Fungicide tolerant *Bradyrhizobium japonicum* mitigate toxicity and enhance greengram production under hexaconazole stress

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**ARTICLE INFO**

Article history:
Received 22 February 2018
Revised 24 June 2018
Accepted 18 July 2018
Available online 26 July 2018

Keywords:
Hexaconazole toxicity
Greengram
*Bradyrhizobium japonicum*
Cellular damage
Nodule ultrastructure
Oxidative stress

**ABSTRACT**

Bacterial strain RV9 recovered from greengram nodules tolerated 2400 μg/mL of hexaconazole and was identified by 16S rDNA sequence analysis as *Bradyrhizobium japonicum* (KY940048). Strain RV9 produced IAA (61.6 μg/mL), ACC deaminase (51.7 mg/(protein·hr)), solubilized TCP (105 μg/mL), secreted 337.6 μg/mL EPS, and produced SA (52.2 μg/mL) and 2,3-DHBA (28.3 μg/mL). Exopolysaccharides produced by strain RV9 was quantified and characterized by SEM, AFM, EDX and FTIR. Beyond tolerance limit, hexaconazole caused cellular impairment and reduced the viability of strain RV9 revealed by SEM and CLSM. Hexaconazole distorted the root tips and altered nodule structure leading thereby to reduction in the performance of greengram. Also, the level of antioxidant enzymes, proline, TBARS, ROS and cell death was increased in hexaconazole treated plants. CLSM images revealed a concentration dependent increase in the characteristic green and blue fluorescence of hexaconazole treated roots. The application of *B. japonicum* strain RV9 alleviated the fungicide toxicity and improved the measured plant characteristics. Also, rhizobial cells were localized inside tissues as revealed by CLSM. Colonization of *B. japonicum* strain RV9 decreased the levels of CAT, POD, APX, GPX and TBARS by 80%, 5%, 13%, 13% and 19%, respectively over plants grown at 80 μg/(hexaconazole·kg) soil. The ability to detoxify hexaconazole, colonize plant tissues, secrete PGP bioactive molecules even under fungicide pressure and its unique ability to diminish oxidative stress make *B. japonicum* an attractive choice for remediation of fungicide polluted soils and to concurrently enhance greengram production under stressed environment.

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**Introduction**

Fungicides are repeatedly and harshly used in various cropping systems including those of legumes to control several soil-borne diseases. Due to extensive and injudicious use, major portion of the fungicides applied in superfluous quantity persists in soils (Gamiz et al., 2016). The accumulation of fungicides within soils and its uptake later on by plants, cause toxicity to legumes (Mohamed and Akladious, 2017). Greengram (*Vigna radiata* (L.) Wilczek) among legumes is an important pulse crop which provides high protein (20%–25%) and carbohydrate (60%–65%) in human foods (Swain et al., 2014). Although greengram is cultivated over a large area, it suffers heavily from several biotic and abiotic factors which

https://doi.org/10.1016/j.jes.2018.07.007
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Rhizobium fungicide tolerant Gram-negative nitrogen fixing bacteria like limit its production (Deepa et al., 2017). Greengram attacked by several pathogens results in extensive yield losses (Tripathy, 2017) which could be up to 44% by fungal pathogens alone (Iqbal et al., 2014). However, to eliminate the fungal diseases, several protective measures such as physical, cultural and mechanical practices along with crop rotation or independently have been employed. These fungicides besides showing inhibitory effects on to fungal phytopathogens also cause major hazards to the existence and metabolic activities of beneficial soil microbiota (Fernández-Calviño et al., 2017). As a result, the fertility and texture of soil is lost (Rani et al., 2017). Hexaconazole among commercial fungicides is used to manage numerous phytopathogenic fungi belonging mainly to ascomycetes and basidiomycetes. On the contrary, hexaconazole destructs the overall soil microbial biomass, respiration, bacterial composition and microbial diversity (Ju et al., 2017). Also, hexaconazole has been found to inversely affect the — (i) nitrogen fixation activity, (ii) ureide levels in legumes, (iii) nitrogen metabolism, and (iv) nitrogen products in plants (Kengar and Patil, 2017).

The regular use of fungicides has however, led to the emergence of resistance among pathogens as well. So, to overcome these problems, some viable and practicable strategies need to be developed. In this context, microbial communities with fungicide tolerance abilities have been identified and explored to eliminate/minimize fungicide threat to sustainable crop ecosystems. For example, some fungicide tolerant Gram-negative nitrogen fixing bacteria like Rhizobium, Azotobacter, and a few Gram-positive bacteria like Bacillus (You et al., 2016) have been reported to play a major role in detoxification of fungicides leading eventually to the enhancement in legume production (Alori et al., 2017). Despite variable impact achieved so far, no systematic study has been conducted to assess the toxic end points of hexaconazole on active biomolecules of Bradyrhizobium japonicum or its influence on growth and oxidative stress of greengram. Also, no study has ever been conducted to understand as to how hexaconazole affects bacterial cell surface, root morphology of greengram plants and Bradyrhizobium–greengram interactions involving localization of rhizobial cells on to root surface and within nodule tissues.

Taking these and other related information into consideration, the present investigations focus on to assess the influence of hexaconazole on — (i) growth of B. japonicum and its ability to secrete bioactive molecules, (ii) biochemical, symbiotic and yield features of fungicide tolerant B. japonicum inoculated greengram plants raised under fungicide pressure, (iii) root morphology and ultrastructure of nodules, (iv) colonization of rhizobial strain in roots and nodules, and (v) antioxidant defense systems, ROS production, lipid peroxidation and proline formation in inoculated plants.

1. Materials and methods

1.1. Nodule bacteria and fungicide tolerance

Healthy and undamaged nodules were detached from greengram plants grown in fields receiving pesticides. Suspensions prepared from surface sterilized (Vincent, 1970) nodules were streaked on YEMA plates and kept at 28 ± 2°C for 3–5 days. Overall, 30 Bradyrhizobium isolates were selected and characterized (Holt et al., 1994). The host specificity was determined by plant infection technique. The isolates were exposed to variable concentrations of hexaconazole (CAS: 79983-71-4, MF: C₆₄H₅₂C₆N₇O, MW (g/mol): 314.21, Purity: ≥99%, MP 111°C) and fungicide tolerant Bradyrhizobium strains (FTRS) were selected. The impact of hexaconazole on growth kinetics was determined further by growing strain RV9 in mineral salt medium (MSM) containing 400, 800 and 1200 μg/hexaconazole/mL. The inoculated but hexaconazole treated flasks were incubated at 28 ± 2°C and growth was recorded at 600 λ.

