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RESEARCH ARTICLE

Biochemical basis of improvement of defense in tomato plant against *Fusarium* wilt by $CaCl_2$

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Abstract The objective of this study was to investigate the effectiveness of calcium chloride ($CaCl_2$), as potential elicitor, on tomato plants against *Fusarium oxysporum* f. sp. *lycopersici*. Foliar application of $CaCl_2$ showed significant reduction of wilt incidence after challenge inoculation. Increased production of defense and antioxidant enzymes was observed in elicitor treated sets over control. Simultaneously, altered amount of phenolic acids were analyzed spectrophotometrically and by using high performance liquid chromatography. Significant induction of defense-related genes expressions was measured by semi-quantitative RT-PCR. Greater lignifications by microscopic analysis were also recorded in elicitor treated plants. Simultaneously, generation of nitric oxide (NO) in elicitor treated plants was confirmed by spectrophotometrically and microscopically by using membrane permeable fluorescent dye. Furthermore, plants treated with potential NO donor and NO modulators showed significant alteration of all those aforesaid defense molecules. Transcript analysis of nitrate reductase and calmodulin gene showed positive correlation with elicitor treatment. Furthermore, $CaCl_2$ treatment showed greater seedling vigor index, mean trichome density etc. The result suggests that $CaCl_2$ have tremendous potential to elicit defense responses as well as plant growth in co-relation with NO, which ultimately leads to resistance against the wilt pathogen.

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Keywords Defense enzymes · Gene expression · High performance liquid chromatography · Seedling vigor index · Wilt

Abbreviations

$CaCl_2$	Calcium chloride
HPLC	High performance liquid chromatography
RT-PCR	Semi-quantitative reverse transcription-polymerase chain reaction
NO	Nitric oxide
NR	Nitrate reductase
CAM	Calmodulin
PGPR	Plant growth promoting rhizobacteria
PO	Peroxidase
GST	Glutathione S-transferases
SNP	Sodium nitroprusside
L-NAME	NG-nitro-L-arginine methyl ester
NOS	Nitric oxide synthase
C-PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
PPO	Polyphenol oxidase
PAL	Phenylalanine ammonia-lyase
CAT	Catalase
APX	Ascorbate peroxidase
DAF-2DA	4,5-Diaminofluorescein diacetate
Prot In	Proteinase inhibitor
GAPDH	Glyceraldehyde phosphate dehydrogenase
ROS	Reactive oxygen species

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the second most economically important vegetable crop cultivated throughout the world. It has immense nutritional value and

antioxidant properties (Nahar and Ullah 2012). The crop is susceptible to over 200 plant diseases of which mostly are of fungal in nature. In field condition, yield of tomato is severely hampered by wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc) Snyder et Hansen (Medeiros et al. 2009; Agrios 2005; Srivastava et al. 2010). This pathogen occurs throughout most tomato-growing areas and infects the secondary root hairs, wherein it penetrates the epidermis and finally it progresses the xylem vessels through the pits and clogs the path for water transport (Zvirin et al. 2010). It is very difficult to eradicate the pathogen from soil because they produce resting spore (chlamydospores) that can survive for many years (Amini 2009). Although, application of fungicides as well as soil fumigators are the regular practice but fruitful results may not come since the disease occurs in the later stages of crop growth when persistence and effectiveness of the applied fungicides are doubtful. Furthermore, biological control by using of plant growth promoting rhizobacterial (PGPR) strains represent a potential alternative disease management approach since PGPR are recognized for growth promotion and disease reduction in crops (Jetiyanon and Kloepper 2002; Acharya et al. 2011a). However, use of a single biocontrol agent often showed conflicting results when applied to the field as it is less liable to be active in diverse soil surroundings and agricultural ecosystems (Raupach and Kloepper 1998; Shanmugam and Kanoujia 2011). So, in the present scenario of global food demand and environmental consciousness, significant research efforts have been shifted to search the alternative non-hazardous compounds, which are capable of triggering plant immune responses for longer period of time (Abdel-Kader et al. 2012; Biswas et al. 2012; Chandra et al. 2014a). According to earlier reports both primary elicitors derived from pathogen and secondary endogenous signals may activate a varied array of plant defense related genes, encoding antioxidant enzymes like peroxidases (PO), glutathione S-transferases (GST), cell wall components, pathogenesis-related proteins, proteinase inhibitors, hydrolytic enzymes, phytoalexin biosynthetic enzymes etc. (Wang et al. 2010). Over expression of defense related genes like thaumatin from rice has been demonstrated to reduce infection of rice by *Rhizoctonia solani* (Grover and Gowthaman 2003) and of carrot by *Alternaria dauci, A. petroselini, A. radicina, Botrytis cinerea, R. solani,* and *Sclerotinia sclerotiorum* (Punja 2005).

Calcium plays an essential function in plant growth and also acts as a universal second messenger involved in various aspects of biotic and abiotic stress responses in plants (Chandra et al. 2014a; Peng et al. 2014). Earlier reports revealed that nitric oxide (NO), calcium (Ca^{2+})/-calmodulin (CaM) and other signaling molecules are required for induced up and down regulation of the expression of plant defense related molecules like PR-protein, phenol, antioxidant enzymes etc. (Sang et al. 2008; Zhang et al. 2009; Ma et al. 2012). Tian et al. (2006) showed that application of $CaCl_2$ as an abiotic elicitor reduces post-harvest damages of pear fruits by induction of the amplified level of defense enzymes activities. Elevation of cytosolic Ca^{2+} signature has been reported as an important event during host pathogen interaction which induces innate immune responses of plant (Dangl et al. 1996). Previously, we also have shown that foliar application of $CaCl_2$ reduces blister blight incidence of tea plants by activating defense related gene expression, accumulation of different enzymes and phenols along with higher production of NO in leaves (Chandra et al. 2014a). However, the interrelationship between $CaCl_2$ and NO needs further investigation.

Under these circumstances, the main objective of this study were to evaluate the potentiality of calcium chloride to induce resistance against *Fusarium* wilt disease in tomato plant by activation of defense enzymes, phenols, flavonoids etc. and also to find out the signaling role of NO in this process.

Materials and methods

Plant material

Seeds of tomato (Globe beauty cultivar) were surface sterilized with 0.1% mercuric chloride for 3 min and then washed with sterile distilled water for three times. Sterilized seeds were then planted in the earthen pots ($6 \times 6 \times 10$ cm) with a potting mixture (clay/coco peat/sand, 3:2:1, v/v). Plants were maintained at 24 ± 2 °C and 12 h photoperiod. The plants watered on every alternate day and with a balanced nutrient solution once a week according to (Chakraborty et al. 2015b).

Pathogen

Fusarium oxysporum f. sp. *lycopersici* was maintained in Potato Dextrose Agar medium. Fungal inoculum was prepared with sterile distilled water according to Manzo et al. (2016). Pathogen was grown on PDA for 15 days at 25 °C. Petri dishes containing pathogen were flooded with 10 ml of sterile distilled water. Conidia were scraped out using sterile spatulas and kept in sterile 50 ml tubes. The conidial suspensions were then adjusted to a final concentration of 1×10^3 conidia ml^{-1} by hemocytometer under light microscope.

Treatment

Forty-five days old tomato plants were sprayed with $CaCl_2$ at a concentration 0.5% (Chakraborty et al. 2015b). The

concentration was selected on the basis of the previous study (Chakraborty et al. 2016). To analyze the participation of NO in the regulation of defense response by $CaCl_2$, the sets were also primed as follows: $CaCl_2$ (0.5%) + L-NAME (10 μM); $CaCl_2$ (0.5%) + C-PTIO (100 μM); SNP (100 mM) and $CaCl_2$ (0.5%) + L-NAME (10 μM) + C-PTIO (100 μM). Here, Sodium nitroprusside (SNP) act as a potential NO donor, NG-nitro-L-arginine methyl ester (L-NAME) acts as nitric oxide synthase (NOS) inhibitor and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO) acts as NO scavenger. NOS inhibitor and NO scavenger were used 8 h prior to the application of elicitor or pathogen in respective sets. Treatment of same-aged plants with distilled water served as control. Each experiment was carried out with three replications and ten plants at time.

