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Elicitation of native bio protective microbial agents associated systemic defense responses and plant growth promotion against bacterial stalk rot pathogen in sorghum (*Sorghum bicolor*)

Sujata Singh Yadav[1*], Anshul Arya[1], Vishal Singh[2] and Yogendra Singh[1]

Abstract

Dickeya dadantii is the causal agent of bacterial stalk rot and one of the most destructive and widespread diseases of the sorghum in the world. Here, we explored microbe-based approaches for managing this destructive pathogen, intending to provide alternatives for integrated disease management. The objective of the research was to decipher the effect of antagonistic microbes on systemic defense enzymes, histochemical changes, plant growth attributes, reduction in disease severity, and interaction of these antagonistic microbes with host. *Trichoderma*, *Pseudomonas*, and *Bacillus* isolates were collected from rhizospheric soil and characterized using morphological and molecular tools. ITS and 16S rRNA sequences were analyzed to determine the molecular characterization of all antagonist microbes, and they were identified as *T. asperellum, T. viride*, *T. harzianum, B. subtilis*, and *P. flourescens*. These isolates were evaluated for antibacterial properties against *D. dadantii* under in vitro conditions and showed the higher inhibition in a dual culture method. Further, the effects of seed bio-priming and soil application of these isolates were tested under glasshouse and field conditions. *T. viride* outperformed the other isolates, significantly enhancing the plant growth parameters and induced resistance to *Dickeya dadantii* (BSR). *T. viride* showed a significantly higher accumulation of defensive enzymes, viz. PAL (1.02), PO (1.70), PPO (1.25), CAT (1.11), and TPC (0.91) at 48 h after pathogen challenge, as compared to the control. Histochemical tests confirmed lignification and callose deposition in the cell walls of the treated plants. Antagonist microbes were further evaluated under field conditions against *D. dadantii* infection. Compared to the control, there is a significant enhancement of plant growth parameters and yield with a simultaneous decrease in disease severity in *T. viride* treated plants. Results showed that the potential benefits of *T. viride* could not only effectively induce resistance in plants, enhance plant growth, increase yield, and suppress pathogen infection but also reduce the use of hazardous pesticides. As a result of correlation, PCA and heat map analyses indicated that *T. viride* is interconnected to determine the crop ability to sustain its growth under pathogen stress.

Keywords Sorghum, *Dickeya dadantii*, Induction of defense enzymes, Disease incidence

*Correspondence:
Sujata Singh Yadav
sujatasingh62@gmail.com
[1] Department of Plant Pathology, College of Agriculture, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263145, India
[2] ICAR- Indian Institute of Maize Research, Punjab Agricultural University, Ludhiana, Punjab 141004, India

Background

Sorghum (*Sorghum bicolor*) is the world's fourth largest cereal crop in production and harvested area (Source: FAOSTAT 2019). It is a staple crop in Asia and Africa, and grown for both food and industrial purposes. This crop's ability in withstanding drought, high temperature and low fertility makes it be an alternative crop to

maize. Because of its resilience to harsh environments, it is a staple crop for household food security in many rural community farming in marginal agroecology, such as in the arid regions of Asia, South Africa, Namibia, and Botswana. The major production constraints of this crop are biotic and abiotic stress factors. Bacterial stalk rot is the most disruptive biotic constraint on sorghum production, which results in yield losses of 60–80% (Saxena et al. 1991). Stalk rot is a devastating bacterial disease incited by *Dickeya dadantii*, (syn. *Erwinia chrysanthemi* (Ech), and it is typically found in the tropics and temperate regions (Pérombelon et al. 1980). Recently, the management of soil-borne bacterial diseases has been mainly based on chemical use. Furthermore, the deployment of resistant cultivars is vital alternative strategy for conferring sustainability to the environment, although resistance collapse has been a problem over the years (Fry 2008). Plants protect themselves from parasitic fungi and microbial pathogens in systemic and localized responses. The activation of defense responses against a range of pathogens, including *D. dadantii*, occurs when signals are initiated at the infection site. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2), and hydroxyl radicals (·OH), are produced as the first line of defense. Various signaling molecules, including superoxide radicals, jasmonic acid, salicylic acid, ethylene, and hydrogen peroxide, have been proposed to coordinate the plant responses. They trigger a number of downstream processes that induce a dynamic defense response, such as reinforcement of cell walls, formation of secondary metabolites, phytoalexin production, callose deposition, and pathogen-related (PR) proteins that inhibit invasion and growth of the pathogen. A number of plant proteins are involved in regulating ROS levels, as well as in the development of induced systemic resistance (ISR) and systemic acquired resistance (SAR). Several species belonging to the genera *Trichoderma*, *Bacillus*, and *Pseudomonas* are extensively applied as biological agents to promote plant growth and enhance the induced systemic resistance (ISR) to several phytopathogens (Nie et al. 2017; Romera et al. 2019). In addition, phylogenetic analysis based on ITS and 16S rRNA has been described as a useful tool for diversity determinations in microbes at the species level (Sawain et al. 2018). *Trichoderma* species has been discovered and described based on sequencing and phylogenetic diversity analysis. *Trichoderma* isolates from native environment have shown the potential to suppress pathogens due to their well-established environments, suggesting that they could be used as biocontrol agents (Yendyo et al. 2017; Konappa et al. 2018).

T. viride and *P. fluorescens* showed a cumulative effect on plant growth, induction of the defense system, and disease suppression (Kloepper 1992). Seed biopriming helps increase the proliferation, colonization, and establishment of biological control agents (BCA) on the seed surface. Additionally, it may trigger the induction defense system of the host plants against the invasive pathogen (Singh et al. 2020). This host–pathogen interaction was responsible for induced signaling molecules in plant systems, leading to antibacterial compounds production. Defensive enzymes, viz., peroxidase (PO), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), and catalase (CAT) are among the most significant and extensively distributed molecules in plants. It has been reported that *Pseudomonas* and *Bacillus* strains are capable of causing systemic resistance to various disease causing pathogens in crops, including bananas, beans, rice, and cucumbers (Harish et al. 2008). PAL is the key enzyme that catalyzes the biosynthesis of lignin and phenolics from the aromatic amino acid phenylalanine (Cartea et al. 2011). Phenolic compounds may accumulate from chloroplasts when PPO leaves the thylakoid membrane. In the existence of oxygen, PPO oxidizes the phenolic compounds in the form of odiphenol to an o-quinone (Raj et al. 2006). Plant peroxidases have been associated in various defense-related processes, including hypersensitivity response, lignification, the crosslinking of phenolics and glycoprotein, and the production of phytoalexins. Cells frequently use catalase to catalyze the breakdown of hydrogen peroxide into less reactive oxygen and water molecules, preventing the cells from disintegrating (Bolwell and Wojtaszek 1997). Higher accumulation of phenolic compounds reduces the chances of disease establishment by increasing physical cellular strength. Accordingly, phenolics act as lignin precursors to facilitate lignification of cell walls (Siddique et al. 2014). During pathogen invasion, the production of callose and lignin acts as a cell wall-strengthening barrier (Desprez et al. 2002).

The primary objective of the present study was to isolate *Trichoderma*, *Pseudomonas*, and *Bacillus* isolates from the soil rhizosphere, to assess the efficacy of bioprimed seeds and soil application to induce systemic resistance and plant growth promotion against *D. dadantii* under glasshouse and field conditions. We found that the antagonist microbes deposite lignin and callose on the hosts cell wall to resist pathogen infection.

Results

Molecular and morphological characterization of antagonistic isolates and bacterial pathogen

The pathogen

The slimy basal or soft stalk rot symptoms of sorghum indicate that the pathogen is a bacterium. Plant bacteria

were identified based on biochemical behaviour, colony morphology, and molecular characterization. On NA media, young colonies looked convex, smooth, circular, and sculptured with irregular margins. Initially, colonies were round with a raised center and lobed edges. After four to eight days, colonies appeared feathery or almost coralloid. Based on the most widely recommended set of biochemical tests, details are given in Additional file 1: Table S1. Molecular characterization was performed using amplification of the 16S rRNA gene from both directions. An accession number of 'MW465754' was obtained from NCBI after sequence analysis.

Antagonistic microbes

Antagonistic microbes isolated from pantnagar, Uttarakhand region were identified based on morphological and molecular identification (ITS1/ITS4), four *Trichoderma* isolates were identified as: *Trichoderma viride*, *Trichoderma harzianum, Trichoderma harzianum* (Th14), *Trichoderma harzianum* (Th19), *Trichoderma harzianum* (ThR), and *Trichoderma asperellum* (TUK42). Whereas, three antagonistic bacteria were identified (16S rRNA) as two isolates of *Pseudomonas* (*Pseudomonas fluorescens* and *Pseudomonas fluorescens*-Psf-2) and one isolate of *Bacillus subtilis*. The bidirectional sequence length range obtained was 578–1329 bp from ITS regions and 817–1357 bp from 16S rRNA. *Trichoderma viride* showed 99.34% homology with MK108412, *T. harzianum* has 98.95% homology with KC330218, *T. harzianum* (Th-14) has 100% homology with OM681173, *T. harzianum* (Th-19) has 100% homology with OM681175, *T. harzianum* (Th-R) has 100% homology with OM681172, *T. asperellum* has 100% homology with OM686854, *Pseudomonas fluorescens* has 99.35% homology with KC495572, *Pseudomonas fluorescens* (Psf-2) has 99.41% homology with JQ691709, and *Bacillus subtilis* has 98.95% homology with OM055790 (Figs. 1, 2). The ITS and 16S rRNA gene sequence data from all nine isolates of antagonist were deposited in GenBank (Additional file 1: Table S2).

