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Differential Reprogramming of Defense Network in *Capsicum annum* L. Plants Against *Colletotrichum truncatum* Infection by Phyllospheric and Rhizospheric *Trichoderma* Strains

Amrita Saxena[1] · Sandhya Mishra[2] · Shatrupa Ray[1] · Richa Raghuwanshi[3] · Harikesh Bahadur Singh[4]

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Abstract

Induction of defense response in host plants by the *Trichoderma* spp. has been attributed as one of the major mechanisms leading to inhibition of the pathogenic ingression. The present study sheds light on the mechanisms employed by the *Trichoderma* isolates, obtained from phyllosphere (BHUF4) and rhizosphere (T16A), to modulate the defense network of chili plant under *Colletotrichum truncatum* challenge. Plants treated with both the *Trichoderma* strains exhibited significant accumulation of phenols under *C. truncatum* challenge with maximum increment recorded for capsaicin (16.1-fold), ferulic acid (5.03-fold), quercetin (5.36-fold), salicylic acid (94.88-fold), and kaempeferol (6.22-fold). Phenol accumulation corresponded to the subsequent defense gene expression pattern. When compared to the pathogen-challenged control plants, enhanced expression of *PR1*, *PIK1*, *CHI*, *GLU*, *Cdef*, and *SAR* genes was recorded in the *Trichoderma*-treated plants acting as a biocontrol agent (BCA). The results of the present study suggest that to strengthen the defense pathways in the host plant, the mechanisms employed by *Trichoderma* isolates differ and depend upon their origin and site of application. While phyllospheric *Trichoderma* isolate (BHUF4) employed the systemic acquired resistance (SAR) pathway, the rhizospheric *Trichoderma* strain (T16A) used the induced systemic response (ISR) pathway for eliciting the defense response in the host plant under *C. truncatum* challenge. The study signifies how *Trichoderma* strains obtained from different origin and when applied at different sites in plant judiciously reprogram the defense network of the host plant to provide robust protection against phytopathogens. In the present case, overall protection is provided to the chili plants against the foliar or underground attack of *C. truncatum*.

Keywords *Capsicum annuum* L. · *Colletotrichum truncatum* · Induced systemic response (ISR) · Phyllosphere · *Trichoderma* spp. · Systemic acquired resistance (SAR)

Introduction

Chili (*Capsicum annum* L.), grown as an annual crop, mainly in tropical and sub-tropical countries is an important global economic commodity. The use of its different varieties as spices and vegetables further enhances its economic profitability. The medicinal and anti-oxidant properties of its main constituent, i.e., capsaicin, further escalate its economic significance (Srinivasan 2016; Sun et al. 2016). Considered as an essential spice, the profitability of its cultivation lies in the fact that both ripened and unripened fruits hold significant market value. However, at the same time, the crop is being affected by a number of phytopathogens like *Phytophthora capsici*, *Xanthomonas campestris*, leaf curl virus, etc. (Saxena et al. 2016b). Among all, chili anthracnose or fruit rot is considered as the most destructive disease owing to

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✉ Harikesh Bahadur Singh
hbs1@rediffmail.com

[1] Department of Botany, Banaras Hindu University, Varanasi 221005, India

[2] CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Yunnan, China

[3] Department of Botany, Mahila Mahavidyalaya, Banaras Hindu University, Varanasi 221005, India

[4] Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi 221005, India

its significant generation of both pre- and post-harvest loss (Saxena et al. 2016b; Sharma et al. 2009).

Huge crop loss (amounting to US$ 491.67 million) has been reported in tropical and sub-tropical countries solely due to this disease (Garg et al. 2014). The reason behind the massive crop loss by this disease owes to its causal organism, *Colletotrichum truncatum* (formerly called as *C. capsici*) (Than et al. 2008; Saxena et al. 2014). This pathogen has been described as hemibiotrophic (O'Connell et al. 2012; Liang et al. 2018) and can also stay dormant in the form of the conidium for long time in the fields. Under favorable conditions like high humidity, moderate temperature, and rain, it spreads widely sometimes leading to an epidemic condition. Being transmitted both by soil and water, its control is tough, and thus heavy dosage of chemical fungicides and pesticides is recommended. This might result in increased crop yield but at the same time due to the high load of chemical fungicides in the fruits, quality gets compromised heavily affecting the export (Rao et al. 2005; Xavier et al. 2014).

Alternate methods of control are considered important for maintaining the optimum quality of fruits suitable for domestic use and export purposes. With a continuous increase in the demands for organically grown crops, biocontrol is considered as an essential alternative in controlling pathogen infestations in crop fields. Many studies have been undertaken to show the efficacy of plant growth-promoting bacteria and fungi (PGPRs and PGPFs) in managing plant diseases (Ray et al. 2016; Singh et al. 2013; Jain et al. 2012; Saxena et al. 2013). As the pathogen is able to spread through both soil and water, an inclusive approach is required to provide complete protection to the host plant against the pathogen intrusion. This was the idea behind a recent study by Saxena et al. (2016a), wherein they have uncovered the efficiency of phyllospheric *Trichoderma* strains which in combination with the rhizospheric *Trichoderma* strain elevates the overall defense response of the plant, thereby providing better protection against the pathogen. The present study is the further extension of the same, in lieu of addressing the mechanisms of induction of defense responses in the host plants, when challenged by the pathogen, i.e., *C. truncatum*.

There are different mechanisms through which a plant protects itself from deleterious pathogens confronting through soil or directly affecting the aerial parts. The hosts have tailor-made defense responses against different types of pathogens, be it biotrophic, necrotrophic or hemi-biotrophic (Koornneef et al. 2008). The PGPRs are known to aid plants in enhancing the induce systemic responses (ISR) via sending signals from the root to the entire aboveground parts (Van Loon et al. 2006; Ray et al. 2018). Similarly, the endophytes and microbes dwelling on the surface of the aerial organs activate the SAR responses via the aerial organs (Van Loon and Van Strien 1999; Shoresh et al. 2010). Salicylic acid (SA) and jasmonic acid (JA) are the two main components of the ISR and systemic acquired resistance (SAR) routes, respectively (Contreras-Cornejo et al. 2011). The two routes are specifically differentiated to induce defense responses which depend on the nature of the pathogen. JA-mediated responses are mostly against plant beneficial microorganisms and herbivores and necrotrophic pathogens (Contreras-Cornejo et al. 2018), while the SA-mediated pathway regulates defense against biotrophic and hemi-biotrophic ones (Koornneef et al. 2008). *Trichoderma* being a well-celebrated biocontrol agent has been reported to elicit the ISR against pathogenic attacks in chickpea, pea, tomato, chili, etc. The mechanisms through which phyllospheric *Trichoderma* strains efficiently elevate the defense response of the host have not yet been explained with clarity. The study puts light on the question, by addressing the molecular changes in the host plant when challenged with the pathogen by using biochemical, molecular, and histochemical approaches. The findings of the study will highlight the mode of action of the phyllospheric BCA which will further strengthen the concept of utilizing more than one BCA for enhanced management of plant diseases.