Enzyme assays

The leaf tissue was collected from different sets of plants 48 h (except the only pathogen treated set) after treatment and was grounded in mortar under liquid nitrogen. 500 mg of fine powder from each set was suspended in 2 ml of extraction buffer containing 0.1% polyvinylpyrrolidone and 20 ml of 1 mM phenylmethylsulfonyl fluoride: 0.1 M of sodium acetate buffer (pH 5.0) for β-1,3 glucanase and chitinase; 0.1 M sodium borate buffer (pH 8.7) for PAL; and 0.1 M of sodium phosphate buffer (pH 7.0) for PO, PPO, CAT and APX. All the extraction procedures were conducted at 4 °C. The sample was centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatants were used as the crude enzyme source for the enzymatic assay. Then it was transferred to a 2 ml microcentrifuge tube and stored at −80 °C for further use.

Peroxidase assay (PO)

PO activity was carried out, following the method of Hemeda and Klein (1990). Substrate was prepared by mixing of 5 ml of 1% guaiacol, 5 ml of 0.3% H_2O_2 and 50 ml of 0.05 M sodium phosphate buffer (pH 6.5). The reaction mixture contained 2.95 ml of prepared substrate and 0.05 ml of enzyme extract and change in absorption was measured at 470 nm for 3 min. PO activity was determined by the increase in the absorbance due to guaiacol oxidation and was expressed as μmol min^{-1} mg^{-1} of protein (E = 26.6 mM^{-1} cm^{-1}).

Polyphenol oxidase assay (PPO)

PPO activity was estimated using the method of Kumar and Khan (1982). The reaction mixture was prepared by 2 ml of 0.1 M sodium phosphate buffer (pH 6.5), 0.5 ml of crude enzyme extract and 1 ml of 0.1 M catechol. The assay mixture was then incubated for 10 min at room temperature. Reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. The absorption of purpurogallin formed was examined at 495 nm. The blank was prepared by adding 2.5 N H_2SO_4 at zero time for the same assay mixture. The PPO activity was expressed in U min^{-1} mg^{-1} protein (U = change in 0.1 absorbance min^{-1} mg^{-1} protein).

Phenylalanine ammonia-lyase assay (PAL)

PAL activity was determined according to the method of Dickerson et al. (1984). 200 μl of crude enzyme extract was incubated with 1.3 ml of 0.1 M borate buffer (pH 8.7) and 0.5 ml of 12 mM L-phenyl alanine for 30 min at 30 °C. The absorbance was measured at 290 nm. Enzyme activity was expressed as production of Transcinnamic acid (in nmol quantities) min^{-1} g^{-1} protein.

β-1,3-glucanase assay

β-1,3-glucanase activity was assayed according to Pan et al. (1991). 50 μl of crude enzyme extract was mixed with equal amount of the substrate (1% Laminarin) and was kept for 1 h at room temperature. After incubation the reaction was blocked by adding 300 μl of Dintrosalicylic acid reagent and followed by boiling for 10 min. The resulting colored solution was diluted with the addition of distilled water to make the total volume up to 2 ml and vortexed and the absorption was measured at 520 nm. The blank set was prepared with equal amounts of crude enzyme and laminarin without incubation. The enzyme activity was expressed as μmol of glucose produced min^{-1} g^{-1} protein.

Chitinase assay

Chitinase assay was performed according to the method of Bansode and Bajekals (2006) with slight modifications. The reaction mixture was prepared with 2 ml sodium acetate buffer (pH-5.0), 2 ml of substrate (1% colloidal chitin) and crude enzyme extract (0.5 ml). The mixture was then incubated for 1 h at room temperature. The reaction was stopped by adding 2.5 ml of 10% dinitrosalicylic acid reagent and followed by heating in a boiling water bath for 5 min. The mixture was then centrifuged at 10,000×*g* for 10 min. The absorption of the supernatant was measured at 540 nm. The enzyme activity was expressed as μmol of glucose equivalent released min^{-1} g^{-1} protein.

Ascorbate peroxidase assay (APX)

APX activity was determined according to Nakano and Asada (1981). The reaction mixture prepared by adding

50 mM potassium phosphate (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 2% H_2O_2, and 0.1 ml enzyme extract in a final volume of 3 ml. The decrease in absorbance at 290 nm for 1 min was recorded and the amount of ascorbate oxidized was calculated using extinction coefficient ($\epsilon = 2.8\ mM^{-1}$ APX was defined as 1 mmol ml^{-1} per min cm^{-1}). One unit of ascorbate oxidized as 1 mmol ml^{-1} ascorbate oxidized per min.

Catalase assay (CAT)

CAT was measured following the technique of Cakmak and Horst (1991) with slight modifications. The reaction mixture prepared with enzyme extract (100 µl), 50 µl of hydrogen peroxide (0.3%) and final volume was made up to 3 ml by adding up of phosphate buffer (50 mM, pH-7.0). Decrease in absorbance was recorded for 3 min for at 240 nm. The enzyme activity was expressed as nmol min^{-1} g^{-1} of protein with help of a molar extinction coefficient $\epsilon = 39{,}400\ M^{-1}\ cm^{-1}$.

Estimation of total protein content

The standard Bradford assay (1976) was employed, using bovine serum albumin as a standard, to test the protein concentration of each extract.

Estimation of Total Phenol

Estimation of total phenol was determined following the method of Zieslin and Ben Zaken (1993). 250 mg of fresh leaf tissue was homogenized in 2 ml of 80% methanol and the material was maintained at 65 °C for 15 min. The sample was then centrifuged at 10,000×*g* for 10 min at room temperature and the supernatant was collected and used to estimate the phenol content. The reaction mixture was prepared by adding up 1 ml of crude sample extract to the mixture of 5 ml distilled water and 250 µl of 1 N Folin ciocalteu reagent. The reaction mixture was incubated for 30 min at room temperature. Phenolic content was measured at 725 nm using standard curve of gallic acid. The amount of total phenol was expressed as µg gallic acid produced g^{-1} tissue.

Estimation of total flavonoid content

Total flavonoid content was determined by following the method of Chang et al. (2002). 150 mg of fresh leaf tissue was grounded in 2 ml of 80% ethanol and the material was incubated in dark place for 30 min. It was then centrifuged at 10,000×*g* for 5 min at room temperature. The reaction mixture was prepared with 1 ml of crude extract (supernatant) mixed with 4.3 ml of 80% aqueous ethanol, 0.1 ml of 10% aluminum nitrate, and 0.1 ml of 1 M aqueous sodium acetate. The reaction mixture was then kept in dark place for 30 min. Absorption was measured at 415 nm. The amount of total flavonoid was expressed as mg g^{-1} of fresh tissue.

Nitric oxide estimation (NO)

Production of NO was measured by haemoglobin assay according to the method of Delledonne et al. (2001). Leaf tissues of control and treated sets were incubated in a reaction mixture containing 10 mM L-arginine and 10 mM haemoglobin in a total volume of 5 ml of 0.1 M phosphate buffer (pH 7.4). Production of NO was measured spectrophotometrically at 401 nm and NO levels were calculated using an extinction coefficient of 38,600 M^{-1} cm^{-1}. After 2 h of incubation, NO content in the reaction mixture was measured as nmol of NO produced g^{-1} tissue h^{-1}.