Evaluation of bacterial and fungal antagonism in vitro

The results revealed a significant reduction in the growth of *D. dadantii* over control treatments in a range of 53.29–85.63% across all the isolates of antagonistic microbes (Additional file 1: Table S2). The isolates of *T. viride* and *P. flourescens* were the most effective and showed growth inhibition of 85.63% and 73.21%, respectively. Other isolates showed moderate to low inhibition against *D. dadantii*.

Effects of seed biopriming and SA with antagonistic microbes on plant growth promotion, and disease dynamics under glasshouse

T. viride and *T. viride* + *P. fluorescens* treatments showed the maximum reduction in disease severity, and better plant growth parameters, in the glasshouse conditions (Additional file 2: Figure S1). In glasshouse conditions, seed biopriming and SA of antagonistic microbes significantly promoted the growth of sorghum plants and reduced the disease index compared to their respective pathogen inoculated plants at 35 and 50 DAS (Additional file 1: Table S3). The maximum increase of all the growth parameters was recorded in *T. viride*. *T. viride* showed the highest shoot length (140.05 cm), root length (23.13 cm), fresh (62.33 g) and dry shoot weight (10.75 g), and fresh (12.49 g), and dry root weight (2.66 g), followed by a combined treatment of *T. viride* + *P. fluorescens*. Further, the effect of antagonistic microbes on disease severity was recorded on 35 and 50 DAS. A minimum PDI was recorded in *T. viride* (35.43%) followed by *T. viride* + *P. fluorescens* (37.52%) over pathogen inoculated control (51.90%) at 50 DAS. The present study's ANOVA test for all parameters is sufficiently large (> 0.05) to make the findings highly reliable. The variable percent disease index allows valid conclusions to be drawn from the ANOVA test under the glasshouse experiment (> 0.05). Principal component analysis (PCA) was carried out using growth parameter variables (shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight,) and potential antagonist microbes (Fig. 3). Two principal components, PCA1, and PCA2, were observed with a variance level of (76.9%) and (12.22%), respectively. The data were distributed in five groups. The first group is composed of *Trichoderma* species, which showed a distinct growth pattern and reduced the disease severity and is far from mock and pathogen-treated control plants. The second group is composed of *Trichoderma* + *P. fluorescens* treated combined and shared the common data growth parameter (axis 1), which is significantly higher than the mock and pathogen-treated control. The third group is composed of only bacterial isolates close to mock and pathogen-treated controls, which shows similar growth parameters and disease severity. Unlike the previous group, the fourth and fifth groups also present no effect on plant growth and showed higher disease severity.

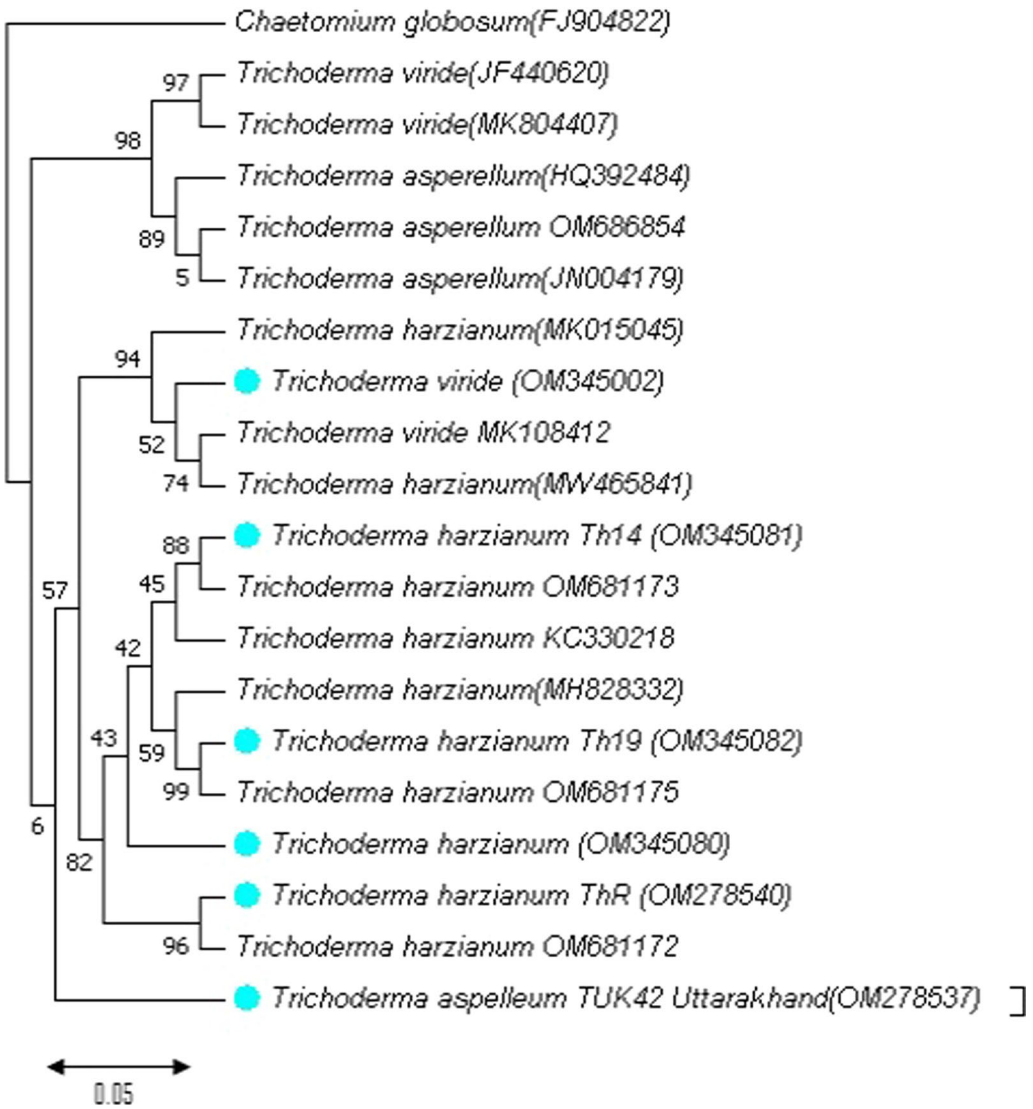

Fig. 1 Maximum likelihood phylogenetic relationship derived by MEGA 7 using analysis of ITS sequences of *Trichoderma* isolates. A bootstrap replication of 1000 was used. The bootstrap value is represented by a numerical value in the node. This comparison was conducted using sequences from NCBI GenBank accession number. To demonstrate the situation of the root the *Chaetomium globosum* (FJ904822) were chosen as an outgroup. The bar indicates 0.05% estimated sequence divergence. *Trichoderma* isolates are marked with •

Effects of seed biopriming with antagonistic microbes on plant growth promotion, disease dynamics, and yield assessment under field conditions

The efficacy of these antagonistic microbes was evaluated under field conditions during 2017–2018. It was observed that *T. viride* significantly reduced the disease severity and enhanced many other growth parameters, including green fodder yield, when compared to the control (Additional file 2: Figure S2). Significant increases in root length (53.52 and 55.62 cm) and shoot length (260.52 and 270.11.20 cm) were recorded for *T. viride* in the two years, respectively. The effect of these antagonistic microbes on disease severity was recorded at 35, 50, 64, and 80 DAS. A higher reduction in PDI at 80 DAS was recorded for *T. viride* (55.62% and 50.36%), followed by *T. viride*+*P. fluorescens* over other treatments and control (75.63% and 86.00%). All the treatments with *D. dadantii* and the antagonists recorded a significantly higher green fodder yield than the pathogen-inoculated treatments. The results suggest that the antagonistic activity of *Trichoderma* isolates minimizes disease severity and produces a substantial increase for the yield of sorghum (400.52 and 463.00 q/h respectively), with *T. viride* during the 2017 and 2018 Kharif seasons. Results

