Crude oligosaccharides mediated resistance and histo-chemical changes in Capsicum annuum against anthracnose disease caused by Colletotrichum capsici

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Abstract
Enhancing the host resistance using biotic elicitors is one of the eco-friendly approaches developed for plant disease management. The Crude Oligosaccharides (CO) extracted from Colletotrichum capsici (Syd.) Butler & Bisby was evaluated for their efficiency to elicit resistance in chilli against anthracnose disease. Among the different concentrations tested, CO treatment at 2.5 mg/ml concentration for 3 h duration significantly enhanced seed germination (90.5%) and seedling vigor (986.7), compared to control which offered 78% of seed germination and 712.5 of seedling vigor. Application of CO at 2.5 mg/ml concentration also reduced the disease severity with a highest anthracnose disease protection of 68% under greenhouse conditions and enhanced the vegetative growth parameters compared to control. The induction of resistance was evident with higher expression of primary defense responses like hypersensitive response, deposition of lignin, callose, hydrogen peroxide and phenol when compared to control plants. There was a one fold increase in defense enzyme activities phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, and lipoxygenase in crude oligosaccharide-treated inoculated seedlings when compared to susceptible inoculated seedlings which were similar to that of resistant inoculated seedlings where a maximum of 1.5-fold increase in enzyme activity was observed.

Keywords: Anthracnose disease, crude oligosaccharides, Capsicum annuum, Defense-related enzymes, histochemical changes, induction of resistance

Introduction
Plants possess a variety of defense mechanisms to protect themselves against microbial attack including pre-existing physical and chemical barriers as well as inducible defense mechanisms (Hematy et al. 2009). The inducible reactions require the perception of either plant-derived (endogenous) or pathogen-derived (exogenous) signal molecules called elicitors. Elicitors are known to induce defense responses in the host by mimicking the infection processes of the pathogen and sensitizing the host against actual pathogen invasion at very low concentrations suggesting the existence of a receptor at the plasma membrane (Ebel & Cosio 1994; Trouvelot et al. 2014).

Pre-treatment of plants with different biotic elicitors such as proteins, lipids, oligosaccharides, phenolic compounds are known to cause structural changes, such as fortifying the physical and mechanical strength of the cell wall, and thereby leading to induction of resistance (Montesano et al. 2003). There are a number of reports on the use of these biotic elicitors for the induction of resistance in plants against various pathogens (Nandini et al. 2013; Anupama et al. 2014). Resistance conferred by various inducing agents is generally expressed first as a hypersensitive reaction (HR) of the penetrated cells followed by other histological changes, like cell wall lignification, callose deposition, and wall thickening due to deposition of compounds like phenolic substances and hydrogen peroxide, thereby barricading the spread of the pathogen (Niranjan Raj et al. 2012; Ge et al. 2013).

Apart from histological changes in plants upon elicitor treatment during induction of resistance, changes also occur at the enzymatic level. There are number of defense-related enzymes (phenylalanine ammonia lyase [PAL], peroxidase [POX], polyphenol oxidase [PPO] and lipoxygenase [LOX], etc.) which are triggered during the induction of resistance in plants (Nandini et al. 2013; Anupama et al. 2014; Murali & Amruthesh 2015). These enzymes play a crucial role with respect to the degree of host resistance, by increasing antimicrobial activity, biosynthetic processes related to wall development such
as phenol, lignification, suberification, polymerization of hydroxyproline-rich glycoproteins, regulation of cell wall elongation, wound healing, and may be directly involved in controlling pathogen development and reduction in disease severity (Kombrink & Somssich 1997; Belkhadir et al. 2004).

Anthracnose disease of chilli caused by Colletotrichum capsici (Syd.) Butler & Bisby is one of the major biotic constraints in chilli production owing to 66–84% loss in India (Thind & Jhooty 1985). The disease is seed or air borne affecting seed germination and vigor to a greater extent. The disease management strategies, which are in practice currently, have their own limitations, and hence newer approaches are being explored and one important option is inducing resistance in the host to exploit its innate immunity. The objective of the present study was to determine crude oligosaccharides (CO) as inducer against C. capsici on chilli plants under greenhouse conditions as there are no reports on the inhibitory effect of CO and its possible mechanism involved in induced resistance in chilli against anthracnose disease. There are reports on application of an elicitor treatment to plants which may trigger defense mechanisms either directly or indirectly upon pathogen attack (Trouvelot et al. 2014). Hence, the present study was intended to isolate the CO as an elicitor from C. capsici which may be considered as possible recognition factors of host for successful disease manifestation and prime the chilli seeds with CO to induce resistance against anthracnose disease.

Materials and methods

Seed samples

Seeds of chilli cultivar (cv), G4 (susceptible) and Ujwala (resistant) to anthracnose disease, were obtained from local seed agencies, in and around Mysore, Karnataka, India.