1.2. Strain identification and construction of phylogenetic tree

Fungicide tolerant Bradyrhizobium strain RV9 characterized primarily was identified to species level by 16S rRNA sequencing using universal primers 785F (5′-GGATTAGATACCTGGTA-3′ and 907R (5′-CCGTCAATTCTTTRAGTT-3′). The sequences so obtained were submitted in GenBank. The BLASTn program available online was adopted to find relatedness with sequence of other bacterial strains deposited in NCBI to accurately identify the species of RV9 strain. Also, sequences were aligned by means of bootstrapped neighbor-joining and tree was created.

1.3. PGP features under hexaconazole stress

1.3.1. Production of IAA, HCN, NH₃, siderophore, ACC deaminase and P solubilization

Indole acetic acid (IAA) secreted by B. japonicum RV9 strain was quantitatively determined using modified procedure of Brick et al. (1991) while Hydrogen cyanide (HCN) was evaluated by growing bacterial culture on glycine induction medium (Bakker and Schippers, 1987). Ammonia and siderophore were determined following the method of Dye (1962) and Atkin et al., (1970), respectively. Further, siderophores was quantitatively assayed by the method of Alexander and Zuberer (1991). Also, salicylates (SAs) and dihydroxy phenols siderophores (DHBAs) were quantitatively assayed (Reeves et al., 1983). 1-amino cyclopropane (ACC) deaminase secreting ability of B. japonicum strain RV9 was qualitatively assessed by the Dworkin and Foster (1958) medium. Mesorhizobium LMS-1 having pRKACC plasmid (Shah et al., 1998) was used as a positive control. Additionally, the quantity of α-ketobutyrate was detected as described by Penrose and Glick (2003). P-solubilization potential was determined using liquid PKV medium (Pikovskaya, 1948). Hexaconazole used under in vitro experiments for all PGP activities was added to each biomolecule specific medium individually at 1× (400 μg/mL), 2× (800 μg/mL) and 3× (1200 μg/mL) concentration.

1.4. EPS extraction, purification and characterization

The release of Exopolysaccharides (EPS) from strain RV9 was analyzed by the employing method of Mody et al. (1989) using 5% sucrose as C source and treated separately with three concentrations of hexaconazole. Total carbohydrate content in
crude EPS was estimated by Duboi’s method while EPS protein was determined by the method of Lowry (1951). The microstructure of dried EPS was observed under Scanning electron microscope (SEM). Elemental, group composition and surface morphology of EPS was determined by Energy Dispersive X-ray spectroscopy (EDX), Fourier Transform Infrared Spectroscopy (FTIR) and atomic force microscopy (AFM), respectively.

1.5. Cellular damage and permeability alteration modulated by hexaconazole

Destruction/damage to rhizobial cell (RV9) was viewed under SEM after growing cells in nutrient broth treated with 1600 μg/mL of hexaconazole. Changes in membrane permeability were observed under Confocal Laser Scanning Microscopy (CLSM) using propidium iodide (PI) as a probe for determination of dead cells. Bacterial cells (non-treated and supplemented with different concentration of hexaconazole) were washed thrice using Phosphate buffer saline (PBS) stained with PI and maintained in the dark to avoid photobleaching of dye, and visualized.

1.6. Plant culture experiments under hexaconazole pressure

1.6.1. Germination efficiency and seedling vigor index

Properly sterilized seeds of greengram (var. K-851) were sown in pots and after five days of sowing (DAS), radicle emergence of 1 mm was scored and germination percentage was calculated as:

Germination% = no.of seeds germinated/total no.of seeds × 100

Seedling vigor index (SVI) was calculated as: SVI = [root length + shoot length] × %seed germination (Abdul-Baki and Anderson, 1973).

1.6.2. Growth, chlorophyll contents and symbiotic features

Nine plants in three pots were detached at 50 DAS after sowing while other three pots were harvested at 80 days after sowing. Greengram plants collected at 50 and 80 DAS were dried at 80°C and dry biomass accumulated inside tissues was recorded. Also, symbiotic characteristics, for example nodule number (NN) and its dry biomass (DBN) were measured both at 50 and 80 DAS. Photosynthetic pigments (‘a’ chl, ‘b’ chl, total chlorophyll and carotenoid contents) in hexaconazole treated foliage (Arnon, 1949) and LHb content (Sadashivum and Manickam, 1992) in fresh nodules were determined 50 days after growth.

1.6.3. Phosphorous content and grain features

The total P content in shoots and roots of greengram plants removed at 80 days after sowing were measured by the method of Jackson (1976). Grain yield (GY) and seed protein (SP) (Lowry et al., 1951) were assessed at 80 DAS.

1.7. Oxidative stress and antioxidant enzymes

1.7.1. Proline accumulation and lipid peroxidation

Accumulation of proline in different organs of plant tissues (root, shoot and leaf) was assayed at 50 days after sowing, whereas, in seeds it was determined at harvest (80 days after sowing) by the method of Bates et al. (1973). Peroxidation of lipid (Malondialdehyde (MDA) content) assayed in leaf by the method of Heath and Packer (1968) was expressed as μ/ (mol-MDA·g·fw). The content of TBARS was calculated by the extinction coefficient = 155 mM/cm.

1.7.2. Antioxidant enzymes activities

Antioxidant defense enzymes like catalase, Guaiacol Peroxidase (GPX), peroxidase and Ascorbate peroxidase (APX) in leaf tissues were determined at 50 days after sowing. For CAT and GPX, leaf foliage (0.1 g) were homogenized in phosphate buffer. After centrifugation of homogenized tissues at 12,000 r/min for 15 min (4°C), CAT activity was estimated (Beer and Sizer, 1952). The drop in the absorbance because of H2O2 (ε = 39.4 mM/cm) breakdown was observed at 240 λ and expressed as Nano molar H2O2 mg/(protein·min) GPX was estimated using crude extract of foliage tissues following the method of Zhang and Kirkham (1996). Intensification in absorbance at 470 λ caused by the development of tetra guaiacol (ε = 26.6 mM/cm) is manifested as μ/ (mol-mg-protein-min). POD and APX activities were assayed by the methods of Leonard et al. (2004) and Hammerschmidt et al. (1982), respectively.

1.7.3. ROS generation under hexaconazole stress

To assess the ROS generation, roots detached from plants grown with hexaconazole were stained using a cell-permeable fluorogenic probe (2’,7’-DCFH-DA) and kept in the dark for some time at RT. After staining, root tissues were washed with PBS (0.1M) to remove extra stain, mounted on slides and were viewed under CLSM.

