, New Delhi for proving INSPIRE Fellowship (IF110422).

Conflict of Interest: None declared

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