Screening, isolation, and identification of C. capsici

The collected susceptible chilli seed samples were washed under running tap water, surface sterilized with 0.2% sodium hypochlorite for one minute and washed repeatedly for 2–3 times with sterile distilled water (SDW). The surface sterilized chilli seed samples were screened to analyze seed-borne infection of C. capsici following standard blotter method (ISTA 2005). After 7 days of incubation, each seed was examined under stereomicroscope and fungal colonies showing typical sporulating structure of C. capsici were picked with a sterile inoculation needle and transferred on to Potato Dextrose Agar (PDA) media under aseptic conditions and incubated at 25 ± 2 °C for 7 days. After incubation, the fungi were further identified based on the morphological, conidial, fruiting bodies and culture characters (Mathur & Kongsdal 2003).

Preparation of inoculum

Conidial suspension of C. capsici was prepared using SDW by harvesting acervuli from freshly sporulating cultures (7 days old) grown on PDA media by scraping the surface with sterile spatula. The concentration of the conidial suspension was adjusted to 4.5 × 10^5 conidia/ml using Haemocytometer and used as standard inoculum throughout the study (Sharma et al. 2005).

Extraction of crude oligosaccharides from C. capsici

Crude oligosaccharides (CO) were extracted by the method of Nita-Lazar et al. (2004). Pure culture of C. capsici was mass multiplied on potato dextrose broth for 15 days at 25 ± 2 °C. After incubation, mycelia were harvested and dried at 60 °C for 48 h. Dried mycelium of C. capsici (100 g) was extracted overnight with acetone (250 ml at 20 °C) and the residual powder was subjected to alkaline treatment involving 100 ml of 0.1 M NaOH at 60 °C for 2 h. The supernatant was collected by centrifugation (16,500 g for 15 min) and was neutralized to pH 7 with 50% acetic acid and stored overnight at 4 °C. The resulting sample was centrifuged (16,500 g for 20 min at 20 °C) and the collected supernatant was lyophilized. Presence of oligosaccharides in the lyophilized sample was confirmed by Molisch test (Sadasivam & Balasubramanian 1985).

Seed priming

The susceptible chilli seeds were surface sterilized and primed with different concentrations of CO (0.5, 2, 2.5, and 4 mg/ml) for different time intervals (3 and 6 h) at 25 ± 2 °C. After incubation, the seeds were air dried aseptically under laboratory conditions and used for further studies. Seeds treated with SDW served as control.

Evaluation of seed priming with CO on seed germination and seedling vigor of chilli

The CO-treated and untreated control seeds were plated equidistantly on three layers of moistened blotter discs placed on Petri plates to evaluate percent germination (Singh & Gopinath 1985) and another set of treated seeds were subjected to between-paper method to record seedling vigor (Abdul Baki & Anderson 1973). The experiment consisted of
four replicates of 100 seeds each and repeated thrice. After fourteen days of incubation at 25 ± 2 °C, percent germination, root length and shoot length were recorded and vigor index was calculated using the formula:

\[
\text{Percent Germination (PG)} = \frac{\text{No. of seeds germinated}}{\text{Total No. of seeds plated}} \times 100
\]

\[
\text{Vigor index} = \frac{\text{Seed Germination} \times \text{Mean Root Length}}{\text{Mean Shoot Length}}
\]

**Evaluation of seed priming with CO on anthracnose disease protection of chilli under greenhouse conditions**

The chilli seeds primed with different concentrations of CO for 3 h duration were sown in earthen pots (9 × 9 inch diameter) containing 2:1:1 red soil, sand (red sandy soil) and farmyard manure (FYM) which was previously autoclaved and was maintained under greenhouse conditions (25 ± 2 °C). Thirty-day-old seedlings were challenge inoculated with *C. capsici* at 4.5 × 10^5 conidia/ml. Each treatment consisted of 20 pots with 10 seedlings per pot and the experiment was repeated thrice. Plants were observed daily and rated diseased when they showed any one of the typical symptoms of anthracnose disease. At the end of 30 days after challenge inoculation, disease protection was recorded using the formula:

\[
\text{Disease Protection} = \frac{\% \text{Anthracnose disease in control plants} - \% \text{Anthracnose disease in treated plants}}{\% \text{Anthracnose disease in control plants}} \times 100
\]

**Evaluation of seed priming with CO on vegetative growth parameters of chilli under greenhouse conditions**

Evaluation of growth promotion under greenhouse conditions was carried out in chilli seeds (cv. G4) primed with CO treatments for 3 h time duration. After priming, the seeds were blot-dried and sown in earthen pots (9 × 9 inch diameter) filled with red soil, sand (red sandy soil) and FYM in the ratio of 2:1:1 which was previously autoclaved and was maintained under greenhouse conditions (25 ± 2 °C). Thirty-day-old seedlings were challenge inoculated with *C. capsici* conidial suspension of 4.5 × 10^5 conidia/ml. Each treatment consisted of 20 pots with 10 seedlings per pot and the experiment was repeated thrice. Plants were observed daily and rated diseased when they showed any one of the typical symptoms of anthracnose disease. At the end of 30 days after challenge inoculation, disease protection was recorded using the formula:

\[
\text{Percent HR} = \frac{\text{Number of seedlings with necrotic spots}}{\text{Total number of seedlings taken}} \times 100
\]

