A novel *Trichoderma* fusant for enhancing nutritional value and defence activity in chickpea

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Abstract In recent years, due to the rise in food consumption, much of the attention has been focused to increase the yield of the agricultural crops which resulted in compromised nutritional quality. Efforts have to be undertaken to enhance the nutritional attributes of legumes, cereals and staple food crops by increasing amino acids and mineral content. In the present study, we evaluated a protoplast fusant (*H. lixii* MTCC 5659) for its ability to enhance nutritional value and defence activity in chickpea. Essential amino acids; methionine (9.82 mg kg⁻¹ dw), cysteine (2.61 mg kg⁻¹ dw), glycine (11.34 mg kg⁻¹ dw), valine (9.26 mg kg⁻¹ dw), and non-essential amino acids; aspartic acid (39.19 mg kg⁻¹ dw) and serine (17.53 mg kg⁻¹ dw) were significantly higher in seeds of fusant inoculated chickpea. Fusant significantly improved accumulation of mineral nutrients i.e. Cu (157.73 mg kg⁻¹ dw), Co (0.06 mg kg⁻¹ dw), Ni (1.85 mg kg⁻¹ dw), Zn (157.73 mg kg⁻¹ dw) and S (16.29 mg kg⁻¹ dw) in seeds. Biocontrol and defence activities of chickpea increased from 20 to 35% in fusant inoculated plants suggesting its potential to ameliorate biotic stress. To the best of our knowledge, this is the first report of an increase in amino acids and mineral content of chickpea by fusant inoculation.

Keywords Protoplast fusant · *Trichoderma* · Amino acids · Nutritional value · Biocontrol

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Introduction

The Food and Agriculture Organization (FAO) estimates that around 850 million people worldwide suffer from malnutrition, due to insufficient protein, essential amino acids (EAAs), iron, zinc in the diet and its deficiency affects especially in women and children of developing countries (Gegios et al. 2010). An efficient way to combat this problem is to enhance nutrient quality of legumes, cereals and staple food crops by increasing essential and other amino acids and mineral nutrient content by using several classical breeding and modern genetic engineering approaches (Hefferon 2015), protoplast fusion being one of them. Through protoplast fusion technology, various efforts have been made towards the development of fusant fungal strains demonstrating higher plant growth promotion, efficient biocontrol against pathogen attack and production of commercial enzymes (Patil et al. 2015).

Trichoderma is a genus of asexually reproducing, free-living fungi that are common in soil and root ecosystems. It is one of the most exploited fungal bio-control agents in the field of agriculture for the management of crop diseases caused by a wide range of fungal phytopathogens (Lamdan et al. 2015). Apart from its ability to enhance growth and yield of crops (Mishra and Nautiyal 2009), *Trichoderma* spp. also alters the activity of industrial enzymes; however, its effect on minerals and amino acids have not been explored in detail. Though the available strains of *Trichoderma* obtained through selection or mutation process, have one or more desirable traits, however, none of them possess most of the attributes to utilize the full potential of the beneficial fungi. To explore the maximum potential of *Trichoderma* spp., protoplast fusion technology constitutes an alternative to the manipulation of *Trichoderma* as potent enzyme producer, and biocontrol agent against diverse

plant pathogens showing enhanced antioxidant activities. It has been demonstrated that intra-strain crossing in *Trichoderma harzianum* via protoplast fusion enhances its chitinase productivity and biocontrol activity (Ahmed and Fatma 2007).

Chickpea (*Cicer arietinum* L.) is one of the major food legumes grown in many countries including India, as a major source of proteins, essential amino acids and minerals. Collar rot disease caused by *Sclerotium rolfsii* is one of the major diseases affecting growth and yield of chickpea (Ojaghian et al. 2014). Though several *Trichoderma* based approaches have been utilized to enhance growth and disease resistance against *S. rolfsii* in chickpea (Saxena et al. 2015), the role of protoplast fusant as more efficient biocontrol agent is yet to be elucidated.

By keeping in mind the potential of protoplast fusion technology, the aim of the present study was to select a novel protoplast fusant, as a future potential non-genetic engineered biotechnological tool to enhance amino acids, mineral contents and yield of the leguminous plant chickpea. The fusant was selected on the basis of carbendazim tolerance up to 10,000 ppm and 70 °C temperature for up to 120 min. In the above background, its potentiality has been evaluated in relation to quantification of nutritional value, yield parameters and biocontrol abilities against collar rot disease of chickpea (*C. arietinum* L.).