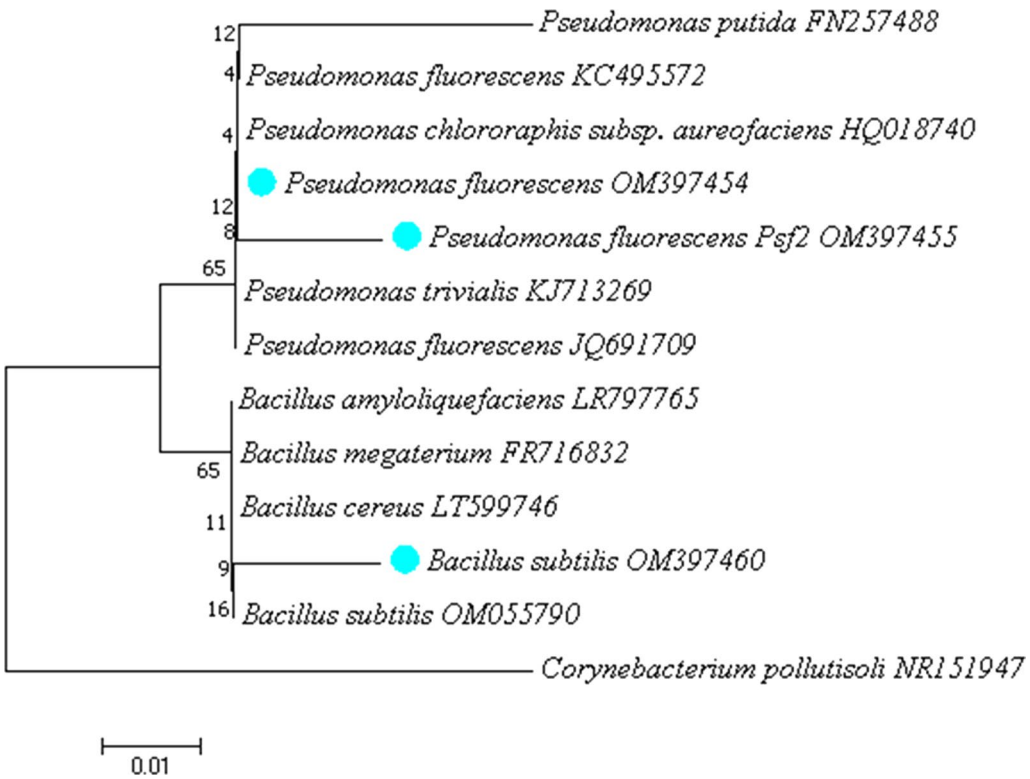

Fig. 2 Maximum likelihood phylogenetic relationship derived by MEGA 7 using analysis of 16S rRNA sequences of Pseudomonas and Bacillus isolates. A bootstrap replication of 1000 was used. The bootstrap value is represented by a numerical value in the node. This comparison was conducted using sequences from NCBI GenBank accession number. The bar indicates 0.05% estimated sequence divergence. Bacterial isolates are marked with •

also showed significant differences in the plants root and shoot length, fresh weight, and dry weight among the treatments (Additional file 1: Tables S4, S5). The ANOVA test showed that *T. viride* significantly affects bacterial stalk rot disease development of plant growth parameters and percent disease index at 35, 50, 65, and 80 DAS.

Based on the heat map, the data were grouped into two clusters, the cluster I and II (Fig. 4). Cluster I was further classified into two sub-clusters. Sub-cluster I consists of an increase in shoot length, root length, reduction in disease severity, and yield percentage; whereas, PDI 35, PDI 50, PDI 65, and PDI 80 were grouped in the sub-cluster II. All the treatments were grouped in cluster II and were further classified into two sub-clusters: the *Trichodrma viride* and *T. viride*+*P. fluorescens* were grouped in sub-cluster I, and the rest of the treatments were grouped in sub-cluster II. As a result of the clustering analysis, antagonistic microbes varied significantly in their ability to improve the different plant attributes and suppress disease severity. Overall, this heat map showed the same pattern of plant growth attributes, percent disease index, reduction in disease severity, and increased yield percentage in both years (2017–2018).

Statistical analysis showed that the treatments effects depicted a high correlation with plant growth attributes and disease suppression in both glasshouse and field conditions (Fig. 5). The correlation analysis of the yield showed a negative correlation with PDI 35, 50, 65, and 80 and a positive correlation with a reduction in disease severity in the field. Thus, in the glasshouse, a reduction in disease severity (RDS_GH) illustrated the statistically significant correlation with all the plant growth parameters and a negative correlation with PDI 35 and 50.

Effect of seed biopriming and SA of antagonistic microbes on induction of defense enzymes related biomolecules under glasshouse conditions

The sorghum seed biopriming and SA with antagonistic microbes were used to study the response of defense-related enzymes, such as PPO, PO, and PAL, as well as TPC and CAT. It was observed that, in all of the treatments, defense-related enzyme activity was significantly increased as compared to pathogen-treated control and mock control (water).

At 48 h after pathogen treatment, all treatments exhibited higher levels of PO activity than

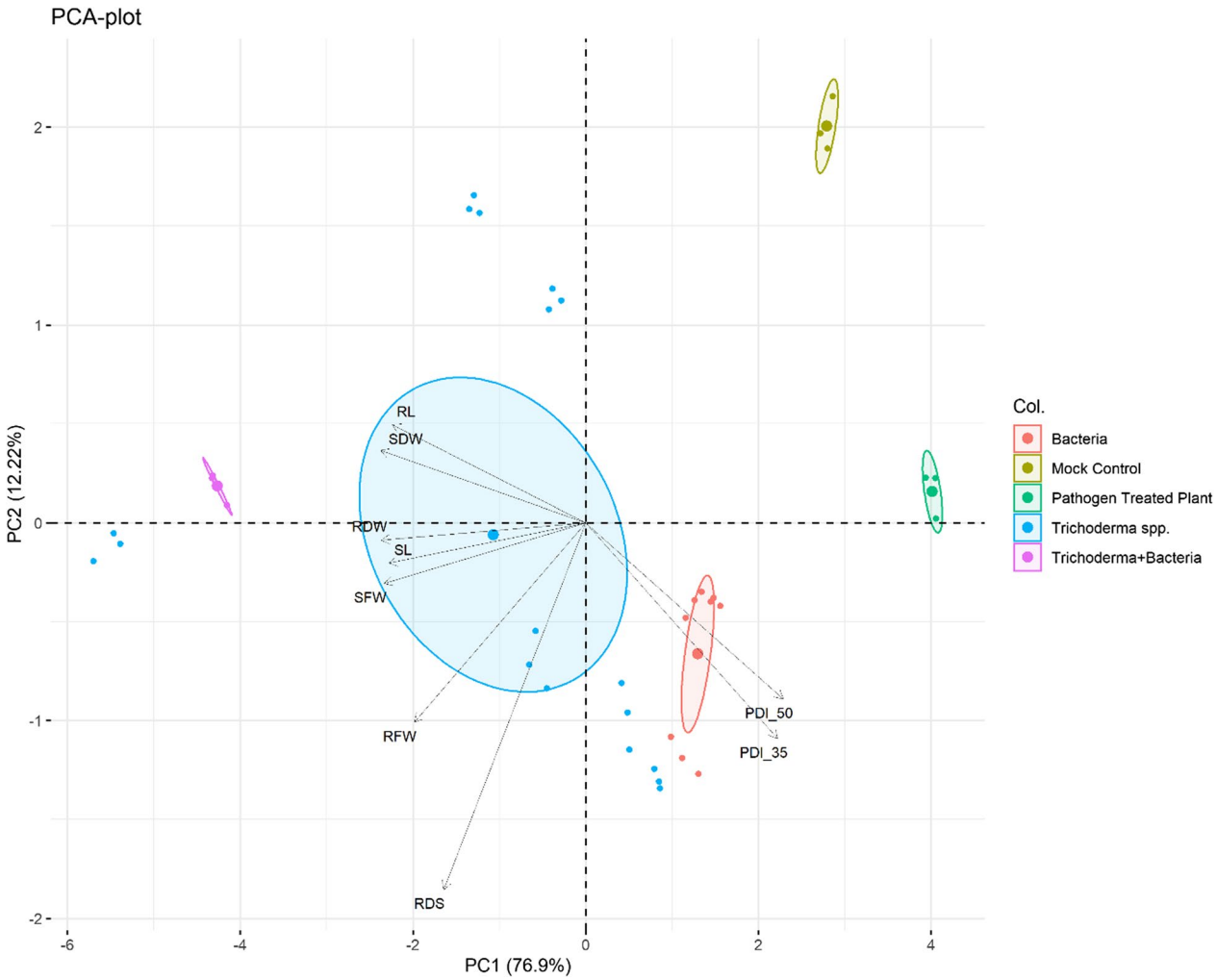

Fig. 3 PCA graph scattered plot of plant growth attributes and disease suppression clustered according to their divergence among the antagonistic microbes in the presence of *D. dadantii*. The vectors derived from biplot origin represent positive and negative association among attributes: SL, shoot length; RL, root length; SFW, shoot fresh weight; SDW, shoot dry weight; RFW, root fresh weight; RDW, root dry weight; PDI, percent disease index and RDS, reduction in disease severity

non-pathogen-treated plants. *T. viride* treatment resulted in the highest PO activity (1.93 DOD min^{-1} mg^{-1} of protein). After 48 h, PO activity declined significantly (Fig. 6a).

The trend of change in PPO activity with time was similar to that of PO in all treatments (Fig. 6b). The PPO activity was higher in *T. viride* treatment (1.93 DOD min^{-1} mg^{-1} protein) at 48 h compared to untreated plants (control).