**Morphological, histological, and biochemical studies**

**Sampling of seedlings.** CO (2.5 mg/ml) treated chilli seeds (cv. G4) and resistant for 3 h along with controls were subjected to between-paper methods and incubated at 25 ± 2 °C for 14 days. After incubation, the seedlings were carefully removed without damaging the roots and dipped in *C. capsici* conidial suspension of 4.5 × 10^5 conidia/ml concentration. The inoculated and uninoculated chilli seedlings (susceptible, susceptible treated, and resistant) were harvested at 0, 3, 6, 9, 12, 18, and 24 h after inoculation (h.a.i.) for HR, lignin, callose, hydrogen peroxide, and phenol deposition studies. For biochemical studies, chilli seedlings were harvested at 0, 3, 6, 12, 24, 36, 48, and 72 h.a.i. and stored at −80 °C until subsequent use. The uninoculated susceptible, susceptible treated, and resistant chilli seedlings served as control.

**Time course analysis of morphological studies**

**Hypersensitive reaction (HR).** Hypersensitive reaction was studied in the test seedlings as described by Kumudini et al. (2001). The inoculated seedlings of chilli were observed at different time intervals for a period of 24 h for the external appearance of necrotic spots or streaks on the coleoptile regions of the test seedlings. The initial time of appearance of HR and the number of seedlings showing the necrotic spots during the experimental period of 24 h was recorded and the percent HR cells were calculated. The experiment consisted of three replicates of 10 seedlings each and repeated thrice.

\[
\text{Percent HR} = \frac{\text{Number of seedlings with necrotic spots}}{\text{Total number of seedlings taken}} \times 100
\]
**Time course analysis of histological studies**

**Lignification.** Lignification study was performed following the method of Sherwood & Vance (1976). Epidermal peelings of inoculated and uninoculated chilli seedlings (susceptible, susceptible treated, and resistant) were placed in 2% phloroglucin in 95% ethanol for 2 h. The tissues were then placed in a drop of 35% HCl on a slide and heated over a low flame until the veins turned reddish purple. The slides were then observed under microscope for the intensity of coloration and the percentage of lignified cells was calculated. The experiment was repeated thrice consisting of three replicates of 10 seedlings each observed over 20 microscopic fields.

**Callose deposition.** Callose deposition studies were carried out following the procedure of Jensen (1962). Epidermal peelings were placed in 0.005% water soluble aniline blue in 0.15 M di-potassium hydrogen phosphate (pH 8.2) for 1 h and mounted in glycerol. The epidermal peelings were then observed under Fluorescence Microscope. The cells with callose-deposition fluoresced and the callose deposited cells were counted and the percent callose cells were calculated. The experiment was repeated thrice consisting of three replicates of 10 seedlings each observed over 20 microscopic fields.

**Hydrogen peroxide deposition (H₂O₂).** Deposition of hydrogen peroxide was studied following the method of Thordal-Christensen et al. (1997). Epidermal peelings of inoculated and uninoculated chilli seedlings were placed in solution of 3,3-diamino benzidine (DAB) at 1 mg/ml, pH 3.8 for 8 h under white light and mounted on a glass microscope slide, rinsed with distilled water, covered with a glass cover slip, and observed using microscope for green-blue staining. The cells showing deposition of phenol were counted and percentage was calculated. The experiment was repeated thrice consisting of three replicates of 10 seedlings observed over 20 microscopic fields.

**Time course analysis of biochemical studies**

**Estimation of PAL activity.** One gram each of harvested chilli seedlings at above-mentioned time intervals was homogenized in 1 ml of ice cold 25 mM Tris buffer, pH 8.8, containing 32 mM of 2- mercaptoethanol in a prechilled 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, 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. 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. Each experiment was repeated thrice taking three replicates each time (Geetha et al. 2005).

**Estimation of POX activity.** One gram each of harvested chilli seedlings was 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. POX activity was determined following the method of Hammes-schmidt and Kuc (1982). The reaction mixture of 3 ml consisted of 0.25% (v/v) guaiacol in 10 mM potassium phosphate buffer (pH 6.9) containing 10 mM hydrogen peroxide. Five microlites of crude extract was added to the reaction mixture to initiate the reaction, which was recorded spectrophotometrically 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.

