Mesorhizobium Ciceri as a Biological Tool for Improving Physiological, Biochemical and Antioxidant State of Cicer Aritienum L. by Lowering the Fungicide Induced Oxidative Stress

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Abstract

The present study demonstrates the interactions of fungicide-tolerant symbiotic bacteria *Mesorhizobium ciceri* with *Cicer arietinum*-kitazin (KITZ) in greenhouse conditions. Under both *in vitro* and soil systems, KITZ imparted deleterious impacts on plants as a function of dose. The three-time KITZ dose detrimentally and maximally reduced germination efficiency, vigor index, dry matter production, symbiosis, leaf pigments and seed attributes of *C. arietinum*. KITZ- induced morphological alterations in root tips, oxidative damage and cell death in root cells of *C. arietinum* were shown by SEM. *M. ciceri* tolerated up to 2400 $\mu\text{g mL}^{-1}$ of KITZ, synthesized considerable amounts of bioactive molecules including indole-3-acetic-acid (IAA), 1-aminocyclopropane 1-carboxylate (ACC) deaminase, siderophores, exopolysaccharides (EPS), HCN and ammonia, and solubilised inorganic phosphate even in fungicide-stressed media. Following application to soil, *M. ciceri* improved performance of *C. arietinum* and enhanced dry biomass production, yield, symbiosis and leaf pigments even in a fungicide-polluted environment. At 92 $\mu\text{g KITZ kg}^{-1}$ soil, *M. ciceri* maximally and significantly ($p \leq 0.05$) augmented whole plant length by 41%, total dry biomass by 18%, carotenoid content by 9%, LHb content by 21%, root N by 9%, shoot P by 11% and pod yield by 15%. Additionally, *M. ciceri* was associated with decreased levels of stressor molecules (proline and MDA) and antioxidant defence enzymes (APX, GPX, CAT and POD) of *C. arietinum* plants when inoculated in soil. The symbiotic strain effectively colonized the plant rhizosphere/rhizoplane. In pesticide- contaminated soils, inoculation of *M. ciceri* may serve as an excellent strategy for augmenting *C. arietinum* productivity.

1. Introduction

Cicer arietinum L. (chickpea) crops often suffer from attack by phytopathogens, which damage the crop and consequently limits crop yield. Fungicides are commonly used to enhance productivity by preventing phytopathogen-related damage (Shahid et al., 2020). However, massive and injudicious use of such chemicals can upset soil fertility and inhibit microbial communities (Walia et al., 2014) and enzymatic activities (Han et al., 2020). Apart from the cytotoxic and genotoxic effect of fungicides on soil microbiota, uptake and translocation of pesticides by different plant organs may severely damage important metabolic activities leading subsequently to death of plants (Eker et al., 2006). Exceptionally high concentrations of pesticides disrupt: (i) cellular organelles and membrane permeability (Shahzad et al., 2018); (ii) respiratory processes and carbohydrate metabolism (Kumar et al., 2012); (iii) physiologically active enzymes (Liu et al., 2006) and proteins (Yin et al., 2016); (iv) photosystems by blocking the effective quantum yield of PSII (ΦPSII) and quantum efficiency of PSII (Fv/Fm) (Niinemets and Kull, 2001); and (v) cause oxidative damage (Singh and Roy, 2017) and genetic makeup of cellular machinery (Gill and Tuteja, 2010). Zablotowicz and Reddy (2007) observed that the pesticide glyphosate considerably decreased nitrogenase activity of rhizobia. As a consequence, symbiotic events leading to nodule formation and root morphogenesis of plants were drastically diminished (Adami et al., 2017). In another study, the fungicide pyrimorph was found to strongly inhibit the electron transport (ET) reactions of chloroplasts and adversely affected the physiology of whole plants (Xiao et al., 2014).

To overcome these problems, certain physico-chemical approaches have been used to destroy pesticides in soil. However, these methods are expensive, and the remediation process often remains incomplete due to the transformation of the parent compound to metabolites which are sometimes more persistent and more toxic for non-target organisms than were the parent compounds. As a consequence, physico-chemical methods of pesticide removal have not been widely accepted. Alternative methods of pesticide degradation/detoxification are therefore necessary. Bioremediation offers some solutions to pesticide detoxification problems. This technique, often referred to as 'microbial remediation' relies on the identification of microorganisms to convert contaminants to simpler and harmless forms and hence, to mitigate pesticide pollution.

To this end, scientists have identified pesticide degrading/detoxifying microbes endowed with potential plant growth promoting activities. Chief among them belongs to genera *Ensifer* (Ma et al., 2017), *Bradyrhizobium* (Romdhane et al., 2016), *Rhizobium* (Hang et al., 2019), *Alcaligenes* (Silambarasan and Abraham, 2013), *Actinobacteria* (Lang et al., 2018) and *Bacillus* (Tang et al., 2018). Apart from degradation of toxic pollutants, plant growth promoting rhizobacteria (PGPR) have the ability to synthesize growth regulating substances. By expressing multifarious physiological activities, PGPR promotes the overall growth and yield of legumes (Matse et al., 2020) raised in soil contaminated during cultivation with pesticides. For instance, inoculation of *Azotobacter* (Gothandapani et al., 2017) and *Bacillus* (Lastochikna et al., 2017) had positive effects on pulses where they supplied N and growth stimulating substances including phytohormones, siderophores and EPS, and solubilized soil P. Some fungicide-tolerant and N₂-fixing bacterial strains such as *Rhizobium* and *Azotobacter* and Gram-positive *Bacillus* sp. detoxified pesticides and enhanced legume production under adverse conditions (Alori et al., 2017). Likewise, pesticide-tolerant free-living PGPR such as *Bacillus* (Roy et al., 2018), *Azotobacter* (Gurikar et al., 2016) and *Stenotrophomonas* (Jaiswal et al., 2019) circumvented the toxicity of pesticides and concurrently improved growth of legumes.

Given the nutritive importance of *C. arietinum* in the diet, the negative impact of fungicides on crop productivity, the lack of adequate information on fungicidal response to *C. arietinum* and the bioremediation potential of PGPR, this study was formulated. The objectives were to: (i) assess the fungicidal toxicity to *C. arietinum* both under *in vitro* bioassays and pot-house conditions; (ii) assess the kitazin-induced distortion, oxidative damage and cell death in *C. arietinum* root cells; (iii) isolate and identify the symbiotic bacterium from *C. arietinum* root nodules; (iv) evaluate fungicidal tolerance by nodule bacteria; (v) determine the production of bioactive molecules under fungicide stress; (vi) evaluate the effects of *M. ciceri* on physiological and biochemical attributes of *C. arietinum*; (vii) determine the stressor molecules and antioxidant enzymes from *C. arietinum* foliage detached from fungicide-treated and bio-inoculated plants; and (viii) evaluate the rhizosphere/rhizoplane colonization potential of *M. ciceri*.

