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RESEARCH ARTICLE

CRUDE OLIGOSACCHARIDES FROM *ALTERNARIA SOLANI* WITH *BACILLUS SUBTILIS* ENHANCE DEFENSE ACTIVITY AND INDUCE RESISTANCE AGAINST EARLY BLIGHT DISEASE OF TOMATO

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ABSTRACT

The present study was aimed to find an eco-friendly approach for the management of tomato early blight disease through seed treatment with different concentrations of crude oligosaccharide elicitor of *Alternaria solani* with PGPR alone and in combination. Seed treatment with the combination of 3 mg/ ml of CO with TN_Vel-35 showed significant increase in germination of 93.33% and 2733 seedling vigor when compared to control as well as individual treatments showing 75% germination and 887 seedling vigor. Under green house conditions maximum disease protection of 83% against early blight disease was recorded in combined treatment of CO with TN_Vel-35 when compared to control which showed less than 10%. At the biochemical level, enhanced accumulation of POX (49.86 U at 24 hpi) and PPO (44.56 U at 48 hpi) was obtained in challenge inoculated seeds treated with combined treatment of CO with TN_Vel-35 when compared to all other treatments and control.

INTRODUCTION

Signaling molecules which activate defense genes in plants are called elicitors (Vidyasekaran, 1997) and have been used widely to find out defense genes in plants. Elicitors from fungi may initiate from surface of germinating zoospores, chlamydospores, cell wall, cellular and intercellular components like proteins, peptides, lipids, oligosaccharides, secondary metabolites etc., which act as host recognition molecules/ toxins/ signal molecules against phyto pathogens (Permberton and Salmond, 2004). Oligosaccharides derived from fungal and plant cell wall polysaccharides are class of well characterized elicitors and in some cases induce defense responses at very low concentration in plants (Shibuya and Minami, 2001). Increased plant defense-related enzyme activities using fungal oligosaccharides have also been reported previously, which are mostly prepared from plant pathogens (Khan *et al*., 2003; Meng *et al*., 2010). Most of the defense reactions induced by oligosaccharide elicitors are also observed when plant cells are treated with non-saccharides elicitors/infected with pathogens. One of the major differences is that, oligosaccharide elicitors in contract to other elicitors, do not induce hypersensitive reaction (HR) leading to cell death (Shibuya and Minami, 2001) but elicit phytoalexin accumulation and lignin or callose formation in plants (Kauss *et al.,* 1989; Lesney, 1989). Nita- Lazer *et al.* (2004) have reported the application of novel oligosaccharides from *F. oxysporum* rapidly induced PAL activity in *Rubus* cells after treatment suggesting an early signal transduction cascade which may be associated with plasma membranes (Nurnberger *et al*., 1997). An Oligosaccharide elicitor of phytoalexin synthesis has been isolated from the soyabean pathogenic fungus *Phytophthora megasperma* and identified as hepta-β glucopyranoside (Sharp *et al*., 1984).

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MATERIALS AND METHODS

Collection of rhizosphere soil samples

Extensive field surveys were conducted covering five states of India namely, Karnataka, Tamil Nadu, Andhra Pradesh, Kerala and Maharashtra during rainy season from July to September, 2009-2011. TN_Vel-35 was isolated from the healthy rhizosphere soil samples of tomato plant.

Seed samples and plant material

Seed samples of tomato cultivar "PKM-1" (highly susceptible) and Abhishek (highly resistant) to early blight disease of tomato were obtained from agro-chemical traders of Mysore district.

Molecular characterization of PGPR

Bacterial genomic DNA was amplified using 16s rRNA conserved primers (1 µl): forward primer (27f) 5'

GAGTTTGATCCTGGCTCAG 3' and reverse primer (1492r) 5' GTATTACCGCGGCTGCTGG 3'. Sequence data were submitted to NCBI (National Center for Biotechnology Information under the Accession number KC438287).