**Estimation of PPO activity.** One gram each of harvested chilli seedlings was macerated in 100 mM potassium phosphate buffer (pH 6.5). PPO activity was assayed spectrophotometrically by following the procedure of Arora & Bajaj (1985) with slight modification. The standard reaction mixture consisted of 3 ml of 10 mM sublimated catechol in 100 mM potassium phosphate buffer (pH 6.5) and 10 μl of enzyme extract. Change in absorbance was recorded at 420 nm for 1 min. The results are expressed as the change in absorbance at 420 nm/min/mg protein. The experiment was repeated thrice taking three replicates each time.
Crude oligosaccharides mediated resistance and histo-chemical changes in *Capsicum annuum*.

Treated with CO for 6 h offered lower seed germination and seedling vigor when compared to 3 h treated seedlings.

**Effect of seed priming with CO on anthracnose disease protection of chilli under greenhouse conditions**

The efficacy of CO was tested under greenhouse conditions for anthracnose disease resistance. The results of the present study offered a varied degree of anthracnose disease resistance ranging from 42 to 68%. Among the different concentrations of CO tested, highest disease protection of 68% was observed in chilli plants treated with 2.5 mg/ml concentration of CO followed by 2 mg/ml which offered 61% disease protection. The SDW-treated control chilli plants recorded 97.3% disease incidence (Figure 1). It was also noted that seed priming with CO at different concentrations offered significant disease protection when compared to untreated control.

**Effect of seed priming with CO on vegetative growth parameters of chilli under greenhouse conditions**

The chilli plants treated with CO for 3 h offered significant enhanced vegetative growth parameters treated with CO for 6 h offered lower seed germination and seedling vigor when compared to 3 h treated seedlings.

**Estimation of LOX activity.** One gram each of harvested chilli seedlings were extracted with 0.2 M sodium phosphate buffer (pH 6.5). LOX activity was carried out by the method of Borthakur et al. (1987). Linoleic acid (70 μl) was mixed with an equal volume of Tween-20 and 3 ml of distilled water and 125 μl of 2 N NaOH was added to obtain a clear solution. The volume of the solution was made up to 25 ml with distilled water. The reaction mixture contained 2.7 ml of sodium phosphate buffer (0.2 M, pH 6.5) and 0.3 ml of substrate. The reaction was initiated by adding 10 μl enzyme extract and the change in absorbance at 234 nm was recorded. The enzyme activity was expressed as a change in the absorbance 234 nm/mg protein/min. The experiment was repeated thrice taking three replicates each time.

**Protein estimation.** Protein content in crude enzyme extracts was estimated by the dye binding method (Bradford 1976) using bovine serum albumin (Sigma) as standard.

**Statistical analysis.** Each experimental data was subjected to analysis of variance (ANOVA) using SPSS Inc. 16.0. 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 seed priming with CO on seed germination and seedling vigor of chilli**

Susceptible chilli seeds treated with CO at different concentrations (0.5, 2, 2.5, and 4 mg/ml for 3 and 6 h) were analyzed for their effect on seed germination and vigor index. Among the different concentrations tested, CO treatment at 2.5 mg/ml concentration for 3 h offered highest seed germination of 90.5% and 986.7 seedling vigor followed by 2 mg/ml which offered 85% seed germination and 974.2 seedling vigor (Table I). The untreated control seedlings offered 78% seed germination and 712.5 seedling vigor. However, chilli susceptible seeds treated with CO for 6 h offered lower seed germination and seedling vigor when compared to 3 h treated seedlings.

![Figure 1](image.png)

**Table I. Effect of crude oligosaccharides of *C. capsici* on seed germination and seedling vigor of chilli.**

| Crude oligosaccharides (mg/ml) | Percent germination | Seedling vigor |
|-------------------------------|---------------------|----------------|
|                               | 3 h | 6 h | 3 h | 6 h | 3 h | 6 h |
| 0.5                           | 82.2 ± 0.8b | 80.7 ± 1.4b | 887.7 ± 5.3b | 827.0 ± 5.7c |
| 2.0                           | 85.0 ± 0.9b | 83.0 ± 0.4b | 974.2 ± 4.3b | 880.0 ± 3.4b |
| 2.5                           | 90.5 ± 0.6b | 85.0 ± 1.0b | 986.7 ± 4.6b | 956.2 ± 2.3b |
| 4.0                           | 84.2 ± 0.4b | 81.2 ± 0.6b | 934.5 ± 5.8b | 873.2 ± 2.4b |
| Control                       | 78.0 ± 1.0b | 78.0 ± 1.0b | 712.5 ± 4.4b | 712.5 ± 4.4b |

Notes: Values are means of four independent replicates. ± indicate standard errors. Means followed by the same letter(s) within the same column are not significantly ($p \leq 0.05$) different according to Tukey’s HSD.
Hypersensitive reaction (HR). Hypersensitive reaction was observed in the form of brown necrotic spots/streaks. A time course study on HR was conducted to analyze the effect of CO on the expression of defense reactions. However the intensity and the number of seedlings showing HR was more in resistant challenge inoculated seedlings when compared to any other treatments and control tested when compared to control plants under greenhouse conditions. Out of the different concentrations of CO tested, the chilli seeds primed with 2.5 mg/ml concentration recorded maximum vegetative growth parameters. There was an increase of 38% plant height, 30% shoot fresh weight, 33% shoot dry weight, 39% increase in leaves/ plant, and 54% of chlorophyll content when compared to SDW-treated control plants (Table II).