Real time NO production was observed by using membrane permeable flurochrome 4-5 diaminofluorescein diacetate (DAF-2DA) dye (Bartha et al. 2005). Lower epidermis of leaf was peeled off and placed in a brown bottle containing 1 ml of loading buffer prepared by 10 mM KCl, 10 mM Tris HCl (pH 7.2) with DAF-2DA at a final concentration of 10 mM. It was then incubated for 20 min in dark. Fluorescence was observed with Lieca DMLS microscope at excitation wavelength 480 nm and emission wavelength 500–600 nm. Green fluorescence indicates the generation of NO.

Quantification of phenolic compounds by HPLC

Leaf samples collected from different sets tomato plants after 48 h of incubation. Samples were crushed in HPLC grade methanol (100%) and prepared for phenolic acid estimation with an HPLC system (Agilant, USA) equipped with a DAAD detector and an Agilent Eclipse plus C18 column (100 mm × 4.6 mm, 3.5 µm). Separation was achieved using a flow rate of 0.8 ml/min at 25 °C. The mobile phase consisted of eluent A (acetonitrile) and eluent B (aqueous phosphoric acid solution, 0.1% v/v). A gradient program was used for elution: 0–2 min, 5% A; 2–5 min, 15% A; 5–10 min, 40% A; 10–15 min, 60% A; 15–18 min, 90% A (Khatua et al. 2015). Gallic acid, *p*-coumaric acid, myricetin, caffeic acid, vanillic acid, ferulic acid, quercetin, cinnamic acid and pyrogallol (M.P. Biomedicals. USA) were used as standards according to Khatua et al. (2015). The DAAD detection was conducted at 278 nm for the quantification (Chandra et al. 2014a). Sample compounds were identified on the basis of absorption spectra and retention times of standard materials. A concentration of each compound was determined by comparing peak areas

of reference compounds with those in the samples run under the same elution conditions.

Analysis of defense-related gene expression by semiquantitative RT-PCR

Expression of defense related and antioxidative genes were analyzed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from different sets of tomato leaves after 48 h of incubation with TRIzol Reagent (Invitrogen, USA). The cDNA was synthesized from the total RNA according to Chandra et al. (2014a). To analyze the expression of a specific gene, 1 µl of the cDNA was taken in a 20 µl PCR mixture containing 1 × DreamTaq PCR buffer, 0.2 mM of each dNTPs, 1 µM of each gene specific primer, and 1 units of Dream Taq DNA polymerase (Fermentas, USA). PCR cycles were carried out under the following conditions: 94 °C for 4 min, then 30 cycles of 94 °C for 30 s, annealing temperature (Tm) for specific primer for 30 s and 72 °C for 60 s with a final extension step of 7 min at 72 °C in a thermal cycler (Applied BioSystem, USA). List of used primers are presented in Table 1. PR-1b (basic PR1), PR-2a (acidic glucanase), PR-2b (basic glucanase), PR-3a (Chitinase 3, acidic), PR-3b (Chitinase 9, basic), PR-5 (Osmotin-like), PR-7 (P69A, subtulisin-like), PAL, Prot In (Proteinase inhibitor), PO, GST (Glutathione-S-transferase), CAM (Calmodulin) and NR (Nitrate reductase) genes were amplified individually (Van Kan et al. 1992; Danhash et al. 1993; Tornero et al. 1996; Rep et al. 2002; Peng et al. 2014; Sanz-Alférez et al. 2008; Jin et al. 2009; Medeiros et al. 2009). The primer pair for the glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as an internal control (Shih et al. 1992). Linearity between the amount of input RNA and the final RT-PCR products was verified and confirmed. The PCR products were resolved in 1% agarose gel, stained with ethidium bromide, visualized and photographed in Bio-Rad Gel Doc EZ

Table 1 List of primers used

Gene of interest	Sequences of primers	Tm (°C)	References
PR-1b (basic PR1)	FOR 5′ CCAAGACTATCTTGCGGTTC 3′	55	Van Kan et al. (1992)
	REV 5′ GAACCTAAGCCACGATACCA3′		
PR2a (acidic glucanase)	FOR 5′ TATAGCCGTTGGAAACGAAG3′	55	Van Kan et al. (1992)
	REV 5′ TGATACTTTGGCCTCTGGTC3′		
PR2b (basic glucanase)	FOR 5′ CAACTTGCCATCACATTCTG 3′	52	Van Kan et al. (1992)
	REV 5′ CCAAAATGCTTCTCAAGCTC 3′		
PR 3a (chitinase 3, acidic)	FOR 5′ CAATTCGTTTCCAGGTTTTG 3′	52	Danhash et al. (1993)
	REV 5′ ACTTTCCGCTGCAGTATTTG3′		
PR 3b (chitinase 9, basic)	FOR 5′ AATTGTCAGAGCCAGTGTCC 3′	59	Danhash et al. (1993)
	REV 5′ TCCAAAAGACCTCTGATTGC 3′		
PR 5 (osmotin-like)	FOR 5′ AATTGCAATTTTAATGGTGC 3′	49	Rep et al. (2002)
	REV 5′ TAGCAGACCGTTTAAGATGC 3′		
PR 7 (P69A, subtulisin-like)	FOR 5′ AACTGCAGAACAAGTGAAGG 3′	50	Tornero et al. (1996)
	REV 5′ AAC GTGATTGTAGCAACAGG 3′		
PAL	FOR 5′ TTCAAGGCTACTCTGGC 3′	52	Peng et al. (2014)
	REV 5′ CAAGCCATTGTGGAGAT 3′		
Prot In (proteinase inhibitor)	FOR 5′ CGGAGAATCTGAATGGGTAAGCGA 3′	63	Medeiros et al. (2009)
	REV 5′ ACAAGCCGTGGTAAAGGTCCACAA 3′		
PO (peroxidase)	FOR 5′ ACGGAGCAAGCGACAATTGACAAC 3′	65	Medeiros et al. (2009)
	REV 5′ CGATTGATTCACCGCAAAGCTCGT 3′		
GST (glutathione-S-transferase)	FOR 5′ TGTCCCAACCTTCTCGTGCAGTTA 3′	65	Medeiros et al. (2009)
	REV 5′ TGAGTGATGCCAGTCCAACACAGA 3′		
Calmodulin	FOR 5′ GCACGGAAGATGAAGGACAC 3′	55	Sanz-Alférez et al. (2008)
	REV 5′ GCAAGCATCATACGGACAAAC 3′		
NR	FOR 5′ CAAGCAATCCATCTCCCAT 3′	57	Jin et al. (2009)
	REV 5′ CATCTCTGTATCGTCTTCAGGA 3′		
GAPDH (glyceraldehyde phosphate dehydrogenase)	FOR 5′ GAAATGCATCTTGCACTACCAACTGTCTTGC 3′	63	Shih et al. (1992)
	REV 5′ CTGTGAGTAACCCCATTCATTATCA TACCAAGC 3′		

Imager system. Densitometric analysis of the photographed gels was carried out by ImageJ software.

Efficacy of $CaCl_2$ against *Fusarium* wilt

Percent disease incidence was measured according to Ramamoorthy et al. (2002). Thirty-five days old seedlings were transplanted in earthen pots filled with sterilized potting soil containing balanced macro and micro nutrient solutions. Ten days after transplanting elicitor solution ($CaCl_2$-0.5%) was sprayed on the leaves until runoff. Two days after foliar application of elicitor, 60 ml of conidial suspension (10^3 microconidia ml^{-1}) of *F. oxysporum* f. sp. *lycopersici* was poured per pot. After 15 days of elicitor application second spray was done. Plants sprayed with sterile water and inoculated with microconidial suspension was considered as control. Wilt incidence was recorded 30 days after inoculation using following formula:

Percent Disease Incidence (PDI) = (Number of diseased plants/Total number of plants) × 100

Ten pots per replication were maintained and there were three replications. The experiment was repeated thrice.