2. Materials And Methods

2.1 Toxicity Assessment of Kitazin to *Cicer arietinum* Under In-Vitro Conditions

2.1.1 Seed Germination, Vigor Index and Plant Length

Healthy and vigorous seeds of *C. arietinum* were soaked in water to imbibe for 24 h. Seeds were disinfected with 3% NaOCl solution and carefully rinsed with sterile water. Soft agar (0.7%) plates supplemented with different concentrations of KITZ (for detail, see supplementary table **S1**) were poured. Soft agar plates without fungicide treatment were served as a control for the sake of comparison. Seeds were placed on the soft agar plates and kept at room temperature (28 ± 2 °C) for 3–4 days. After 6–7 days of incubation, germination percentage, vigor index, radicle and plumule lengths of the plantlets were recorded.

2.1.2 Percent phytotoxicity, Tolerance Index (TI) and Root-Shoot Length Ratio

Additionally, the phytotoxicity (%), tolerance index and ratio of root and shoot length was recorded in fungicide treated/untreated *C. arietinum* seedling. (See the electronic supplementary information for details).

2.2 *Micro-morphological Alteration, Oxidative Damage and Cytotoxicity in C. arietinum Root*

2.2.1 Alteration in Root Tip Morphology analyzed by SEM

Morphological analysis of *C. arietinum* roots was done under scanning electron microscopy (JSM 6510 LV, JEOL, Japan) following the treatment of roots on soft agar (0.7% w/v) amended with $1000 \mu\text{g mL}^{-1}$ KITZ. After seven days of germination, roots were picked up from the agar plates and rinsed at least thrice with sterile phosphate buffer saline (PBS) and then fixed and processed following our previously described protocol for tissue fixation for SEM (Ahmed et al., 2018). Fixed and ethanol dehydrated tissues were examined under SEM at 10 kV accelerating voltage to check the fungicide induced distortion/damage in root tips, if any. A control set was also included for comparison.

2.2.2 Oxidative Damage and Cell Death by CLSM analysis

Confocal laser scanning microscopy of KITZ treated root was performed to check the cytotoxic potential and membrane damage. For the assay, seedlings were grown on soft agar as described for SEM analysis. Roots from seven days grown seedlings were carefully detached from soft agar and washed with buffer. Roots were tagged with a mixture of two fluorescent tags (i) acridine orange ($10 \mu\text{g mL}^{-1}$) and (ii) propidium iodide ($25 \mu\text{M}$) and visualized for any membrane compromised dead cells under LSM-780 Confocal Microscope (Zeiss, Germany). In order to differentiate between metabolically active and inactive cells, loss of plant cell membrane was used as a toxicity indicator. A well-adapted Evans blue staining

procedure was followed (Ref). *C. arietinum* roots grown in the presence of three concentrations (1X, 2X, and 3X) of KITZ were allowed to take up the 0.25% w/v solution of Evans blue stain for at least 15 min. After gentle washing with DDW thrice, emission of fluorescence was examined under LSM-780.

2.3 Isolation of Root Nodule Bacteria and Kitazin Tolerance

Fresh and un-damaged healthy nodules were detached from *Cicer arietinum* (chickpea) plants and surface sterilized by dipping nodules in 4% NaOCl for 2 min., washed three times with sterile double distilled water (DDW) and crushed gently. A-100 μ L freshly extracted nodule suspensions were streaked on YEMA plates and incubated at 28 ± 2 °C for 3–5 days. A total of 20 *Mesorhizobium* strains were isolated and morphologically and biochemically identified (Holt et al., 1994). Plant infection technique was carried out to determine the host specificity (Vincent, 1970). Mesorhizobial isolates were exposed to varying concentrations of kitazin using minimal salt agar (MSA) in order to select pesticide tolerant *Mesorhizobium* strain. Colonies grown on YEMA plates and efficiently surviving at the highest concentration of pesticides were chosen and referred to as pesticide tolerant mesorhizobial strains (PTMS). Of the total 20 *Mesorhizobium* isolates, BRM5 expressing maximum tolerance to pesticides was selected.

2.4 Molecular Identification of BRM5 Isolate

For the identification of isolate to genus level, 16S rRNA sequencing was performed (Shahid et al. 2020). (See the supplementary method section for detailed methods of DNA isolation, PCR and sequencing).

2.5 Production of PGP Active Biomolecules under Fungicidal Pressure

2.5.1 Indole acetic acid (IAA), Siderophore Production and ACC Deaminase Activity

The IAA produced by *Mesorhizobium ciceri* BRM5 was quantitatively assessed by modified method of Brick et al. (1991). For the assay, strain *M. ciceri* was cultured in LB broth containing a fixed amount ($100 \mu\text{g mL}^{-1}$) of tryptophan and amended with 600 (1X), 1200 (2X) and 1800 (3X) $\mu\text{g mL}^{-1}$ of KITZ (See supplementary methods for details).

The isolate was spot inoculated on kitazin supplemented universal chrome azurol S (CAS) agar plates followed by incubation at 28 ± 2 °C for detection of orange colour halo around the bacterial colonies. Also, the siderophore was quantitatively assessed by growing the bacterial strain in fungicide amended iron (Fe) free succinate liquid medium as suggested by Barbhuiya and Rao (1985). The estimation of siderophore was done according to universal chrome azurol liquid assay (Schwyn and Neilands 1987). Siderophore units were calculated as follows:

$$\% \text{ Siderophore unit} = \frac{\lambda \text{ of reference (Ar)} - \lambda \text{ of test (As)}}{\lambda \text{ of reference (Ar)}} \times 100$$

For ACC deaminase activity, *M. ciceri* was cultured in broth supplemented with various kitazin concentrations and amount of α -ketobutyrate produced by strain was determined following the method of Honma and Shimomura (1978) and Penrose and Glick (2003) (See supplementary methods).

2.5.2 EPS production, HCN and Ammonia Production

Exopolysaccharide (EPS) produced by *M. ciceri* under fungicide stress was scrutinized by culturing the cells into liquid medium supplemented with variable doses of KITZ (see supplementary method for details). The production of cyanogenic compound (HCN) and ammonia were assayed using the methods of Bakker and Shipper (1987) and Dye (1962), respectively.