In our previous study (Saxena et al. 2016a), two different *Trichoderma* isolates were used, which belonged to two separate species. BHUF4 (*T. asperellum* (KJ636986)) was isolated from the phyllospheric region of the chili plant, while T16A (*T. harzianum* (KC609758)) was isolated from the rhizospheric region of the chili plant. To check their biocontrol efficiency, BHUF4 was applied on the aerial surface of the host plant by spraying and T16A was applied directly to the soil by the soil drenching method. Interestingly, comparable biocontrol efficiency was observed for both the isolates, in spite of the difference in their origin of isolation and site of application. The present study extends the previous results to highlight the different mechanisms employed by the *Trichoderma* isolates to strengthen the defense pathway of the host plant.

Materials and Methods

Maintenance and Multiplication of Pathogen and Bioagents

The pathogen *Colletotrichum truncatum* reported in the previous study (Saxena et al. 2016a) was used for challenging the chili plants. The pathogen culture was maintained at 4 °C on PDA slants and was revived after every 30 days.

The bioagents BHUF4 (*Trichoderma harzianum*) and T16A (*Trichoderma asperellum*) isolated from the phyllosphere and rhizosphere of chili plants were used in this study. Their characterization and identification have been

previously described (Saxena et al. 2016a). The cultures were maintained at 4 °C on PDA slants and were revived after every 30 days.

Experimental Design

The pot experiments were carried out under greenhouse conditions (28 ± 2 °C and 80% RH with 12 h alternate light and dark conditions) at Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, BHU, Varanasi (25.32°N, 82.97°E). The experiment was divided into two sets, (1) pathogen challenged and (2) pathogen unchallenged. Each set was comprised of four treatments, i.e., untreated plants as control, BHUF4-treated plants, T16A-treated plants, and BHUF4 + T16A-treated plants. For each treatment, six pots containing three seedlings were maintained. The experiment was repeated twice and was designed in a completely randomized manner.

Inoculum Preparation of Bioagents and Pathogen for Treatment of Chili Plants

Seven-days-old cultures of the two selected *Trichoderma* isolates (BHUF4 and T16A) were used for preparing spore suspension. Spores from the fungal colony (T16A and BHUF4) were collected with sterile and distilled water and were filtered through the layers of muslin cloth. The conidia count was adjusted to 2×10^7 ml^{-1} using hemocytometer.

Twenty-one-days-old culture of *C. truncatum* was taken for preparing the conidial suspension of the pathogen. The culture of *C. truncatum* profusely growing in Petri dishes was flooded with sterile and distilled water, and the mycelia were scraped using a sterile spatula. The suspension formed was filtered through the layers of cheesecloth and the conidial count was adjusted to 10^6 conidia ml^{-1} using hemocytometer.

Greenhouse Experiment

Twenty-one-days-old chili seedlings (Var: Surajmukhi) obtained from sterilized seeds were transplanted in pots containing sterile soil mixture. Soil mixture containing sandy soil, vermicompost, and farmyard manure (2: 1: 1) was sterilized in an autoclave at 15 lbs pressure for 30 min on three consecutive days, and 1.5 kg of the mixture was filled in each plastic pot (15 cm × 10 cm). For treating the plants with BHUF4, seedlings were sprayed with the spore suspension of BHUF4 till complete drenching of the plant (phyllospheric application of the BCA). Roots of 21-days-old seedlings were dipped in the spore suspension of T16A isolate for 2–5 min and were then transplanted into the pots (rhizospheric application of BCA). After transplantation, soil drenching was performed to treat the plants with T16A, while spraying was carried out to treat the plants with BHUF4 isolate. The treatment with the BCAs was repeated five times in the entire life cycle of the plant. Untreated plants served as control (C). Further, after 1 week of treatment with bioagents, the seedlings were sprayed with conidial suspension of *C. truncatum* and covered using sterile poly bags for 48 h to maintain humidity. Untreated and unchallenged plants served as positive control while untreated but challenged plants served as the negative control. The negative control plants were first sprayed with sterile water and then infected with *C. truncatum* conidial suspension followed by covering for 48 h to maintain humidity.

Histochemical Staining

Chili plants infected with pathogen were collected 4 days after pathogen infection from each treatment separately. Transverse sections of internodal regions were obtained with a freezing microtome (Fielabo Equipment Works, Haryana, India). Further, sections were examined under a light microscope (Dewinter, India) after fixing in 95% (v/v) ethanol and mounting in presence of a saturated solution of aqueous phloroglucinol in 20% HCl. Each examination was done twice. The lignified areas were indicated by the appearance of a red–violet color as described in earlier reports (Saxena et al. 2015).

In Vitro Pathogenicity Assay

The conidial suspension of *C. truncatum* was prepared as described earlier. Fruits from plants of each treatment were used for the experiment. The fruits were surface sterilized with 1% sodium hypochlorite solution for 5 min and rinsed with sterile distilled water for two to three times. Ten microlitre of the conidial suspension was injected at the center of each fruit using a sterile syringe. The fruits were then kept in moist chambers maintained at 25–26 °C and 98% relative humidity. Un-inoculated but wounded fruits served as the negative control, while fruits from untreated plants with *C. truncatum* infection served as the positive control. Ten fruits from each treatment were considered for the assay.

Inoculated fruits were evaluated for anthracnose symptoms after 9 days of incubation on the basis of lesion size relative to the overall size of the fruit. The length of the lesion was measured (L_l) and the length of the whole fruit (L_f) was measured. The formula $((L_l/L_f) \times 100)$ was used to get the % area of infected fruit. Disease severity was scored on a 0–9 scale (0 = no infection, 1 = 1–2%, 3 = 3–5%, 5 = 6–10%, 7 = 11–25% and 9 = > 25% infected fruit area) as described by Montri et al. (2009). The experiment was repeated three times to confirm the results. The pathogen was re-isolated after 10 days using direct isolation and was cultured on PDA

plates to morphologically identify and compare with the original isolate for confirmation of Koch's postulates.

Sample Collection for HPLC and RNA Isolation

From each treatment, randomly 12 plants were uprooted carefully and collected in sterile poly bags after 0, 24, 48, and 72 h of pathogen infection. Nodal leaves (third to fifth nodes) from the bottom were collected as samples for HPLC analysis. Collected leaves were washed in running tap water, dried with blotting paper, and stored at − 80 °C until used for HPLC analysis. For further gene expression analyses, leaves were harvested in sterile falcons in presence of liquid N_2 and were stored at − 80 °C till the extraction of RNA.

