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Tolerance of pesticides and antibiotics among beneficial soil microbes recovered from contaminated rhizosphere of edible crops

Mohammad Shahid *, Mohammad Saghir Khan

Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh-202002, Uttar Pradesh, India

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ABSTRACT

A total of 45 beneficial soil bacterial isolates (15 each of *Pseudomonas, Azotobacter* and phosphate solubilizing bacteria: PSB) recovered from polluted rhizosphere soils were morphologically and biochemically characterized. Bacterial isolates produced indole-3-acetic acid (IAA), phenolate siderophores; SA (salicylic acid) and 2, 3-dihydroxy benzoic acid (2, 3-DHBA), 1-amino cyclopropane 1-carboxylate (ACC) deaminase, solubilised insoluble phosphate (Pi), secreted exopolysaccharides (EPS) and produced ammonia and cyanogenic compound (HCN). Isolates were tested for their tolerance ability against 12 different agrochemicals (chemical pesticides) and 14 antibiotics. Among *Pseudomonas*, isolate PS1 showed maximum (2183 μg mL^{-1}) tolerance to all tested agrochemicals. Likewise, among all *Azotobacter* isolates ($n = 15$), AZ12 showed maximum (1766 μg mL^{-1}) while AZ7 had lowest (950 μg mL^{-1}) tolerance ability to all tested agrochemicals. Moreover, among phosphate solubilizing bacterial isolates, maximum (1970 μg mL^{-1}) and minimum (1308 μg mL^{-1}) tolerance to agrochemicals was represented by PSB8 and PSB13 isolates, respectively. The antibiotic sensitivity/resistance among isolates varied considerably. As an example, *Pseudomonas* spp. was susceptible to several antibiotics, and inhibition zone differed between 10 mm (polymyxin B) to 34 mm (nalidixic acid). Also, isolate PS2 showed resistance to erythromycin, ciprofloxacin, methicillin, novobiocin and penicillin. The resistance percentage to multiple antibiotics among *Azotobacter* isolates varied between 7 and 33%. Among PSB isolates, inhibition zone differed between 10 and 40 mm and maximum and minimum resistance percentage to multiple antibiotics was recorded as 47% and 20%, respectively. The persistence of pesticides in agricultural soil may contribute to an increase in multidrug resistance among soil microorganisms. In conclusion, plant growth promoting (PGP) substances releasing soil microorganisms comprising of inherent/intrinsic properties of pesticides tolerance and antibiotics resistance may provide an attractive, agronomically feasible, and long-term prospective alternative for the augmentation of edible crops. However, in future, more research is needed to uncover the molecular processes behind the development of pesticide tolerance and antibiotic resistance among soil microorganisms.

1. Introduction

Agrochemicals, a growing part of contemporary agriculture, are a variety of compounds used in agricultural operations to increase crop yield. It includes pesticides, synthetic fertilizers, hormones, antibiotics, liming and acidifying agents and soil conditioners, etc. An agrochemical is any substance used by humans to help in the management of an agricultural ecosystem. Generally, agrochemicals often refer to a broad range of chemical pesticides (herbicides, fungicides and insecticides) commonly used to counteract the harm caused by a variety of pests. Though the use of pesticides has been found critically important in optimizing the agricultural productivity, yet excessive or injudicious usage of such substances has been reported to causes microbiological (Mandal et al., 2020), ecological (Koli et al., 2019) and environmental damage (Cederberg et al., 2019). After, their application, agrochemicals may be utilized as a nutrient source by soil microorganisms. After consumption, these chemicals are degraded by microbes that results in synthesis of other novel metabolites which might be considerably more harmful to plants than the original molecules (Magnoli et al., 2020). The microbial activities such as- N_2-fixation, bacterial virulence factor and organic matter breakdown are negatively influenced by chemical pesticides. In contrast, excessive use of such agrochemicals leads the development of chemical resistance among beneficial soil microorganisms. Therefore, to resolve these problems, bacteria resistant to

* Corresponding author.
E-mail address: shahidfaiz5@gmail.com (M. Shahid).

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pesticides that might be utilized as microbiological agents in order to boost crop output in polluted soil has been recovered by various workers (Shahid and Khan, 2020; Khan et al., 2020; M. Shahid et al., 2019; Shahid and Khan, 2019). As an example, N_2- fixing strains of *Azotobacter* isolated from various rhizosphere represented the tolerance toward the different group of pesticides (Chennappa et al., 2014). Additionally, numerous species of *Bacillus* have reported to tolerate higher concentrations of pesticides in addition to augmenting the yield and productivity of crops (Radhakrishnan and Lee, 2016). The capacity to withstand even at higher rates of pesticides is though, a unique property among microorganisms including N_2-fixers and phosphate solubilizers which may either be due to constitutive or induced mechanisms (Kirubakaran et al., 2019). The PGPR's capacity to withstand at high level of pesticides could be encoded with plasmid or mediated with chromosome or could be due to some other mechanisms (Shafiani and Malik, 2003). Principally, physiological activity and genetic composition of microbiota affect the pesticide resistance (Herman et al., 2005). As a result, bacteria that can tolerate greater levels of pesticides have been identified as frequent pesticide degraders.