Materials and methods

Fungal isolates

Trichoderma isolates used in this experiment were obtained from diesel contaminated soil around CSIR-NBRI farm workshop, Lucknow. *Trichoderma* isolates, parent 1 (carbendazim tolerance up to 10,000 ppm and temperature tolerance up to 30 min at 60 °C), and parent 2 (temperature tolerance up to 120 min at 70 °C and cannot grow at 10 ppm carbendazim) were identified according to the identification key (Rifai 1969), as *H. lixii* (MTCC 5660) and *T. viride* (MTCC 5661), respectively. The fusant (*H. lixii* MTCC 5659) obtained from the protoplast fusion of parent 1 and 2 showed growth on the carbendazim (100 ppm) and tolerated temperature tests at 70 °C (Mishra and Nautiyal 2014). Fungal isolates were maintained on potato dextrose agar (PDA, HI-MEDIA Laboratories Pvt. Ltd., Mumbai, India) as described earlier (Mishra and Nautiyal 2009).

Plant growth promotion assay

The trials were carried out in the greenhouse grown seeds of chickpea (*C. arietinum* cv.116 Radhey) in pots (30 cm in diameter) and inoculums of parental strains and fusant were individually inoculated at the rate of 1% w/w, while un-inoculated seeds served as control, as described earlier (Mishra and Nautiyal 2009). The unhealthy seedlings were removed from the pot and maintained two seedlings per pot on the 15th day. For chlorophyll estimation, plants were grown for 4 months, while for rest of the parameters evaluated, plants were grown up to 5 months, as described earlier (Mishra and Nautiyal 2009). Six pots constituted a replicate and there were six replications per treatment.

Estimation of amino acids

The pico tag method was followed for the estimation of amino acids on HPLC system (Water, USA). Homogenized 200 mg seeds were hydrolyzed in 10 ml of 6 N HCl for 1 h at 150 °C in an oven. Samples were then filtered for further analysis. Samples (10 µl) and standard (2.5 µmol ml⁻¹ in 0.1 N HCl) were dried in a vacuum oven at 55 °C for 30 min at 75 millitorr. This was re-dried twice by adding 20 µl of redrying solution (Ethanol: Triethylamine: Water, 2:1:2). Then samples were derivatized by adding 20 µl of derivatization reagent (Ethanol: Triethylamine: Water: Phenylisothiocyanate, 7:1:1:1) and again vacuum dried. These samples were diluted to 1 ml with pico tag sample diluent, filtered (0.22 µm syringe filters) and injected (20 µl) into the system. The samples were analyzed on a pico tag column eluted with a gradient of (A) containing sodium acetate trihydrate (19 g), triethylamine (0.5 ml) and (B) acetonitrile (60%) in a 1 litre Milli Q water (Bidleingmeyer et al. 1984).

Estimation of mineral elements

For analysis of macro and micro elements viz. Fe, Cu, Se, Ni, Zn and Co in chickpea seeds, samples were oven dried at 70 °C and wet digested in HNO₃ (69%, ACS quality Germany) at 120 °C as described earlier (Dwivedi et al. 2010). The multi-element calibration standard -2A (MECS-2A, part no. 8500-6940) was used for standardization of Fe, Cu, Se, Ni, Zn and Co. Rhodium (Rh) was used as an internal standard during analysis. These elements were quantified by Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7500 cx). The recoveries of the standard reference material were Fe 92 ± 6%, Cu 92 ± 6%, Se 86 ± 6%, Ni 84 ± 6%, Zn 88 ± 5% and Co 80 ± 8%. Phosphorus and sulphur were quantified through a spectrophotometric method by using the procedure of Jackson (Jackson 1967).

Biocontrol activity of fusant and parents against *S. rolf sii*

Antagonistic effect of the parents and fusant strain were evaluated against *S. rolf sii* (Mishra et al. 2011). A bid of 6 mm of parents and fusant *Trichoderma* strains, and *S. rolf sii* was cut and placed at opposite edge of the PDA plate separately. Control plates were prepared for each fungus by inoculating the plates with the respective fungus. The plates were incubated at 28 °C till the control plate attained maximum growth and subsequently the reduction in *S. rolf sii* growth was compared with control plate.