The PAL is a major enzyme in phenylpropanoid pathway and plays a significant role in plant defense during pathogen infection. The maximum accumulation of PAL was recorded in the *T. viride* treatment (1.40 µmol of trans-cinnamic acid/mg protein/h) at 24 h of the untreated control plants (Fig. 6c). TPC activity was found to be significantly higher in all treatments after 48 h than in untreated plants (the control). TPC activity showed the maximum induction with *T. viride* (1.08 µg/g) compared to untreated plants (Fig. 6d). Maximum CAT activity was observed in the treatment of *T. viride* (1.01 µmol of H_2O_2/min/mg of protein) at 48 h, followed by *T. viride* + *P. flourescens* (0.95 µmol of H_2O_2/min/mg of protein). The CAT activity in all the treatments was the highest at 48 h, and it declined gradually with time after the pathogen inoculation (Fig. 6e). The defense enzyme activity data were sufficiently large enough (> 0.5) to be subjected to analysis of variance (ANOVA) to make the findings highly reliable based on the present investigation under glasshouse conditions.

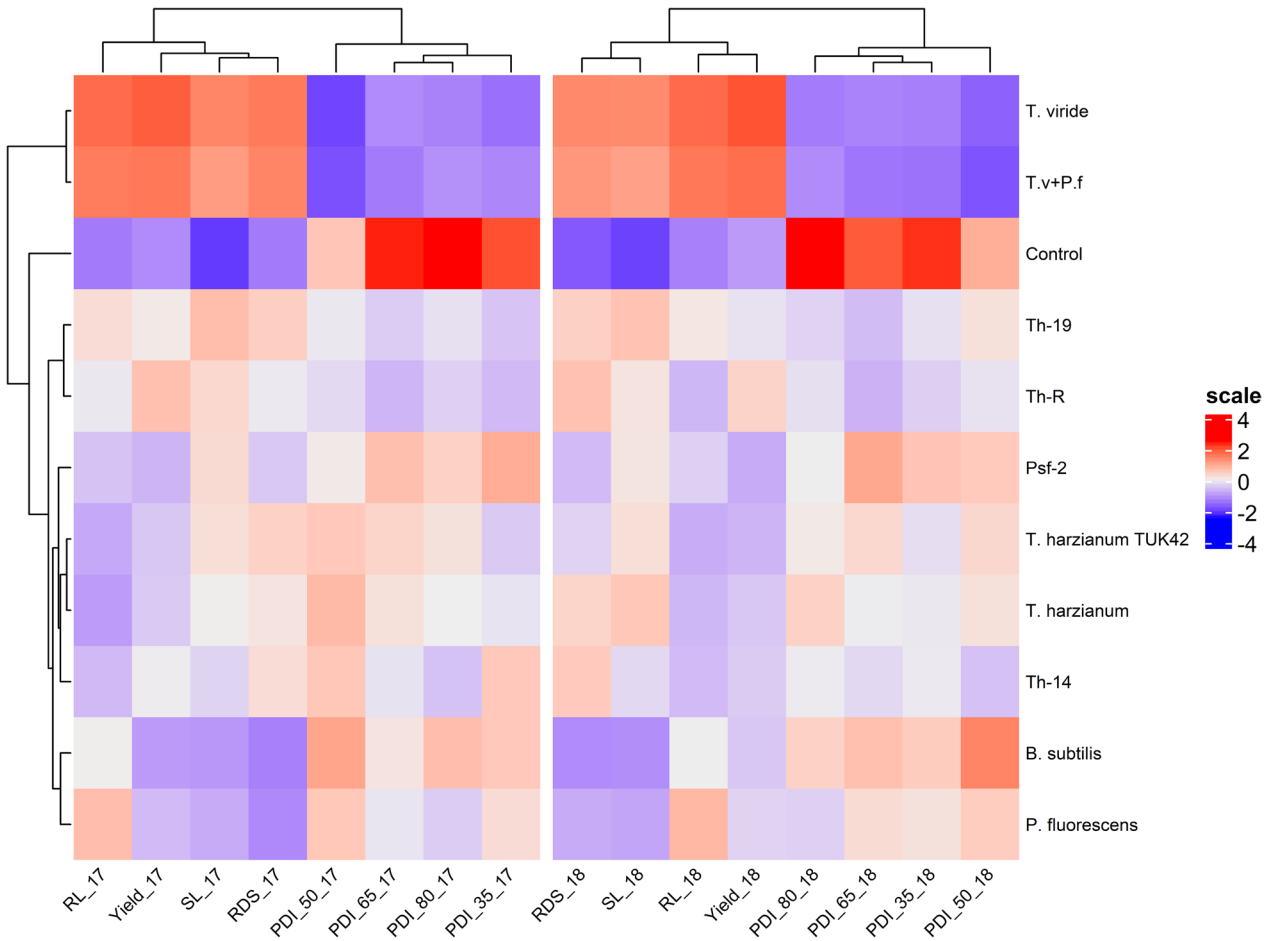

Fig. 4 Cluster heat map analysis showing the responses of antagonistic microbes on plant growth attributes and disease suppression under field condition of two consecutive years (2017 and 2018) at 80 DAS. The colour gradient from blue to red indicates an increasing effectiveness of treatment

Scanning electron microscopic (SEM) observations

Primary root sections were examined the morphology of the *T. viride* and sorghum root interaction by scanning electron microscope. It observed that cells of isolate *T. viride* were consistently distributed on the surface of roots when compared to the uncolonized sorghum roots (Fig. 7). The results of SEM showed that *Trichoderma* interacts with sorghum roots directly by developing a plant fungus interaction system.

Histological detection of lignin and callose deposition

Lignin deposition was evaluated seven days post-inoculating the pathogen in both bioprimed and unprimed plants. The pink-colored staining of Phloroglucinol- HCL stain lignified tissues was shown to have intense coloration (Fig. 8e, f). Histochemical tissue sections of sorghum plants treated with an antagonist showed significant variations in lignin deposition. Seed bioprimed and soil application of *T. viride* showed the highest lignification in all the root sections, interfascicular cells, vascular bundles, sclerenchyma cells, endodermis, pericycle, and protoxylem. Moreover, *T. viride* bioprimed plants co-inoculated with pathogen showed a higher accumulation of lignified tissues, suggesting that the defense response becomes more robust in the presence of antagonist microbes.

The results of callose deposition were in accordance with that of lignin, wherein the effects of antagonist microbes were evaluated on the sorghum immune response under pathogen stress. Aniline blue staining of cells showing a bright greenish fluorescence was considered positive for callose deposition in the leaf and root tissues. The *T. viride* bioprimed samples had a comparatively higher amount of callose deposition than unprimed controls upon a pathogen inoculation when they were visualized under a fluorescence microscope (Fig. 8a–d).

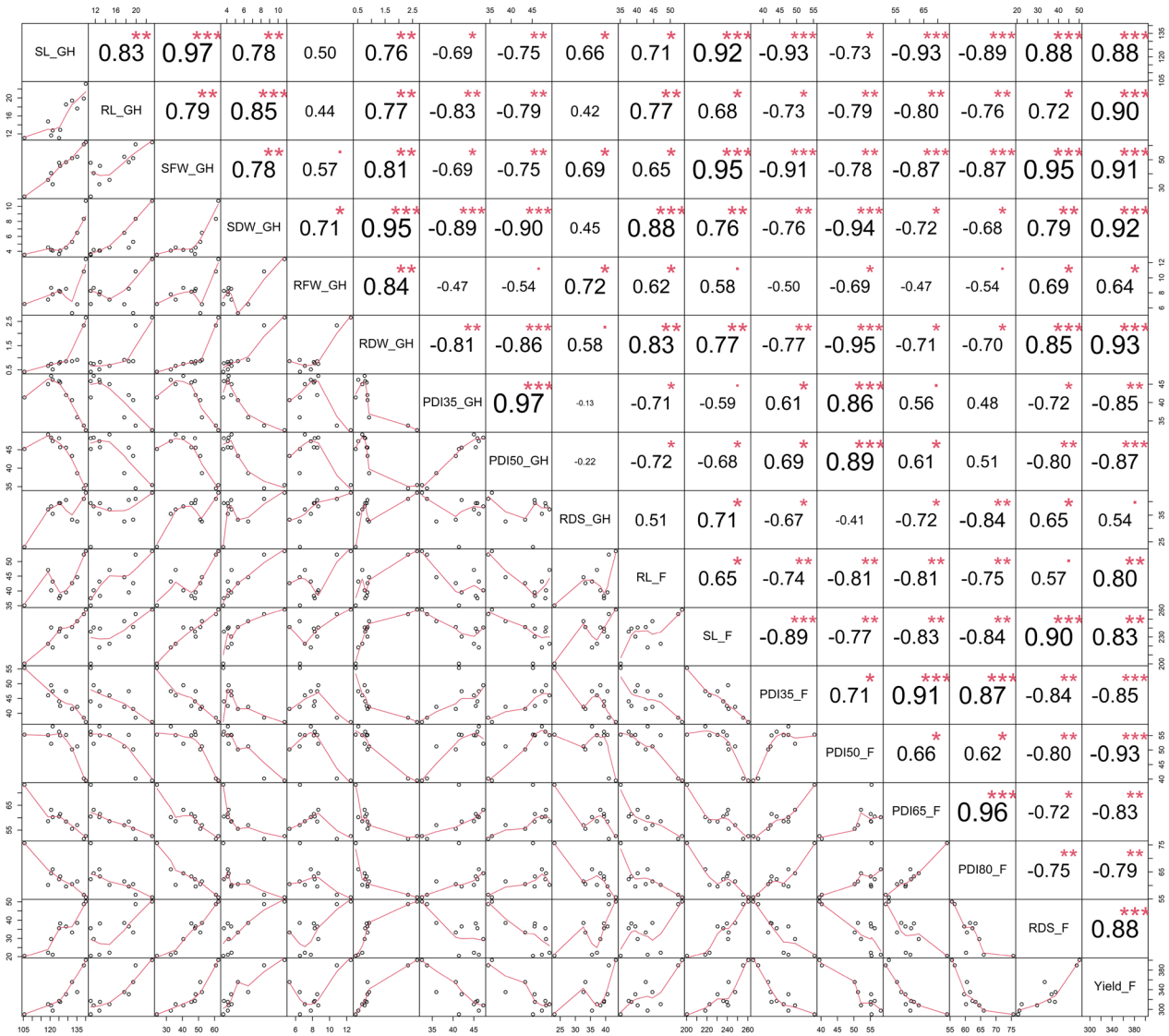

Fig. 5 Significance of correlation among the plant growth attributes and disease suppression and yield in the glasshouse and field among the treatments. *** = Significant at $P < 0.001$; ** = Significant at $P < 0.01$; * = Significant at $P < 0.05$. SL_GH, shoot length, RL_GH root length, SFW_GH, Shoot fresh weight, RFW_GH, Root fresh weigh, SDW_GH, shoot dry weight, RDW_GH, root dry weight, PDI_GH, Percent disease index, RDS_GH, reduction in disease severity. GH, glasshouse, F, Field