Preparation of TN_Vel-35 inoculum

Bacterial cell suspensions were prepared by streaking loop full of culture on NA medium and incubated at 28 ± 2° C for 24 h. The pure colonies obtained were transferred to Tryptic Soya Agar (TSA). After 48 h of incubation, culture broth was centrifuged at 10,000 rpm for 10 min. The pellets were re-suspended in SDW and washed thrice. The washed bacterial pellet was then reconstituted with SDW to obtain a turbid solution, whose optical density at 610 nm was adjusted to 0.45 using a spectrophotometer to obtain a final density of $1x10^8$ cfu/ ml (Sudisha *et al*., 2006) which was further used for seed treatment.

Extraction of cell wall crude oligosaccharides (CO)

The fungus *A. solani* was mass multiplied on potato dextrose broth (PDB) for ten days. Dried mycelium of the fungus was extracted overnight with acetone (250 ml at 20° C) and the residual powder was subjected to alkaline treatment involving 100 ml of 0.1M NaOH at 60° C for 2 h. The supernatant collected by centrifugation (16,500 g, 15 min) was neutralized to pH 7 with 50% acetic acid and stored overnight at 48° C. The resulting sample was centrifuged (16,500 g, 20 min at 20° C) and the supernatant was collected and lyophilized (Nita-Lazar *et al.,* 2004). Presence of oligosaccharides in this powder was confirmed by Molisch test (Sadasivam & Balasubramanian, 1985), and reducing sugars were quantified by phenol-sulfuric acid method (Dubois *et al.,* 1956).

Seed priming with mixture of CO and TN_Vel-35

Susceptible tomato seeds (PKM-1) were surface sterilized with 0.2% sodium hypochlorite, rinsed in SDW for 10 min and coated with carboxy methyl cellulose (CMC) as a polymer. Polymer coated seeds were soaked with constant ratio of TN_Vel-35 ($1x10^8$ cfu/ ml), CO with different concentrations of 0.5, 1, 3, 5, 7 and 9 mg /ml and a combined treatment of 1 ml of TN_Vel-35 ($1x10^8$ cfu/ ml) with 0.5, 1, 3, 5, 7 and 9 mg/ ml of CO. Treated seeds were kept at 28 ± 2° C in a rotary shaker (90 rpm) 9 h, then dried overnight aseptically in laminar air flow and used for both laboratory and greenhouse conditions. Seeds soaked in SDW followed by CMC severed as control.

Determination of seed germination and seedling vigor

Treated and untreated seeds as explained above were used for the experiment. The germination and vigor analysis was carried out by the paper towel method (Abdul Baki and Anderson (1973). After seven days of incubation, the percent germination was calculated. The experiment was repeated three times with four replicates of hundred seeds each. Distilled water treated seeds served as control.

$$\% \text{ Germination} = \frac{\text{No. of seeds germinated}}{\text{Total No. of seeds plated}} \times 100$$

To assess the vigor, the length of the root and shoot of each individual seedling was measured. The vigor index (VI) was calculated using the formula

Seedling Vigor = (Mean Root Length + Mean Root Length) x % germination

Disease protection studies

Treated seeds were sown in plastic pots, with four seeds per pot (susceptible, resistant and control) containing soil, sand and manure at 2:1:1 ratio. Pots were watered regularly and maintained in insect-proof screen house. Three-week-old tomato plants raised from seeds induced/ primed with different treatments were challenge inoculated with 100 ml suspension of *A. solani* (5×10^4 cfu/ ml) by hand sprayer till runoff at 28° C. Similarly, challenge inoculation was given to plants after 1st, 2nd, 3rd and 4th day (correspondingly 22, 23, 24 and 25-day-old plants) as described by Sudisha *et al*. (2013). The pots were then arranged in a randomized complete block design and maintained under greenhouse conditions. The inoculated plants were covered with plastic bags for two days to maintain humidity and to avoid cross contamination. Each treatment consisted of 16 plants in four replications and repeated twice. To evaluate the efficacy of combined inducer against early blight disease, after 21 days of challenge inoculation each plant was scored for disease symptoms and percent protection was tabulated using the formula,

$$\text{Disease protection} = \frac{C - T}{C} \times 100$$

Where, C represents percentage (%) of early blight disease incidence in the control and T represents percentage (%) of early blight disease incidence in induced plants.