**Time course analysis of morphological studies**

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### Table II. Effect of crude oligosaccharides of *C. capsici* on vegetative growth parameters of chilli under greenhouse conditions after 30 DAS.

| Treatments (mg/ml) | Height (cm) | Shoot fresh weight plant⁻¹ (g) | Shoot dry weight plant⁻¹ (g) | No. of leaves plant⁻¹ | Chlorophyll content (mg/g) |
|--------------------|-------------|--------------------------------|-----------------------------|-----------------------|---------------------------|
| 0.5                | 25.0 ± 1.1  | 3.7 ± 0.6                      | 2.3 ± 0.2                   | 28 ± 1.1              | 2.4 ± 0.3                 |
| 2.0                | 35.6 ± 0.8  | 5.2 ± 0.2                      | 3.6 ± 0.7                   | 37.8 ± 0.5            | 5.4 ± 0.4                 |
| 2.5                | 40.6 ± 1.4  | 5.4 ± 0.7                      | 3.7 ± 0.8                   | 41.0 ± 0.5            | 8.2 ± 0.4                 |
| 4.0                | 32.0 ± 0.5  | 4.6 ± 0.4                      | 2.9 ± 0.6                   | 31.6 ± 0.8            | 3.5 ± 0.5                 |
| Control            | 10.6 ± 0.8  | 1.8 ± 0.1                      | 1.1 ± 0.4                   | 10.5 ± 0.8            | 1.5 ± 0.6                 |

Values are means of four independent replicates, ± indicate standard errors. Means followed by the same letter(s) within the same column are not significantly (*p* ≤ 0.05) different according to Tukey’s HSD.

Figure 2. Light microscopic pictures showing the deposition of lignin in the epidermal peelings of coleoptile regions of chilli seedlings upon treatment with crude oligosaccharides of *C. capsici* after 24 h.a.i. (a) Resistant uninoculated; (b) resistant inoculated; (c) susceptible treated uninoculated; (d) susceptible treated inoculated; (e) susceptible uninoculated; (f) susceptible inoculated. Arrows indicate lignin deposition.
seedlings. HR was observed as early as 3 h.a.i. and reached maximum at 24 h.a.i. in resistant and treated seedlings, while in control seedlings HR was noticed at 9 h.a.i and reached maximum at 24 h.a.i. A total of 19.2% resistant inoculated seedlings offered the presence of HR at 3 h.a.i., while 16% CO-treated inoculated seedlings offered HR at same time interval. At the end of 24 h.a.i., 83.2% of resistant, 70.8% of treated inoculated seedlings offered the HR response but the susceptible seedlings recorded a poor HR of 21.5% at same time interval.

Time course analysis of histological studies

Lignification. Chilli seedlings treated with CO were subjected for identification of lignin deposition in cell wall in response to inoculation with the \textit{C. capsici} by differential staining. Microscopic observations of the epidermal peelings showed lignin as reddish brown depositions along the cell wall and observations were recorded up to 24 h.a.i. (Figure 2). Lignification was observed as early as 3 h.a.i. in CO treated and resistant inoculated seedlings, whereas there was no significant lignin deposition in susceptible inoculated seedlings up to 6 h.a.i. Rapidity of lignification in resistant, induced resistance, and susceptible seedlings of chilli in both pathogen inoculated and uninoculated varied with time. Resistant inoculated seedlings offered maximum lignification when compared to inducer and susceptible inoculated seedlings. A maximum of 83% cells of resistant inoculated seedlings showed lignification, followed by inducer treated inoculated seedlings where 74.2% cells were lignified. The susceptible inoculated seedlings showed a slight increase in lignifications in cells by the end of 24 h.a.i. but no lignification was noticed in susceptible uninoculated seedlings.