Staining of lignin

To measure lignin production in the stems of elicitor treated plants, fresh sections were left for 5 min in 2% phloroglucinol in 95% ethanol and mounted in 6 N HCl (Vallet et al. 1996). Appearance of reddish-pink product ensures the presence of lignin in the tissue. Phloroglucinol in acidic condition reacts with mainly the cinnamaldehyde groups present in lignin and give colored product.

Effect of elicitor on physiological parameters of tomato

To study the effect of elicitor on certain physiological parameters tomato seeds were first surface sterilized with 0.1% $HgCl_2$ for 2 min and followed by three washings with the sterile distilled water. Sterilized seed were dipped in the solution of $CaCl_2$ (0.5%) and SNP (100 µM) for 8 h and were placed on a wet blotting paper to study their effect on seed germination and seedling vigor index. The blotting papers were incubated at 24 °C with relative humidity above 85%. Number of germinated seedling was recorded after 14 days and mean seed germination was calculated according to Raut et al. (2014). The root and shoot length (cm) of randomly selected 10 normal seedlings was measured and seedling vigor index was calculated (Raut et al. 2014). Total chlorophyll of elicitor treated and untreated plants was estimated following Arnon's method (Arnon 1949) with slight modification as described by Chakraborty et al. (2015a, b). Mean trichome density of elicitor treated and untreated plants was measured according to Boughton et al. (2005). Yield of the elicitor treated and untreated control plant was compared according to Nahar and Ullah (2012).

Statistics

All data presented were mean ± standard deviation (S.E.) of three replicates. Statistical analyses were performed by analysis of variance (ANOVA) using SPSS software version 20 (Chakraborty et al. 2015b) and the significance of difference between the treatments was determined using Duncan's Multiple Range Test ($p < 0.05$).

Results

Effects of $CaCl_2$ on defense enzyme activity in tomato plant

Foliar application of $CaCl_2$ at a concentration 0.5% showed increased amount of all the defense enzymes production in tomato leaves compared to water treated control. Elicitor treatment showed higher amount of PO, PPO, PAL, β-1,3-glucanase and chitinase production along with elevated level of antioxidative enzymes like CAT and APX. Among all the treatments, tomato plants pre-treated with $CaCl_2$ (0.5%) showed highest inductive ability for all the defense enzymes. The induction of the PPO and chitinase was significantly higher, and about 4 and 3.6 fold increase in enzyme production was observed in tomato plants treated with $CaCl_2$ over the water treated control set (Table 2). Similarly, accumulation of PO, PAL and β-1,3 glucanase activity was noted 2.71, 2.16 and 3.1 fold higher in elicitor treated plants than control, respectively (Table 2). This results also coincide with increased production of antioxidative enzymes. In $CaCl_2$ treated plants 4.83 and 1.61-fold increased amount of APX and CAT enzyme activity was recorded when compared to water treated control plant (Table 2). Compare to other sets plants treated with both the NO modulator (C-PTIO and L-NAME) and elicitor showed significant reduction for all the enzymes studied compared to control plant.

Effects of $CaCl_2$ on defense-related gene expression in tomato plant

To justify spectrophotometric results and also to elucidate effect of elicitor treatment on defense related genes, expression in the transcript level was determined by semi-quantitative RT-PCR analysis. Differential

Table 2 Effect of foliar application of $CaCl_2$ (0.5%), SNP (100 μM), C-PTIO (100 μM), L-NAME (10 μM) and pathogen inoculation alone or in different combinations on the production of defense enzymes, Phenol and flavonoid in leaves of tomato

Enzymes	Control	$CaCl_2$	$CaCl_2$ + L-NAME	$CaCl_2$ + C-PTIO	SNP	$CaCl_2$ + L-NAME + C-PTIO
Peroxidase (PO) [μmol min^{-1} mg^{-1} protein]	2.53 ± 0.2^{c}	6.11 ± 0.54^{b}	2.01 ± 0.16^{cd}	1.56 ± 0.28^{de}	6.1 ± 0.43^{b}	1.3 ± 0.2^{de}
Polyphenol oxidase (PPO) [U min^{-1} mg^{-1} protein]	33.96 ± 4.1^{c}	124.1 ± 16^{ab}	22 ± 6.69^{c}	26.18 ± 5.5^{c}	104.96 ± 13.62^{b}	19.14 ± 3.51^{c}
Phenylalanine ammonia-lyase (PAL) [nmol of transcinnamic acid min^{-1} g^{-1} protein]	114.35 ± 9.9^{c}	224.79 ± 16.43^{a}	90.22 ± 10.29^{d}	74.69 ± 6.31^{de}	192.28 ± 12.23^{b}	48.09 ± 9.07^{f}
β-1,3-glucanase [μmol glucose produced min^{-1} g^{-1} protein]	28.8 ± 5.33^{c}	88.51 ± 11.88^{a}	26.21 ± 6.67^{c}	20.71 ± 4.29^{c}	66.37 ± 11.83^{b}	17.52 ± 2.09^{c}
Chitinase [μmol glucose equivalent released min^{-1} g^{-1} protein]	0.85 ± 0.05^{d}	2.37 ± 0.24^{b}	0.62 ± 0.08^{de}	0.53 ± 0.14^{e}	2.08 ± 0.09^{c}	0.38 ± 0.06^{e}
Ascorbate peroxidase (APX) [μmol min^{-1} g^{-1} protein]	0.18 ± 0.01^{c}	0.69 ± 0.03^{b}	0.15 ± 0.03^{cd}	0.12 ± 0.02^{cd}	0.85 ± 0.05^{a}	0.11 ± 0.03^{d}
Catalase (CAT) [nmol min^{-1} g^{-1} protein]	6.66 ± 0.25^{c}	10.34 ± 1.1^{b}	4.86 ± 0.53^{d}	3.97 ± 0.64^{d}	12.27 ± 0.68^{a}	3.35 ± 0.35^{d}
Total phenol [μg gallic acid produced g^{-1} tissue]	283.43 ± 35.19^{b}	564.11 ± 41.98^{a}	210.15 ± 13.24^{bc}	157.36 ± 15.71^{c}	550.74 ± 43.14^{a}	225.38 ± 61.81^{bc}
Total flavonoid[mg g^{-1} of the tissue]	740.73 ± 87.2^{c}	1254.01 ± 12.79^{b}	474.42 ± 85.53^{d}	425.96 ± 59.73^{de}	1381.17 ± 128.25^{b}	327.66 ± 30.88^{e}

Values represent mean ± SE of three separate experiments, each in triplicate

$CaCl_2$ Calcium chloride, *SNP* sodium nitroprusside, *C-PTIO* 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, *L-NAME* NG-nitro-L-arginine methyl ester

Different letters within the row indicate significant difference ($p < 0.05$) from the control set using Duncan's multiple range test. Same letter within the row denotes no significant difference between the groups

alteration of defense-related genes like PR-1, PR-2a, PR-2b, PR-3a, PR-3b, PR-5, PR-7, PAL, Prot In, PO, GST, CAM and NR expression in different treated tomato plants (Fig. 1). Elevated defense enzyme productions were also reflected in the gene expression analysis in different treated tomato plants. From the Fig. 1 it is clearly observed that for all the genes examined, marked change observed in $CaCl_2$ sprayed tomato plants. Higher amount of GST gene expression in elicitor treated set implies greater protection of plants against oxidative stress. Furthermore, the expression analysis of NR gene revealed that $CaCl_2$ also have inductive role in overexpression of nitrite reductase gene for the higher accumulation of NO which might act as a signaling compound in downstream gene expression system. Among all the sets NO scavenger and NOS inhibitor treated plants showed significant decreased expression of mRNA for all the tested genes.