The callose deposition count and percentage area after inoculation with *T. viride* significantly increased compared to the control.

Discussion

Bacterial stalk rot caused by *Dickeya dadantii* in sorghum is a destructive disease that causes a severe threat to its productivity and nutritional quality. As a soil-borne disease, it is challenging in managing the disease. Antagonistic microbes (biocontrol agents) are one of the most suitable substitutes to enhance the innate immunity of plants due to their fast-growing, extensive availability, and compatible nature. *Trichoderma* is a universal opportunist in ecosystems and interacts directly or indirectly with roots by developing a plant-fungus interaction system. Most of the researches have reported on biocontrol strategies to manage the variety of pathogens, such as *Pectobacterium atrosepticum*, *Pectobacterium carotovorum* subsp. *carotovorum*, and, to a lesser extent, *Dickeya* sp. pathogens (Lim et al. 2013; Song et al. 2016). Previous studies have explored and applied the strategies of using antagonistic microbes to control *Dickeya*. However, the use of *Trichoderma* as a fungal antagonist against the

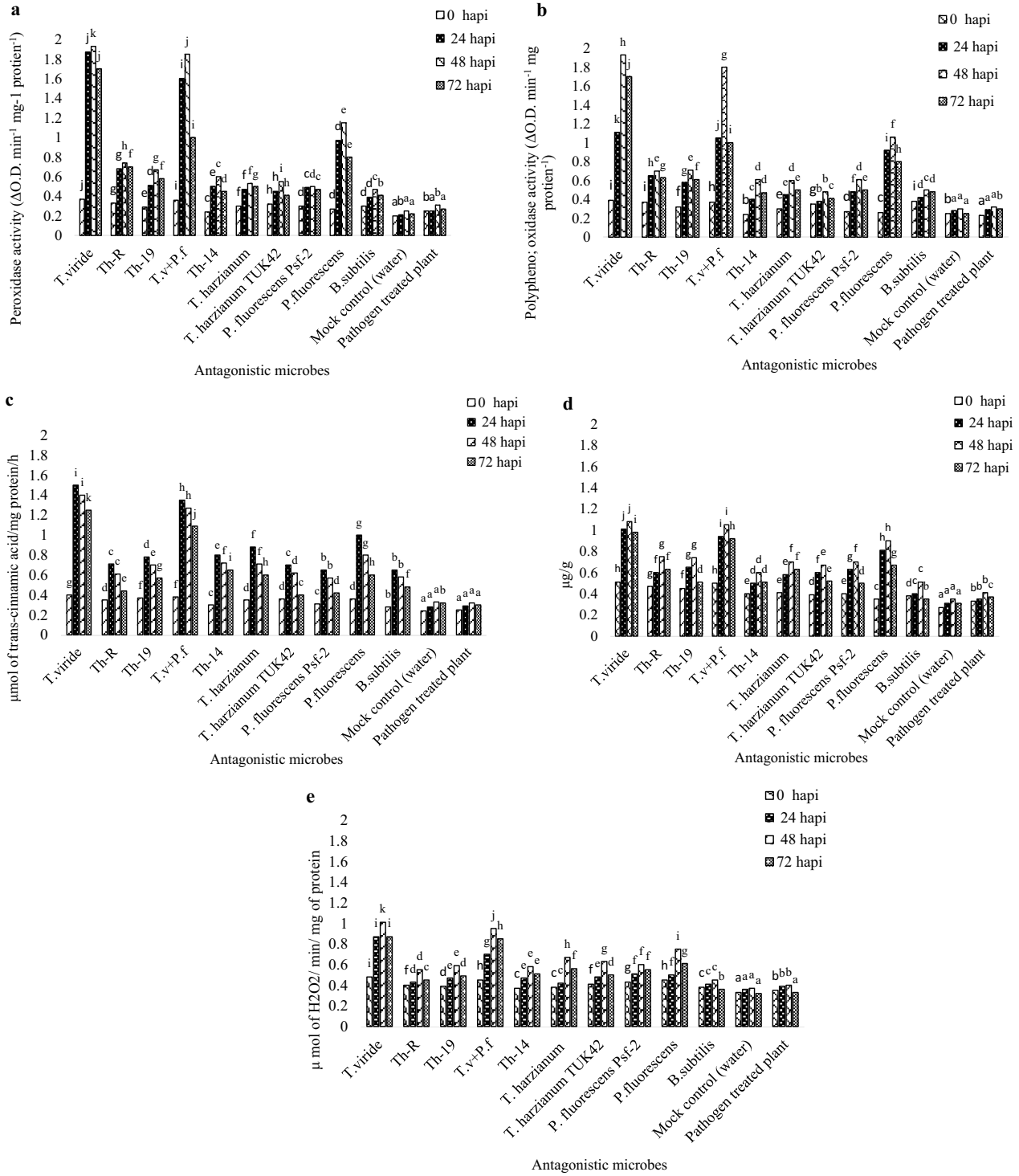

Fig. 6 The effect of seed biopriming and soil application on the induction of defense enzymes activities against *D. dadantii* in glasshouse conditions; observation was recorded on 30 DAS. Results are expressed as mean of three replication and vertical bars indicate standard deviation of the mean. Letters above error bars (**a**–**k**) indicate superscripts significant difference among treatments according to Duncan's multiple range test at $P \leq 0.05$. **a** Peroxidase (PO). **b** Polyphenol oxidase (PPO). **c** Phenyl ammonium lyase (PAL). **d** Total phenolic content (TPC). **e** Catalase (CAT)

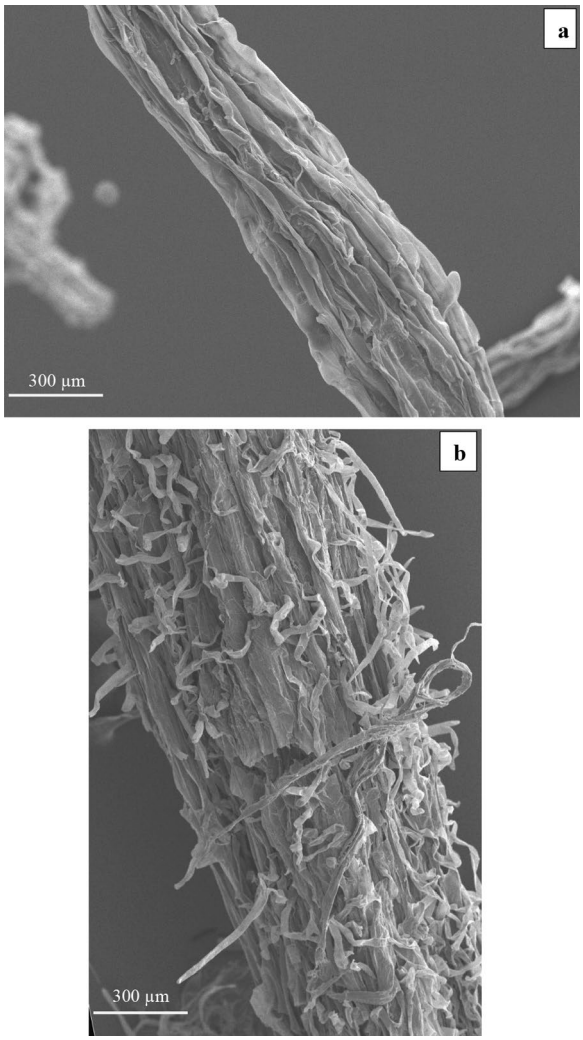

Fig. 7 SEM images of sorghum roots colonized by *T. viride*. **a** Root surface of non-inoculated seedlings. **b** The surface of the root intact with hyphae of *T. viride*

bacterial stalk rot pathogen has not been reported. The present study showed that seed biopriming and soil application with antagonistic microbes isolated from rhizosphere ecosystems protected host plants against phytopathogens by activating the induced defense system and suppressing disease progression under both greenhouse and field conditions. The molecular identification of these antagonist isolates was performed using ITS1 and ITS4, and 16S rRNA gene sequencing approaches. Based on the sequencing, two bacterial isolates were identified as *P. fluorescens*, and one as *B. subtilus*, whereas four fungi were identified as *T. harzianum* (Th-R, Th-14, Th-19, and *T. harzianum*), one *T. viride,* and one *T. asperellum* (TUK42) (Singh et al. 2016; Mukherjee et al. 2017). The phylogenetic tree was constructed based on sequence analysis of 16S rRNA and ITS genes of the bacterial and fungal antagonists.