1.7.4. Cytotoxicity assay, anatomical and cytological destruction of nodules

Loss in cell survivability of plants was observed by employing Evan’s blue dye method. To observe the viability loss, hexaconazole treated roots were stained with Evan’s dye (0.2% w/v) for 10 min. and successively washed using DDW for 30 min. Samples were then viewed under CLSM to record cell death. Anatomical and cytological changes in hexaconazole stressed nodules were observed under SEM following standard methods.

1.8. Root tip distortion and bacterial colonization

The toxic and damaging effect of hexaconazole on morphological structure of test plant roots were observed by allowing the seeds to grow on 0.7% soft agar plates supplemented with 1600 μg/mL hexaconazole and monitored under SEM. Uninoculated and RV9 inoculated roots of greengram were further used to observe the B. japonicum establishment inside root tissues. For this, roots were washed carefully with H2O and PBS. Roots were fixed for overnight in 2% (w/v) glutaraldehyde prepared in 0.1 M PBS (pH 7.0), washed thrice and dehydrated, dried in CPD, fixed in gold stubs and viewed under SEM. The aggregation of rhizobial strain RV9 within nodule tissues was also assayed and viewed under CLSM.
2. Results and discussion

2.1. Characterization, identification and fungicide tolerance of rhizobial strain

Strain RV9 recovered from greengram nodules was recognized as Gram-ve bacterium and exhibited different biochemical properties (Supplementary Table S1). Accordingly, strain RV9 was found to belong to genus Bradyrhizobium while 16S rDNA gene sequence analysis confirmed it as B. japonicum. A phylogenetic tree constructed by neighbor joining method using MEGA 6.0 software employing 16S rRNA partial gene sequence is presented in Supplementary Fig. S1.

Fungicides are generally applied in agronomic practices to offset diverse soil borne disease causing phytopathogenic fungi to safeguard crops and hence, to enhance food production. However, random and excessive use of fungicides and their excessive use in soils leads to the decrease in productivity of crop and emergence of pesticide resistance among microbial communities. These are greatly worrying for agronomists and require urgent remedy to overcome such a daunting problem. Targeting these, we endeavored to discover fungicide tolerant root nodulating bacteria which could be helpful in completely eliminating/reducing the fungicide input in legume production. To this end, B. japonicum strain RV9, when exposed to different concentrations of hexaconazole, was found to exhibit a strangely higher tolerance (2400 $\mu$g/mL) to hexaconazole. Since this bacterium was grown on minimal salt medium which was deficient in C and N but had only varying concentrations of hexaconazole, it is presumed that the strain RV9 might have degraded this fungicide and used it as a nutrient for its growth. Consequently, the strain RV9 displayed higher tolerance against hexaconazole. Fungicide tolerance that among nodule bacteria is indeed an interesting feature which could be used to facilitate legume production in fungicide polluted soils. Mechanistically, the fungicide detoxification is a complex physiological process which is controlled largely at genetic level (Jahn et al., 2017). And hence, the microorganisms that possess this property (fungicide tolerance) are likely to destruct/degrade fungicides and in effect may be used to remediate the contaminated soils (Sang et al., 2016).

2.2. Hexaconazole modulates growth kinetics, cell surface structure and membrane permeability

After evaluating the influence of fungicide on solid medium, growth pattern of fungicide tolerant B. japonicum strain RV9 was determined by growing this bacterium in liquid MSM treated with varying concentrations of hexaconazole (Fig. 1 iii). Usually, the growth of strain RV9 was sluggish in the beginning which however, increased somewhat linearly with increasing growth periods and decreased sharply thereafter. Hexaconazole at 1200 $\mu$g/mL in general had more devastating effects on the growth of bacteria than other rates of fungicide. While comparing the growth behaviour of strain RV9 on solid and liquid MSM, strain RV9 survived better on solid medium. In contrast, strain RV9 grew in liquid medium even at 1200 $\mu$g/mL hexaconazole, but the growth was comparatively poor.

Fig. 1 – (i) SEM micrograph of B. japonicum strain RV9 (a) and control (b & c) treated with 1600 $\mu$g/mL hexaconazole (yellow arrows indicate the damage/rupture in cells after exposure to hexaconazole. (ii) CLSM images of live/dead cells of B. japonicum (RV9) stained with PI (Propidium iodide) & DCF (di chloro fluorescein) indicating the cell viability (A) and control cells (B, C & D) treated with 0, 400, 800 and 1200 $\mu$g/mL of hexaconazole. (iii): Growth pattern of B. japonicum strain RV9 grown in MSM medium treated with increasing concentrations of hexaconazole as a function of time of incubation at 28 ± 2°C. Data represent the mean ± S.D. of three independent experiments done in triplicate.
The inconsistency in tolerance to hexaconazole, however, may be due to the variations in nature of medium used. For instance, in liquid medium, the uptake of fungicide by the rhizobial cell is easy due to the greater solubility and easy availability compared to solid medium where the movement of fungicide could be restricted due to the polymeric nature of solid agar which is used as a solidifying agent. And hence, there were greater tolerance on solid medium than in liquid culture medium. However, despite these, strain RV9 showed maximum tolerance to hexaconazole which gives this organism an edge over other sensitive strain while growing under harsh (stressed) environment.

After assessing the lethal influence of hexaconazole and tolerance ability of _B. japonicum_ RV9 under in vitro, the impact of hexaconazole on bacterial cell surface was assessed. The micrographs showed a dissimilar but intact cell when raised in hexaconazole free environment (Fig. 1i A). But, cells of strain RV9 treated with hexaconazole were found damaged (Fig. 1i B, C) which confirmed the toxicity of fungicide under in vitro conditions. Also, the cell permeability of _B. japonicum_ cells RV9 observed under CLSM was lost when exposed to 1200 μg/mL hexaconazole (Fig. 1 ii). Metabolically inactive/dead cells of bacterial strains were red colored short rods due to propidium iodide staining of nucleic acid (DNA) of bacterium. As the concentration of hexaconazole increased, red fluorescence was also increased due possibly to the binding of propidium iodide with DNA in cellular membrane. Once the fungicide molecules attach to the cellular membrane by electrostatic interaction, it (interaction) triggers the initiation of the oxidative stress leading to the formation of free radicals (ROS). The ROS so generated disrupts the bacterial cell membrane and eventually propidium iodide is taken up by bacterial cells. Similarly, reduction in cell permeability of bacterial cells as a result of increased red fluorescence has recently been reported (Shahid and Khan, 2018).