2.6 Crop-Based Experiments

2.6.1 Planting, Fungicide Treatment and Application of Mesorhizobium

Seeds were disinfected/sterilized with NaOCl (2%), washed, cleaned and desiccated at room temperature. Commercial grade fungicide kitazin (**Table S1**) [recommended dose: $1 \times (96 \mu\text{gKg}^{-1})$, $2 \times (192 \mu\text{gKg}^{-1})$ and $3 \times (288 \mu\text{gKg}^{-1})$] of soil were applied to moist experimental soils before sowing of seeds (at least one day before sowing). The soils were filled in 20×24 cm clay pots of having approximately 5 kg soil per pot. Seeds were then coated/bacterized with freshly prepared inoculum of *M. ciceri* after dipping the seeds in liquid culture medium for 2 h using 10% gum arabic as a sticker to achieve 1×10^8 cells seed⁻¹ which was confirmed by viable cell count. The un-inoculated sterilized seeds submerged in sterile water only were taken as control. Non-bacterized and bio primed seeds ($n = 10$) were sown in respective earthen pots containing 5 kg of conventional soils. Two controls were run in parallel; one was un-inoculated and untreated control (without bacteria and without fungicides) and another was inoculated (only bacteria but no fungicides). Each test concentration was replicated thrice and pots were arranged in a completely randomized block design. After germination, seedlings were thinned and two uniform healthy seedlings of *C. arietinum* were maintained in each pot, 15 days after emergence (DAE). Pots were watered regularly and were kept in open field condition. The crop experiments were carried out regularly for two succeeding years to achieve the consistency in results.

2.6.2 Germination Efficiency, Plant height, Dry biomass and Photosynthetic Pigment in the presence of *M. ciceri* and KITZ

The KITZ treated and bacterized *C. arietinum* plants were removed at 80 and 120 DAS and germination efficiency, root and shoot length, weight and dry biomass was measured. For dry biomass, plants were dried in oven (Yorco, York Scientific Industries, Pvt. Ltd. India) at 80 °C for 2 days and then weighed using an electronic scale balance (BL-220 H, Shimadzu, Japan), and average was calculated. Leaf photosynthetic molecules (chlorophyll and carotenoid) accumulated in fungicide treated/untreated and bacterized *C. arietinum* foliage was estimated following the methods of Arnon (1949) and Kirk and Allen (1965), respectively (See supplementary methods in electronic supporting information).

2.6.3 Symbiosis, Nutrient Uptake and Seed attributes

Symbiotic features of *C. arietinum* were assayed by carefully removing the nodules from root systems. Nodules were counted and oven dried (80 °C) in a ventilated oven for 48 h. After drying, nodule dry biomass (mg plant^{-1}) was weighed using an electronic scale balance (BL-220 H, Shimadzu, Japan) and average was calculated. Furthermore, leghaemoglobin (LHb) content was quantitatively assayed following the earlier demonstrated method of Shahid and Khan (2018). (see electronic supporting information).

The nutritional content (nitrogen and phosphorous) in fungicide treated and bacterized *C. arietinum* plants was estimated at harvest as previously described by Jackson (1976) and Lindner (1944), respectively. Seed yield was recorded. Grain protein was extracted and estimated following the method of Lowry (Lowry et al., 1951). (See supplementary methods).

2.7 Assessment of Oxidative Stress Parameters in Bio inoculated and treated *C. arietinum*

2.7.1 Estimation of Proline and MDA Content (Lipid Peroxidation)

The free proline and MDA content in various organs of *C. arietinum* cultivated with/without the amendment of fungicide was assayed as demonstrated earlier (Bates et al., 1973) (See supplementary methods).

2.8 Extraction and Determination of Antioxidant Enzymes

For antioxidant enzyme activity, foliage was crushed in 4 mL of enzyme extraction buffer [(50 mM phosphate buffer (pH = 7.8)] containing 1 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVP). For GPX (E.C. 1.11.1.7), foliage tissues (100 mg) were homogenized in tris-buffer and the homogenate was centrifuged at 12,000 rpm for 20 min. at 4 °C. Increase in absorbance at 470 nm due to formation of tetra guaiacol ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) is expressed as $\mu \text{ mol mg protein}^{-1} \text{ min}^{-1}$. The reaction mixture (3 mL) consisted of 100 mM phosphate buffer (pH = 7.0), 0.1 mM EDTA and 20 mM H_2O_2 . The reaction was initiated by adding 100 μL of enzyme extract. The POD (E.C. 1.11.1.7) and APX (E.C. 1.11.1.11) activities

were determined following the modified methods of Leonards et al. (2004) and Hammerschmidt et al. (1982), respectively. All enzyme assays were performed three times with three replicates of each assay.

2.9 Rhizosphere and Rhizoplane Colonization by *M. ciceri* under Stress

The colonization of root surface by *M. ciceri* was determined in the presence/absence of fungicide. For the examination, roots were rinsed with DDW and PBS. The scanning electron microscopy was performed following the method of Shahid et al. (2019). Furthermore, the colonization of roots in term of CFU g⁻¹ of root material was determined at 40 and 80 DAS after exposure with different concentrations of fungicides.

2.10 Statistical analyses

The data were statistically analyzed using Sigma Plot 12.0 and Minitab17 software. Complete randomized block design (CBRD) for pot experiments was followed with at least three pots per individual test concentration. Crop experiments were conducted for two consecutive years to confirm the reproducibility of data. The data recorded in each year were pooled and analyzed. The mean of the data within a single column was calculated and compared with control treatments. The data represented either in figures or tables is the mean $\pm$ standard deviation (S.D.) of at least three replicates (n = 3). Different alphabets in graphs and tables show a significant difference among the treatments at a confidence level of $p \leq 0.05$. The least significant difference (LSD) among treatment means was calculated by two-way analysis of variance (ANOVA) at $p \leq 0.05$.

3. Results And Discussion

3.1 Toxic Impact of Fungicide on *C. arietinum* under *In-Vitro* Conditions

3.1.1 Germination Percentage, Vigor Index and Plant Length

The impact of dose of KITZ on germination efficiency and seedling attributes of *C. arietinum* seed developed on fungicide-amended agar plates was variable but negative (Fig. 1) The 3X concentration showed pronounced toxicity and significantly ($p < 0.05$) reduced the germination %, vigor index (SVI), and radicle (RL) and plumule (PL) length by 40%, 47%, 66% and 79% compared to control, respectively (Figs. 1 panel a-c). The reduction in germination efficiency and vigor index may possibly due to the distressed germination metabolism caused by the fungicide. Pesticides detrimentally influenced the germination ability of different legumes as reported by various workers. In this regard, lethal effect of fungicides on seedling germination and biological attributes of *P. sativum* under *in vitro* conditions has been reported (Shahid et. al., 2018).