Induction of defense mechanism

One gram leaf samples were harvested at 0, 4, 12, 24, 48 and 96 hours post inoculation (hpi), from three-week-old treated and untreated control plants for each enzyme assay. The collected leaf samples were immediately homogenized with liquid nitrogen and 1 g of powdered sample was extracted with 2 ml of sodium phosphate buffer, 0.1 M (pH 7.0) at 4° C. The homogenate was centrifuged for 20 min at 8000 g at 4° C and the collected supernatants were used for enzyme assays.

Protein estimation

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

Peroxidase (POX)

POX enzyme was extracted in 10 mM potassium phosphate buffer (pH 6.9) and the activity was measured according to the procedure of Hammerschimdt *et al.* (1982) using 0.25% v/v guaiacol as the hydrogen donor. The POX activity was expressed in terms of the changes in A_{470}/ min/ mg protein.

Polyphenol oxidase (PPO)

PPO enzyme was extracted with 100 mM potassium phosphate buffer (pH 6.5) by following the procedure of Arora and Bajaj (1985) using 3 mL of 10 mM sublimated catechol as standard. The results were expressed in terms of µmol quinine formed in A_{420}/ min/ mg protein.

Statistical analysis

All data from three replicates collected from laboratory and green house experiments were analyzed separately for each experiments and were subjected to arcsine transformation and analysis of variance (ANOVA) (SPSS, version 16). Significant effects of treatments were determined by F-test ($P \leq 0.05$). Treatment means were separated using Tukey's HSD.

RESULTS

Effect of seed priming with inducers on seed germination and seedling vigor of tomato

Seed germination and seedling vigor was significantly ($P \leq 0.05$) increased with combined treatment of CO with TN_Vel-35 when compared to individual treatments as well as control (Table 1). CO with TN_Vel-35 at the concentration of 3 mg/ ml of CO with 1 ml of TN_vel-35 ($1x10^8$ cfu/ ml) recorded a maximum seed germination of 93.33% and seedling vigor of 2733 when compared to other combined/ individual treatments. The control seedlings recorded 75% germination and 887 seedling vigor (Table 1).

Table 1. Effect of seed priming with different concentration of inducer on seed germination and seedling vigor

Treatments	Concentration	Germination	VI
TN_vel-35	$1x10^8$ cfu/ ml	89.00 ± 0.23^{b}	2584 ± 0.32^{i}
CO from *A. solani*	0.5	77.00 ± 1.00^{ef}	894 ± 2.60^{i}
	1	$78.66\pm0.33d^{ef}$	893 ± 1.66^{i}
(mg/ ml)	3	82.00 ± 1.00^{bcd}	1043 ± 1.52^{g}
	5	81.33 ± 0.33^{bcd}	993 ± 0.33^{g}
	7	81.66 ± 0.66^{bcd}	994 ± 0.57^{h}
	9	80.66 ± 0.33^{bcd}	952 ± 1.33^{h}
CO from *A. solani* +	0.5 + 1	82.00 ± 1.00^{bcd}	1014 ± 3.17^{e}
TN_vel-35	1 + 1	80.00 ± 0.57^{bcde}	1030 ± 3.84^{f}
	3 + 1	93.33 ± 0.66^{ab}	2733 ± 0.71^{f}
(mg/ ml + $1x10^8$ cfu/ ml)	5 + 1	79.33 ± 0.33^{cde}	2214 ± 2.00^{b}
	7 + 1	82.66 ± 0.66^{bc}	2214 ± 0.33^{d}
	9 + 1	80.33 ± 0.33^{bcde}	2233 ± 1.52^{c}
Control	-	75.66 ± 0.66^{f}	887 ± 0.00^{i}
Resistant	-	98.00 ± 0.57^{a}	2947 ± 1.45^{a}

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

Effect of seed priming with inducers on tomato early blight disease incidence under greenhouse conditions

The effectiveness of early blight disease resistance from inducers was tested under greenhouse conditions.