### 2.3. Plant growth bioactive molecules under hexaconazole stress

#### 2.3.1. IAA, cyanogenic compounds, NH₃, phosphate solubilization, siderophores and ACC deaminase

_B. japonicum_ strain RV9 revealed a variable amount of PGP bioactive molecules when grown in the presence and absence of hexaconazole (Table 1). Usually, the quantity of plant growth promoting substances secreted by RV9 dropped with cumulative rates of hexaconazole. For example, RV9 produced 61.6 μg/mL IAA when grown in fungicide devoid medium. On the contrary, the IAA declined gradually with consistent increase in the rates of hexaconazole reaching to a maximum of 26.9 μg IAA/mL at 1200 μg/mL. In support of this finding, Nithyakalyani et al. (2016) reported the production of similar phytohormone by Bradyrhizobium strain. The progressive decline in phytohormones with constantly enhancing hexaconazole concentration is indeed disturbing on one hand while the secretion of IAA even at higher concentration (even though lesser quantity) is agronomically encouraging. This finding therefore is likely to boost the legume production even under fungicide rich soil when such rhizobium will be used as bacterial inoculant (biofertilizer). From this study it is

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<th>Treatments</th>
<th>Dose rate (μg/mL)</th>
<th>IAA (μg/mL)</th>
<th>P-solubilization (μg/mL)</th>
<th>Catalase activity (U/mL)</th>
<th>ACC deaminase activity (μM α-Ketobutyrate mg⁻¹ protein hour⁻¹)</th>
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**All values indicate the mean of three independent replicates ± standard deviation. Mean values (±) followed by different letters are significantly (p ≤ 0.05) different from each other according to DMRT.**
apparent that when applied under field, bradyrhizobial strains are likely to continue secreting phytohormone even under fungicide stress. The IAA so released will facilitate various biological functions like cell growth and division, morphogenetic changes, symbiotic attributes, growth stimulation and movements of plants under fungicide polluted soils (Deinum et al., 2016). Cyanogenic compound produced by beneficial microbes is yet another important secondary metabolite that suppresses the growth of fungal pathogens inhabiting soil. Hydrogen cyanide and NH₃ as detected in this study at lower concentration was completely abolished at three times more rates of hexaconazole. Similar inhibition in HCN and ammonia production by Bradyrhizobium and Pseudomonas species under herbicide stressed environment is reported (Shahid and Khan, 2017).

The P-solubilizing activity (PSA) of *B. japonicum* strain RV9 grown with different concentrations of hexaconazole varied considerably (Table 1). Generally, as the dose of hexaconazole increased, a decline in PSA both on/in PVK medium was noticed. The toxic impact of hexaconazole on P-zone forming ability of *B. japonicum* RV9 was less pronounced at smaller concentration compared to higher dose rates. In liquid culture medium, the maximum decrease (53%) in PSA was recorded at 3× of hexaconazole (49.3 μg/mL) compared to untreated control (105 μg/mL). In many studies, it has been reported that PSA of rhizobacteria is mediated by different low molecular weight organic acids such as α-keto gluconic, oxaloacetic gluconic, acetic, maleic, citric and succinic etc. secreted by *P* solubilizers (Zaidi et al., 2017). Like other plant growth regulators, the production of siderophores including SA and 2,3 DHBA also diminished noticeably under changing concentrations of hexaconazole. For example, SA and 2,3-DHBA produced by strain RV9 grown in controlled condition was 52.2 and 38.3 μg/mL respectively, which however, decreased by 53 and 41%, respectively at 3× of hexaconazole. The secretion of siderophores, a low molecular mass compound (iron-chelators) by microbes under iron starved/deficient environment is considered important (Grandchamp et al., 2017), for many plants growing under iron limited environment (Verbon et al., 2017). Also, under aerobic environments, siderophores form a complex with iron and make it inaccessible/unavailable to other harmful microbial communities. Due to these, the release of siderophores by nodule bacteria under iron limited condition could be extremely useful in the management of phytopathogens.

ACC (1-amino cyclopropane 1-carboxylate) deaminase produced by numerous bacteria belonging to *Plant growth promoting rhizobacteria* (PGPR) group is an imperative biochemical feature that converts thenoxious constituents of ethylene into safe and harmless form and permits the floral vegetations to grow normally under the adverse environments (Han et al., 2015). In the present study, *B. japonicum* RV9 strain even under the fungicide stressed condition produced/secreted ACC deaminase reaction (Table 1). However, like other growth regulators, the concentration of α-ketobutyrate also declined gradually with increasing concentrations of fungicide (Table 1). Hexaconazole at 3×, exhibited the maximum inhibitory impact and reduced the production of α-ketobutyrate maximally by 69% over untreated control. The secretion of ACC deaminase by *B. japonicum* RV9 even under fungicide pressure might agriculturally be an advantageous feature for optimizing the yield and efficiency of crops under fungicide stressed condition (Glick et al., 2007). Overwhelmingly, the ability of *B. japonicum* RV9 to persist under hexaconazole stress and to maintain its potential to produce the active biomolecules even under fungicide pressure makes this organism an attractive and best valuable option for enhancing the yield of crops even under fungicide contaminated environment. However, efforts should be directed to prevent losses in growth promoting activity of *B. japonicum* RV9 while growing under fungicide stressed environment for extended periods. This could be achieved by evolving mechanism where bacteria can drive out fungicides as it come into the cell or by altering the fungicide target site. Also, identifying hexaconazole tolerant genes from other PGPR and inserting it into rhizobia could solve these problems. However, all these approaches require participation of microbiologists, plant protectionists and molecular biologists to find a suitable strategy for maintaining the secretion of growth regulators even under fungicide stress.

**2.3.2. Extraction, purification and characterization of EPS**

In order to better understand the importance of EPS in BNF (Arnold et al., 2018), soil compaction (Harahap et al., 2018) and competence of soil bacteria (PGPR) to adjust to the environmental stressor molecules, strain RV9 was further scrutinized for its capacity to synthesize EPS under fungicide pressure. Interestingly, RV9 released a considerable quantity of exopolysaccharide that may disturb the growth and physiological functioning of various plants directly/indirectly under harsh environmental conditions (Dimkpa et al., 2009). In the absence of hexaconazole, strain RV9 produced 337.6 μg/mL EPS which however, declined gradually with increasing concentrations of fungicide. The bacterial exopolysaccharide might be beneficial both for producer (bacterial strain) and crops while growing under stress. Bacterial EPS are reported to protect bacterial strains from unsuitable environments like desiccation, heavy metals, drought (Naseem and Bano, 2014) and salinity (Meneses et al., 2017) by developing a polymeric network around bacterial culture. Also, bacterial EPS safeguards the plant system from the attack of phytopathogens (Zheng et al., 2016) and bacteriophages (Whitfield et al., 2015). Apart from these, EPS positively influence incursion procedure, formation of infection threads, bacteroid development, and nodular morphogenesis throughout symbiotic process. Therefore, the search for exopolysaccharide producing PGPR strains has greatly been intensified (Kaushal and Wani, 2016).