3.1.2 Phytotoxicity %, Tolerance index (TI) and Root-Shoot Length Ratio

A 19%, 42% and 88% phytotoxicity was recorded for *C. arietinum* when grown with 96, 192 and 288 $\mu\text{g KITZ kg}^{-1}$, respectively, compared to control (Fig. 1e). The higher KITZ concentration (3X) maximally affected the root-shoot length ratio and reduced it by 0.9 – 0.4 (55% reduction over control) (Fig. 1d). The tolerance index (TI) in *C. arietinum* decreased with increasing KITZ doses and confirmed a negative correlation between fungicide and TI. The TI of *C. arietinum* was recorded at 70, 56 and 26% at 1X, 2X and 3X dose of KITZ respectively; over the untreated control (Fig. 1 panel f). These results indicate that lower fungicide concentrations resulted in maximum TI, whereas the 3X dose exhibited the minimum TI in *C. arietinum*. Similarly, root-shoot length ratio and tolerance index of chickpea were negatively influenced by the higher concentrations of two neonicitnoid group of pesticides (Shahid et al., 2020).

3.2 Micro-morphological Root Tip Distortion, Oxidative Stress and Cell Death

SEM images reveal the inhibitory effects of fungicide to the radicle regions of root tips and their surfaces, which are in the form of cracks/fractures, breakages, crumbling and spears relative to an indistinct, smooth/even and unbroken shape (Fig. 2 panel II B, B1) as shown in the control root tip and surface (Fig. 2 panel II A, A1). The alterations in root tip morphology further validate the fungicide toxicity which, in turn, might have reduced uptake of water and nutrients from soil causing altered root as well as reduced plant growth (Fig. 2 panel I). Similar damage to the micro-morphological structure of roots tips due to the toxicity of pesticides and other toxic pollutants has reported (Shahid et al., 2020; Zeyad et al., 2019; Kumar and Pandey, 2015; Tripathy et al., 2013).

Fungicide-induced oxidative stress in root membranes was also visualized. AO/PI stained and fungicide-treated *C. arietinum* roots were observed using confocal laser scanning microscopy (CLSM). A concentration-dependent increase in dead/injured cells observed as red/orange color occurred in roots exposed to 3X of KITZ (Fig. 2 panel III B1 B2 and B3). Untreated root tissues exhibited maximum intensity of green fluorescence resulting from AO reaction representing little or no damage (Fig. 2 panel III B). This is an indication that pesticide exposure was arbitrated by ROS-mediated damage to membrane lipids which therefore increased fluorescence of DNA-bound propidium iodide in membranes. Cortés-Eslava et al. (2018), using CLSM, reported similar oxidative stress, oxidative damage and apoptosis in two model plants grown in insecticide- stressed conditions. The loss/damage of plasma membrane in fungicide-treated root tissue was obvious when *C. arietinum* roots were stained with Evans blue dye. The uptake of dye by root tissues increased three- to four-fold with increasing KITZ concentrations (Fig. 2 panel IV C1, C2 and C3). In contrast, dye was not taken up by untreated roots (Fig. 2 panel IV C) and hence, the root margin remained smooth signifying its functional integrity.

3.3 Biochemical and Molecular Identification of *Mesorhizobium* and Fungicide Tolerance

Strain *M. ciceri* was characterized morphologically and biochemically (Table S2). Based on biochemical and cultural characteristics, the genus of the symbiotic bacterium was confirmed and strain was presumed as *Mesorhizobium*. Isolate BRM5 showed the maximum base sequence similarity (> 96.7%) to type strain *Mesorhizobium jarvisii* ATCC 233669^T (Accession number NR135858.1), (Fig. S1). Based on this relatedness, isolate BRM5 was identified as *Mesorhizobium ciceri*.

The tolerance of *M. ciceri* to KITZ was assessed while grown in minimal salt (MS) broth added with variable concentrations of fungicide; strain BRM5 survived up to 2400 µg mL⁻¹ of KITZ (Table S2). *Achromobacter spanium* and *Serratia plymuthica* tolerated exceptionally high concentrations of different group of pesticides recovered from pesticide-polluted rhizospheres (Aroua et al., 2019). Various workers have isolated the pesticide-tolerant bacterium recovered from nodules of different legumes raised in pesticide-polluted soil (Shahid and Khan, 2020; Shahid et al., 2019a; Shahid et al., 2019 b; Shahid and Khan, 2017; Ahemad and Khan, 2012).

3.4 PGP Active Biomolecules Produced by *M. ciceri* under Fungicide Stress

3.4.1 IAA, Siderophores and ACC Deaminase

Pesticide-tolerant *M. ciceri* revealed inconsistent secretion/production of active biomolecules when cultured in both stressed and controlled (fungicide-free) environments. Under controlled conditions *M. ciceri* synthesized 43.3 ± 3.2 µg IAA mL⁻¹ which decreased with increasing doses of KITZ. This strain synthesized indole-3-acetic acid even when cultured in LB broth supplemented with higher concentrations of fungicide. For example, secretion of IAA was reduced from 43.3 ± 3.2 to 32.2 ± 3.1 µg IAA mL⁻¹ (25.6% decrease) at 1800 µg KITZ mL⁻¹ (Fig. 3A panel a). The impact of different groups of pesticides on IAA and other plant growth regulating active biomolecules of nodule bacterium *Bradyrhizobium* sp. (Romdhane et al., 2016), *B. japonicum* (Shahid et al., 2019a) and *R. Leguminosarum* (Shahid et al., 2019b) have also been reported. Secretion of IAA by the fungicide-tolerant BRM5 strain even at higher levels of pesticide is a promising feature of soil microbes, because such pesticide-tolerant PGPR strains, when used in harsh environments are likely to endure producing/releasing phytohormones such as IAA. This crucial growth-augmenting plant hormone will thus be accessible to plants even at high levels of pesticides. A trend similar to IAA was observed for siderophore production. *M. ciceri* synthesized siderophores even under stressed conditions (Fig. 3A panel b-d). Similar production of siderophores by *Rhizobium* sp. (Kang et al., 2020; Sijilmassi, et al., 2020), *Mesorhizobium* sp. (Ahemad and Khan, 2012), *Pseudomonas* sp. (Khan et al., 2020) and *A. vinelandii* (Shahid et al., 2019) under stressed conditions are reported.

Bacterial ACC deaminase is an outstanding biological attribute which can substantially decrease levels of ethylene (C₂H₂) in plants and thus, accelerates the functioning of developing plants in harsh environments (Belimov et al., 2015). *M. ciceri* exhibited a positive response to ACC deaminase even when cultured in fungicide-supplemented medium. Maximum (40.9 ± 1.8 µM α-Ketobutyrate mg⁻¹ protein hour⁻¹) and minimum (27.0 ± 0.87 µM α-Ketobutyrate mg⁻¹ protein hour⁻¹) amounts of ACC were synthesized

at 0 and 1800 µgKITZmL⁻¹, respectively (Fig. 3A panel e). Secretion of ACC deaminase by the tolerant strain, however, even in stressed environments is agronomically a beneficial feature for increasing productivity of crops under pesticide stress. This intrinsic property of ACC deaminase production even under pesticide pressure makes them a promising choice for crop production even in soils polluted with excess pesticide.