Effects of $CaCl_2$ on production of total phenol and flavonoid in tomato plant

Studies on induction of phenolic compounds revealed that higher accumulation total phenol and flavonoid levels were

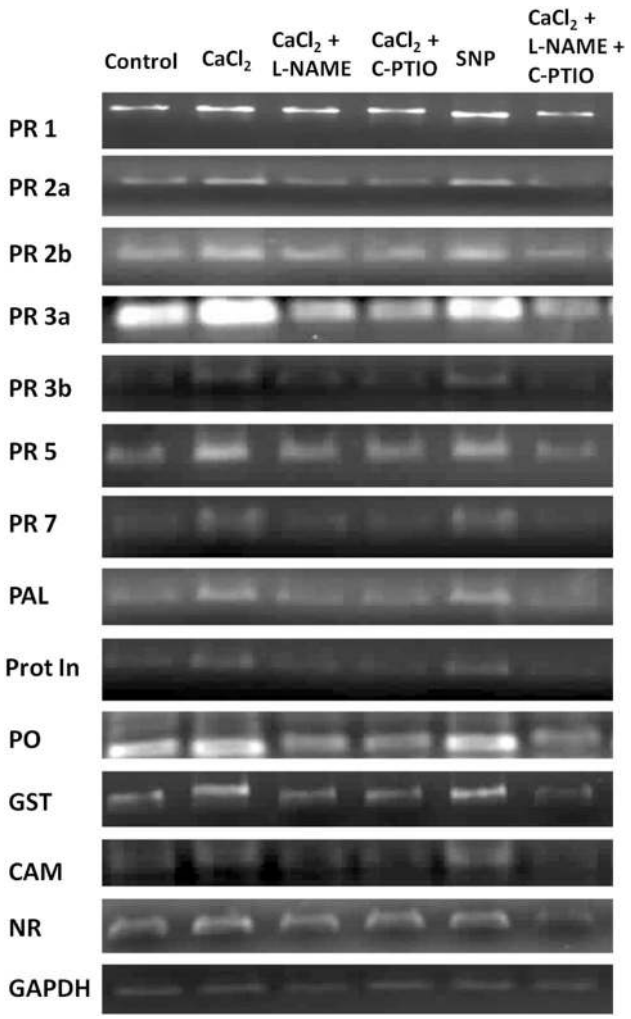

Fig. 1 Semi-quantitative RT-PCR analyses of different sets treated with $CaCl_2$, Pathogen and NO modulators either alone or in combinations. Expression of different defense related PR genes, antioxidant enzyme-coding genes (PO, GST), calcium sensor gene (CAM) and gene of nitrate reductase (NR) are represented in the subsequent order of treatment. GAPDH band represents internal control

observed in $CaCl_2$ treated tomato plants challenge inoculated by *F. oxysporum* f. sp. *lycopersici* as well as in the plants solely sprayed with $CaCl_2$. The maximum accumulation of total phenol and flavonoid was recorded as 2.1 and 2-fold higher in elicitor treated sets than control plants, respectively (Table 2). However, NO modulator treated plants showed minimum amount of phenolic compound production. Furthermore, through HPLC analysis specific phenolic acids were quantified from the total phenol extracts of different sets of treated tomato plants. Phenolic compounds present in the samples were identified by comparing both retention times and UV–Vis spectra with those of pure standards (Table 3). Production of gallic acid, *p*-coumaric acid and vanillic acid were not found in control plants but those compounds were observed in different sets of treated plants (Table 3). Among all the sets significant increase of all the phenolic compounds were found to be higher in the plants sprayed with $CaCl_2$. Production of caffeic acid, ferulic acid, myricetin, quercetin, cinnamic acid and pyrogallol were noticed 26.33, 55.56, 8.46, 26.11, 27.69 and 2.17 fold higher than untreated control in $CaCl_2$ treated set.

Effects of $CaCl_2$ on production of NO in tomato plant

It has already been well-known that NO plays a key role to modulate the immune responses in plants during elicitor treatment (Chakraborty et al. 2015b; Chandra et al. 2014a, 2015). To investigate whether increase of defense in our model plant by $CaCl_2$ is NO mediated, we checked the NO production in both $CaCl_2$ and various combinations of NO modulator treated tomato leaves and compared them with the water treated control. In $CaCl_2$ treated plants, an increased level of NO production was observed when compared to the control. However, significant reduction of NO production was observed in the plants treated with C-PTIO and L-NAME (Fig. 2I). Almost around 2.7 fold increase in NO production was observed in elicitor treated set compared to control.

NO production was further justified by using a NO specific fluorophore DAF-2DA on leaf peals, which converts fluorescent triazol derivative upon reaction with NO. Similar kind of increase in NO production was observed in elicitor treated sets as monitored by spectrophotometry (Fig. 2II).

Effect of NO donor (SNP) and NO modulators (L-NAME and C-PTIO) on defense response

Induction of defense response and production of signaling molecule NO in the $CaCl_2$ treated tomato plants are clear from the above results. To further substantiate the involvement of NO in the observed $CaCl_2$-mediated defense stimulation, we treated tomato plants with NO donor (SNP) as well as NO scavenger (C-PTIO) and NOS inhibitor (L-NAME) either singly or in combination with elicitor. In SNP treated sets, a significant increase of defense enzymes namely, PO (2.41-fold), PPO (3.9-fold), PAL (1.68-fold), β-1,3-glucanase (2.3-fold) and chitinase (2.4-fold) along with the induced activity of antioxidant enzymes CAT (1.84-fold) and APX (4.72-fold) was observed (Table 2). Simultaneous higher accumulation of total phenolics (1.94-fold) and total flavonoids (1.86-fold) were detected (Table 2). Interestingly, increased amount of different phenolic acids were also increased compared to water treated control plant (Table 3). Similarly, expression of defense related genes and antioxidant enzyme-coding

Table 3 Quantitative analysis of phenolic acid content in tomato leaves due to foliar application of $CaCl_2$ (0.5%), SNP (100 μM), C-PTIO (100 μM), L-NAME (10 μM) and pathogen inoculation alone or in different combinations

Standard compounds	Quantity (μg/g of fresh weight)					
	Control	$Cacl_2$	$CaCl_2$ + L-NAME	$CaCl_2$ + C-PTIO	SNP	$CaCl_2$ + L-NAME + C-PTIO
Gallic acid	NF	25.84 ± 1.36^{b}	NF	NF	20.89 ± 0.59^{c}	4.62 ± 0.70^{d}
Caffeic acid	2.58 ± 0.30^{f}	67.95 ± 0.87^{c}	6.64 ± 0.79^{d}	3.77 ± 0.18^{ef}	80.03 ± 1.07^{a}	5.02 ± 0.42^{e}
Vanillic acid	NF	36.12 ± 0.86^{a}	12.36 ± 0.68^{c}	10.69 ± 0.72^{d}	27.22 ± 0.34^{b}	NF
P-Coumaric acid	NF	79.57 ± 2.48^{b}	26.03 ± 0.40^{f}	22.05 ± 0.36^{g}	59.86 ± 0.79^{c}	4.53 ± 0.17^{h}
Ferulic acid	2.67 ± 0.30^{h}	148.36 ± 3.95^{b}	55.91 ± 0.58^{d}	45.21 ± 0.60^{e}	111.28 ± 1.33^{c}	5.47 ± 1.07^{g}
Myricetin	6.74 ± 0.71^{d}	57.07 ± 1.81^{a}	NF	NF	14.81 ± 0.64^{c}	NF
Quercetin	4.92 ± 0.28^{f}	128.47 ± 2.0^{b}	15.02 ± 0.40^{d}	11.44 ± 0.87^{e}	130.47 ± 0.98^{a}	4.4 ± 0.32^{f}
Cinnamic acid	2.57 ± 0.28^{e}	71.17 ± 4.11^{b}	8.01 ± 0.68^{d}	4.79 ± 0.45^{e}	56.70 ± 0.86^{c}	NF
Pyrogallol	3.62 ± 0.31^{d}	7.88 ± 1.84^{b}	12.93 ± 0.71^{a}	9.06 ± 0.44^{b}	6.28 ± 0.36^{c}	4.88 ± 0.23^{d}