The physical properties, functional moieties and super molecular structure of EPS produced by bacterial strain was further characterized using SEM, FTIR and AFM techniques. The microscopic structure and surface topology images of exopolysaccharides produced by RV9 are depicted in Fig. 2 (B and A1). Under SEM, EPS seems overlaid polysaccharide sheet appearing as smooth exterior (at 10,000× magnification) and exhibited a very compact structure. Like this, SEM image of EPS produced by *Lactobacillus* strains isolated from cabbage and cucumber is reported (Singh et al., 2017). Furthermore, elemental composition of EPS was quantitatively assayed by EDX (Fig. 2B).

Among other techniques employed in detection of biomolecules, the FTIR spectroscopy is considered a very sensitive,
specific, and non-destructive technique. The IR spectrum of EPS recorded in ATR mode (Fig. 2E) showed a very common and broad signal highest at 3417 cm$^{-1}$, which indicates broad stretching phenolic group ($\text{-OH}$). Polysaccharides when observed under FTIR were found to have a number of $\text{-OH}$ groups at various sites and exhibited a stretching peak at around 3400–3300 cm$^{-1}$. Therefore, the absorption at 3417 cm$^{-1}$ is suggestive of polysaccharide in the sample as also reported by other workers. The signals at 2926 and 2850 cm$^{-1}$ correspond to C–$\text{H}$ asymmetric stretching of $\text{CH}_3$ in fatty acids moieties and C–$\text{H}$ asymmetric stretch of $\text{>CH}_2$ (Maquelin et al., 2002). No signal around 1700–1775 cm$^{-1}$ was observed suggesting the absence of either glucuronic acid or diacyl ester in EPS, which is consistent with the findings of Wang et al. (2010). A strong absorption peak at around 1621 cm$^{-1}$ also specified the distinguished feature of IR signal for polysaccharides. A weak signal at 1539 cm$^{-1}$ could be allocated to N–$\text{H}$ winding vibrations in amide II of proteins. The peak at 1455 cm$^{-1}$ can be attributed to unequal twist of CH$_3$ and CH$_2$ in proteins (Lu et al., 2011). The signal in the region of 1413 cm$^{-1}$ well matched $\text{>C=O}$ stretch and C–O bond of carboxyl (COO$^-$) groups. The presence of a relatively strong signal at 1238 cm$^{-1}$ corroborates the findings of Wang et al. (2010) due to the existence of O-acetyl ester. Another broad stretch in the fingerprint region (1000–1200 cm$^{-1}$) due to C–O–C and C–O stretch corresponds to carbohydrates and hence the absorption highest at 1055 cm$^{-1}$ is assigned to polysaccharides. Specifically, the signal at 887 cm$^{-1}$ determines glycosidic linkages of polysaccharides. The FTIR spectrum of EPS isolated from $B$. japonicum represents the presence of COO$^-$ groups which is likely to influence the binding positions of divalent cations (Bramhachari et al., 2007). Furthermore, the presence of prominent signals for ($\text{-OH}$), COO$^-$ and glycosidic bonds could be accountable for highly cross-linked polysaccharides in EPS formation.

The structural analysis of exopolysaccharides by AFM is shown in figure. EPS of $B$. japonicum deposited from aqueous solution in ultrapure water on cover slip showed spherical lumps of variable sizes. The maximal height of polymer aggregates was 300 nm (Fig. 2C & C1). The viscosity could also be attributed to the glycosidic linkages between monomer units in the polysaccharide structure. The AFM–SMFS technology is a unique device which is used to explain the various properties of EPS such as conformational transitions, the dynamics, and molecular structures (Lei et al., 2017). The AFM image of polysaccharides extracted from strain RV9 are shown in Fig. 3C & C1. The height of EPS lumps was 13 nm. However, at 100 g/mL of EPS, the lumps and chains were
irregular in shape. Furthermore, the three-dimensional structure of EPS extracted from *B. japonicum* viewed under AFM was variable. The variable size of particles found inside EPS represented surface roughness (mean value 191 mm) of EPS.

### 2.4. Plant growth suppression and toxicity chopdown

#### 2.4.1. Germination, SVI and promotion of greengram plants under hexaconazole stress

Generally, a germination test is conducted to determine the germination potential, or viability, of a seed and can subsequently be used to estimate the seedling vigor. Germination proficiency of greengram seeds grown up in earthen pots ranged from 80% to 93% (Fig. 3A). Furthermore, hexaconazole used at three varied concentrations caused significant reduction in SVI which was calculated as 29, 38 and 63% over control (Fig. 3B). The highest SVI (6409) was however, observed for plants inoculated with strain RV9. Similar SVI has also been used as a phytotoxicity index to evaluate the effect of heavy metals on *Allium cepa* seedling growth (Fatma et al., 2017).

The *Bradyrhizobium* inoculated and non-inoculated greengram plants cultivated in soils supplemented with variable rates of hexaconazole had inconsistent plant growth (Table 2). Over all, the measured biological parameters decreased progressively with consistently increasing concentrations of hexaconazole. Bio-inoculated plants even though, demonstrated better development relative to non-inoculated plants but biological features of such bacterized plants decreased markedly when treated with fungicide related to plants cultivated in hexaconazole devoid soils (Table 2). For instance, *B. japonicum* RV9 when applied as a microbial inoculant with two times more dose (80 μg/kg) of hexaconazole, increased the dry biomass of roots and shoots by 5 and 7% at 50 days after sowing and 6 and 13% at 80 days after sowing respectively related to non-inoculated greengram plants. While comparing the effect of microbial inoculant (strain RV9) applied with 120 μg/kg concentration of hexaconazole, a maximal enhancement of 18 and 16% in root and shoot biomass, respectively was observed at 80 days after sowing relative to uninoculated and fungicide (3×) supplemented soils.

The two-way ANOVA of the data measured at 50 DAS and 80 DAS suggested that the impact of bacterial inoculation and hexaconazole treatment on greengram plants was significant (*p* ≤ 0.05). Like many other PGPR applied under conventional soils, the FTRS RV9 applied as bioinoculant also caused a noteworthy increase in root and shoot growth of plants which could be attributed to various reasons like — (i) supply of growth enhancing active biomolecules especially IAA. Phyto-hormone (indole acetic acid) stimulates the root morphogenesis directly by invigorating expansion and division of plant cells (Etesami et al., 2015). Consequently, prolonged roots, uptake of H_{2}O and other solutes/reserves by plants is increased (Kashyap et al., 2017). Other supplementary reasons
which adequately bestowed in the growth and improvement of plant could possibly be the vital plant nutrients such as N and P supplied by bacterial strain.