High-Performance Liquid Chromatography (HPLC) Analyses of Phenylpropanoid Derivatives in Leaves of the Chili Plant

For analysis of phenylpropanoid derivatives, 500 mg of fresh tissue was harvested 48 h after pathogen challenge (hapi) and the extract was obtained with 50% methanol (10 ml). The solvent was evaporated under reduced pressure on rota evaporator (Eyela N–Nseries, Tokyo, Japan). The residue was dissolved in HPLC-grade methanol and subjected to HPLC for qualitative and quantitative analysis of specific phenolics. The HPLC system Shimadzu LC-10A (Japan) was equipped with a dual pump LC-10A binary system, UV detector SPD-10A, Phenomenex (Torrance, USA) C18 column (RP-Hydro, 4X, 250X4.6 mm). The data were integrated by the Shimadzu Class VP series software. Separation of compounds was achieved with acetonitrile/water (1:1 v/v) containing 1% acetic acid in a linear gradient program, starting with 18% acetonitrile, followed by changing to 32% in 15 min and finally to 50% in 40 min (Singh et al. 2009). The solvent flow rate was maintained at 1.0 ml min^{-1}. Results (µg ml^{-1}) were obtained by comparing the peak areas (max 254 nm) of the samples with those of standards (Class VP series software, Shimadzu, Japan).

Total RNA Extraction and Gene Expression Study

Total RNA was isolated from liquid N_2 frozen leaf tissues of each treatment using the RiboZol™ RNA Extraction reagent (AMRESCO, LLC, US) following the manufacturer's instructions. The RNA quality was tested by OD260/OD280 ratio and gel electrophoresis. The same total RNA was used for first-strand cDNA synthesis using iScript™ cDNA Synthesis Kit (BIORAD, Hercules, CA). Single-strand cDNA of all the four treatments was PCR amplified using selected primers after equalization with internal control actin.

Primer pairs were synthesized at Scigenome Laboratories, Bengaluru, India. The sequences of primer pairs considered for this study have been summarized in Table 5.1. Semi-quantitative PCR of the respective genes was performed using equalized cDNA (25 ng) from each RNA sample (data not shown).

Following the effective expression of the five genes, viz; *PR1*, *GLU*, *PIK1*, *Cdef*, *SAR8.2* during semi-quantitative analysis, real-time expression analysis of the above mentioned genes was carried out for the individual treatments at 48 hapi using iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). The thermal cycle profile comprised of 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. All the five genes were amplified using iQ™ SYBR® Green Supermix (Bio-Rad) in the PCR reaction of 20 µl according to the manufacturer's instructions. The actin gene was used as internal control. All samples were assayed in triplicate on the same plate. The relative amount of target mRNA expression was calculated by the delta delta Ct method (Livak and Schmittgen, 2001).

Statistical Analysis

All the experiments were repeated twice unless otherwise stated. The data presented are means of three replications ± standard deviation (SD). The data were subjected to a one-way variance analysis (ANOVA) using SPSS Ver. 16 (SPSS Inc., Chicago, IL) to test the significance of the observed differences. The differences between means of the parameters were evaluated by Duncan's Multiple Range Test (DMRT) and P values ≤ 0.05 were considered as statistically significant.

Results

Histochemical Assay

The treated chili plants exhibited enhanced lignin deposition upon challenge with *C. truncatum* as compared to untreated and challenged plants. Interestingly, hosts primed with BHUF4 expressed a similar intensity of lignification as compared to the treatments primed with T16A. However, plants treated with both the strains resulted in an intensified lignin deposition around the vascular bundles (Fig. 1a–d) (Supplementary Fig. S1). Increased lignin deposition was evident in the secondary xylem and phloem cells, with moderate deposition in sclerenchyma cells of the treated plants. The increased lignin deposition was also evident by the increased thickness of the secondary vascular tissues, especially thick-walled meta-xylem cells (Fig. 1e–h).

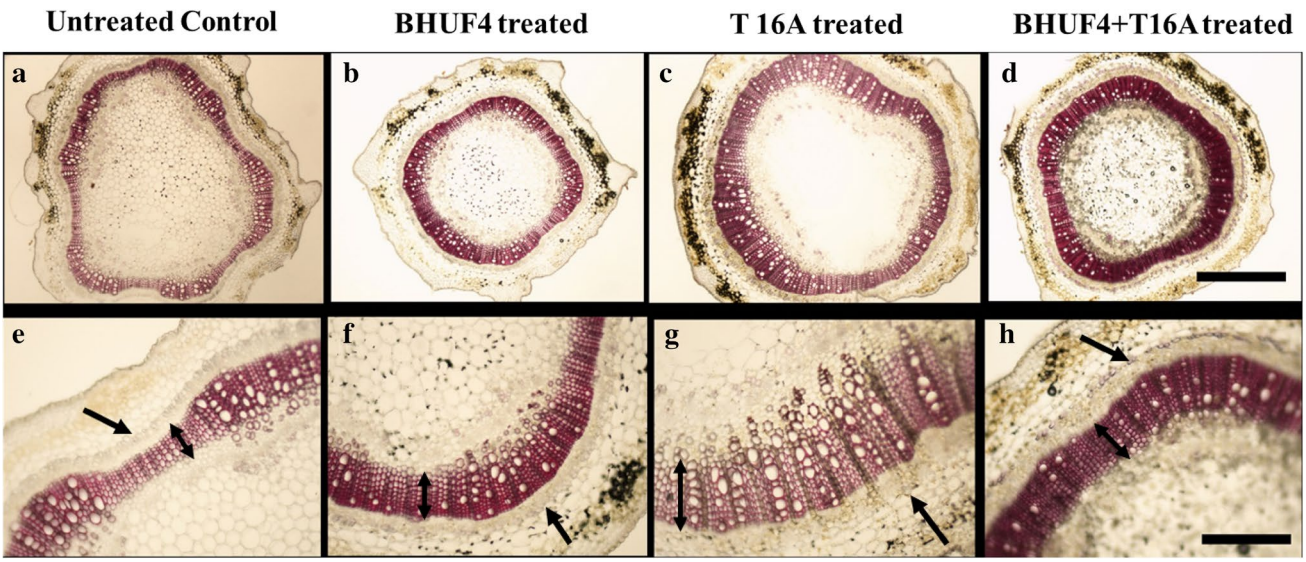

Fig. 1 Influence of different treatments on the lignification pattern of internodal regions of 21-days-old chili plants under *C. truncatum* challenge. Bar indicate 0.5 mm (**a**–**d**) and 0.2 mm (**e**–**h**)

In Vitro Pathogenicity Assay

Further, to access the beneficial effect of applying bioagents on the fruits of the treated plants to restrict the pathogen ingression, the in vitro pathogenicity assay with unripe chili fruits was carried out. The fruits that were obtained from the treated plants expressed enhanced resistance to pathogen infection as compared to the fruits obtained from untreated plants. Most effective protection from *C. truncatum* progression was exhibited in the fruits obtained from plants that were given the consortial treatment of both BHUF4 and T16A *Trichoderma* strains (Fig. 2a) wherein the lesion diameter on the fruits was restricted to 8.7% of the total fruit area with a corresponding disease severity scale of 3. On the contrary, a lesion diameter of 26% (disease severity score-9) was observed in the infected fruits of the untreated control. BHUF4-treated fruits displayed significant management of pathogen control by restricting the infected fruit area to 12.1% with a corresponding disease severity score of 5 as opposed to 15.7% fruit area infection by T16A isolate (Fig. 2b).