In vitro results demonstrated that the antagonist isolates had variable antagonistic effects on *D. dadantii*. *T. viride,* and Th-R. *P. fluorescens* had a more antagonistic effect against stalk rot bacteria than the others in the dual culture assays, which is likely due to the production of antibiotics and other metabolites, *viz*., diketopiperazines, peptaibols, polyketides, and alky pyrones (Sharma and Dohroo 1991; Howell et al. 2000). The effect of *Trichoderma* can result from direct parasitism on *Erwinia* cells followed by penetration of the cells and producing enzymes that degrade the host cell wall, convergently leading to cell death (Al-Jarah et al. 2013). Several studies reported the activity of *P. fluorescens* and *T. viride* against *Erwinia carotovora* subsp. *Carotovora* in the *in vitro* tests (Sandipan et al. 2015; El-Hendawy and Abo-Elyousr 2016; Sulaiman et al. 2020). Similar results revealed that *T. viride*, *T. harzianum, P. fluorescents,* and *B. subtilis* reduced tissue maceration and protected the potato slices against the development of soft rot in the *in vivo* tests (Nayral et al. 2019; Khair et al. 2021). In addition, *Trichoderma koningiops* showed antagonistic activity against *Erwinia mallotivora* in papaya (Tamizi et al. 2022).

In this study, the antagonistic microbes promoted plant growth and systemically stimulated the phenylpropanoid pathways, leading to resistance against *D. dadantii* infection through the accumulation and activation of various phenolic compounds and the production of enzymes associated with ISR in sorghum. A greenhouse experiment was performed to evaluate the seed biopriming and SA of *Trichoderma*, *Pseudomonas*, and *Bacillus*, against *D. dadantii* of sorghum plants. In this research, seeds primed with *T. viride* had significantly improved growth attributes, including the root length, root dry weight, shoot dry weight, shoot length, and decreased disease severity. Similarly, it was reported that members of *Trichoderma* produce phosphate solubilizing enzymes, siderophores, and phytohormones (Doni et al. 2014), which might contribute to enhance the plant growth and development. Several studies demonstrated the antagonist potential and growth promotion ability of *Pseudomonas fluorescens*, *T. harzianum*, and *B. subtilis* against *Erwinia* soft rot in potatoes (Brotman et al. 2010). Previously, *Trichoderma* spp. were shown to enhance the plant growth attributes of various crops, such as strawberries, soybeans, tomato, apples, mangroves, and cotton (John et al. 2010; Raman 2012; Jambhulkar et al. 2018). Some antagonists were investigated for controlling *Erwinia chrysanthemi*, the causal agent of

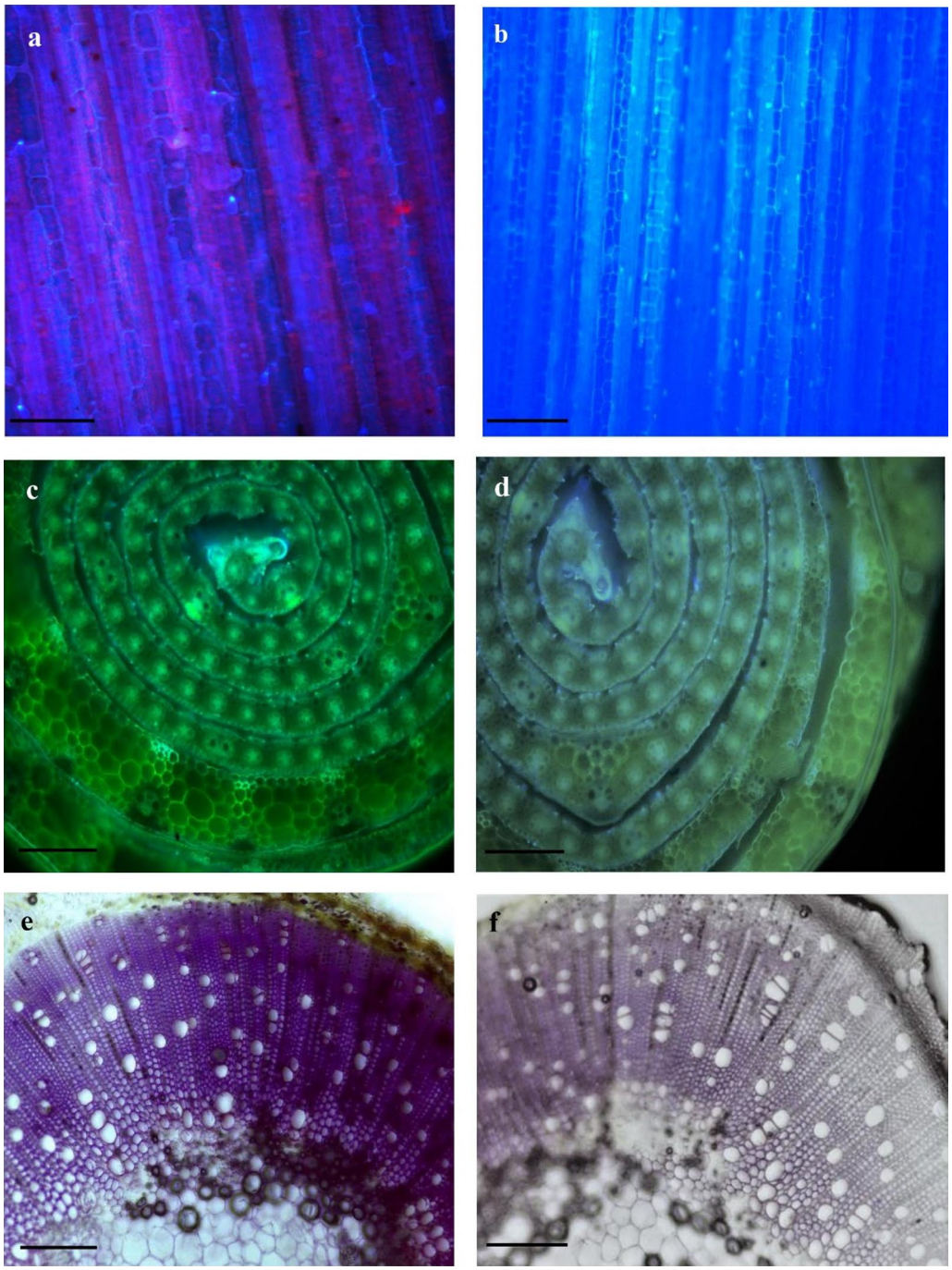

Fig. 8 An assessment of histological plant defense responses such as callose and lignification. The figure shows the transverse sections of stem and root tissues with callose and lignin deposition in sorghum tissues. Callose deposition: **a** Sorghum leaves tissue taken from an unprimed plant (control). **b** Leaves tissue sections from *T. viride* challenged plant. **c** Sorghum stem tissue taken from an unprimed plant (control). **d** The stems sections from *T. viride* challenged plant. Lignin deposition: **e** The *T. viride* challenged stem tissue section of sorghum. **f** Lignin deposition of control stem tissue. Scale bars = 50 µm

soft rot on tomato under glasshouse conditions (Aysan et al. 2003).

The seed biopriming with antagonistic microbes colonizes the plant system and induces plant defense against abiotic and biotic stresses to participate in the induced

systemic resistance/tolerance (ISR/IST) defense signalling pathway (Jogaiah et al. 2013; Konappa et al. 2020). The present study found that seed biopriming and SA with *T. viride* plants exhibited a significantly higher accumulation of defense enzymes (PO, PPO, PAL, TPC, and CAT) compared to other treatments. All the *Trichoderma* isolates exhibited higher PO, PPO, PAL, TPC, and CAT activities than the pathogen-treated control and untreated plants (healthy control) and, which consequently restricted the progression of the disease. In addition, *T. viride* outperforms the others.

In plants, phenolic compounds are usually enacted in response to biotic and abiotic stresses through the phenylpropanoid pathway, including xylanase, cellulose, lignin, and other plant metabolites. Consequently, PPO catalyses the synthesis of phenolics to produce quinines through a secondary reaction, which further results in the formation of a complex polymer, melanin, and serves as a physical barrier against the ingression of microbes (Taranto et al. 2017). These results corroborate with Velmourougane et al. (2017), who suggested the high accumulation of PPO, PO, PAL, and SOD in chickpea when the plants were treated with *Pseudomonas*, *Rhizobium*, and *Trichoderma*. The antagonist activities of the two bioagents tend to stimulate the defense system in plants, which leads to the production of phytoalexins, PR proteins, and activation of induced systemic resistance (ISR) by enhanced synthesis of ethylene, jasmonic acid, and NPR-1 regulatory genes (Mohapatra and Mittra 2017; Konappa et al. 2020).