Further, green house experiment was performed to evaluate the biocontrol ability of fusant and parent *Trichoderma* isolates. The following treatments were examined: (a) Control, (b) fusant + *S. rolf sii* (c) parent 1 + *S. rolf sii* (d) parent 2 + *S. rolf sii* (e) *S. rolf sii* infection. The pathogen *S. rolf sii* was isolated by collecting sclerotia produced on infected collar region of chickpea plants. The freshly grown culture of *S. rolf sii* was inoculated in sterile cornmeal and incubated for 4 weeks at 25 °C for proper proliferation (Abeygunawardena and Wood 1957). After incubation, the cornmeal based *S. rolf sii* inoculum (50 gm per kg of soil) was mixed with soil (as per treatment). Plastic pots (15 × 10 cm) were used for sterile soil assay and 1.5 kg of the potting mixture was filled in each pot. For biocontrol treatment, fusant and parent isolates of *Trichoderma* were grown in potato dextrose broth (PDB) individually and incubated for 1 week at 24°C. After incubation, the broth culture of fusant and parent strain was centrifuged at 12,000×g for 5 min to obtain cell culture. The cell suspension was maintained up to 3×10^6 - spore ml⁻¹ in sterile distilled water. Surface sterilized chickpea seeds were treated with the suspension of fusant and parent *Trichoderma* isolates.

Biochemical assays of defence activities

After observing superior biocontrol ability against *S. rolf sii*, plant test was carried out with chickpea as a host plant. Further disease indices were assessed in terms of physical parameters, chlorophyll content, and defence activities of plants. Total chlorophyll content in plants was determined by the protocol of Wellburn (Wellburn 1994). Defence parameters of plants including lipid peroxidation (LPX), total phenolics and antioxidant enzymes, superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX), guaiacol peroxidase (GoPX), phenylalanine ammonia lyase (PAL) and polyphenol peroxidase (PPO) were measured following the protocols of Heath and Packer (1968), Mallik and Singh (1980), Beauchamp and Fridovich (1971), Nakano and Asada (1981), Lavania et al. (2006) respectively.

Physiological performance of chickpea in the presence of fusant and parents during biotic stress

The gas exchange parameters like stomatal conductance (gs), transpiration rate (E), and net photosynthesis rate (A) were monitored in fully expanded chickpea leaves with Li-Cor 6400 gas exchange portable photosynthesis system (Li-Cor, Lincoln, Nebraska, USA). The CO₂ levels inside the leaf cuvette were maintained at 400 ppm, photosynthetic photon flux density (PPFD) was 600 μmol m⁻² s⁻¹, leaf temperature was 30 °C, and leaf-air vapour pressure deficit was < 3.0 kPa.

Scanning electron microscopy

SEM analysis was performed to confirm the biocontrol activity of fusant against collar rot disease. Transverse sections of roots were cut and transferred to a small cap tube vials, fixed for 1 h in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma). This was followed by 40 min treatment in 1% (w/v) osmium tetroxide (OsO₄, Sigma). The root cells were dehydrated by sequential passage through increasing concentration of ethanol (30–100%) in 10% increments, dried in a critical point dryer, coated with gold-palladium for 60 s in a Pelco 3 sputter coater (SC 7620, mini sputter coater, Quorum Technology Ltd., UK), and visualized using a scanning electron microscope (Quanta 450FEG, FEI, The Netherlands) as described earlier (Pathan et al. 2008).

Statistical analysis

All the results reported are means of six replicates. One way analysis of variance (ANOVA) was done using the SPSS 16 computer software and Critical Difference (CD) was calculated at 0.05 probabilities.