2.4.2. Structural damage/distortion and bacterial colonization in root surface

Since hexaconazole demonstrated toxicity to root growth, it was decided to further examine its impact on morphology of water/nutrient absorbing component (root tips) of greengram plants using SEM while growing under hexaconazole treated soils (Fig. 4). The inhibitory effect of hexaconazole to roots was distinct at radical areas of developing plants. The hexaconazole application showed a significant aberration, fissures, crumble, fracture and spikes on the root surface compared to a clear, smooth and intact (Fig. 4B, B1) root surface under SEM. The destruction of root surface further validated the inhibitory potential of hexaconazole which in turn might have impaired the uptake of water and nutrients from soils causing improper/altered root growth. Similarly, morphological distortion/alteration in root structure of *Cicer arietinum* plants grown in pesticide stressed condition has previously been reported (Mondal et al., 2013).

Despite the toxicity of hexaconazole to both uninoculated and inoculated plants, there were considerable improvements

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose rate (μg/kg soil)</th>
<th>Plant length (cm/plant)</th>
<th>Fresh weight (g/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shoot 50 DAS 80DAS</td>
<td>Root 50 DAS 80DAS</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>0</td>
<td>30 ± 2.5b 34 ± 3b 26 ± 0.9b 32 ± 2b</td>
<td>13 ± 0.8b 13.9 ± 3b 2.0 ± 0.33a 3.9 ± 0.12b</td>
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<tr>
<td></td>
<td>40</td>
<td>21 ± 2c 24 ± 1cd 24 ± 0.9b 25 ± 2de</td>
<td>9 ± 0.57 11 ± 1.5bcd 1.6 ± 0.36b 3.3 ± 0.4b</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>19 ± 2.5c 21 ± 1ef 20 ± 1.6c 22 ± 1.9ef</td>
<td>5 ± 0.42 7 ± 1.7def 1.5 ± 0.26b 2.1 ± 0.4c</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>13 ± 2d 17 ± 1.5e 14 ± 1.2d 17 ± 2d</td>
<td>2 ± 0.1f 4.7 ± 0.4f 0.7 ± 0.2c 1.6 ± 0.2c</td>
</tr>
<tr>
<td>Inoculated</td>
<td>0</td>
<td>34 ± 1e 37 ± 1a 34 ± 1.2a 35 ± 2a</td>
<td>17 ± 1.4f 20 ± 1.3d 3.0 ± 0.36a 5.5 ± 0.6a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>18 ± 3f 27 ± 1c 26 ± 0.8bid 28 ± 1.1c</td>
<td>10 ± 1.03f 13 ± 1.9hce 1.0 ± 0.32b 4.0 ± 0.4b</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>21 ± 2.0g 22 ± 1def 20 ± 1.6c 27 ± 1def</td>
<td>6.0 ± 0.9d 9 ± 0.6eide 1.6 ± 0.21b 2.3 ± 0.4c</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>18 ± 0.5h 19 ± 1g 17 ± 1.8c 22 ± 1l</td>
<td>4.0 ± 1.3g 6.5 ± 0.8fect 1.5 ± 0.4b 1.7 ± 0.2f</td>
</tr>
<tr>
<td>LSD</td>
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<td>3.34 2.57 2.67 2.74</td>
<td>1.02 3.899 0.576 0.7044</td>
</tr>
<tr>
<td>F value (treatment)</td>
<td></td>
<td>31.305 62.58 40.737 37.22</td>
<td>86.716 14.9 13.389 36.58</td>
</tr>
</tbody>
</table>

Each value is a mean of three replicate where each replicate constituted three plants/pot. Mean values (a) followed by different letters are significantly different within a row or column at $p \leq 0.05$ according to DMRT test.

Fig. 4 – SEM micrograph of greengram roots: Panel A and A1 shows root tip and surface of untreated root tissues (control), respectively, while panel B and B1 represents the hexaconazole treated root surface showing fissures and fracture, respectively.
in the growth (root and shoot) of greengram plants. In order to understand this, we attempted to assess the actual impact of strain RV9 on greengram plants. For this, we decided to witness the colonization (aggregation) and colonizing efficiency of microbial inoculant. To achieve this, the roots of inoculated plants were analyzed under SEM. Interestingly, strain RV9 was found to aggregate onto root tips and at zone of extension (Fig. 5). This colonization of strain RV9 on the roots surface consolidated our facts that the enhancement in plant growth was due largely to the bacterial aggregation and subsequent secretion of plant growth promoting metabolites for extended time periods under fungicide stressed soils. Similar colonization of bacteria on the chickpea root surface and consequent increase in plant growth has recently been reported by Alekhya and Gopalakrishnan (2017).

2.4.3. ROS production and cell death in root tissues

The effect of hexaconazole on ROS production by fungicide treated and untreated greengram roots were measured by a cell-permeable fluorogenic probe (di chloro fluorescein) and were observed under CLS microscope. Results revealed a steady and sharp upsurge in dichlorofluorescein (DCF) fluorescence which could be due to the generation of ROS by roots, as the doses of hexaconazole were increased over the untreated control (Fig. 6 i). The level of ROS was, however, maximum among the roots detached from greengram treated with $3 \times$ dose rate of hexaconazole suggesting a strong physical interaction of hexaconazole with roots. In a similar experiment, Ahmed et al. (2017) reported a significant improvement in DCF fluorescence due to ROS production when the roots of A. cepa were exposed to ZnO nanoparticles. Similarly, pesticide dimethoate accumulated in the root organs of greengram, increased the ROS, which in turn interacts with several biological molecules (lipids and proteins) and concurrently destructs the stability of membrane. Also, Parween et al. (2012) have reported that chlorpyrifos increased the level of ROS antioxidant enzymes and other stress biomarkers in greengram plants. Region wise, meristematic zone of roots in general, was severely affected by ROS production. Also, a loss of plasma membrane under hexaconazole stress was evident when roots were stained with Evans blue. As the concentration of fungicide increased, the uptake of dye by the root tissues increased 3–4-fold resulting in losses in integrity of plasma membrane. As an example, the roots treated with $3 \times$ concentration displayed an increased intensity of fluorescence of blue color compared to untreated roots. On the contrary, untreated roots (without hexaconazole) did not take up the dye and therefore, the root periphery remained smooth suggesting a better and functional plasma membrane integrity (Fig. 6 ii). These findings further suggest that hexaconazole while acting on roots may rupture the cells leading to cell death in both the differentiation and elongation zones of the roots. Similarly, programmed cell death in roots of Oryza sativa have been reported (Wang et al., 2017).