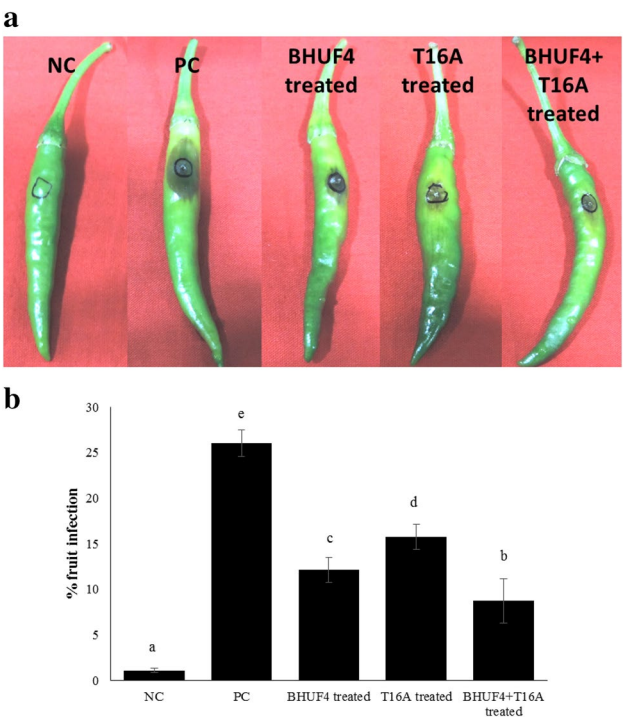

Fig. 2 Fruit infection assay. **a** The fruits from treated chili plants showing the lesion development under different treatments on inoculation with *C. truncatum* spores under in vitro conditions; **b** quantitative estimation of the percentage fruit infection as seen under different treatments on *C. truncatum* challenge. Data are mean ± SE of three separate measurements and significantly different at $P \leq 0.05$. Different letters indicate significant differences among treatment results taken at the same time interval according to Duncan's multiple range test at $P \leq 0.05$

Phenol Accumulation in Treated Chili Plants With and Without Pathogen Inoculation

Significant variation in phenol accumulation was recorded in the treated chili leaves in the presence and absence of pathogen when observed through HPLC. In absence of pathogen inoculation, a significant augmentation of shikimic acid (1313.62 ± 27.12 µg gFW^{-1} to 1663.81 ± 60.42 µg gFW^{-1}), gallic acid (27.55 ± 1.74 µg gFW^{-1} to 40.42 ± 2.60 µg gFW^{-1}), ferulic acid

(91.66 ± 3.55 µg gFW^{-1} to 152.8 ± 2.82 µg gFW^{-1}), and kaempferol (0.91 ± 0.05 µg gFW^{-1} to 2.14 ± 0.19 µg gFW^{-1}) was observed in BHUF4-treated host plants as compared to T16A-treated plants (Fig. 3a, Table 1). However, their dual application resulted in maximum accumulation of gallic acid (55.62 ± 1.34 µg gFW^{-1}), chlorogenic acid (59.52 ± 0.89 µg gFW^{-1}), syringic acid (11.40 ± 0.63 µg gFW^{-1}) and daidzein (0.107 ± 0.02 µg gFW^{-1}), when compared to the single *Trichoderma* isolate treated and also untreated control plants.

However, in the presence of the pathogen, additional accumulation of caumeric acid, capsaicin, myrcetin, salicylic acid, and quercetin was recorded in the chili leaves (Fig. 3b, Table 2). Maximum phenol accumulation was recorded in chili plants treated with the consortium of both the *Trichoderma* isolates (BHUF4 + T16A) upon *C. truncatum* challenge. The consortium-treated plants recorded a 2.59-fold increment in shikimic acid content, 1.6-, 1.13-, 2-, 1.16-, and 2.22-fold increment in concentrations of gallic acid, chlorogenic acid, syringic acid, p-caumeric acid, and myrcetin, respectively, as compared to positive control plants. The maximum accumulation of capsaicin (16.1-fold) was recorded in dual consortia-treated chili plants under the challenge of *C. truncatum*. The significant increment was recorded for ferulic acid (5.03-fold) and quercetin (5.36-fold) in chili plants treated with both BHUF4 and T16A *Trichoderma* isolates. However, the negative control plants recorded the absence of salicylic acid and kaempferol which was induced in treated plants;

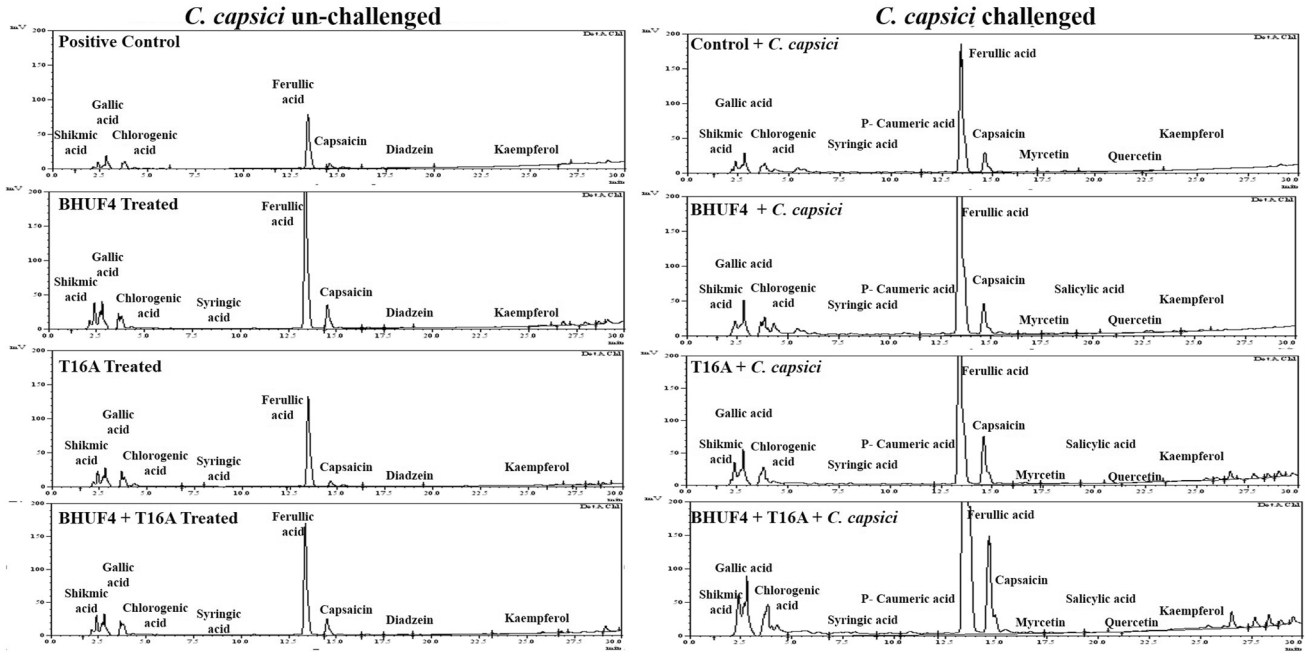

Fig. 3 The phenolic profile of treated and untreated chili leaves at 48 h after pathogen inoculation (hapi) in the absence (**a**) and presence (**b**) of *C. truncatum* challenge