Results and discussions

Plant growth promotion

All the three *Trichoderma* strains, parent 1, 2, and fusant were able to increase chlorophyll content, shoot weight, root weight, the number of pods and total seed weight as compared to control (Fig. S1). Among the three inoculated *Trichoderma* spp., fusant was able to enhance the physiological efficiency of chickpea to the maximum. Dry weight of shoot (43.2 and 43.1%), root (12.9 and 19.6%), number of pods (18.1 and 34.4%), seed weight (101.8 and 90.2%), and yield (6.2 and 3.2%) were increased in plant inoculated with fusant, as compared to parent 1 and parent 2

respectively (Fig. 1). Chlorophyll content (23.6%) was higher in fusant treated plant in comparison to parent 1; however, no significant difference in chlorophyll content of fusant and parent 2 treated plant was observed (Fig. 1). *Trichoderma* spp. is well known plant growth promoter and biocontrol agent (Brotman et al. 2013). They can enhance the productivity of crops by several direct and indirect mechanisms including mineral solubilization, phytohormone production and increasing nutrient uptake efficiency (Contreras-Cornejo et al. 2016). Our results suggested that to enhance the capabilities of *Trichoderma* spp., protoplast fusion technology can emerge as a potential non-genetically engineered approach to increase plant yield. Apart from this study, in a recent study, a consortium of microbes

which includes *Trichoderma asperellum* as one of its constituents was applied to chickpea and a similar result regarding plant growth and uptake of minerals along with enhancement of nutrient quality was obtained (Yadav et al. 2017).

Estimation of amino acids

Inoculation of parental strains and fusant affected amino acids (AA) profile differentially, the effects were more pronounced in fusant inoculated seeds, as compared to parental strains (Fig. 2). In general, the levels of Met, Cys, Asp, Gly, Ser and Val were significantly higher in seeds of fusant inoculated chickpea plants than the control and both

Fig. 1 Enhancement of plant biomass and chlorophyll content by fusant as compare to parental strains. All the values are the mean of six replicates $\pm$ SD ANOVA at $p \leq 0.01$ significance level. Different

letters indicate significant difference between control, parental strains and fusant (DMRT, $p < 0.05$)

Fig. 2 Effect of *Trichoderma* parental strains and its fusant inoculation on amino acids content in chickpea seeds. All the values are the mean of six replicates $\pm$ SD ANOVA at $p \leq 0.01$ significance level.

the parental strains, while the essential amino acid (EAAs); Met (24.14 and 20.49%), Gly (20.25 and 36.79%), and Cys (57.52 and 107.1%), significantly increased as compared to

Different letters indicate significant difference between control, parental strains and fusant (DMRT, $p < 0.05$)

parent 1 and 2 respectively. The percent induction of nonessential amino acids (NEAAs); Val (47.21 and 76.04%), Asp (29.8 and 20.6%) and Ser (7 and 3.4%) were

found in fusant inoculated seeds as compared to parent 1 and 2 respectively (Fig. 2). Chickpea proteins are considered to be a suitable source of dietary protein in several developing countries due to the excellent balance of EAAs composition.

Amino acids are the building blocks of proteins. The protein content is influenced by genetics, environment, nitrogen (N) fertilization and application of plant growth promoting microbes (Munees and Kibret 2014). Thus, it seems that parental strains and fusant may potentially enhance the N accumulation in chickpea plants and subsequently, the amino acids composition. In the present study it was noticed that the sulphur content of the chickpea seeds increased, thereby increasing the content of sulphur containing amino acids (Met and Cys). To the best of our knowledge, the influence of fusant inoculation on level of S and sulphur containing amino acids in chickpea is novel observation as legumes are often poor in S containing amino acids such as Met and Cys. Arg, Leu and Lys form a major part of amino acids of chickpea. Lys content (41%) was higher in fusant in comparison to control and at par with parent 2 further indicating its more N enhancing capability subsequently leading to higher level of amino acids in chickpea.

Food insecurity is primarily caused by low productivity and poor access to farming resources and technology, as well as poor access to and consumption of key nutrients. Enhanced key amino acids by inoculation of protoplast fusant of *Trichoderma* bear the potential to bring changes in agricultural practice and human nutrition interventions.

Estimation of mineral elements

Both parental strains and the fusant significantly affected the mineral nutrients uptake in chickpea seeds. In general, the sulphur and micronutrients such as Cu, Ni, Zn and Co were significantly higher in seeds of fusant inoculated chickpea plants than control and parental strains. The induction percentage of nutrients like Cu (ca. 5 and 109.98%), Co (ca. 172.7 and 100%), Ni (ca. 311.1 and 83%), Zn (ca. 94.7 and 26.9%) and S (14.9 and 45.9%) were significantly higher in fusant inoculated seeds as compared to the parent 1 and parent 2 respectively (Fig. 3). Interestingly, induction of Ni and Zn was more pronounced (Ni, 96% and Zn, 39%) in fusant inoculated chickpea plants (grains) as compared to other treatments. Although P accumulation increased in all parental strains and fusant treated chickpea seeds in comparison to control, however, S was always higher in the fusant inoculated seeds than in control and parents.