2.4.4. Photosynthetic pigments, symbiosis, grain attributes and total P content

In uninoculated and hexaconazole treated plants, photosynthetic pigments and symbiotic features of greengram plants dropped continually with independent and increasing concentrations of hexaconazole (Fig. 7 A). Hexaconazole at 120 μg/
kg decreased chlorophyll a, chlorophyll b, total chlorophyll and carotenoids contents maximally by 51%, 33%, 41% and 40%, respectively, in comparison to non-inoculated and untreated greengram plants. Additionally, a highest upsurge of 6%, 17%, 8% and 9% in chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content, was noticed when inoculated plants was grown with 120 μg/kg hexaconazole.

When applied with hexaconazole, RV9 strain detoxified the inhibitory effect of hexaconazole and consequently enhanced the symbiotic attributes of greengram plants. Nodule number, DBN and LHb were decreased expressively \((p \leq 0.05)\) by 51, 56 and 48%, respectively at 120 μg/kg concentration of hexaconazole at 50 DAS (Fig. 7 A). Nodules collected from bio-primed and hexaconazole treated plants were however, relatively more in number and had greater NDB than those recovered from non-inoculated plants. Conversely, inoculant RV9 even in the presence of hexaconazole \((3\times)\), reduced fungicidal toxicity and consequently enhanced the NN, DBN and LHb content by 16, 45 and 3%, respectively at 50 DAS. The enhancement in symbiotic attributes is a strong and rich indication of microbial colonization and existence inside the host legume. Similarly, positive effect of bioinoculant *Bradyrhizobium* on different growth parameters of some leguminous crops cultivated under fungicide treated environment has recently been investigated (Shahid and Khan, 2017).

Grain yield and seed protein of plants displayed a steady decline with consecutive increase in doses of hexaconazole (Supplementary Fig. 2A & B). The fungicide tolerant *B. japonicum* RV9 maximally augmented the GY and SP by 26 and 43%, respectively, at 80 μg/(hexaconazole·kg·soil), which however, was only related to non-inoculated plants cultivated in soil amended just with identical rates of hexaconazole. Whereas, fungicide tolerant strain *B. japonicum* RV9 improved the GY and SP by 8 and 4% respectively, at \(3\times\) dose rate of hexaconazole, compared to the non-inoculated plants grown up at the similar concentration of hexaconazole. Similarly, an increase in nodulation and yield of *Rhizobium* inoculated faba bean has been reported (Kebene and Tamiado, 2017). Two-way ANOVA showed the significant \((p \leq 0.05)\) interactive effect of inoculation and hexaconazole \((inoculation \times hexaconazole)\) on the measured parameters.

Owing to the deleterious impact of hexaconazole on nutritive value of leguminous crops, the efficiency of RV9 strain to increase the P uptake of greengram was evaluated. Interestingly, strain RV9 enhanced the P content in roots and shoots by 8% and 6%, respectively compared to those recorded in root and shoots of plants grown in just three times more concentration of hexaconazole (Supplementary Fig. 2C). Moreover, the single impact of bioinoculant and its interface with fungicide \((bioinoculant \times hexaconazole)\) was statistically significant \((p \leq 0.05)\) when analyzed by two factor ANOVA.

2.4.5. Anatomical/ultrastructural changes in nodules and localization of RV9

Nodulation involves a series of complex events which are likely to be easily disrupted by deleterious environmental factors. These effects may be detectable both at anatomical and ultrastructural level. So, to understand the toxic impact of hexaconazole on *Bradyrhizobium*-legume interaction...
especially on anatomical and ultrastructural structure of nodules, nodules collected from fungicide treated plants were examined under SEM. Nodules of untreated and \textit{Bradyrhizobium} inoculated plants revealed that nodules contained bacteroids surrounding the vacuoles. Also, SEM showed that infested portion of fungicide un-supplemented nodules was enlarged and contained a condensed mass of bacteroid (Fig. 8i). Contrary to this, infested cells in hexaconazole supplemented nodules were smaller in size, distorted/damaged and in a disordered form. This could probably be due to the toxic action of hexaconazole added to soil prior to sowing. However, the infested regions had comparatively less bacteroid inhabitants while major section of cellular content comprised of vacuoles (Fig. 8ii). Likewise, the untreated but infested cells comprised of a thick population of strong and vigorous bacteroid with a characteristic T and Y shaped bacteroid. On the contrary, mature-infested nodules detached from fungicide supplemented plants had severely damaged bacteroids. Our SEM analysis however, conclusively revealed that hexaconazole caused a noticeable structural change in nodule tissues including the intracellular deterioration of cytoplasmic machineries, and disintegration of bacteroids, which might limit eventually the BNF. Similar anatomical/ultrastructural alterations in nodular tissues after exposing faba bean- \textit{Rhizobium leguminosarum} pairing to silver nanoparticles has been reported (Abd-Alla et al., 2016). However, despite reports here and there, to the best of our information, this is first finding highlighting the damaging effect of hexaconazole on anatomical and ultrastructural changes in nodules of greengram plants inoculated with \textit{B. japonicum}. These observations further proved that even though the \textit{Rhizobium} inoculant could reduce fungicide toxicity to certain extent but at higher concentration this fungicide can hamper greengram-\textit{Bradyrhizobium} symbiosis leading eventually to the destruction in N supply to plants due to adverse impact on bacteroids.

Fig. 7 – Influence of three concentrations of hexaconazole on uninoculated and \textit{B. japonicum} strain RV inoculated greengram plants on symbiotic attributes; number and dry mass of nodules (A), LHb content (B) and photosynthetic pigments (chl a, chl b, total chl and carotenoid content). Here, UC is uninoculated and untreated control, IC is inoculated with RV9, H1X, H2X and H3X are recommended, two times and three times more than recommended doses of hexaconazole, respectively.
Like many legumes, nodules produced on the root systems of inoculated greengram plants were healthy and pink in color which was suggestive of effective BNF. However, in order to monitor the entry and aggregation of *Bradyrhizobium* inside nodule tissues, and to assess the role of strain RV9 in nodule formation, nodules were thoroughly analyzed under CLSM. For this, numerous root nodules (~36–45 dpi) were arbitrarily chosen and LS of the nodules were cut to see the presence of *B. japonicum* cells under CLSM. The inoculated bacteria were found to successfully localize in different zones of nodules, but they were dominant particularly in infection thread and cortical tissues (zone of bacteroid) (Supplementary Fig. 3). This was clearer in LS section that was stained with fluorescent dye DCF. This study is hugely important and supported our observation that rhizobia used in this study established inside nodules leading to enhanced BNF even
under fungicide stress. This study thus probably helps to better understand the mechanistic basis of Rhizobium–legume interactions (symbiosis) even under stressed environment. Similar to this observation, Benito et al. (2017) showed the infection and localization of Micromonospora with rhizobia in two species of legumes Trifolium and Lupinus.