Values represent mean ± SE of three separate experiments, each in triplicate

$CaCl_2$ Calcium chloride, *SNP* sodium nitroprusside, *C-PTIO* 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, *L-NAME* NG-nitro-L-arginine methyl ester, *NF* not found

Different letters within the row indicate significant difference ($p < 0.05$) from the control set using Duncan's multiple range test. Same letter within the row denotes no significant difference between the groups

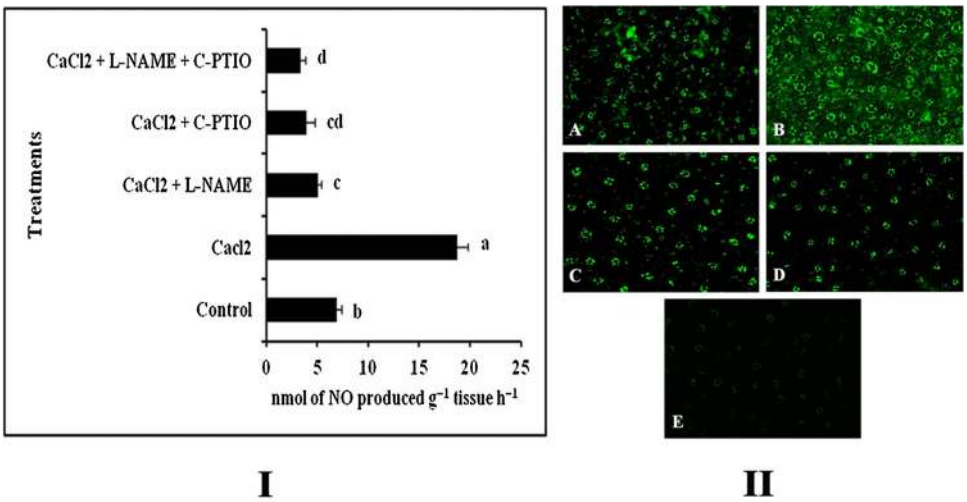

Fig. 2 Effect of foliar application of $CaCl_2$ (0.5%) along with different NO modulators on the production of NO. **I** Spectrophotometric analysis of NO production in the leaf tissue. **II** Real-time detection of NO. NO production in leaf epidermal cells was stained by DAF-2DA. (**A**) Control; (**B**) $CaCl_2$ (0.5%); (**C**) $CaCl_2$ (0.5%) + L-NAME (10 μM); (**D**) $CaCl_2$ (0.5%) + C-PTIO (100 μM); (**E**) $CaCl_2$ (0.5%) + L-NAME (10 μM) + C-PTIO (100 μM). Results are mean ± SE of three separate experiments done in triplicate. *Different letters* in the bar graph specify significant difference ($p < 0.05$) from the control set using Duncan's multiple range test whereas *same letter* denotes no significant difference between the groups

gene as well as the gene of CAM and NR were also increased (Fig. 1). It was evident that $CaCl_2$ treatment showed higher accumulation of NO in tomato plants. However, tomato plants co-treated with elicitor as well as NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) or NO scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO) in various combinations, restricted the NO production noticeably, as detected by spectrophotometrically or microscopically (Fig. 2). Consequently, accumulation of defense enzymes, antioxidant enzymes, total phenolic and flavonoid contents (Table 2) and different phenolic acids (Table 3) along with

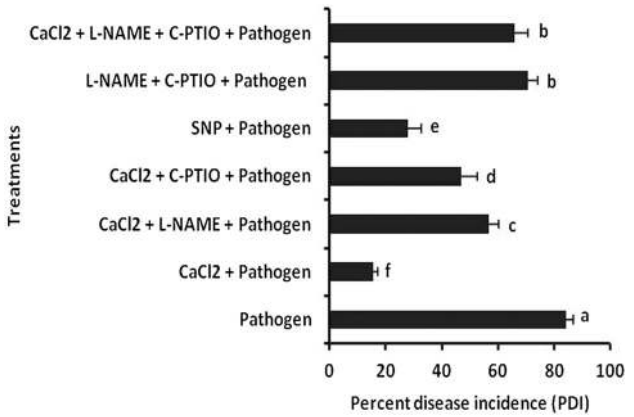

Fig. 3 Effect of exogenous application of $CaCl_2$ (0.5%) and pathogen inoculation along with different NO modulators on percent disease incidence on tomato plants. Results are mean ± SE of three separate experiments done in triplicate. *Different letters* in the bar graph specify significant difference ($p < 0.05$) from the control set using Duncan's multiple range test whereas same letter denotes no significant difference between the groups

the expression profiles of different defense related genes, including genes of antioxidant enzyme and CAM and NR gene were found to be observed reduced or at basal level like control plants (Fig. 1).

Effects of $CaCl_2$ treatment on incidence of *Fusarium* wilt disease

The treatments of tomato seedlings with $CaCl_2$ significantly reduced wilt development by *F. oxysporum* f. sp. *lycopersici* (Fig. 3). The amount of reduction from *F. oxysporum* f. sp. *lycopersici* treated plant was recorded 84.24%. However, SNP treated set showed 66.80% reduction than pathogen treated control. Furthermore, co-treatment of NO scavenger (C-PTIO) and elicitor as well as NO synthase inhibitor (L-NAME) and elicitor or in a combination of both scavenger and inhibitor with elicitor showed significant increase in disease incidence compared to plants treated with $CaCl_2$ alone. In those sets, the amount of reduction of disease incidence was observed as 44.26, 32.81 and 21.73% than pathogen treated control plant respectively.

Effects of $CaCl_2$ on production of lignin in tomato plant

Histochemical observations demonstrate that $CaCl_2$ also have inductive role for the production of lignin which starts to deposit in primary cell walls prior to the secondary walls on secondary xylem and other tissues. From Fig. 4, it was clear that elicitor treated plants showed higher amount of lignifications in the tissue 10 days after elicitation compared to water treated control plants. However, plants