3.4.2 EPS and NH₃ Production

EPS synthesized by *M. ciceri* increased with increasing concentrations of fungicide. For example, at 1800 µgKITZmL⁻¹ (3X dose), *M. ciceri* secreted the maximum EPS which was 29% (165 ± 12 µgmL⁻¹) greater than that released under fungicide-free conditions (117 ± 5.4 µgmL⁻¹) (Fig. 3A panel f). Furthermore, the EPS secreted by *M. ciceri* BRM5 (Fig. 3B panel A) showed an enhancement with increasing concentrations of FIP (Fig. 3B panel B). Additionally, the EPS produced by strain was quantified (Fig. 3B panel C) using standard protocols. The structural morphology and topography of dried powder of EPS was done using SEM (Fig. 3B panel D) and AFM (Fig. 3B panel E) techniques. Also, the elemental analysis indicated the presence of some major and trace elements (Fig. 3B panel F). Release of EPS by nodule bacterium both in the absence or presence of stressor molecules (pesticides) could be advantageous for producing bacteria and for growing crops. When EPS are liberated from bacterial cells into their surroundings, EPS may influence the growth of plants even under stressed conditions. Exopolysaccharides protects bacteria from desiccation, phagocytosis and phage attack (Angelin and Kavitha, 2020) by forming a polymeric network around growing cells, while it protects plants from pathogen attack (Rodríguez-Navarro et al., 2014). In addition, the rhizobia invasion process, formation of the infection thread, bacteroid, and nodules during *Rhizobium*–legume interactions is greatly influenced by EPS (Gage, 2004). By synthesizing substantial quantities of EPS, bacterial strains can be safeguarded from the noxious effects of contaminants by masking their effects (Ghosh et al., 2019). Due to these benefits, interest in identifying EPS-producing nodule bacteria has increased in recent years (El-Ghany et al., 2020). Similarly, EPS production of PGPR strains under pesticide stress was reported by other workers (Mukherjee et al., 2019; Fatima et al., 2019). Bacterial strain showed the activity of ammonia production at all the concentrations of KITZ (Table 1).

Table 1
Ammonia, HCN and siderophore production by *M. ciceri* BRM5 under kitazin stressed condition

Treatment	Dose rate (µgmL ⁻¹)	^a NH ₃ Production	^b HCN Production	Siderophore production (FeCl ₃ Test)
Control	0	++	ND	++
Kitazin	600*	+	ND	+
	1200**	+	ND	+
	1800***	+	ND	+

In this table, *, ** and *** represents the 1X, 2X and 3X concentrations of kitazin, respectively. ^aammonia, ^b HCN production. ++ and ND indicate 'positive reaction' and 'not detected', respectively.

3.5 *C. arietinum*-Fungicide-*Mesorhizobium* Interactions: Comprehensive Toxicity and Bioremediation Studies

Bioinoculation impact of *M. cicero* on Biochemical Characteristics of *C. arietinum*

3.5.1 Seed Germination

The kitazin tolerant *M. ciceri* improved the growth of plants when applied to *C. arietinum* plants in soil system treated with variable level of fungicide (Fig. 4 panel a). The impact of *M. ciceri* BRM5 on germination efficiency of *C. arietinum* seedlings grown in earthen pots supplemented separately with varying doses of KITZ was variable (Fig. 4 panel b). Generally, strain BRM5 had a positive impact on germination and vigor index relative to un-inoculated seeds. *M. ciceri* BRM5 exhibited a maximum increase of 5% and 6% in germination and SVI at 96 $\mu\text{gKITZkg}^{-1}$ (Fig. 4 panel b) compared to the un-inoculated but similar dose of fungicide. *Microbacterium hydrocarbonoxydans* BHUJP-P1, *Stenotrophomonas rhizophila* BHUJP-P2, *B. licheniformis* BHUJP-P3 and *B. cereus* BHUJP-P4 increased germination efficiency and growth of crops even in the presence of varying concentrations of monocrotophos and chlorpyrifos (Jaiswal et al., 2019).

3.5.2 Length and Weight of Plant Organs

A gradual increase in length of roots and shoots of *M. ciceri* BRM5-bacterized plants was observed (Fig. 4 panel c) both at 80 and 120 DAS in the presence of variable doses of KITZ. *M. ciceri* BRM5, when grown with KITZ, imparted maximum benefits on plant organs which decreased considerably at 3X concentration. *M. ciceri* BRM5 at 96 $\mu\text{gKITZkg}^{-1}$ increased the lengths of roots, shoots and whole plants maximally by 11, 7 and 41% (at 80 DAS) respectively, over sole application of 1X of KITZ (Table S3, Fig. 4 panel c). Similarly, *M. ciceri* BRM5 increased the fresh weight of roots, shoots and whole plant by 29, 18 and 22%, respectively (120 DAS) when used with 96 $\mu\text{gKITZkg}^{-1}$ compared to un-inoculated plants at the identical dose of KITZ (Table S3, Fig. 4 panel d). Enhancement in plant growth may be due to increased availability of IAA, soluble P, siderophores, and ACC deaminase secreted by the microbial symbiont. Of these, growth regulators like IAA promote root growth directly by stimulating cell elongation or cell division (Pacheco-Villalobos et al., 2016). The well-developed and expanded roots absorb more water and minerals from soil (Rijavec and Lapanje, 2016) which in turn enhance the growth of the plant. Kumar et al. (2017) reported that pesticide-tolerant PGPR strains *P. putida* and *B. amyloliquefaciens* alleviated the adverse effect of pesticides and increased soil enzyme activities and seed germination efficiency, elongated plant organs and enhanced other parameters of *C. arietinum*. These findings corroborate our facts that inoculated PGPR degrade/detoxify pesticides and thus, improve various parameters of legumes grown in the affected soil.

3.5.3 Dry Biomass Accumulation

The bacterized and un-inoculated *C. arietinum* plants cultivated in soil treated with varying levels of fungicide had variable dry biomass of *C. arietinum*. A gradual increase in root and shoot biomass of *M. ciceri* BRM5-inoculated plants treated with different doses of KITZ was observed both at 80 and 120 DAS. *M. ciceri* BRM5 maximally increased root, shoot and total dry biomass by 12, 17 and 18%, (at 80 DAS) when applied in soil treated with $96\mu\text{gKITZkg}^{-1}$ compared to dry biomass of un-inoculated but treated with the same dose of KITZ (Table S3, Fig. 4 panel e). There was a significant ($p \leq 0.05$) interaction between application of symbiotic

bacterium and fungicide. The effect of bio-priming and fungicide on biological and chemical characteristics of test plants was significantly correlated both at 80 DAS and 120 DAS as revealed by regression analysis and principal component analysis (PCA) (Fig. S1 and S2).