Callose deposition. Callose deposition in the cell wall of all the test seedlings was studied by differential staining under Fluorescence Microscope and the

Figure 3. Fluorescent microscopic pictures showing the callose deposition in the epidermal peelings of coleoptile regions of chilli seedlings upon treatment with crude oligosaccharides of \textit{C. capsici} after 24 h.a.i. (a) resistant uninoculated; (b) resistant inoculated; (c) susceptible treated uninoculated; (d) susceptible treated inoculated; (e) susceptible uninoculated; (f) susceptible inoculated. Arrows indicate callose deposition.
cells with callose deposition appeared as bright greenish yellow fluorescence (Figure 3). Rapidity of callose deposition in resistant, induced resistant, and susceptible seedlings of chilli in both pathogen inoculated and uninoculated varied significantly \((p \leq 0.05)\). The resistant seedlings recorded maximum callose deposition (80.2%) at 24 h.a.i. followed by CO treated chilli seedlings (72.2%) at same time interval. Callose deposition was noticed as early as 3 h.a.i. and increased gradually up to 24 h.a.i. in resistant and CO treated inoculated and uninoculated seedlings. There was no significant callose deposition up to 6 h.a.i. in susceptible inoculated and uninoculated seedlings but increased gradually and reached a maximum of 35.2 and 29% at 24 h.a.i., respectively.

**Hydrogen peroxide deposition \((H_2O_2)\).** Chilli seedlings raised from CO-treated seeds along with resistant and SDW control were subjected for identification of \(H_2O_2\) deposition in cell wall in response to inoculation with \(C. capsici\) pathogen. Microscopic observations of the epidermal peelings showed \(H_2O_2\) depositions as brown staining along the cell wall and observations were recorded up to 24 h.a.i. (Figure 4). \(H_2O_2\) deposition was observed as early as 3 h.a.i. in CO-treated and resistant inoculated seedlings, whereas there was no significant \(H_2O_2\) deposition in susceptible inoculated seedlings up to 6 h.a.i. Resistant inoculated seedlings showed maximum localization of \(H_2O_2\) when compared to CO-treated and susceptible inoculated seedlings. A maximum of 84.7% localization of \(H_2O_2\) was recorded in the cells of resistant inoculated seedlings, followed by CO-treated inoculated seedlings which recorded 78% at 24 h.a.i. In susceptible inoculated seedlings, a maximum of 38% \(H_2O_2\) localization was observed at the end of 24 h.a.i.

**Phenol deposition.** Phenol deposition in chilli seedlings (resistance, induced resistant, and susceptible) with response to inoculation and

![Figure 4. Light microscopic pictures showing the deposition of hydrogen peroxide in the epidermal peelings of coleoptile regions of chilli seedlings upon treatment with crude oligosaccharides of \(C. capsici\) after 24 h.a.i. (a) resistant uninoculated; (b) resistant inoculated; (c) susceptible treated uninoculated; (d) susceptible treated inoculated; (e) susceptible uninoculated; (f) susceptible inoculated. Arrows indicate hydrogen peroxide deposition.](image)
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from 0 h.a.i. and reached maximum at 72 h.a.i. in all the test seedlings (Figure 6). However, maximum PAL activity was noticed in resistant inoculated seedlings (67 U) followed by CO-treated inoculated seedlings (64.2 U). The uninoculated resistant, inducer treated, and susceptible seedlings offered 55.2, 52, and 21 U of PAL activity, respectively, at 72 h.a.i. which was lesser when compared to challenge inoculated seedlings. An increase of one-fold in PAL activity was observed between the susceptible inoculated, resistant, and inducer treated inoculated seedlings.

Estimation of POX activity.

Significant differences in POX enzyme activity was observed in resistant, CO-treated, and control seedlings at different time intervals tested (Figure 7). The chilli seedlings raised upon CO treatment and challenge inoculation offered higher level of POX activity (40 U) at 48 h.a.i. when compared to susceptible inoculated seedlings which

uninoculation with *C. caprisci* was carried out. The microscopic observations of the stained epidermal peels showed phenol as green blue staining along the cell wall and observations were recorded up to 24 h.a.i. (Figure 5). Phenol deposition was observed as early as 3 h.a.i. in CO-treated and resistant inoculated seedlings, whereas there was no significant phenol deposition in susceptible inoculated seedlings up to 6 h.a.i. A maximum of 85% phenol deposition was noticed in resistant inoculated seedlings, followed by 75.7% in CO-treated inoculated seedlings, while least (32.2%) phenol deposition was observed in susceptible uninoculated seedlings. The rapidity of phenol deposition in pathogen inoculated and uninoculated resistant, induced resistant and susceptible seedlings varied with time interval.

**Time course analysis of biochemical studies**

**Estimation of PAL activity.** The results of the present study showed a progressive increase in PAL activity from 0 h.a.i. and reached maximum at 72 h.a.i. in all the test seedlings (Figure 6). However, maximum PAL activity was noticed in resistant inoculated seedlings (67 U) followed by CO-treated inoculated seedlings (64.2 U). The uninoculated resistant, inducer treated, and susceptible seedlings offered 55.2, 52, and 21 U of PAL activity, respectively, at 72 h.a.i. which was lesser when compared to challenge inoculated seedlings. An increase of one-fold in PAL activity was observed between the susceptible inoculated, resistant, and inducer treated inoculated seedlings.