Myriads of mechanisms by which *Trichoderma* promotes plant growth have been suggested, such as the

production of plant growth regulators, solubilization of insoluble trace nutrients in the soil (Verma et al. 2014) and increased uptake and translocation of less available minerals (Mehetre and Mukherjee 2015). In the present study, micronutrients such as Fe, Zn, Cu, Co and Ni were found to be significantly increased in the chickpea seeds following fusant strain inoculation. In chickpea, *Trichoderma* inoculation has been reported to increase AA and mineral content by modulating arsenic speciation and accumulation (Tripathi et al. 2015). Enhanced sulphur accumulation in chickpea seeds obtained through fusant inoculation is reported here for the first time. During the present study, we also observed that the induction of most of the trace minerals was higher in the fusant treated chickpea seeds, which may be due to more solubilisation of insoluble nutrients in soil facilitating its availability to chickpea (Verma et al. 2014). Fusant strain had greater potential for improved Cu (ca. 5 and 109.98% as compared to parent 1 and 2) uptake and it seems that this property is likely to be derived from the higher Cu accumulating inoculants parent 1. In the present study, Fe accumulation in seeds has been found to be significantly higher in fusant inoculated plants than inoculated either with parents or un-inoculated chickpea. Our observations in chickpea seeds are consistent with Fe uptake in wheat where a *Trichoderma* T-34 strain facilitates the accumulation of Fe in the aerial parts of the plants (de Santiago et al. 2011). It may appear that mineral nutrient content such as Fe, Cu, Ni, Zn, Co and S were always higher in chickpea seeds in fusant inoculated plant as compared to parent strains. The higher mineral content of chickpea inoculated with fusant can enrich nutritional value to combat malnutrition.

Biocontrol activity of fusant and parents against *S. rolfii*

In plate assay, all the three *Trichoderma* strains were able to inhibit the growth of *S. rolfii*, however, the inhibition was maximum by fusant, followed by parent 2 and parent 1 (Fig. S2). Further, plant test was also conducted to evaluate the biocontrol activity of all the three *Trichoderma* strains *in vivo* with chickpea as a host plant. In accordance with the earlier studies, all the three strains of *Trichoderma* were potent enough for controlling the collar rot disease of chickpea caused by *S. rolfii* (Fig. 4a). Least mortality rate was observed in fusant (16.7%) which was followed by parent 2 (37.5%) and parent 1 (45.8%), highest mortality rate was observed in infected control (75.0%) (Fig. 4b). It was very clear from the visual observation that fusant inoculation was not only able to suppress the occurrence of collar rot successfully but was also able to enhance plant growth effectively, while the parent strains could only

Fig. 3 Effect of *Trichoderma* parental strains and its fusant inoculation on mineral nutrient contents in chickpea seeds. All the values are the mean of six replicates $\pm$ SD ANOVA at $p \leq 0.01$ significance

control the severity of disease. A significant increase in fresh weight (43.27 and 29.58%), dry weight (56.05 and 33.15%) and chlorophyll content (14.02 and 18.16%) in

level. Different letters indicate significant difference between control, parental strains and fusant (DMRT, $p < 0.05$)

fusant inoculated chickpea was observed in comparison with parent 1 and parent 2 inoculated chickpea plants (Table 1).

Fig. 4 **a** Biocontrol effect of *Trichoderma* strains against *S. rolfisii* in chickpea (**a**) Control, (**b**) fusant inoculation, (**c**) parent 1 inoculation, (**d**) parent 2 inoculation, (**e**) *S. rolfisii* infection. **b** Mortality rate

Trichoderma spp. are well known biocontrol agents which apply a myriad of mechanisms including mycoparasitism, induction of induced systemic resistance in plants, production of antimicrobial secondary metabolites and compounds to kill the plant pathogen (Mukherjee et al. 2008). The protoplast fusants have widely been employed to enhance the biocontrol and extracellular enzyme activities of parent *Trichoderma* strains (Fahmi et al. 2012). As a result of protoplast fusion, the content of sulphur and amino acids increased, which might be utilized in the synthesis of glutathione and secondary metabolites involved in plant defence. This is probably one of the reasons which enhanced the biocontrol potential of fusant with respect to the parents. In an earlier study in cotton, an enhanced biocontrol activity against *R. solani* was obtained in *T. koningii* and *T. virens* fusant (Hanson and Howell

2002). Similarly, in another study, a *T. harzianum* fusant showed a higher growth inhibition rate (100%) as compared to the parent strain against *Rhizoctonia solani* (Kowsari et al. 2014).