Table 1 Effect of different *Trichoderma* isolates treatment on the accumulation of phenol derivatives in chili leaves without the *C. truncatum* challenge

Treatment	Shikimic acid[#]	Gallic acid[#]	Chlorogenic acid[#]	Syringic acid[#]	Ferulic acid[#]	Daidzein[#]	Kaempferol[#]
Untreated control	896.69 ± 1.75^{a}	18.31 ± 0.55^{a}	22.15 ± 1.59^{a}	N.D.	38.18 ± 1.34^{a}	0.10 ± 0.02^{a}	0.83 ± 0.03^{ab}
BHUF4 treated	1163.81 ± 60.42^{d}	40.42 ± 2.60^{c}	29.72 ± 1.31^{b}	8.23 ± 0.48^{a}	152.80 ± 2.82^{d}	0.30 ± 0.11^{b}	0.42 ± 0.19^{c}
T16A treated	1313.62 ± 27.12^{c}	27.55 ± 1.74^{b}	50.32 ± 1.00^{c}	8.81 ± 0.70^{a}	91.66 ± 3.55^{c}	0.97 ± 0.06^{c}	0.91 ± 0.05^{b}
BHUF4 + T16A treated	1165.83 ± 18.23^{b}	55.62 ± 1.34^{d}	59.52 ± 0.89^{d}	11.40 ± 0.63^{b}	71.39 ± 1.64^{b}	0.107 ± 0.02^{d}	0.62 ± 0.08^{a}

Different letters indicate significant differences among treatment results taken at the same time interval according to Duncan's multiple range test at $P \leq 0.05$

ND not detected

[#]Data represent mean ± standard error (SE) of three replicates

Table 2 Effect of different *Trichoderma* isolates treatment on the accumulation of phenol derivatives in chili leaves under *C. truncatum* challenge

Treatment	Shikimic acid[#]	Gallic acid[#]	Chlorogenic acid[#]	Syringic acid[#]	*P*-Caumeric acid[#]	Ferulic acid[#]	Capsaicin[#]	Myrcetin[#]	Salicylic acid[#]	Quercetin[#]	Kaempferol[#]
Untreated control	1145.65 ± 8.99^{a}	50.27 ± 4.05^{a}	49.16 ± 2.18^{a}	21.65 ± 1.61^{a}	28.04 ± 1.56^{a}	140.34 ± 2.16^{a}	6.37 ± 1.07^{a}	0.61 ± 0.17^{a}	N.D.	0.48 ± 0.05^{a}	N.D.
BHUF4 treated	1523.28 ± 31.12^{c}	67.17 ± 4.17^{b}	60.13 ± 2.20^{c}	23.55 ± 1.77^{a}	68.27 ± 4.07^{b}	311.51 ± 9.92^{c}	46.76 ± 1.69^{b}	2.27 ± 0.27^{c}	4.17 ± 0.35^{a}	2.50 ± 0.15^{b}	2.13 ± 0.19^{a}
T16A treated	1835.05 ± 41.82^{b}	54.98 ± 1.80^{a}	51.20 ± 3.45^{ab}	38.28 ± 1.84^{b}	65.17 ± 2.17^{b}	274.86 ± 6.48^{b}	76.64 ± 1.68^{c}	2.08 ± 0.46^{c}	3.75 ± 0.04^{a}	2.38 ± 0.15^{b}	5.61 ± 0.26^{b}
BHUF4 + T16A treated	2962.96 ± 39.25^{d}	80.44 ± 4.92^{c}	55.72 ± 1.63^{bc}	43.48 ± 2.05^{c}	32.56 ± 2.21^{a}	705.53 ± 6.92^{d}	102.54 ± 9.80^{d}	1.37 ± 0.17^{b}	4.89 ± 0.36^{b}	2.58 ± 0.66^{b}	6.22 ± 0.98^{b}

Different letters indicate significant differences among treatment results taken at the same time interval according to Duncan's multiple range test at $P \leq 0.05$

ND not detected

[#]Data represent mean ± standard error (SE) of three replicates

maximally, recorded in dual consortia-treated plants (4.88- and 6.22-fold increase, respectively).

The difference in the phenol accumulation due to different treatments in the chili plant with and without *C. truncatum* challenge has been compared in Table 3.

Gene Expression Study

The semi-quantitative expression studies for the genes corresponding to the defense response activation in *Capsicum* spp. depicted effective variations in expression of *PR1* (basic pathogen resistance gene) (AF053343), *PIK1* (pathogen-induced kinase gene) (GU295436), *GLU* (β-1, 3-glucanase gene) (AF227953), *Cdef* (chili defensive protein) (AF442388), *CHI* (chitinase), *CaSAR8.2* (*Capsicum* SAR gene) (AF313766), and *ABR1* (abscisic acid-responsive gene) (GQ373000) among the treatments as compared to the control plants. The expression profiles of the BCA-treated plants without pathogen challenge, exhibited almost similar gene expression profiles. Upon *C. truncatum* challenge, a significant increase was recorded in the expression profiles of the above-said genes with a maximum elevation in plants treated with the dual consortia of *Trichoderma* isolates (Supplementary Fig. S2).

Rt PCR for *PR1*, *PIK1*, *SAR*, *Cdef*, and *GLU* genes was carried out, to elucidate the mechanisms through which the two *Trichoderma* isolates aid in the differential enhancement of defense network of chili plant upon challenge with *C. truncatum*. In this context, the *SAR* and *Cdef* genes were not found to yield a significant difference in the expression pattern under different treatments (Supplementary Fig. S3). The phyllospheric *Trichoderma* isolate (BHUF4) recorded a 5.02-fold induction in *PR1* gene, 2.06-fold induction in *GLU* transcript, and 10.5-fold increment in the formation of *PIK1* transcript as compared to untreated and unchallenged control (Fig. 4). The significant fold induction by the rhizospheric *Trichoderma* isolate in all the genes was at par with the phyllospheric *Trichoderma* isolate except in the case of *PR1* transcript in which 79.3-fold increment was recorded in the treated chili plants.

The maximum induction of *GLU* and *PIK1* transcript was recorded in dual consortia-treated chili plants (5.45- and 23.95-fold increment, respectively). The data discussed here are for the pathogen-challenged treatments due to the involvement of the genes in the defense network of chili plants under pathogen stress.