**Estimation of POX activity.** Significant differences in POX enzyme activity was observed in resistant, CO-treated, and control seedlings at different time intervals tested (Figure 7). The chilli seedlings raised upon CO treatment and challenge inoculation offered higher level of POX activity (40 U) at 48 h.a.i. when compared to susceptible inoculated seedlings which
pathogen inoculated seedlings when compared with uninoculated ones. The resistant inoculated seedlings recorded highest PPO activity of 46.1 U followed by CO-treated inoculated seedlings (38.4 U) at 48 h.a.i. (Figure 8). It was also observed that there was up to 1.4-fold increase in PPO activity in resistant inoculated and one-fold increase in CO-treated inoculated seedlings over the susceptible inoculated control.

**Estimation of LOX activity.** The time course study of CO-treated seedlings followed by pathogen inoculation recorded maximum LOX activity at 36 h.a.i and decreased at other time points tested (Figure 9). The LOX activity in challenge inoculated CO-treated, resistant, and control seedlings was evident as early as 0 h.a.i. and reached maximum at 36 h.a.i. and decreased thereafter. An increase of 22 U was observed in resistant inoculated chilli seedlings followed by CO-treated inoculated seedlings.
The statistical analysis of the results of plant growth between the pathogen and inducer treatments. Effective in promoting plant growth and induced disease resistance both directly or indirectly (Montesano et al. 2003). The CO tested in the study was efficient in elevating plant growth parameters and defense responses in plant cells and their involvement in the detection of potential pathogens in plants has been discussed (Ebel & Cosio 1994; Shibuya & Minami 2001; Hindumathy et al. 2006; Nandini et al. 2013; Anupama et al. 2014; Manjula et al. 2015). Elicitors provide an excellent model system to study the perception and transduction of external signals to induce defense responses in plant cells. In the present study, CO was extracted from C. capsici and further CO was evaluated for its efficiency in elevating plant growth parameters and defense response in chilli against anthracnose disease.

Oligosaccharides are known to inhibit microbial pathogens in various crop plants (Shibuya & Minami 2001; Nandini et al. 2013; Anupama et al. 2014). During induction of resistance, these oligosaccharides are known to activate multiple defense signaling pathways in the signaling events that activate the expression of defensive genes in response to both herbivore and pathogen attacks (Doares et al. 1995). The study revealed that seed quality parameters were enhanced in all the concentrations of CO-treated chilli seedlings and maximum seed germination of 90.5% and seedling vigor of 986.7 was observed in chilli seedlings treated at 2.5 mg/ml when compared to control. The results are in accordance with earlier reports, where pearl millet seedlings treated with crude oligosaccharide of Trichoderma spp. (Nandini et al. 2013) and in tomato by crude oligosaccharides of Alternaria solani (Anupama et al. 2014) enhanced seed quality parameters when compared to control. Oligosaccharides have been shown to act as potent elicitor signals in several plant systems as they induce lignification, callose formation, membrane depolarization, expression of unique early responsive genes and typical defense-related genes which indicate that these oligosaccharides can regulate plant immune responses both directly or indirectly (Montesano et al. 2003). The CO tested in the study was effective in promoting plant growth and induced disease resistance was systemic as there was no direct contact between the pathogen and inducer treatments. The statistical analysis of the results of plant growth promotion and disease protection showed that treatment with CO under greenhouse conditions had a significant enhancement of growth parameters and anthracnose disease suppression in comparison with the control. The results corroborates with the studies of Kishimoto et al. (2010), where they reported that chitin oligosaccharides, a component of fungal cell wall, induced disease resistance against Magnaporthe oryzae, the casual agent of rice blast disease. The vegetative growth parameter studies of chilli plants treated with CO significantly enhanced plant height, shoot fresh weight, shoot dry weight, number of leaves per plant and chlorophyll content maximum effect was seen in plants grown in CO treated at 2.5 mg/ml concentration followed by 2 mg/ml, respectively. Likewise, pearl millet treated with ns-LTPs of rice enhanced the vegetative growth parameters (Manjula et al. 2015).

It is well known that all plants are endowed with some kind of defense mechanisms which are quiescent in nature and need appropriate stimuli or signals to activate them. Elicitors are usually capable of inducing various modes of plant defense systems including the production of reactive oxygen species, the hypersensitive response and the production of antimicrobial compound, phytoalexins, etc. (Montesano et al. 2003). Induced resistance in plants is correlated with defense reaction markers which include both biochemical markers like involvement/induction of pathogenesis related (PR) proteins, morphological and histological markers, such as HR, deposition of lignin, callose, H₂O₂, phenolic compounds, etc. in various plant pathogen interactions (Hindumathy et al. 2006; Kishimoto et al. 2010; Chang et al. 2015). The present study provides direct evidence that chilli plants treated with CO respond to C. capsici infection by triggering the morphological, histological, and biochemical mechanisms of cell defense. The degree and expression of resistance among chilli seedlings varied greatly as evidenced from the histological data.