Biochemical assays of defence activities

For evaluation of plants stress responses during the infection of *S. rolfisii*, LPX was assessed. It was highest in infected chickpea plants as compared to other treatments at different time intervals of 1, 2, 3 and 4 days (Fig. 5a). LPX is one of the most damaging processes known to occur in the living organism and is frequently used to assess the stress regarding production of thiobarbituric acid reactive substances (TBARS) (Gill and Tuteja 2010). Though inoculation of all the three *Trichoderma* strains to chickpea was able to ameliorate biotic stress by reducing LPX activity, the maximum reduction (50.56%) in comparison with infected plants was demonstrated by fusant at day 4 of infection.

SOD, POX, APX, and GoPX activities of parents and fusant inoculated plants were compared with *S. rolfisii* infected plants at different time intervals of 1, 2, 3 and 4 days. Fusant inoculated plant showed a maximum increase in stress enzyme activities after the second day of *S. rolfisii* infection. SOD (17.04 and 7.9%), APX (31 and 23%), GPX (31.49 and 23.06%), and POX activities (26.12 and 3.2%) were maximum in fusant inoculated chickpea plants, as compared to parent 1 and 2 respectively (Fig. 5b–e). Though defence activities were found minimum in *S. rolfisii* infected plants.

To combat the pathogenic attack and subsequent production of the high level of ROS generated, plants are equipped with many antioxidant enzymes. SOD, APX, GPX and POX are the major enzymes involved in amelioration of stress in plant defence machinery (Gill and Tuteja 2010; Singh et al. 2013). The cascade of enzymes having ROS scavenging potential was significantly up-regulated in *Trichoderma* inoculated plants, however the fusant inoculation increased the defence enzyme activity to the maximum among all the treatments. Higher accumulation of sulphur and other nutrients in fusant might have resulted in enhanced production of glutathione and

Table 1 Effect of *Trichoderma* parental strains MTCC 5660, MTCC 5661 and its fusant MTCC 5659 inoculation on total chlorophyll, fresh weight and dry weight of infected chickpea plants

Treatments	Total chlorophyll (mg g ⁻¹ FW)	Fresh weight (in gm)	Dry weight (in gm)
Control	3.195 ± .083	2.005 ± .51	1.295 ± .63
Infected control	2.281 ± .26	1.37 ± .30	0.893 ± .54
Parent 1	5.422 ± .524	2.417 ± .79	1.570 ± .80
Fusant	6.184 ± .53	3.461 ± 1.3	2.456 ± 1.6
Parent 2	5.23 ± .64	2.679 ± 1.01	1.840 ± .99

All the values are the mean of six replicates ± SD ANOVA at $p \leq 0.01$ significance level

Fig. 5 Enhancement of defence activities in chickpea by fusant as compared to parents during biotic stress. **a** LPX, **b** SOD, **c** APX, **d** GoPX, **e** POX. All the values are the mean of six replicates

± SD ANOVA at $p \leq 0.01$ significance level. Different letters indicate significant difference between control, parental strains and fusant (DMRT, $p < 0.05$)

cofactors involved during scavenging of ROS, making it more potent to ameliorate biotic stress as compared to the parents.

Phenylpropanoid activities

Accumulation of total phenolic compounds (344.7%) and PPO activity (33.52%) was maximum in plants treated with fusant on day 3 after *S. rolfisii* infection (Fig. 6a, b). On day 2 after *S. rolfisii* infection, a significant increase of 41.10 and 47.40% was observed in the activity of PAL (Fig. 6c) in plants treated with fusant in comparison to parent 1 and 2 respectively.