3.5.4 Bio-inoculation Impact of *M. ciceri* on Photosynthetic Molecules

The effect of varying concentrations of KITZ on photosynthetic pigments of *C. arietinum* in the presence of *M. ciceri* BRM5 at 80 DAS was variable. On comparing the effect of the 1X concentration of KITZ on inoculated and non-inoculated plants, a maximal increases of 14% (0.28 mgg^{-1}), 11% (0.28 mgg^{-1}), 5% (0.40 mgg^{-1}) and 9% (1.21 mgg^{-1}) in Chl a, Chl b, total chlorophyll and carotenoids content, respectively, was noted in *M. ciceri* BRM5-inoculated *C. arietinum* plants over untreated and non-inoculated control plants (Table S3). Likewise, photosynthetic pigments of *C. arietinum* plants improved following the inoculation of pesticide-tolerant PGPR strains. In this context, enhanced symbiotic performance and productivity of *Phaseolus vulgaris* in harsh environmental conditions was observed following the inoculation of tolerant native PGPR (Yanni et al., 2016). Increased nodulation and seed yield in *V. faba* due to inoculation of *Rhizobium* has been reported (Kebeneand, 2017).

3.5.5 Effect of *M. ciceri* on Symbiotic Features of *C. arietinum*

3.5.5.1 Nodulation: Nodule Numbers and Nodule Dry Biomass

The roots detached from un-inoculated and KITZ treated *C. arietinum* showed the poorly developed root system and weak/unhealthy nodular systems. In contrast, a better root system having healthy and more pink-colored showing wavy margin was recorded in bio-inoculated *C. arietinum* plant (Fig. 5 panel a, b). Generally, the symbiotic attributes [nodule number (NN) and nodule dry biomass (NDB)] of *M. ciceri* BRM5 bacterized *C. arietinum* plants grown in the presence of KITZ was greater compared to those recorded for un-inoculated plants supplemented with the identical dose of fungicide. *M. ciceri* BRM5 maximally increased NN and NDB by 23% and 22% at 80 DAS and 16% and 14%, respectively at 120 DAS

when used with $96 \mu\text{gKITZkg}^{-1}$ compared to un-inoculated but KITZ-treated plants (Fig. 5 panel c and d). Ullah et al. (2016) also reported that PGPR strains in combination with *M. ciceri* increased the growth and nodulation of *C. arietinum* even in pesticide-stressed conditions.

3.5.5.2 Leghaemoglobin (LHb) Content and nutrient uptake in nodule

The LHb content in fresh nodules of *C. arietinum* declined in the presence of symbiotic bacteria similar to that in plants grown in fungicide only-supplemented soils. A considerable

Figure.5: Bio-inoculation impact of *M. ciceri* BRM5 on symbiotic features of *C. arietinum* plants; attachment of nodules with inoculated and treated roots (panel a), morphology of nodule (panel b) nodule number (panel c), nodule dry biomass (panel d) and LHb content (panel e) and nutrient uptake in nodules (panel f) grown in sandy clay loam soil treated with 96 (1X), 192 (2X) and 288 (3X) $\mu\text{gKITZkg}^{-1}$ soil and harvested at different intervals. The bar and line diagrams represent the mean $\pm$ S.D ($n = 3$) of three replicates where each replicate constituted three plants/pot. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to DMRT test.

improvement in LHb content of bacterized plants was, however, observed at 80 DAS relative to plants grown in the pesticide-only treatment. *M. ciceri* BRM5 significantly ($p \leq 0.05$) and maximally enhanced LHb content by 21% [0.34 to $0.43 \text{ mM (gfw)}^{-1}$] at $96 \mu\text{gKITZkg}^{-1}$ over LHb content of un-inoculated but KITZ-treated plants (Fig. 5 panel e). Furthermore, the nutritional uptake in nodule was assessed and it was recorded that N and P was maximally increased by *M. ciceri* when root systems were detached from plants treated with 1X dose of KITZ (Fig. 5 panel f).

3.5.6 Impact of *M. ciceri* on Grain Attributes and Nutrient Uptake of *C. arietinum*

3.5.6.1 Seed Yield (SY) and Grain Protein

Number of seeds, yield and grain protein of *C. arietinum* declined significantly in the presence of varying doses of KITZ. In contrast, seed yield (SY) of *M. ciceri* BRM5-inoculated *C. arietinum* plants improved significantly ($p \leq 0.05$) in the presence of different doses of KITZ. A maximum increase of 13%, 15%, 11% and 6% in pod number, pod weight, seed number and seed yield was recorded when *M. ciceri* BRM5 was used with $96 \mu\text{gKITZkg}^{-1}$ soil over un-inoculated but pesticide-treated control (Fig. 6 panel a and b). Likewise, BRM5 increased the grain protein by 7% when used with the 1X dose of KITZ compared to un-inoculated plants treated with the identical dose of fungicide (Fig. 6 panel c). The decrease in protein content of grain is likely to be due to the binding of pesticides to R-SH groups of proteins which, in turn, alters protein structure. However, seed features of *C. arietinum* were generally improved following inoculation with *M. ciceri* BRM5 even in the presence of pesticide. *R. leguminosarum* strain PS1, when

used as bio-inoculant in pesticide-treated pea plants, increased SY by 43% compared with fungicide-treated but un-inoculated plants (Tariq et al., 2016). Enhancement in growth attributes and yield of atrazine-treated *Phaseolus vulgaris* when grown in the presence of a consortium containing *Rhizobium* sp. and *Trichoderma* have been reported (Madariaga-Navarrete et al., 2017). Similar improvements in growth and nutrient levels were observed when stress-resistant PGPR strains of *P. aeruginosa* and *Burkholderia gladioli* were applied to plants grown under stressed conditions (Khanna et al., 2019).

3.5.6.2 Nutrient Uptake

Bio-inoculation impact of *M. ciceri* BRM5 on N and P content of *C. arietinum* plant organs at 80 DAS differed in a concentration-dependent manner. The N content in *C. arietinum* roots increased from 20.6 to 22.6 $\mu\text{g g}^{-1}$ whereas in shoot tissue it increased from 11.5 to 13.3 $\mu\text{g g}^{-1}$ when *M. ciceri* BRM5 was used in the presence of 96 $\mu\text{gKITZkg}^{-1}$ (Fig. 6 panel d). Similarly, *M. ciceri* BRM5 at 96 $\mu\text{gKITZkg}^{-1}$ soil improved root and shoot P by 7 and 11%, respectively, compared to non-inoculated plants treated with similar concentrations of KTZ (Fig. 6 panel e). Uptake of nutrients (N and P) by *C. arietinum* raised in pesticide-enriched soils was also enhanced following inoculation with fungicide-tolerant symbiotic bacteria. In a similar study, ACC deaminase positive PGPR strains *Pseudomonas brassicacearum* Am3, *P. marginalis* Dp1 and *Rhodococcus* sp. Fp2 were found to improve growth and uptake of both major and trace nutrients viz., N, P, K, Ca, S and Fe in different varieties of legumes raised in stressed soils (Safronova et al., 2006). In another study, *Phaseolus vulgaris* plants inoculated with stress-tolerant PGPR belonging to a group of phosphate-solubilizing bacteria significantly lowered electrolyte leakage, LPO level, SOD, hydrogen peroxide and proline phosphatase activities and improved physio-biochemical attributes, nutrient uptake, and protein and carbohydrate content by relieving the stress (Rady et al., 2019).