2.5. Extraction of proline, lipid peroxidation (MDA) and antioxidant defense enzymes

Proline (water-soluble amino acid) produced by plants under harsh environment is considered an important biomolecule which has been found to protect the membranous sheaths from destructive impacts of higher doses of ions by acting as a scavenger. And hence, the boost in cellular proteins secreted under variable environmental stresses has been reported to play multifarious protective roles in majority of plant species. Considering these, the accumulation of proline in different parts of greengram plants raised under fungicide stressed soils was assessed. Here, we recorded a considerable accumulation of stressor molecule in various organs of greengram plants cultivated under hexaconazole stress. Expectedly, the concentration of proline in roots, shoots and leaves estimated at 50 days after sowing and seeds (at 80 DAS) improved with enhancing hexaconazole concentration (Table 3). A maximum of 43.5, 42.6 and 26.3 mg/(g·fw) of proline was recorded in roots, shoots, leaves and grains, of plants grown at 120 μg/kg of hexaconazole. In accordance with this finding, an increase in the concentration of proline in fungicide treated soybean has been reported (Renukaswamy et al., 2016). In contrast, the bioinoculant RV9 substantially reduced the level of proline in all four organs of plants grown at all three concentrations of hexaconazole. However, the reduction in proline was maximum at 40 μg/kg hexaconazole where it was 28% (24.5 mg/(g·fw)), 38% (18.7 mg/(g·fw)), 53% (9.8 mg/(g·fw)) and 30% (10.5 mg/(g·fw)) in roots, shoots, leaves and grains, respectively. (Table 3). The decline of proline level in various organs of bio primed plants grown in fungicide treated soil could possibly be because of the detoxification or bioremediation potential of RV9 strain. Similar reduction in proline concentration due to metal tolerant bioinoculant P. aeruginosa in wheat cultivated under heavy metal stress has been reported by Rizvi and Khan (2017). The MDA contents in fungicide treated mungbean plants increased progressively with cumulative rates of hexaconazole. For instances, hexaconazole at 3× concentration, produced the maximum amount (17.4 μmol/(g·fw)) of malondialdehyde in leaves tissues. Besides this, strain RV9 inoculated greengram plants exhibited a marginal decrease (14.7 μmol/(g·fw)) in MDA content when applied with 120 μg/kg soil fungicides (Table 3). Similarly, Li et al. (2017) have recorded a substantial increase in malondialdehyde content (lipid peroxidation) of two cultivars of kidney bean under cadmium stress.

Reactive oxygen species are another extremely sensitive molecule that can disrupt the biomolecules like lipids, proteins and nucleic acids. To diminish/lessen the influence of oxidative stress, plants have evolved well-organized defense (antioxidant) systems to safeguard itself from shocking effect. Some of the most common ROS scavenging enzymes present in plant organelles such as chloroplast and
mitochondria are superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase etc. The production of ROS and amplified antioxidant enzymatic action in abiotic stressed conditions (such as pesticides and heavy metals) has been reported in many plants. In our study, the antioxidant enzymes (CAT, POD, APX and GPX) detected in leaf tissues varied greatly among the bio-primed and untreated greengram plants raised in fungicide amended soil. Usually, the amount of antioxidant defense enzymes increased with increasing rates of fungicides. Besides this, a significant reduction (51%) in CAT activity was found in B. japonicum RV9 inoculated plants grown at 3× of hexaconazole. However, at lower concentration of hexaconazole, the reduction in antioxidant enzymes of each plant organ was comparatively poor (Table 3). The activity of POD improved marginally by 8% when greengram was cultivated with 3× of hexaconazole, related to uninoculated control. Additionally, APX activity increased by 20% at 3× dose of hexaconazole. Guaiacol peroxidase activity of leaf tissues was increased by 9% at 3× dose rate of hexaconazole over un-inoculated and control plants. Conversely, strain RV9 induced a 19 and 7% decline in APX and POD activity, respectively, even when greengram plants were grown in soils treated with 3× dose of hexaconazole. Similar to our observation, the increased level of catalase activity in different organs of leguminous seedlings has previously been reported. Also, increased level of antioxidant enzymes like SOD, CAT, APX, PPO and lipid peroxidation have been reported in greengram and Phaseolus vulgaris while growing under abiotic and biotic stresses (Park et al., 2017; Alderfasi et al., 2017; Fernandes et al., 2018).

3. Conclusions

Hexaconazole used in this study both in the presence and absence of microbial inoculant demonstrated variable toxicity to greengram plants. However, the toxicity to greengram plants was reduced considerably by B. japonicum (RV9) when applied even with hexaconazole. The toxicity was obvious when bacterial cells and different organs of greengram plants like root tips and nodules collected from fungicide treated plants were observed under SEM and CLSM. Hexaconazole also altered the concentration of ROS scavenging molecule proline, MDA content and antioxidant enzymes in greengram plants. On the contrary, FTRS B. japonicum (RV9) when tested under in vitro conditions continued to express many fold PGP activities even under hexaconazole pressure. The fungicide tolerance and PGP abilities of strain RV9 were further validated when it was applied as bioinoculant and allowed the greengram to grow normally even under hexaconazole stress and concurrently increased the overall performance of plants. The inclusive development in the measured parameters was attributed due to firm colonization of rhizobial cells onto root surface and inside nodule tissues, secretion of growth regulators, and reduction in the level of antioxidant defense enzymes. This study is therefore, of great practical importance and is sure to boost the sustainable production of greengram when grown even under fungicide stressed soils.

Acknowledgment

The authors are grateful to National Bureau of Agriculturally Important Microorganisms (NBAIM) Mau, (India), Macrogen Seoul, Korea, for providing 16SrRNA gene Sequencing analysis, Institute Instrumentation Centre, IIT, Roorkee for AFM analysis, University Sophisticated Instrument facility (USIF) for providing SEM and CLSM facilities.

The author (Mohammad Shahid) would like to acknowledge the financial support received in the form of UGC Non-NET fellowship granted by University Grants Commission (D. O.No.F.1993/2006 (CU) dated 01.02.2007), New Delhi.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2018.07.007.

References