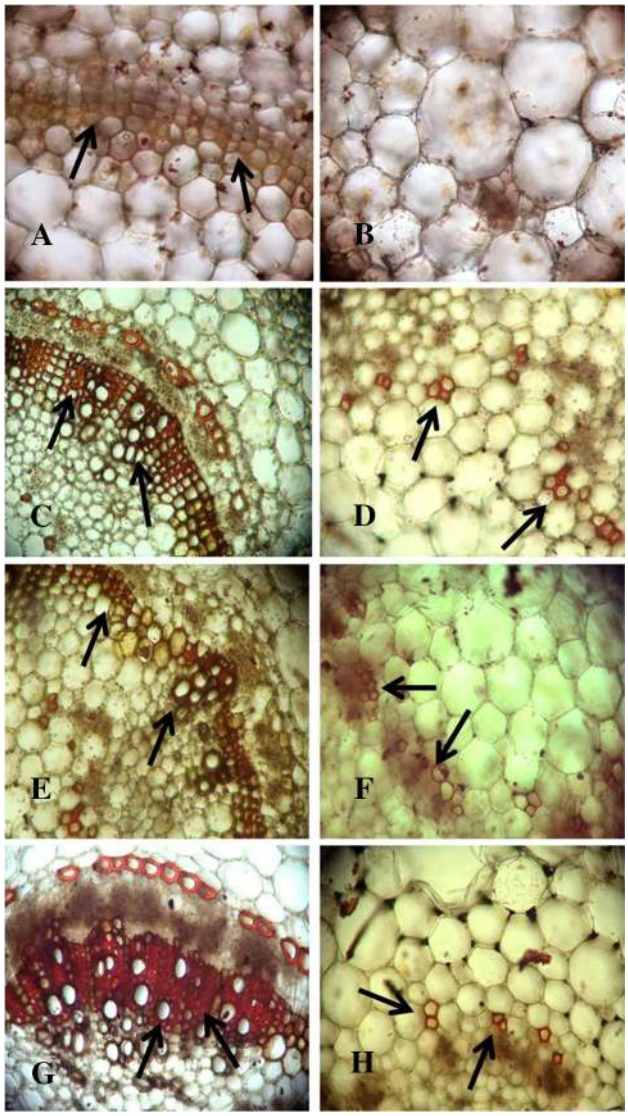

Fig. 4 Effect of exogenous application of $CaCl_2$ (0.5%) on lignin production in the stem of tomato plants. **A** and **B** Control (water treated); **C** and **D** $CaCl_2$-0.5% (observed after 10 days of elicitor application); **E** and **F** Pathogen inoculated (observed after 10 days); **G** and **H** $CaCl_2$ (0.5%) + pathogen inoculated plant (observed after 10 days). *Arrows* indicate the sites of lignin synthesis

inoculated with pathogen also showed greater lignifications in the stem tissue after 10 days of incubation. Interestingly, highest lignifications occurred in the plants treated with $CaCl_2$ (0.5%) and challenge inoculated with pathogen.

Effects of $CaCl_2$ on seed germination, shoot length, root length, seedling vigor, chlorophyll content, mean trichome density and yield in tomato plant

The increase in seed germination by $CaCl_2$ and SNP was recorded 9.39 and 7.66% respectively (Table 4). Shoot length and root length was also significantly increased in

Table 4 Effect of $CaCl_2$ (0.5%) and SNP (100 μM) as seed and foliar treatment on seed germination, shoot length, root length, seedling vigor index, total chlorophyll content, trichome density and yield in tomato plants

Sets	% Seed germination	Shoot length (cm)	Root length (cm)	Seedling vigor index	Total Chlorophyll [mg of total chlorophyll g fresh tissue^{-1}]	Mean trichome density (no./ microscopic field)	Yield (g/pot)
Control	80.33 ± 1.52^b	6.53 ± 0.35^c	5.2 ± 0.5^c	941.93 ± 46.41^c	0.70 ± 0.08^b	9.66 ± 1.52^c	115.66 ± 4.04^b
$Cacl_2$	88.66 ± 1.15^a	9.56 ± 0.85^a	7.1 ± 0.26^a	1478.4 ± 110.26^a	1.11 ± 0.15^a	17 ± 1.73^a	175 ± 7.21^a
SNP	87 ± 1^a	8.33 ± 0.51^b	6.16 ± 0.35^b	1261.7 ± 43.15^b	0.73 ± 0.08^b	13.66 ± 1.52^b	167 ± 6.24^a

Values represent mean ± SE of three separate experiments, each in triplicate

$CaCl_2$ Calcium chloride, *SNP* sodium nitroprusside

Different letters within the row indicate significant difference ($p < 0.05$) from the control set using Duncan's multiple range test. Same letter within the row denotes no significant difference between the groups

elicitor treated sets compared to water treated control. In comparison to SNP, seeds treated with $CaCl_2$ showed greater shoot and root length. Amount of increase in shoot and root length for both $CaCl_2$ and SNP treated seeds showed 1.46, 1.27, 1.36 and 1.18 fold compare to control respectively (Table 4). The extent of seedling vigor was observed highest in $CaCl_2$ treated seeds. Value was recorded 1.56 and 1.33-fold increase in $CaCl_2$ and SNP treated seeds respectively compared to control (Table 4). Furthermore, chlorophyll content in $CaCl_2$ treated plants was 1.58-fold increased. However, in SNP treated plants it remains same like control (Table 4). Mean trichome density was significantly higher in both $CaCl_2$ and SNP sprayed plants and recorded 1.75 and 1.41-fold increase compared to water treated control plants (Table 4). Yield of tomato plants were significantly altered with the application of $CaCl_2$ and SNP compared to control plant. 1.51 and 1.44-fold increase in total yield was recorded in $CaCl_2$ and SNP treated plants compared to control respectively.

Discussion

Plants acquire an array of mechanisms to shield themselves against regular assault of potential pathogens and other stresses (Chakraborty and Acharya 2017). During course of time they have advanced their defense strategies that include both constitutive as well as pathogen-induced molecules (Vanitha et al. 2009). In fact, natural defense response of plants against various pathogens depends upon early recognition of intra or extracellular components of pathogen (Chandra et al. 2015). Plants treated with different abiotic or biotic elicitor molecules have been shown to encourage plant's innate immune system to over express different defense related enzymes and genes, increased accumulation of phenolic compounds, cell wall material synthesis, over production of different signaling molecule etc. (Nürnberger et al. 2004; Acharya et al. 2011a; Chandra et al. 2014b).

In this study, we examined the efficacy of $CaCl_2$, as an abiotic elicitor, on tomato plants against *Fusarium oxysporum* f. sp. *lycopersici*. Biochemical, molecular and enzymatic studies revealed that all the parameters examined were significantly altered due to $CaCl_2$ treatment. Notable elevation of defense response was observed in tomato plants by foliar application of $CaCl_2$. There has been quantitative increase in the levels of biochemicals such as phenols and flavonoids and an increase in defense enzymes like PO, PPO, PAL, β-1,3-glucanase and chitinase activities in $CaCl_2$ treated plants was recorded after 48 h of incubation. Furthermore, transcript analysis of $CaCl_2$ treated plants showed higher expression of PR 1, PR 2a, PR 2b, PR 3a, PR 3b, PR 5, PR 7, PAL, Prot In and PO gene compared to control. In which PR 2a and PR 2b represents β-1,3-glucanase; PR 3a and PR 3b represents chitinase; PR 5 represents osmotin like proteins. Among these defense molecules, PO and PPO are recognized to be involved in the strengthening of the plant's cell wall by accumulation of lignin which leads to protection against different invading pathogens (Lamb and Dixon 1997; Bruce and West 1989; Acharya et al. 2011b; Chandra et al. 2015). These observations were reflected in our study as the higher accumulation of PO and PPO ultimately leads to the greater production of lignin in the $CaCl_2$ treated plants. On the other hand, induced expression of β-1,3-glucanase beside its well known antifungal activities would also enhance varied physiological roles (Balasubramanian et al. 2012). Thus over expression of β-1,3-glucanase by foliar application of $CaCl_2$ might become valuable at the time of pathogen foray as this enzyme is directly implicated in hydrolyzing the components of fungal cell wall. Another PR protein, chitinases (PR 3) also has the potential to hydrolyze major components of fungal cell wall like chitin (Leah et al. 1991). Application of $CaCl_2$ have been shown

to induce better expression of PO, PPO, PAL, β-1,3-glucanase, chitinase and other defense molecules in different host plants (Chandra et al. 2014a; Tian et al. 2006; Chakraborty et al. 2015a). Thus induction of these enzymes in the host plant during pathogenesis possesses a great importance as shown in our result.