The phenylpropanoid metabolism in plants generates a wide range of secondary metabolites (Dicko et al. 2005),

which can further be utilized against the pathogens. Phenylalanine ammonia-lyase (PAL) is the first enzyme of this pathway which converts phenylalanine into trans-cinnamic acid (Singh et al. 2013). PPO is also an important enzyme involved in phenol metabolism which oxidizes endogenous phenolic compounds into o-quinones, which are toxic to invading pathogens and pests. Increase in content of amino acids after inoculation of fusant to plant can play a key role in enhancing synthesis of phenolics. Amino acids are the precursors of proteins and secondary metabolites. An elevated level of AA, in fusant treated plants, enhanced the activity of enzymes involved in secondary metabolites synthesis that helped the plant to cope up with biotic stress in a more efficient manner than the parental strains treated plants.

Fig. 6 Effect of fusant in phenolics enhancement as compared to parents in chickpea during biotic stress **(a)** total phenolics, **(b)** PAL activity, **(c)** PPO activity of infected chickpea plants. All the values

are the mean of six replicates ± SD ANOVA at $p \leq 0.01$ significance level. Different letters indicate significant difference between control, parental strains and fusant (DMRT, $p < 0.05$)

Fig. 7 Physiological performance of chickpea in presence of fusant and parent strains, during biotic stress. All the values are the mean of six replicates $\pm$ SD ANOVA at $p \leq 0.01$ significance level. Different

letters indicate significant difference between control, parental strains and fusant (DMRT, $p < 0.05$)

Fig. 8 A Scanning electron micrographs of epidermal layer of vascular bundle at collar region (a) control, (b) infected control, (c) infected plant inoculated with fusant MTCC 5659. **B** Scanning electron micrographs of suppression of *S. rolfii* mycelia by fusant spores localized at different regions of vascular bundles of collar region in chickpea. (a) Control, (b) *S. rolfii* infected, (c) infected plant inoculated with fusant

FM= Fungal mycelium; RM= Ruptured Mycelium; TS= *Trichoderma* spores

Physiological performance of chickpea in the presence of fusant and parent strains, during biotic stress

Inoculation of fusant also resulted in maximum increase in stomatal conductance (42.22 and 34.75%), transpiration rate (34.54 and 25.45%) and net photosynthetic rate (21.08 and 16.21%) in comparison with the plants inoculated with parent 1 and 2 respectively (Fig. 7), which resulted in better amelioration of biotic stress. Naveed et al. (2014) in their study also found that increase in stomatal conductance and net photosynthetic rate enhanced abiotic stress resilience of maize through endophytic colonization by

Burkholderia phytofirmans PsJN and *Enterobacter* sp. FD17.

Scanning electron microscopy

Collar rot disease of chickpea is one of the most devastating fungal diseases which damage the collar region resulting in significant loss in grain yield (Ojaghian et al. 2014). After observing the highest biocontrol activity of fusant in comparison with the parent strains (Fig. S2), the collar region of chickpea was observed in scanning electron microscopy to determine the anatomical differences between control, pathogen challenged and infected plants inoculated with fusant.

A well-arranged epidermal layer was observed in control plant (Fig. 8Aa), while the epidermal layer was completely damaged after infection of *S. rolfsii* (Fig. 8Ab). A clear halo zone formed in endodermis while the outer epidermis was completely deformed in infected collar region. The epidermal structure of fusant inoculated plants was similar with the control, showing the efficacy of fusant to act as a potent biocontrol agent (Fig. 8Ac).

To get a detailed insight into the anatomical differences among the three treatments, sections of vascular bundles were observed. It was found that control plant had a well arranged xylem, metaxylem, phloem and pith regions (Fig. 8Ba). The invasion of *S. rolfsii* was observed in vascular bundles of the plant by presence of mycelia and structural aberrations caused (Fig. 8Bb). As evident from the SEM images, inoculation of fusant was efficient enough to ameliorate biotic stress by colonization in vascular bundles and disrupting mycelia of *S. rolfsii* (Fig. 8Bc).

Conclusions

The focus on value-added traits, especially improved nutrition is undoubtedly one of the areas of great interest. Obtained fusant has properties to enhance amino acids and nutrient content, plant growth, defence activities in chickpea which could become a powerful tool to combat biotic stress for plant development. Further application of this fusant may open new opportunities in pharmaceutical and agricultural industries for sustained release of nutrients, pathogen control in an economical approach.

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