Discussion

Biocontrol Treatment Strengthens the Mechanical Barrier

Plants are equipped with a plethora of defense mechanisms involving several lines of defenses for protecting them from

Table 3 Comparison of the accumulation of phenol derivatives due to different treatments of *Trichoderma* isolates in *C. truncatum* challenged and unchallenged chili leaves

Phenolic acids	Untreated control plants		BHUF4 treated		T16A treated		BHUF4 + T16A treated	
	Unchallenged	Challenged	Unchallenged	Challenged	Unchallenged	Challenged	Unchallenged	Challenged
Shikimic acid[#]	896.69 ± 1.75[a]	1145.65 ± 8.99[a]	1166.81 ± 60.42[d]	1523.28 ± 31.12[c]	1313.62 ± 27.12[b]	1835.05 ± 41.82[b]	1165.83 ± 18.23[c]	2962.96 ± 39.25[d]
Gallic acid[#]	18.31 ± 0.55[a]	50.27 ± 4.05[a]	40.42 ± 2.60[c]	67.17 ± 4.17[b]	27.55 ± 1.74[b]	54.98 ± 1.80[a]	55.62 ± 1.34[d]	80.44 ± 4.92[c]
Chlorogenic acid[#]	22.15 ± 1.59[a]	49.16 ± 2.18[a]	29.72 ± 1.31[b]	60.13 ± 2.20[c]	50.32 ± 1.00[c]	51.20 ± 3.45[ab]	59.52 ± 0.89[d]	55.72 ± 1.63[bc]
Synergic acid[#]	N.D.	21.65 ± 1.61[a]	8.23 ± 0.48[a]	23.55 ± 1.77[a]	8.81 ± 0.70[a]	38.28 ± 1.84[b]	11.40 ± 0.63[b]	43.48 ± 2.05[c]
p- Caumeric acid[#]	N.D.	28.04 ± 1.56[a]	N.D.	68.27 ± 4.07[b]	N.D.	65.17 ± 2.17[b]	N.D.	32.56 ± 2.21[a]
Ferulic acid[#]	38.18 ± 1.34[a]	140.34 ± 2.16[a]	152.80 ± 2.82[d]	311.51 ± 9.92[c]	91.66 ± 3.55[c]	274.86 ± 6.48[b]	71.39 ± 1.64[b]	705.53 ± 6.92[d]
Capsaicin[#]	N.D.	6.37 ± 1.07[a]	N.D.	46.76 ± 1.69[b]	N.D.	76.64 ± 1.68[c]	N.D.	102.54 ± 9.80[d]
Salicylic acid[#]	N.D.	N.D.	N.D.	4.17 ± 0.35[a]	N.D.	3.75 ± 0.04[a]	N.D.	4.89 ± 0.36[b]
Kaempferol[#]	0.83 ± 0.03[ab]	N.D.	0.42 ± 0.19[c]	2.13 ± 0.19[a]	0.91 ± 0.05[b]	5.61 ± 0.26[b]	0.62 ± 0.08[a]	6.22 ± 0.98[b]

Different letters indicate significant differences among treatment results taken at the same time interval according to Duncan's multiple range test at $P \leq 0.05$

ND not detected

[#]Data represent mean ± standard error (SE) of three replicates

copious phytopathogens. Phenols play an important role in the defense structure of plants. They act as antimicrobial compounds as well as precursors to structural polymers like lignin and signal molecules for expression of defense-related genes (Dakora, 1996; Madhavan et al. 2010). Previously, Saxena et al. (2016a) reported the elicitation of phenols in treated chili plants on *C. truncatum* attack, by using BHUF4 and T16A *Trichoderma* isolates. We further analyzed the effect of the treatments with these BCAs in the overall architecture of the plant, when attacked by *C. truncatum*. It was interestingly found that the treated plants exhibited enhanced lignin deposition upon *C. truncatum* attack, in comparison to the untreated control plants. This signifies that the bioagents strengthen the immunity of host plant by fortifying the mechanical barrier which further aids in enhanced protection against pathogenic attack. Increased lignin and suberin deposition in plants have been previously shown to be related to the strategy of the plant to protect itself from pathogen ingression (Rogers and Campbel 2004; Hano et al. 2006). The robust secondary growth in the stem by lignin deposition clearly indicates the probable might of the plant toward its protection from pathogen attack.

Trichoderma Treatment Reduces Disease Severity

The significant decrease in the disease severity scale and lesion development in the in vitro pathogenicity assays signifies that the bioagents primed plants were effective in providing protection against disease development. The results also relate to an important aspect of priming the plants with beneficial microbes, which signifies that the induced systemic response is incorporated even in the organs that develop later, rather than being limited to the source of its application (Shoresh et al. 2010). When the efficiency of T16A and BHUF4 was compared in terms of % fruit infection, it was interesting to find that BHUF4 was able to restrict the pathogen growth to 12%, than the 15% fruit infection by T16A isolate. The independent application of both the isolates significantly reduced the % fruit infection when compared with fruits from untreated control plants. However, the phyllospheric application of the BCA was more potent in restricting pathogen growth. The results support the proposed strategy of using dual consortia for better management of *C. truncatum* infection, as the dual consortia of the isolates were most effective in managing the fruit infection.

Phenol Accumulation was Recorded in Plants Treated with Trichoderma Strains

Elevation in the level of phenols in *Capsicum annum* L. upon treatment with biocontrol agents or their byproducts when challenged with different pathogens has been reported previously (Anand et al. 2009; Madhavan et al. 2010, Naveen