3.5.7 Proline, MDA and Antioxidant Enzymes

3.5.7.1 Proline and MDA Content

The BRM5-inoculated *C. arietinum* plants had low levels of proline, MDA and antioxidant enzyme activity in organs even in the presence of different concentrations of KITZ. In general, strain BRM5 minimized the proline level even in the presence of fungicide. *M. ciceri* BRM5 significantly ($p \leq 0.05$) and maximally reduced the proline content in roots, shoots and seeds by 27%, 26% and 33% at the 1X dose of KITZ (Fig. 7 panel a and b) compared to un-inoculated plants treated with the identical dose of fungicide. Similarly, it was observed that *M. ciceri* BRM5 reduced the MDA content from 5.4 to 3.07 $\mu\text{moles g}^{-1}\text{fw}$ compared to un-inoculated plants treated with 96 $\mu\text{gKITZkg}^{-1}$ (Fig. 7 panel c). Information is lacking as to how and why proline levels decline in legumes bio-inoculated with pesticide-tolerant PGPR and raised in pesticide-contaminated soil. The accumulation of proline declined significantly ($p \leq 0.05$) in bio-primed *C. arietinum* grown in soil supplemented with high doses of pesticide. A similar reduction in proline content in foliage was recorded following inoculation of three phosphate-solubilizing strains of *Pseudomonas* (*Pseudomonas* sp. *P. putida* and *P. fulva*) under chlorpyrifos and pyriproxyfen stress (Munir et al., 2019). The bio-inoculation impact on MDA level in the presence of higher doses of KITZ

varied considerably among the bacterial species. In another study, inoculation of plants with *P. putida* caused substantial reduction in lipid peroxidation biomarkers, MDA content and electrolyte leakage and gradually improved chlorophyll, carotenoid and carbohydrate contents and growth of plants under norflurazon (herbicide)-stressed conditions (Bourahla et al., 2018). Fungicide-tolerant *M. ciceri* BRM5 used in this study resulted in a significant decrease in MDA concentrations in pesticide-stressed *C. arietinum* plants. The inoculant ameliorated pesticide pressure, relieved oxidative damage in plants and allowed them to grow pesticide-polluted soil. These results indicate that the bacterial strains could serve in bioremediation strategies which lead to overall improvement in performance of leguminous crops while being cultivated under pesticide stress.

3.5.7.2 Antioxidant Enzymes

Antioxidant enzymes of *C. arietinum* foliage treated with different doses of KITZ decreased substantially in the presence of bacterial inoculants. CAT activity of *C. arietinum* foliage was reduced maximally and significantly ($p \leq 0.05$) by (8.3%) by *M. ciceri* BRM5 in the presence of 96 KTZ μgkg^{-1} soil (Fig. 7 panel d). Similarly, bacterial strains maximally lowered the POD activity by 21% (from 2.78 to 2.2 $\mu\text{mol min}^{-1}\text{mg}^{-1}\text{fw}$) in the foliage system when detached from 1X concentration of KITZ (Fig. 7 panel d). Likewise, the APX and GPX activities of *C. arietinum* were increased by 19.6% (from 2.09 to 1.68 $\mu\text{mol min}^{-1}\text{mg}^{-1}\text{fw}$) and 9% (from 0.99 to 0.90 $\mu\text{mol min}^{-1}\text{mg}^{-1}\text{fw}$) respectively at the 3X dose of KITZ following application of symbiotic bacterium (Fig. 7 panel e). In a similar study, strain SRB02 of *B. aryabhatai* considerably reduced levels of oxidative stress and antioxidant enzymes CAT, POD and SOD in soybean plants grown in stressed soil, and promoted overall growth of plants (Park et al., 2017). The declines in antioxidants due to inoculation with pesticide-tolerant bacterial strains consequently resulted in a substantial upsurge in overall growth of *C. arietinum* even under pesticide stress.

3.5.8 Rhizosphere and Rhizoplane Colonization by *Mesorhizobium* under Stress

Root colonization is a critical initial component of plant-microbe interaction in the plant rhizosphere. This mutualistic interaction is helpful in growth and development of plants as well as in protecting the crops from various biotic and abiotic insults (Verma et al., 2018). Considering this, halotolerant PGPR strain *M. ciceri* was checked for its root colonizing ability using SEM in the absence (Fig. 8A) and presence of KITZ (Fig. 8B). SEM images revealed that fungicide-untreated roots resulted in dense/compact colonization whereas treated roots showed lesser bacterial populations. Similar colonization of bacteria on *C. arietinum* root surfaces and consequent increase in plant growth was reported by other workers (Alekhya and

Figure. 7: Bio-inoculation impact of *M. ciceri* BRM5 on proline content (panel a) antioxidant enzymes: GPX (panel b), APX (panel c) CAT (panel d) and MDA content (panel e) of *C. arietinum* plants grown in sandy clay loam soil treated with 96 (1X), 192 (2X) and 288 (3X) μgKTZkg^{-1} soil. The bar and line diagrams represent the mean $\pm$ S.D ($n = 3$) of three replicates where each replicate constituted three

plants/pot. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to DMRT test.

Gopalakrishnan 2017; Wheatley and Poole, 2018; del Gallo et al. 2017). Bacterial components such as EPS, cell wall polysaccharides and extracellular bacterial proteins can provide for attachment onto the root surface. Bacterial counts from the rhizosphere and rhizoplane at varying intervals of seeding, i.e., 80 and 120 DAS with different doses of KITZ, were determined. The CFU counts were significantly reduced over untreated control with increasing level of fungicide; however, strain BRM5 survived and colonized even at the higher dose of fungicide. The lower doses imparted lesser impacts on viable counts of bacteria. At 3X KITZ, the rhizospheric CFU count of *M. ciceri* was 3.2 and 4.3 log CFU/mL as compared to 6.7 and 5.1 log CFU/mL (untreated control) at 80 DAS and 120 DAS, respectively (Fig. 8C). Similarly, viable populations of rhizoplane bacteria had declined over the untreated control. As PGPR colonize root surfaces, they multiply and reproduce by receiving key signalling compounds and nutrients from root exudates which subsequently leads to biofilm formation on the root system, a prominent indicator of successful plant-microbe interaction.