This glasshouse experiment was further carried out in a field experiment in two consecutive seasons in Kharif during 2017 and 2018. A variation in climatic conditions including the temperature, humidity, and rainfall during the planting year affected yield and disease severity. Our field study showed that bio-priming of the seeds and successive SA with *T. viride* significantly reduced the bacterial stalk rot disease severity and percent disease index and increased green fodder yield compared with untreated plants.

The histological observations showed that extensive lignin and callose deposition in sorghum plants treated with *T. viride* positively impacted this plant. Lignin and callose are formed due to the interaction with *T. viride* in the sorghum cell wall, which constitutes a physical barrier for better defense against pathogens. Our results depicted that the content of lignin and callose was shown significantly increased in treated plants compared to the untreated plants. Similarly, callose and lignin are also used by plants to strengthen their cell walls in response to external stress (Desprez et al. 2002). Under stress regimes, plants frequently accumulate lignin subunits and phenolic (Sattler and Funnell-Harris 2013). Previous studies investigating tomato and rice plant protection against vascular pathogens have also revealed the increased deposition of lignin and callose (Nawrocka et al. 2018). In this work, it was observed that seed biopriming with antagonist microbes is a very important factor for plant growth promotion and induction of defense enzymes. A root derived polysaccharide and monosaccharide facilitate *Trichoderma* growth and the development of positive interactions with plants (Druzhinina et al. 2011). *Trichoderma* strains are well known for colonizing roots and controlling plant diseases. The SEM study revealed that entire roots were colonized by *Trichoderma viride* but not other microbes. According to previous observations, our findings agree with previous observations which suggest that some *Trichoderma* strains colonize only local root sites. Rhizosphere-competent strains, however, colonize the entire root surface for several weeks or months at a time (Metcalf and Wilson 2001; Harman et al. 2004). The root colonized by *Trichoderma* increased defense-related enzymes and strongly inhibits a range of pathogens (Howell et al. 2000). As a root-colonizing microorganism, *Trichoderma* spp. also increases plant growth and productivity and activates the resistance genes in plants (Kloepper 1992). Results of this study suggest that it may be possible to use *Trichoderma* spp. as a biological agent to control stalk rot caused by *Dickeya dadantii* in sorghum.

Conclusions

Seed biopriming and soil application with *T. viride* is one of the potential antagonistic microbes against *D. dadantii*. The *Trichoderma viride* may be used to reduce disease incidence, and to promote the plant growth parameters under glasshouse and field conditions. *T. viride* depicts intrinsic stress tolerance by employing the expression of stress-related enzymes and increases the production of physical defense barrier. However, *T. viride* colonized the root surface and protect the plant from the invading by pathogens. One strain, namely *Trichoderma viride* may be promoted in sustainable crop management for their beneficial role.

Methods

Plant pathogen and antagonistic microbes

A virulent strain of *Dickeya dadantii* was isolated from plants with typical symptoms of bacterial stalk rot at Govind Ballabh Pant University of Agriculture and Technology Pantnagar, Uttarakhand, latitude, and longitude (29.00N, 79.48E) respectively. To confirm the identity, the pathogen was subjected to PCR amplification using universal primers for the 16S rRNA region (Edwards et al. 1989). The *Trichoderma* spp., *Pseudomonas fluorescens*, and *Bacillus subtilis* were freshly isolated from

different pantnagar region. The Uttarakhand rhizosphere soil samples were plated on Kings B medium, nutrient agar, and Trichoderma-selective agar medium (T.S.M.) by serial decimal dilutions. *Trichoderma* species were identified by examining the distinctive conidiophores structure under a microscope according to I.S.T.H. guidelines (Gams and Bissett 2002). The molecular characterization of fungal and bacterial isolates was done by amplifying Internal Transcribed Spacer (ITS) regions and 16S rRNA gene, respectively, using universal primers set ITS1 (5′TCCGTAGGTGAACCTGCGG3′), ITS4 (3′TCCTCCGCTTATTGATATGC5′) and 16S rRNA gene (5′AGA GTT TGA TCC TGG CTCAG 3′) (5′AAG GAG GTG ATC CAG CCG CA 3′) (Edwards et al. 1989; Photita et al. 2005). Bidirectional Sanger sequencing was used to sequence the obtained PCR products, which were analyzed using chromatograms and assembled using Bio Edit Version 7.0.5. The sequences were identified using the *blastn* (https://blast.ncbi.nlm.nih.gov/Blast.cgi) program of the National Center for Biotechnology Information (NCBI) database, and sequences with an identity greater than 99% were considered belonging to the same species level. The 16S rRNA and ITS partial sequence data were deposited in the GenBank public sequence repository at the NCBI to obtain accession numbers (Additional file 1: Table S2). The pathogenicity was conducted under glasshouse conditions on three-week-old healthy seedlings of sorghum plants against *D. dadantii* (Singh et al. 2019). Homologous 16S rRNA and ITS sequences were used to construct a phylogenetic tree using the MEGA 7 package by the maximum likelihood algorithm using the Tumura Nei modal parameter distance with 1000 bootstraps. To determine the extent of similarity among sequences in proximity, we aligned them individually within the same branch of the dendrogram. Sequences with a similarity greater than 95–99% were considered to belong to the same species (Tamura et al. 2011).

Antagonistic activity of antagonist microbes to *Dickeya dadantii*

The antagonistic activities of all the bacterial isolates *Pseudomonas fluorescens, Pseudomonas fluorescens* (Psf-2), and *Bacillus subtilis* against the pathogen *Dickeya dadantii* was evaluated by dual culture technique (Singh et al. 2016). The bacterial culture (*Dickeya dadantii*) was inoculated to nutrient broth (NB) medium for 24 h at 30 ± 1°C with 1×10^7 CFU/ml at (0.1 OD600). This fresh culture (100 µL) of *D. dadantii* was spread evenly using a spreader on NA plates. Thereafter, 0.5 cm diameter wells were created using a sterilized cork borer in each petri plate. A 60 µL suspension of antagonistic bacterial isolates of 0.1 OD at 600 nm was poured into the wells. As a negative control, the same amount of NB medium was added to the well. All these plates were incubated at 30°C for 48 h to determine the diameter of the bacteriostatic zones (Singh and Singh 2012). These potent antagonist bacteria were stored in 20% glycerol at −80°C for long-term storage. The antagonistic activity of *Trichoderma* isolates on *D. dadantii* was measured in vitro as per the methods described elsewhere (Sinclair and Dingra 2017) with slight modifications. On a PDA plate, individual isolates of *Trichoderma* and the pathogen were placed equidistantly from the edges and were incubated at 28°C for 5 days. A control plate was maintained by inoculating *D. dadantii* without antagonistic fungal culture at the second edge. Reduction in the colony of *D. dadantii* was measured by clear inhibition in the treated samples as compared to the control plate, and percent inhibition was calculated using the following formula

$$Percentage\ inhibition = \frac{(R_1 - R_2)}{R_1} \times 100$$

where R1 is the growth of the pathogen in control plate and R2 is growth of the pathogen in the test plate.

Preparation of talc based bioformulation of microbial antagonists

The isolates of *Trichoderma* species were mass cultured on the grain of barnyard millet (*Echinocloa frumentacea* L; local name: jhangora). After soaking the grains in water for 12 h, they were placed in 500-ml Erlenmeyer flasks (at 100 g/flask). These flasks were autoclaved for 30 min at 121.1°C, 15 lbs psi. The flasks were cooled to room temperature, and the mycelial discs were inoculated with a 4–5-day-old culture of *Trichoderma* isolate and incubated at 28°C for 12 days. *Trichoderma* colonized barnyard millet grains were air-dried in the open shade and ground into a fine powder using a Willy Mill. The powder was passed through a sieve of 50 and 80 mesh sizes in order to obtain a pure powder of the spores. The formulation was prepared by mixing talcum powder (mesh = 360 with 95% whiteness) with 1% carboxy methyl cellulose (CMC) to obtain the desired concentration of biocontrol agents.

Formulations of *Pseudomonas fluorescens* and *Bacillus subtilis* were prepared using talcum powder as a carrier material. One kg of talc powder was placed in a sterile metal tray, and the pH was adjusted to 7.0 with 15 g/kg (w/w) $CaCO_3$. Ten grams of Carboxy Methyl Cellulose (CMC) were added to 1 kg of talc as an adhesive and mixed well. Finally, the mixtures were packed into a polythene bag and autoclaved for 1 h at 121°C (15 lbs). After autoclaving, 400 ml of bacterial suspension (1×10^8 CFU/

mL) was added to the sterilized carrier material (1 kg) and thoroughly mixed under sterile conditions (Subash et al. 2014). The prepared formulations were allowed to dry aseptically and were ground to powder. The powder was then packed in sterile polythene bags and stored at 4°C.