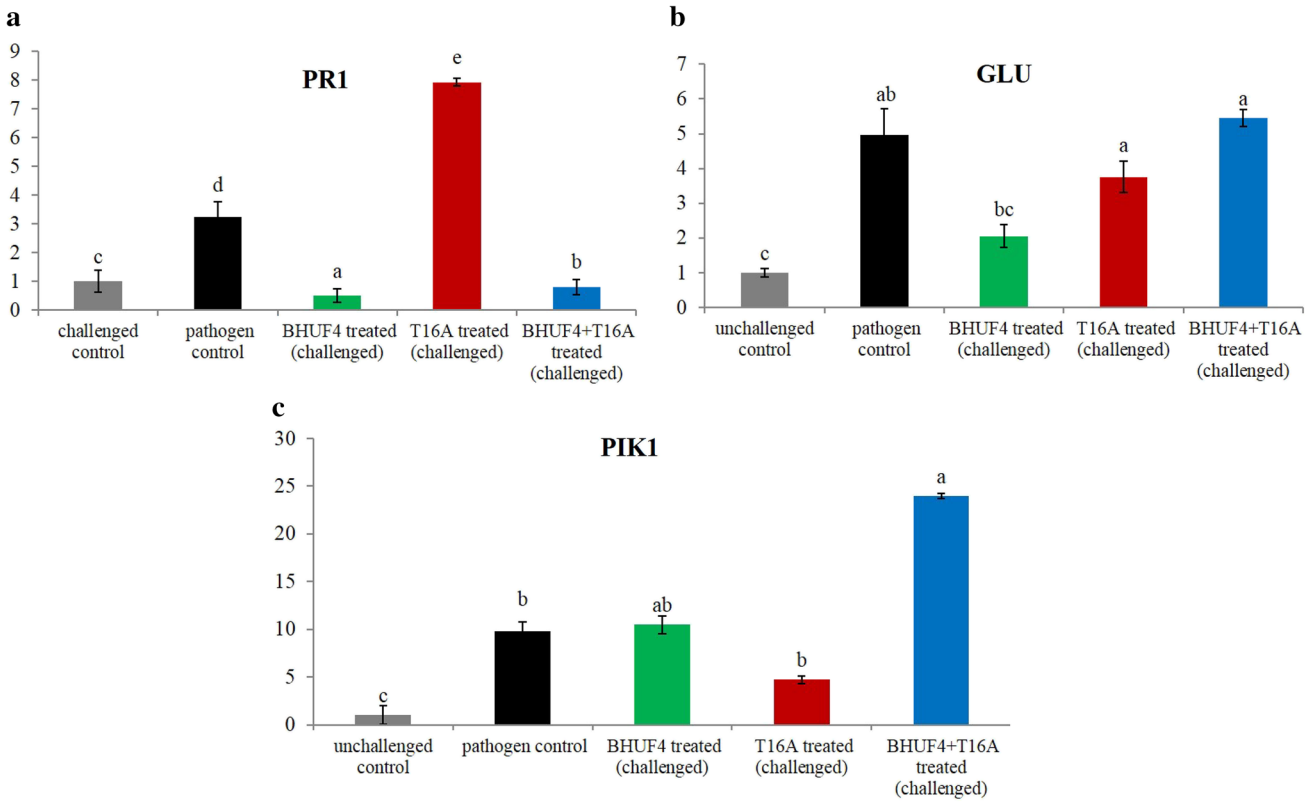

Fig. 4 RT-PCR determination of expression of selected genes in chili under different treatments. Data are mean ± SE of three separate measurements and significantly different at $P \leq 0.05$. Different letters indicate significant differences among treatment results taken at the same time interval according to Duncan's multiple range test at $P \leq 0.05$

et al. 2012). Most of the studies have focused on host–BCA interaction at the rhizospheric region with only a few reports targeting the study of host–microbe interaction at the phyllospheric region (Madhavan et al. 2010; Nantawanit et al. 2010).

The current study was aimed at assessing the role *of Trichoderma* isolate obtained from the phyllospheric region of chili plants in the enhancement of host immunity, particularly upon challenge with *C. truncatum*. The proposed strategy could be beneficial for managing the foliar diseases more effectively in chili, which has been a matter of concern for chili cultivators. This study supports the use of foliar sprays involving both phyllospheric and rhizospheric *Trichoderma* isolates for augmented plant growth and defense.

The elevated levels of accumulated biochemical enzymes involved in phenylpropanoid and shikimic acid pathway (Saxena et al. 2016a) supported the observed levels of phenols in the leaves of treated chili plants under *C. truncatum* challenge when observed in HPLC phenolic profiling. When the host chili plants were subjected to *C. truncatum* challenge, additional accumulation of p-caumeric acid, capsaicin, myrcetin, salicylic acid, and quercetin was recorded. All of these play a pivotal role in modulating the defense pathway of the plant, with some possessing anti-microbial properties or some being part of the shikimic acid pathway of plant defense (Makoi and Ndakidemi 2007; Chan et al. 2007).

Though phenols like shikimic acid, gallic acid, t-chlorogenic acid, syringic acid, ferulic acid, and kaempeferol were recorded in both *C. truncatum* challenged and unchallenged plants, their levels were significantly increased under the pathogen-challenged condition signifying their eminent role in plant defense response. Shikimic acid pathway is one of the important pathways linked with plant defense, the elevated levels of shikimic acid in the treated and challenged plants directly corelate with enhanced defense network of the host plant (Lattanzio et al. 2006). The elevated gallic acid content in the leaves further signified the ability of both rhizospheric as well as phyllospheric *Trichoderma* strains in strengthening the cell wall of the host by probable accumulation of lignin, suberin, and other polyphenolics meant to check pathogen ingression by acting as barriers (Sarma et al. 2002; Singh et al. 2014). This could further relate to the increased lignin accumulation recorded for dual-consortia-treated plants. The increased accumulation of t-chlorogenic acid and syringic acid in treated and challenged chili plants

indicates their role in elevating the defense response of the host plant (Maddox et al. 2010; Singh et al. 2013). The role of ferulic acid has been advocated as a precursor for phenylpropanoid derivatives like hydroxycinnamic acid amides (HCAA) and flavonoids which possess antimicrobial properties and fall in the category of phytoalexins (Dixon et al. 2002; Facchini et al. 2002). In the present study, maximum accumulation of ferulic acid in dual-consortia-treated plants supports the synergistic action of the microbes in eliciting the defense pathway of the chili plant against pathogen attack. Similarly, the most important alkaloid of *Capsicum* genus possessing antimicrobial capacity, capsaicin, displayed the highest accumulation in dual-consortia-treated plants under pathogen challenge. This strengthens the concept of the involvement of capsaicin in defense networking of pepper plants against pathogen attack (Stoessl et al. 1973; Naveen et al. 2012).

Defense-Related Genes Expression on Biocontrol Agent Treatment

The defense network in plants is cross linked to multiple components that get activated after pathogen attack. The efficient performance of the network demands a substantial commitment at the cellular level which includes genetic reprogramming. This incorporates the induction of defense-related genes in the plants including pathogen resistance genes (PR proteins) along with the expression of genes encoding specific metabolites or proteins responsible in defense setup of the plant system.

The most studied and characterized class of protein known to get induced in response to a pathogenic attack or any abiotic stress conditions constitutes the PR protein family (Kim and Hwang, 2011; Sarowar et al. 2005). Fourteen different families of PR proteins have been categorized in plants, some of which have been characterized for their biochemical properties like β-1, 3- glucanases (*PR-2*), chitinase (*PR-3, PR-4, PR-8, PR-11*), proteinase inhibitors (*PR-6*), and peroxidase (*PR-9*), all involved in defense induction under pathogen attack (van Loon and van Strien, 1999; van Loon et al. 2006). The results of the study are in agreement with the previous reports that illustrate the involvement of basic PR1 protein in response to pathogen stress (Silvar et al. 2009; Kim and Hwang 2011). Interestingly, T16A strain treatment resulted in an eight-fold increase in *PR1* expression, while BHUF4 treatment did not cause any significant increment in the *PR1* expression. Also, treatment with both BHUF4 + T16A showed an insignificant expression of *PR1*. The differential expression pattern of *PR1* in the treatments suggest that the two *Trichoderma* strains belonging to two different species (*T. harzianum* (T16A) and *T. asperellum* (BHUF4)) have dissimilar modes of eliciting defense responses in the host plant. Previous reports have also indicated that different species of *Trichoderma* may employ different strategies to check pathogen growth. For instance, Shoresh et al. (2005) have shown that the application of *T. asperellum* T203 strain modulates the genes involved in ethylene signaling while salicylic acid has been shown to regulate the proliferation of *T. harzianum* into the roots of *Arabidopsis thaliana* (Alonso-Ramírez et al. 2014).