Table 2. Effect of seed priming with different concentration of inducer on early blight disease protection

Treatments	Concentration	Protection
TN_vel-35	$1x10^8$ cfu/ ml	77.00 ± 0.88^{cde}
CO from *A. solani*	0.5	63.00 ± 1.00^{h}
	1	67.00 ± 1.00^{efg}
(mg/ ml)	3	71.66 ± 0.33^{cde}
	5	67.66 ± 1.33^{efg}
	7	68.33 ± 0.33^{defg}
	9	66.00 ± 1.00^{gh}
CO from *A. solani* + TN_vel-	0.5 + 1	68.66 ± 0.66^{defg}
35	1 + 1	71.33 ± 0.33^{cdef}
	3 + 1	83.00 ± 1.00^{b}
(mg/ ml + $1x10^8$ cfu/ ml)	5 + 1	72.00 ± 1.00^{cde}
	7 + 1	72.66 ± 0.66^{bcd}
	9 + 1	74.33 ± 0.33^{bc}
Control	-	9.00 ± 1.45^{i}
Resistant	-	94.33 ± 0.33^{a}

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

All the tested inducers on tomato plants showed significant ($P \leq 0.05$) disease protection when compared to untreated control. Among the inducers tested, a maximum of 83% disease protection was offered by seeds treated with combination of CO with TN_Vel-35 (3 mg/ ml of CO with 1 ml of TN_vel-35 at $1x10^8$ cfu/ ml) followed by 77% disease protection in seeds treated with TN_vel-35 ($1x10^8$ cfu/ ml). In control plants 93% disease incidence was recorded (Table 2).

Elicitation of defense enzymes POX and PPO by seed priming with inducers

The results from the above experiments suggested that seed treatment TN_vel-35, CO (3 mg/ml) and combination of TN_vel-35 and CO (3 mg/ ml + 1 ml of TN_vel-35 at $1x10^8$ cfu/ ml) offered maximum seed germination, vigor and disease protection and hence these treatments were further subjected for its effect on enzyme activity during induction of disease resistance. All the tested inducers had a varying degree of enzyme activity. Among the inducers tested combined treatment of TN_vel-35 and CO offered a maximum of POX activity of 49.86 U at 24 hpi and there was a 3-fold increase in POX activity when compared to control plants which recorded 16.06 U at same time interval. The tomato plants treated with individual treatment of CO and TN_Vel-35 showed considerable level of activity of 20.86 U and 39.10 U, respectively at 24 hpi (Table 3). The results of PPO activity were in line with the results of POX activity, where a maximum of 44.56 U was obtained at 48 hpi in seedlings raised from the combined treatment of CO and TN_Vel-35 followed by individual treatments. The control seedlings recorded a maximum of 25.2 U PPO activity at same time interval (Table 4).

DISCUSSION

In 1970's the precise and effective biological effects of cell wall derived oligosaccharides on living plant cells was demonstrated earlier by plant pathologists. Both plant and fungal oligosaccharides have been revealed to activate a large range of genes encoding proteins involved in plant defense (Lawton & Lamb, 1987; Ryan, 1988). The pathogen recognition supposed to be mediated by chemical substances secreted or present on the surface of the pathogen. Different molecules like lipids, proteins, glycopeptides and oligosaccharides have been shown to induce defense responses in plants, and their role in detecting pathogen (Hindumathy, 2012). Oligomers of chitin and glucan released from fungal cell wall are known to be the primary signaling compounds inducing plant defense reactions (Lamb *et al*., 1989) which elicit phytoalexin accumulation, callose formation and lignin in plants (Kauss *et al.,* 1989; Lattanzio *et al.,* 2006). In this work we have reported that, CO with TN_Vel-35 act as potent inducers for growth promotion, increased enzyme activity correlated with decreased disease incidence. Under green house conditions, considerable disease protection of 83% was observed indicating the CO's with TN_vel-35 where as individual treatments of TN_vel-35 showed 77% and control plants showed less than 10% protection. Here, the combination of oligosaccharides and TN_vel-35 gave considerable growth improvements in germination of 93.33% and seedling vigor 2733 when compared to individual treatment and control which showed 75% germination and 887 seedling vigor as well as in individual treatments which recorded 89%