Experimental preparation of inoculum and seed treatments for pots and fields

These antagonistic microbes were evaluated under glasshouse and field conditions to test the efficacy of seed biopriming and soil application (SA). The experiments were laid out with three replicates per treatment in a randomized block design (RBD) and a completely randomized design (CRD) at the department of plant pathology, G.B.P.U.A. & T., Pantnagar, Uttarakhand, India. For seed biopriming, the sorghum seeds were surface sterilized with 95% (v/v) ethanol and shaken in 10% (v/v) Clorox bleach for two to three minutes. The seeds were then rinsed thoroughly three times with sterile distilled water. For the protection assay, biocontrol agents or a corresponding mock (0.85% NaCl) were bioprimed (10 g/Kg of seeds, conidia 10^7 CFU/g in 0.5% aqueous carboxy methyl cellulose) for 12 h prior to sowing (Kumar et al. 2014). These bioprimed seeds were sown with sterilized soil in pots. Further, seedlings were drenched by SA of antagonistic microbes at 5 g talc-based formulation/lit at rhizospheric root zone after twenty days of sowing. The untreated seeds served as controls and were also maintained in the experiment (Udai et al. 2016). *D. dadantii* was grown on Luria–Bertani (L.B. Himedia) agar plates at 37°C for 24 h. Subsequently, the bacterial cells were pelleted by centrifugation and were re-suspended in sterile 0.85% NaCl at a final concentration of 2×10^8 CFU/mL (Additional file 2: Figure S3).

Evaluation of antagonistic effects on plant growth promotion and disease dynamics under glasshouse and field conditions

Glasshouse experiment

A glasshouse experiment was performed to evaluate the seed biopriming and SA in the rhizospheric zone with antagonistic microbes for their effects on the growth parameters, disease suppression, and induction of defense enzymes. The seeds hydrated with water alone were considered as a mock control. *D. dadantii*-inoculated plants were considered as the pathogen-treated control. In sterilized 30 cm plastic pots, soil and sand were mixed at a ratio of 3:1 (w/w), pH 7.0. Ten seeds were sown in three pots in each treatment and thinned to six plants in each pot after germination. Plants were grown at an average temperature of 30°C during the day (10–13 h) and 26°C at night. After twenty-one days, sorghum plants were inoculated with *D. dadantii* suspension (2×10^8 CFU/mL). The observations were recorded over fifty days after sowing on plant growth attributes, including root length, root fresh weight, root dry weight, shoot length, shoot fresh weight, and shoot dry weight. Three replicates per treatment were used, and followed a CRD in 2016. The symptom of bacterial stalk rot was recorded based on the visible scale (0–5) of Teh (1983), and the percent disease index (PDI) was calculated as described previously (Wheeler 1969).

0 = No symptoms 1 = Initial small necrotic areas/ partial rotting at the base of the whorl/stalk, 2 = 25–49% dark brown, water-soaked, soft or slimy at the base of the whorl, the disintegration of pith tissues at a single internode, premature wilting of uppermost leaves, 3 = 50–74%, decay spreading rapidly crossing 2–3 internodes in a collapsed plant, 4 = 75–100% of tissue rotted with foul smell at the base of whorl/ extensive necrosis; soft rotting with visible external symptoms, 5 = lodging accompanied by extensive necrosis/ rotting of leaf /stalk tissue, usually having a very strong.

$$\begin{aligned}\text{PDI}(\%) = &\Sigma(\text{Scale} \times \text{Amount of plants})\\ &/(\text{Maximum grade}\\ &\times \text{Total number of plants})\end{aligned}$$

Field experiment

Sorghum seeds were sown in three rows of 6 m in length in the plots at the Livestock Research Centre, G.B. Pant University of Agriculture and Technology, Pantnagar, during the Kharif seasons of 2017 and 2018 with a randomized block design (RBD) with three replications. The dimensions of the spacing were 40 cm × 15 cm. The seed was sown at a rate of 15 kg/ha and a depth of 3–4 cm. The urea, single superphosphate (P_2O_5), and potash muriate (K_2O) were applied at rates of 120, 50, and 40 kg/ha, respectively. Nitrogen was treated equally to urea in two divided doses. The first half is applied as a basal dose along with phosphate and potash, and the second half was applied as a top-dressing 40 days after sowing. After 25 days after sowing (DAS), the plants were thinned to maintain a space of 15 cm in between. The pathogen and antagonistic application were described in experimental design and sampling. The observations on root length, shoot length, and yield biomass were recorded at 80 DAS. The symptom of bacterial stalk rot was recorded based on the visible scale (0–5), and PDI was calculated as described above under glasshouse conditions. Five replicates were used in each treatment.

Effects of seed biopriming and soil application on the induction of defense enzymes under glasshouse condition

To study the defense enzymes, 500 mg of leaf tissue from sorghum seedlings was treated with antagonistic microbes and water were collected at various time intervals, viz., pre-inoculation (PI) and 24, 48, and 72 h after pathogen inoculation (HAI). The samples were quickly frozen in liquid nitrogen and kept at −80°C until further analysis. Enzyme activities were quantified as per the procedures described for PAL (Amrhein et al. 1992), polyphenol oxidase (PPO) (Hammerschmidt et al. 1982), and peroxidase (PO) (Hills and Swain 1959), total phenolics content (TPC) (Hills and Swain 1959), and catalase (CAT) (Dhindsa et al. 1981).

Histological studies

Lignification: Sorghum seedlings treated with antagonistic microbes were collected seven days after pathogen inoculation (DAI) under glasshouse conditions. The transverse section of the roots was examined to investigate in situ lignin deposition. The staining of five root sections with a 1% saturated aqueous solution of phloroglucinol in 20% hydrochloric acid was placed on a slide and fixed with 95% (v/v) ethanol. The observations were captured by a compound light microscope (Nikon, Japan). The presence of lignin is indicated by the development of a pink color (Saxena et al. 2015).

Callose deposition: To detect the callose deposition, antagonists-treated and control plants were assayed after 48 h of pathogen challenge following a method described previously (Asselbergh and Höfte 2007). The leaves were incubated in a 3:1 ratio of ethanol: acetic acid overnight for decolonization. Hand-cut leaves sections were stained for 2 h with 0.01% aniline blue (Sigma-Aldrich, Merck), and prepared in 1 mM sodium phosphate buffer (pH 8.0). The stained sections were mounted on a slide and examined under a fluorescence microscope (Leica DMI3000 B) using a UV filter and the excitation and emission wavelengths are 480 and 570 nm, respectively with a 4′,6-diamidino-2-phenylindole (DAPI) filter. Images were collected, processed, and analyzed using ImageJ software (http://rsbweb.nih.gov/ij/) (Piršelová et al. 2012).

Electron microscopic studies

Twenty days old sorghum seedlings were randomly selected from the growth pot for scanning electron microscopy examination. Tissue samples of non-inoculated and inoculated seedling roots of sorghum were fixed overnight in 2% glutaraldehyde (5 mM phosphate buffer pH 7) in the refrigerator (4°C). For dehydration, samples were washed twice in the same buffer for 15 min, and post-fixed in 1% OsO4 for 5 h. The samples were dehydrated in 20, 30, 50, 70, 80, and 90% ethanol for 10 min, and 100% ethanol twice for 20 min (Kim et al. 2005). Scanning electron microscope (SEM) imaging was conducted using the Critical Point Drying (CPD) method with sputter coating and 20 kV scanning electron microscopes. A SEM analysis revealed root vascular systems and patterns of antagonists colonization.

Statistical analyses

ANOVA was used to analyze the experimental data followed by Duncan's Multiple Range Test (DMRT) as a post hoc analysis at 5% ($P \leq 0.05$) confidence interval, using the Statistical Package for Statistical Product and Service solutions (SPSS 20.0) program. As shown in the figures, the values of in vitro (dual culture assay) and glasshouse experiments are the mean of three replications ± standard deviation. Heat map, correlation, and principal component analysis (PCA) analyses were performed in RStudio version 1.1.463 (RStudio Team 2018), using the factoextra, PerformaceAnalytics, and complexHeatmap packages of R version 4.1.0 (R Core Team 2018). The whole experiments were repeated twice in year 2017–2018.

Abbreviations

G.B.P.U.A. & T	Govind Ballabh Pant University of Agriculture and Technology Pantnagar, Uttarakhand
DMRT	Duncan's multiple range test
DAI	Days after pathogen inoculation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00202-z.

Additional file 1. Table S1. Biochemical, physiological, and morphological characterization of isolates of *Dickeya dadantii* isolated from sorghum plants. **Table S2.** Details of Origin, Identification, GenBank accession numbers, and confrontation assay of antagonistic isolates. **Table S3.** Effect of seed biopriming and soil application antagonist microbes on growth promotion and disease severity under glasshouse conditions at 35 and 50 DAS. **Table S4.** Effect of seed bio priming and soil application antagonist microbes on growth promotion and disease severity under field conditions at 35, 50, 65, and 80 DAS 2017. **Table S5.** Effect of seed bio priming and soil application antagonist microbes on growth promotion and disease severity under field conditions at 35, 50, 65, and 80 DAS 2018.

Additional file 2 Figure S1. Effects of seed biopriming and soil application antagonist microbes on disease development in sorghum under pathogenic stress conditions. **Figure S2.** Rotting symptom of bacterial stalk rot in sorghum under field conditions. **Figure S3.** Flow chart of the experimental design.

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Author contributions
SY designed and conducted the experiments, and prepared the manuscript. YS supervised the experiment and provided the facility for the experiment. VS analyzed the data. AA contributed to perform the experiments. All authors read and approved the final manuscript.

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Availability of data and materials
All datasets generated from the current study are included and cited in the manuscript. Further datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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