Furthermore, PAL is the entry-point enzyme in the phenylpropanoid biosynthesis pathway and plays an important role in phenolic compound synthesis (Parmar and Subramanian 2012). The vital role of phenolic compounds is to induce disease resistance. Their accumulation due to application of different elicitors have already been reported (Nicholson and Hammerschmidt 1992; Sánchez-Estrada et al. 2009; Dong et al. 2010; Chandra et al. 2015). Phenolic and flavonoid compounds are also known to have free radical scavenging, antimicrobial activity, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (Mandal et al. 2009; Srivastava et al. 2013). In this study, higher accumulation of phenolic compounds has been recorded in elicitor treated plants as a consequence of increased amount of PAL activity. Previously, it was reported that higher accumulation of ferulic acid in tomato roots may become esterified and cross linked to form lignin-like polymers which in turn provide defense against impending pathogens (Mandal et al. 2009). In addition to this, 4-coumaric acid and ferulic acid has been found to be effective to reduce the growth of *Fusarium* species in vitro at all concentrations tested (McKeehen et al. 1999). However, reduced level of ferulic acid, 4-hydroxybenzoic acid and 4-coumaric acid along with lower level of PAL and PO enzyme activity, increase the susceptibility of tomato plant against wilt pathogen (Mandal et al. 2009). Our results agree with this view as HPLC analysis showed that in elicitor treated sets production of phenolic compounds like gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, cinnamic acid and pyrogallol was significantly increased. These compounds are helpful to plants to surmount adverse environmental conditions as well as to give resistance against pathogens (Gould and Lister 2005; Chandra et al. 2015; Ruiz-García and Gómez-Plaza 2013). According to Ruiz et al. (2003) foliar application of $CaCl_2$ (10 μM) in *Citrus* increases PAL activity which leads to the accumulation of phenolic compounds and provide resistance against *Alternaria alternata*. In our work, increased amount of phenol accumulation by foliar application of $CaCl_2$ significantly reduce *Fusarium* wilt disease incidence in tomato plants.

Generation of reactive oxygen species (ROS) is thought to be one of the earlier event towards the recognition of a pathogen by the plants (Baker and Orlandi 1995; Chakraborty et al. 2015b). To cope up with this, plants have developed different scavenging system including production of antioxidant enzymes (Grob et al. 2013). APX and CAT are two main antioxidant enzymes produced by the plants in response to ROS. Increasing production of these two enzymes were already reported in tomato plants by foliar application of sub-lethal dose of $CuCl_2$ (Chakraborty et al. 2015b). In this study, amount of APX and CAT was increased in elicitor treated plants. Not only that the mRNA expression of GST, another antioxidant enzyme, was also elevated moderately due to elicitor application. These results signify that $CaCl_2$ might also provide the requisite guard to the plants from the oxidative stress coupled with pathogenic attack.

Downstream signal transduction pathways after perception of elicitor are an important subject of investigation (Baenas et al. 2014). Beside many other roles, Ca^{2+} is a well documented important intracellular messenger in plant defense signaling, which is relayed by the calcium sensor like CaM that quickly converts the signal to second messengers like NO and cyclic nucleotides (Chandra et al. 2014a; Peng et al. 2014). Over the last few years, NO has been popularized as an important signaling molecule behind several patho-physiological events and also involved in the activation of plant defense during pathogen attack (Klessig et al. 2000; Wendehenne et al. 2001; Lecourieux et al. 2006; Acharya and Acharya 2007; Romero-Puertas and Delledonne 2003). Ca^{2+} dependent NO production was recorded in few plant system in response to different elicitors like cryptogein, $CaCl_2$ etc. (Foissner et al. 2000; Lamotte et al. 2004; Chandra et al. 2014a). Calcium induced NO production and induction of antioxidative enzymes in response to different stresses was hampered by application of EGTA (an extra cellular calcium chelator) (Qiao et al. 2015; Rahman et al. 2016). Moreover, characterization of a plant nitric oxide synthase (NOS) from *Arabidopsis thaliana* showed that the enzyme contains CaM-binding motifs and that signifies complete activation of NOS needs both Ca^{2+} and CaM (Guo et al. 2003; Lecourieux et al. 2006). We have shown elevation in NO level in several plants like *Raphanus sativus, Camellia sinensis* and in *Capsicum annuum* by foliar application of different biotic and abiotic elicitors (Chandra et al. 2014a, b, 2017; Chakraborty et al. 2015b, 2016; Chakraborty and Acharya 2016), showing its involvement in the signal transduction process leading to induced defense responses. In this study, $CaCl_2$-treated tomato plants showed greater NO production than the control set. Nitrate reductase (NR) has been shown to be one of the major source NO in plants including tomato (Meyer et al. 2005; Jin et al. 2009) and NO was found to be one of the major positive regulator of NR (Du et al. 2008). This fact was reflected in our study where elicitor and SNP both differentially modulate the expression of NR gene in treated tomato plants. Furthermore, to establish the connection between NO and $CaCl_2$ induced resistance, separate tomato

plant was treated with NO donor, SNP, a strong NOS inhibitor, L-NAME and a potent NO scavenger C-PTIO alone or in combinations.

Production of the defense related enzymes (i.e., PO, PPO, PAL, β-1,3-glucanase and chitinase) including the antioxidant enzymes (CAT and APX) and the expression level of different defense genes (PR 1, PR 2a, PR 2b, PR 3a, PR 3b, PR 5, PR 7, PAL, Prot In and PO) and that of the antioxidant enzyme (GST), NR and CAM as well as total phenol and flavonoid contents were comparatively lower in $CaCl_2$ + L-NAME, $CaCl_2$ + C-PTIO and $CaCl_2$ + L-NAME + C-PTIO treated sets compared to the plants treated with $CaCl_2$ and SNP. Production of NO was also validated by using DAF-2DA stain which showed significant reduction of fluorescence in L-NAME and C-PTIO treated plants than the plants treated with $CaCl_2$. Our results coincide with the results of some previous studies where co-treatment with L-NAME or C-PTIO and elicitor have showed reduced production of NO and as a consequence, reduction in defense induction was observed in pearl millet, *Rauvolfia serpentina,* tea etc. (Manjunatha et al. 2009; Gupta et al. 2013; Chandra et al. 2015). However, sole treatment of SNP induces defense responses in several plants (Hu et al. 2003; Hasanuzzaman and Fujita 2013; Chandra et al. 2014b). In present work application of SNP showed significant induction for all the defense molecules including defense enzymes, defense gene expressions, antioxidant enzymes production and activation of phenolic compounds which signifies the role of NO in this process.

Furthermore, according to earlier findings some of resistance inducing chemicals like salicylic acid, β-aminobutyric acid, chitosan and 2, 6 dichloroisonicotinic acid are also known to enhance the seed germination and other physiological aspects including, root and shoot length, yield, chlorophyll content etc. (Zhou et al. 2002; Rajaei and Mohamad 2013; Jayalakshmi et al. 2010; Raut et al. 2014). In present study both $CaCl_2$ and SNP showed significantly higher amount of seed germination, root and shoot length, seedling vigor, chlorophyll content and yield compared to control which implies, both elicitor and NO have inductive effects on those parameters also. Trichomes, are one of the defensive structure, which occur on the surfaces of many plants and provide protection to the plants against herbivores (Simmons and Gurr 2004; Boughton et al. 2005). Foliar applications of abiotic elicitors such as methyl jasmonate (MJ) or Benzothiadiazole (BTH) induces increased densities of defense-related glandular trichomes on new leaves of tomato plants (Boughton et al. 2005). In our case also we have also found mean trichome density becoming much higher in $CaCl_2$ and SNP treated sets than untreated control.

From these results, it could be concluded that foliar application of $CaCl_2$ could provide promising integrated alternatives in suppression of *Fusarium* wilt disease of tomato. In this connection, Ca^{2+} ion acts as an external signal which induces to generate an internal signature signal like NO that ultimately leads to the over expressions of different defensive responses to the tomato plant. Furthermore, $CaCl_2$ also have the potential to improve plant growth. So, this work presents an alternative, safer and user friendly approach for the management of *Fusarium* wilt disease of tomato. Further studies regarding the use of $CaCl_2$ alone or in combination should be examined under different combinations of host and pathogen for better understanding and to establish it as a multiuser compound for sustainable agriculture in a broader range.

Author's contribution KA designed whole research. NC and SC conducted experiments and analyzed data. NC wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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