In the present study, an increased accumulation of GLU mRNA transcript corresponding to the expression of β-1, 3 glucanase in leaves has been recorded in the dual-consortia-treated plants that highlight the ability of the beneficial microbes in eliciting the defense networking of the plant, providing it better protection against the pathogenic ingression (Esposito et al. 2000; Emani et al. 2003).

The protein kinases are crucial for pathogen recognition and subsequent activation of signal transduction accompanied by protein phosphorylation, thereby activating different transcription factors for inducing the pathogenesis-related (PR) genes leading to local and systemic response in different host plants like chili, rice, *Arabidopsis* (Veronese et al. 2006; Zhang et al. 2007; AbuQamar et al. 2008; Afzal et al. 2008; Li et al. 2009; Kim and Hwang 2011). The highest accumulation of *PIK1* mRNA level was obtained in the leaves of the dual-consortia-treated plants in comparison to pathogen-challenged control plants.

This supports our hypothesis, wherein the treatment of host plants at two different zones could provide rapid identification of the pathogen attack, thereby switching an array of gene expressions, leading to strengthened defense mechanism. Further, a significant increase in *PIK1* expression in BHUF4 treatment as opposed to T16A treatment suggests that foliar application of *Trichoderma* aids in prompt recognition of the pathogen as compared to rhizospheric application. The subsequent increase in phenol accumulation in the leaves further substantiates the reported role of protein kinases in strengthening the early events of plant defense responses (Kim and Hwang 2011).

Thus, from the phenolic profiling assays and gene expression studies, it may be hypothesized that treatment of host plants at two different spheres (rhizosphere and phyllosphere) leads to the elicitation of defense mechanisms by two different modes. The phyllospheric treatment induces the immediate effect by recognizing pathogen ligands and, thereby, triggering the *PIK1* gene cascading leading to probable activation of the SAR pathway, which eventually induces salicylic acid accumulation leading to accumulation of important phenols (Fig. 5). On the other hand, the rhizospheric treatment of *Trichoderma* induces the ISR pathway which is mediated by PR proteins, which also leads to the accumulation of important phenols and flavonols (Fig. 5). Thus, the results of the study propose that depending upon the application of the BCA, either the SAR or the ISR modes

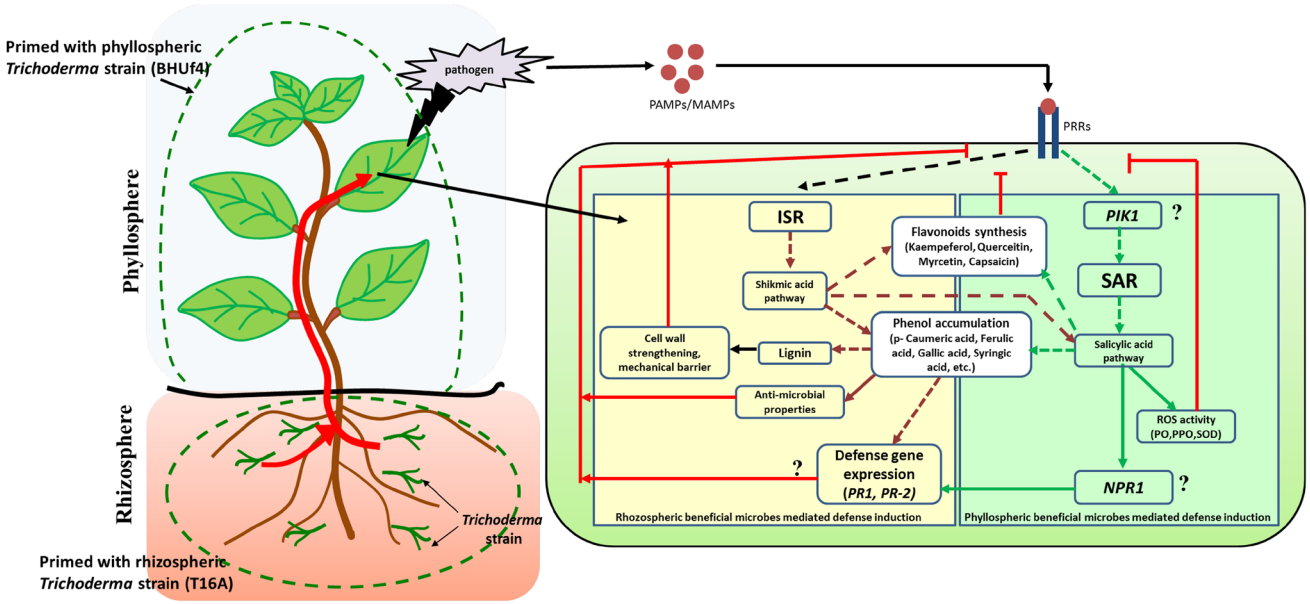

Fig. 5 A proposed model showing the elicitation of defense responses by beneficial *Trichoderma* strains when applied at the rhizospheric region and phyllospheric region of the host plant. *ISR* induced systemic responses, *SAR* systemic acquired resistance, *PIK1* pathogen-induced kinase1, *PR1, PR2* pathogen resistance genes; *NPR1* natriuretic peptide receptor 1

of defense signaling gets activated which further strengthens the host plant against the pathogenic attack.

Conclusion

Anthracnose or fruit rot caused by *C. capsici* (Sydow), Butler and Bisby have been major restraints in chili production causing a noteworthy loss in profitable cultivation and seed production among the major chili producing Asian countries including India. Being one of the ten most destructive pathogens of chili affecting crop yield worldwide, *C. truncatum* has been linked with anthracnose of chili leading to both pre- and post-harvest losses. Anthracnose may affect the aerial parts of the crop which may then act as inocula for the spread of the disease in fields either through water splashes or through winds. Many management strategies have been employed for its control yet maximum reduction has been obtained only through the use of fungicides. The use of BCAs for its control has not been so effective. The prevailing concept of sustainable agriculture further necessitates the need for developing integrated management strategies that use BCAs. The results of the present study suggest the use of foliar sprays of phyllospheric competent *Trichoderma* strains in combination with seed treatment to increase the expression of defense-related genes of a complex signaling network against the pathogen. This strategy could prove useful in managing the spread of the disease as the plants would be reinforced with the beneficial cover of *Trichoderma* spp. even at the aerial regions. Also, better prevention could be obtained by direct check on the growth of the pathogen on the leaf and fruit surfaces. Also, the results of the study hint at the different mechanisms by which the differential application of the BCA can be perceived by the host plant, to elevate its defense program against the pathogen attack. However, a further molecular study is required to delineate the different modes by which the BCA application actually reprograms the defense network of the host plant.

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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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