Niranjan Raj et al. (2012) reported that hypersensitive response which is visually observed as brown necrotic spots or streaks representing rapid and localized cell death is associated with resistance responses in interaction between plants and pathogen. In the present study, seed treatment with CO led to the advancement in HR, which may have resulted in reduced disease incidence. There was a rapid expression of HR in the resistant and CO-treated chilli seedlings, while the susceptible seedlings expressed HR at later hours of pathogen inoculation. Zapata et al. (1994) observed similar results as they observed hypersensitive-like response in suspension cell cultures of grapevine treated with an elicitor (cellulase, Onozuka R-10) isolated from T. viride which also increased endogenous level of H₂O₂ and was
accompanied by localized cell death. Increase in HR was also observed in rice against rice blast disease by chitin oligosaccharides (Kishimoto et al. 2010).

Lignification and callose depositions acts in plant defense by establishing mechanical barriers to invasion of pathogens through modification of cell wall-degrading enzymes or may possess toxic properties which will reduce the movement of nutrients from the host cell to the pathogen (Hindumathy et al. 2006). The results of the study also evidenced the deposition of lignin and callose as early as 3 h.a.i. and reached maximum at 24 h.a.i. in resistant and induced resistant seedlings after challenge inoculation with the pathogen, thereby inducing resistance in chilli plants. There are reports on lignification and callose deposition during induction of resistance in pearl millet against downy mildew pathogen (Niranjan Raj et al. 2012). Likewise, OPEL, a secretory protein from \textit{P. parasitica}, elicit basal defense responses, including callose deposition, ROS accumulation and cell death in tobacco plants, and enhance resistance against \textit{P. parasitica} (Chang et al. 2015).

It was also observed that deposition of hydrogen peroxide and phenol varied depending on the susceptible, resistant, and induced resistance nature of the seedlings and increased with time after pathogen inoculation and maximum deposition was observed in resistant and induced resistant inoculated seedlings. Our results corroborate with earlier studies, where \( \text{H}_2\text{O}_2 \) localization and rapid esterification of phenolic compounds into the plant cell wall during pathogen attack in melon against \textit{C. lagenarium} (Ge et al. 2013).

Studies have also demonstrated that PR proteins are induced in pathological situations in plants, produced via salicylic-dependent pathway and considered a part of the multiple defense systems of plants (Kombrink & Somssich 1997). Different kinds of proteins are found to play certain roles in the plant defense mechanism and resistance to plant pathogens (Belkhadir et al. 2004). The temporal pattern of PAL, POX, PPO, and LOX activities in the present study revealed that there was an increase in enzyme activities upon pathogen inoculation in fourteen-day-old inducer treated (CO of \textit{C. capsici}) chilli seedlings. It is noted that these enzymes play a vital role in the induction of resistance during host-pathogen interaction (Nandini et al. 2013; Anupama et al. 2014; Manjula et al. 2015). The results of the present study revealed that there was an increase in all the defense enzyme activities upon CO treatment. PAL activity increased as early as 3 h.a.i. and reached maximum at 72 h.a.i., while POX and PPO activities reached maximum at 48 h.a.i. and decreased thereafter, but LOX activity reached maximum at 36 h.a.i. and maintained thereafter. The maximum defense enzyme activities was observed in resistant inoculated seedlings followed by inducer treated inoculated seedlings. There was an increase of 1 to 1.5-fold in enzyme activities in resistant inoculated seedlings when compared to susceptible inoculated seedlings. Likewise, there was an increase of 1 to 1.1-fold in enzyme activities in inducer treated inoculated when compared to susceptible inoculated seedlings. Increased 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 defense enzymes during pathogen infection and host resistance. The results are in accordance with the findings of Nandini et al. (2013) and Anupama et al. (2014) wherein seed treatment of pearl millet and tomato with crude oligosaccharide from \textit{Trichoderma} and \textit{A. solani} was found to stimulate POX, LOX, and PPO. Likewise, trehalose, a carbohydrate elicitor, induced activation of PAL and POX activity in wheat after challenge inoculation with \textit{B. graminis}, the causal agent of powdery mildew disease (Reignault et al. 2001).

In the present study, treatment of CO isolated from \textit{C. capsici} was effective in morphological, histological, and biochemical defense responses which reduced the incidence of anthracnose disease. Our results suggested the histochemical and biochemical defense responses like HR, deposition of lignin, callose, hydrogen peroxide and phenolic compounds and expression of vital defense enzymes (PAL, POX, PPO, and LOX) might be the important mechanisms by which CO imparts resistance against chilli anthracnose disease. Further, it can be noted that the results of the study confirm that CO of \textit{C. capsici} can be used as a source of inducer in chilli against anthracnose disease and the findings have also evidenced the interconnection of Induced Systemic Resistance and defense responses.

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Supplemental data

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