4. Conclusion

Fungicide applied in the study detrimentally affected the germinative ability of *C. arietinum* leading to the decrease in plant growth parameters as recorded both under *in vitro* and soil system. *M. ciceri* could tolerate higher level of kitazin and synthesized growth regulating bioactive molecules even in fungicide supplemented media. Following application to soil, *M. ciceri* improved performance of *C. arietinum* and enhanced dry biomass production, yield, symbiosis and leaf pigments even in a fungicide-polluted environment. Additionally, pesticide-tolerant nodule bacterium *M. ciceri* declined the stressor metabolites and antioxidant status of plant. The present study creates a new perspective for understanding the mechanistic basis of declines in stressor molecules and antioxidant defense enzymes in symbiotic bacterium-inoculated *C. arietinum* grown in fungicide-contaminated soil. Furthermore, symbiotic strain effectively colonized the plant rhizosphere/rhizoplane. Conclusively, in pesticide- contaminated soils, inoculation of *M. ciceri* may serve as an excellent strategy for augmenting *C. arietinum* productivity.

Declarations

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Compliance with ethical standards

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Credit Author Statement

M.S performed the experiment, statistically analyzed the data and prepared the draft of the manuscript. M.S.K, supervised, designed, corrected and improved the original draft of the manuscript. All the authors revised and approved the final version of manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Effect of kitazin on germination percent (panel a), plant length (panel b) vigor index (panel c) root-shoot length ratio (panel d) percent phytotoxicity (panel e) and tolerance index (panel f) of *Cicer arietinum* seedlings germinated on 0.7% soft agar plates treated with three concentrations of KITZ under in vitro condition. Each bar represents the mean $\pm$ S.D (n = 3) of three replicates where each replicate constituted three plants/pot. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to Duncan's multiple range (DMRT) test whereas error bars represent standard deviation (S.D).

Figure 2

Effect of KITZ on *C. arietinum* plants grown with 96 (KITZ 1X), 192 (KITZ 2X) and 288 $\mu\text{gKITZkg}^{-1}$ (KITZ 3X) soil (Panel I). Scanning electron microscopic (SEM) images of *C. arietinum* roots demonstrating distortion/damage induced by KITZ exposure: A and A1 represents the root tip and root surface of untreated/control. B and B1 represent the distorted/ruptured root tips and root tip surfaces treated with KITZ (Panel II). The Z-stack images of PI/AO stained *C. arietinum* roots using CLSM. Images reveal an increase in red/orange fluorescence as concentrations of KITZ increase. Untreated control root showing no red color (B), while roots treated with various doses of KITZ (B1, B2 and B3) (Panel III). The Z-stack image of chickpea using CLSM representing the cytotoxicity (Evans blue dye exclusion) assay in root tissues induced by fungicide. Figures show uptake of Evans blue dye by root cells; untreated control root showing no blue color (C), while C1, C2 and C3 represents the roots treated with various doses of KITZ (Panel IV).

A

B

Figure 3

A) Plant growth regulating bioactive molecules; indole-3-acetic acid (panel a), phenolate type siderophore [salicylic acid and 2,3-DHBA] (panel b), siderophore % unit (panel c), chrome azurol-S agar (panel d), ACC deaminase (panel e) and exopolysaccharide (panel f) produced by *M. ciceri* BRM5 in the absence and presence of different doses of KITZ. In this figure, bar diagrams represent the mean values (mean ± S.D.) of three independent replicates, whereas error bars depict the standard deviation (S.D.). Different letters on bars denote that mean values are significantly different (at $p \leq 0.05$) according to DMRT. B) Exopolysaccharide (EPS) producing cultures of *M. ciceri* on YEMA plate (A), Bar diagrams represent the EPS synthesized by strain BRM5 in the presence of varying concentrations of KITZ (B), quantification of EPS; carbohydrate and protein content (C), morphological analysis of dried powder of EPS under SEM (D), topographical analysis of dried powder of EPS under Atomic force microscope (AFM) (E), EDX analysis of dried powder of EPS showing the presence of various elements (F)

Figure 4

Inoculation impact of kitazin tolerant *M. ciceri* BRM5 on *C. arietinum* plants grown in sandy clay loam soil treated with 96 (1X), 192 (2X) and 288 (3X) $\mu\text{gKITZkg}^{-1}$ soil developed in greenhouse conditions (panel a), germination efficiency and vigor index (panel b), total plant length (panel c) total fresh weight (panel d) and total dry biomass (panel e). The bar and line diagrams represent mean $\pm$ standard deviation (S.D) ($n = 3$) of three replicates where each replicate constituted three plants/pot. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to DMRT test.

Figure 5

Bio-inoculation impact of *M. ciceri* BRM5 on symbiotic features of *C. arietinum* plants; attachment of nodules with inoculated and treated roots (panel a), morphology of nodule (panel b) nodule number (panel c), nodule dry biomass (panel d) and LHB content (panel e) and nutrient uptake in nodules (panel f) grown in sandy clay loam soil treated with 96 (1X), 192 (2X) and 288 (3X) $\mu\text{gKITZkg}^{-1}$ soil and harvested at different intervals. The bar and line diagrams represent the mean $\pm$ S.D ($n = 3$) of three replicates where each replicate constituted three plants/pot. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to DMRT test.

Figure 6

Bio-inoculation impact of *M. ciceri* BRM5 on *C. arietinum* plants on: pod number and yield (panel a), seed number of seed yield (panel b), grain protein (panel c), N content (panel d) and P content (panel e) grown in sandy clay loam soil treated with 96, 192 and 288 $\mu\text{gKITZkg}^{-1}$ soil. The bar and line diagrams represent the mean $\pm$ S.D (n = 3) of three replicates where each replicate constituted three plants/pot. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to DMRT test.

Figure 7

Bio-inoculation impact of *M. ciceri* BRM5 on proline content (panel a) antioxidant enzymes: GPX (panel b), APX (panel c) CAT (panel d) and MDA content (panel e) of *C. arietinum* plants grown in sandy clay loam soil treated with 96 (1X), 192 (2X) and 288 (3X) $\mu\text{gKITZkg}^{-1}$ soil. The bar and line diagrams represent the mean $\pm$ S.D (n = 3) of three replicates where each replicate constituted three plants/pot. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to DMRT test.

Figure 8

Colonization of *M. ciceri* strain BRM5 on root surface of *C. arietinum* plants using scanning electron microscopy; uninoculated control showing no colonization on roots (panel A) inoculated and treated with kitazin showing adherence of bacteria on root surface (panel B). Rhizoplane and rhizosphere colonization in the presence of different concentrations of kitazin at two different seeding stages (80 and 120 DAS) (panel C).

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