Table 3. Time course study of Peroxidase (POX) activity in three-week-old tomato seedlings. SU- Susceptible Uninoculated, SI- Susceptible Inoculated; RU- Resistant Uninoculated; RI- Resistant Inoculated; STU-1: Susceptible Treated Uninoculated (TN_Vel-35); STI-1: Susceptible Treated Inoculated (TN_Vel-35); STU-2: Susceptible Treated Uninoculated (CO); STI-2: Susceptible Treated Inoculated (CO); STU-3: Susceptible Treated Uninoculated (TN_Vel-35 + CO); STI-3: Susceptible Treated Inoculated (TN_Vel-35 + CO)

Plants	0 hpi	4 hpi	12 hpi	24 hpi	48 hpi	96 hpi
SU	1.90± 0.14^{h}	3.20 ± 0.28^{g}	5.00 ± 0.07^{h}	11.50 ± 0.28^{h}	7.00 ±0.44^{h}	7.80 ±0.59^{g}
SI	3.90 ± 0.10^{g}	5.40 ± 0.20^{f}	13.80± 0.37^{g}	16.06 ± 0.61^{g}	25.20±0.64^{d}	19.70 ±0.39^{f}
RU	6.60 ± 0.10^{e}	13.50 ± 0.17^{cd}	19.36 ±0.08^{ef}	21.66 ± 0.21^{e}	23.16 ±0.12^{e}	25.36 ±0.14^{e}
RI	11.86 ± 0.06^{a}	21.63 ± 0.18^{a}	28.30 ± 023^{a}	53.53 ± 0.06^{a}	56.6±0.40^{a}	60.36 ±0.88^{a}
STU- 1	3.90 ± 0.13^{g}	11.50 ± 0.33^{e}	17.00 ± 0.36^{f}	19.90 ± 0.57^{f}	21.90 ±0.51^{f}	24.30 ±0.57^{e}
STI- 1	7.20 ± 0.15^{c}	19.10 ± 0.62^{b}	24.30 ± 0.31^{c}	39.10± 0.28^{c}	47.70 ±0.40^{b}	47.50 ±0.61^{b}
STU- 2	4.03 ± 0.03^{g}	12.46 ± 0.88^{de}	16.40 ± 0.30^{g}	17.30 ± 0.25^{g}	19.16 ±0.17^{f}	20.53 ±0.14^{f}
STI- 2	6.96 ± 0.08^{d}	14.70 ± 0.75^{c}	17.80 ± 0.10^{e}	20.86 ± 0.36^{ef}	23.6 ±0.15^{ef}	24.76 ±0.31^{e}
STU- 3	5.60 ± 0.10^{f}	13.79 ± 0.14^{cd}	16.83± 0.16^{d}	25.03 ± 0.12^{d}	35.33 ±0.12^{c}	36.46 ±0.26^{d}
STI- 3	8.86 ± 0.03^{b}	22.43 ± 0.06^{a}	31.83 ± 0.42^{b}	49.86 ± 0.03^{b}	46.93 ± 0.61^{b}	45.33± 0.88^{c}

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

Table 4. Time course study of Polyphenyl oxidase (PPO) activity in three-week-old tomato seedlings. SU- Susceptible Uninoculated, SI- Susceptible Inoculated; RU- Resistant Uninoculated; RI- Resistant Inoculated; STU-1: Susceptible Treated Uninoculated (TN_Vel-35); STI-1: Susceptible Treated Inoculated (TN_Vel-35); STU-2: Susceptible Treated Uninoculated (CO); STI-2: Susceptible Treated Inoculated (CO); STU-3: Susceptible Treated Uninoculated (TN_Vel-35 + CO); STI-3: Susceptible Treated Inoculated (TN_Vel-35 + CO)

Plants	0 hpi	4 hpi	12 hpi	24 hpi	48 hpi	96 hpi
SU	1.00 ± 0.08^{i}	1.10 ±0.21^{j}	1.70 ±0.34^{i}	7.70 ±0.18^{h}	5.40 ± 0.27 ^{i}	4.90 ±0.34^{i}
SI	2.10 ±0.10^{h}	3.00 ±0.10^{h}	13.80 ±0.32^{f}	24.30 ±0.42^{e}	25.20 ± 0.29^{e}	19.70 ±0.36^{g}
RU	7.16 ±0.33^{e}	8.4 ±0.33^{e}	16.66 ±0.88^{e}	20.30 ±0.57^{f}	22.10 ±0.57^{f}	25.06 ±0.33^{f}
RI	15.16 ±0.57^{a}	20.53 ±0.33^{b}	29.73 ±0.66^{a}	45.00 ±0.41^{a}	49.56±0.33^{a}	52.30 ± 0.10^{a}
STU-1	2.00 ±0.23 ^{h}	2.60 ±0.21^{i}	9.80 ±0.44^{h}	13.30 ±0.24^{g}	14.00 ±0.52^{h}	14.00 ± 0.30^{h}
STI-1	9.50 ±0.19^{d}	15.20 ±0.18^{d}	23.30 ±0.0.27^{d}	39.00 ±0.28^{b}	38.10 ±0.17^{c}	36.70 ± 0.40^{c}
STU-2	4.56 ±0.1^{f}	4.83 ±0.33^{g}	11.26 ±0.14^{g}	14.30 ±0.57^{g}	18.60 ±0.30^{g}	20.36 ± 0.33^{g}
STI-2	11.4 ±0.33^{c}	16.26 ±0.66^{c}	24.26 ±0.66^{c}	27.46 ±0.33^{d}	30.13 ±0.33^{d}	34.40 ± 0.10^{d}
STU-3	4.26 ±0.33^{g}	6.466 ±0.33^{f}	10.13 ±0.66^{h}	13.53 ±0.33^{g}	18.53 ±0.03^{g}	26.70 ± 0.10^{e}
STI-3	12.43 ±0.33^{b}	22.8 ±0.57^{a}	28.53 ±0.33^{b}	31.26 ±0.66^{c}	44.56 ±0.66^{b}	40.53 ± 0.33^{b}

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

germination and 2584 vigor (TN_vel-35) and different concentrations of CO's which showed approximately 77-82% germination and 893-1043 vigor. The levels of defense related enzymes (PAL, POX, PPO, LOX etc.) play a crucial role with respect to the degree of host resistance (Ralph *et al*., 2006). In the present study enhanced defense activity of POX and PPO were observed significant with the challenge inoculated seedling showing 3-fold increase in POX activity at 24 hrs and 1.76 fold increase of PPO. Highest POX activity was observed in STI-3 which gave 49.86 U followed by STI-1 (39.10 U) STI-2 (20.86 U) and SI (16.06 U). Enhanced PPO activity was recorded in STI-3 which showed 44.56 U followed by STI-1 (38.10 U), STI-2 (30.13 U) and SI (25.20 U). Similar observations were done by using CO's in inducing resistance against pearl millet against downy mildew disease Nandini *et al*. (2012). Similarly enhanced defense related enzyme activity was observed by Pushpalatha *et al*. (2011) in Oligosaccharide treated pearl millet seedling upon challenge inoculation with *Sclerospora graminicola*. These are highly important in the defense mechanism against pathogens, by increasing antimicrobial activity and may be directly involved in controlling pathogen development (Melo *et al*., 2006). Biosynthesis of secondary metabolites is due to elicitor induced defense responses (Cote and Hahn, 1994).

Conclusion

Crude oligosaccharides of cell wall from *A. solani* shows potential effect in the management of tomato early blight disease along with TN_Vel-35 they have shown to be good plant growth promoters. Though our work was confined only to laboratory and green house which showed significant results, further we will concentrate on purified fraction of crude elicitor present with suitable formulation in large scale to carry out field trials. In the current investigation it was not possible to find out the exact role of the combination of CO with TN_Vel-35 in inducing resistance. Although in a particular concentration this gave better disease protection as well as plant growth than other treatments.

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