The extensive use of antibacterial medicines (antibiotics) has resulted in an increase in frequency of antimicrobial resistance, even among bacteria that are not directly targeted by antibiotics. Antibiotics may pose alteration in microbial community and biomass (Karishma and Prasad 2016), modification in ratio of total soil bacterial and fungal population and N-transformation (Thiele-Bruhn and Beck, 2005) and disruption in natural cycle of elements (Kotzerke et al., 2008). Bacterial species may have resistance to single or multiple antibiotics. The multiple antibiotic resistances in soil bacteria may possibly be due to alterations in their chemical, biological or genetic makeup. Genes responsible for antibiotic resistance in bacteria are preserved in plasmids, transposons, and mobile genetic elements (Böhme et al., 2005). Antibiotic-resistant genes may be rapidly transmitted from donor bacteria to the recipient bacteria during horizontal transfer process, resulting in a rise in antibiotic-resistant microbial communities (Chee-Sanford et al., 2009).

It is worth noting that for many years antibiotic resistance genes survive in the soil, whereas in several months the antibiotics is itself destroyed (Kang et al., 2017). It has recently been of significant scientific interest to disseminate antibiotics or resistance genes in agricultural soils (Heuer et al., 2011), as this affects public-santé. Various workers have been reported antibiotic sensitivity/resistance behavior of beneficial soil microbes (Fang et al., 2014). For instance, Asmiran et al. (2018) in an observation found pesticides and tetracycline resistant *Azotobacter* isolated from paddy rhizosphere. The bacterial isolates exhibiting both agrochemicals and antibiotics tolerance/resistance has been found to adapt faster in the contaminated environment due to the presence of resistance factors (R-factors) and not by the mutation and natural selection (Wani and Khan, 2014). However, deviation in resistance pattern to various tested antibiotics as detected/observed here may probably be due to the variations in growth conditions, intrinsic property of cells, and rhizobacterial exposure to stressful or hazardous circumstances, and the existence or lack of resistance mechanisms encoded by chromosomal and/or R-plasmid (Dipta and Kaushal, 2018). In light of these considerations, the current research was undertaken to: (i) isolate the different group of soil bacteria from pesticide contaminated rhizosphere soil and their characterization using various biochemical tests (ii) evaluate the plant growth regulating substances produced/synthesized by bacterial isolates (iii) assess the pesticide (herbicides, fungicides and insecticides) tolerance ability of rhizobacterial isolates and (iv) assess the antibiotic sensitivity/resistance traits in rhizobacteria.

2. Material and methods

2.1. Soil sample collection and their physico-chemical analysis

The composition and microbial density were determined in rhizospheric soils obtained from different vegetables crops like baqla (*Vicia faba*), cabbage (*Brassica oleracea*), wheat (*Triticum estivum*), mustard (*Brassica campestris*), chickpea (*Cicer arietinum*), tomato (*Solanum lycopersicum*) and chili (*Capsicum annuuam*) raised in agricultural fields (S1), pesticide polluted sites of Industrial estate of Ghaziabad, U.P (S2) and Khadar region of Yamuna river, New Delhi (S3) (Table S1; Fig. 1). The samples collected from rhizospheric soils of various crops grown in different regions of North India, were analyzed to determine their physicochemical makeup. The pH of soil samples were determined using L1 glass electrode pH meter (Thomas, 1996). Furthermore, for estimation of electrical conductivity (EC), the method of Rhoades et al. (1989) was followed. The content of percent OC (organic carbon) in soil samples was analysed by the method of (Walkley and Black, 1934). Available N, P and K was assayed by alkaline potassium permanganate distillation method, ammonium molybdate method and flame photometer (Subbiah and Bajaj, 1962; Fang et al., 1985), respectively. While, available S was assessed following the method of Chesnin and Yien (1950). Further, using atomic absorption spectrophotometer (ASS), the micronutrients (zinc, iron, manganese and copper) present in soil samples were detected (Isaac and Kerber, 1971). Available boron (B) was determined by hot water treatment method of Berger and Truog (1944). All analyses were done from Krishi Vigyan Kendra (KVK), Aligarh, U.P, India.

2.2. Isolation of soil bacteria

For quantitative enumeration of microbial diversity, soil samples were serially diluted (10^{-1} to 10^{-7}) in normal sterile saline solutions (NSS) and 100 μL of each diluted soil suspension was spread plated on King's B (for *Pseudomonas* sp.), Ashby's mannitol agar (for *Azotobacter* sp.) and Pikovskaya's (PKV) agar (for phosphate solubilizers) (Hi-media Pvt. Ltd. Mumbai. India). The uniformly spread plates were incubated at 28±2 °C for- (i) two days (for *Pseudomonas* sp.) (ii) seven days (for *Azotobacter* sp.) (iii) and three to seven days (for phosphate solubilizers).

2.3. Morphological features of bacterial cells

2.3.1. Gram reaction and phenotypic characterization

Bacterial cultures were cultivated overnight and exposed to Gram reaction to classify heterogeneously dispersed bacterial populations into Gram positive and Gram negative groups. Gram positive (Gram +ve) and Gram negative (Gram -ve) rhizobacteria were identified that appeared purple and red under a basic microscope. Bacterial cell colony parameters such as size, shape, border, and color, among others, were also observed.

2.4. Biochemical characterization

2.4.1. Indole, methyl red, voges-proskauer and citrate utilization

For estimation of indole production, 2–3 drops of Kovac's reagent were added to freshly grown bacterial cultures (MacWilliams, 2012). In order to assess MR-VP Methyl red-Voges Proskaur (MR-VP) test, MR-VP broth were inoculated with tested cultures, incubated at 30±2 °C for 24–48 h. After incubation, methyl red solution was added and checked the reaction whether Gram positive (Gram +ve)/Gram negative (Gram -ve). Likewise, to detect the Voges-Proskauer activity, five drops each of Baritt's reagent A and Baritt's reagent B was added to MR-VP broth and examined the reaction. For citrate utilization, Simmons's citrate agar plates were prepared; spot inoculated with freshly grown bacterial cells, incubated at 30±2 °C for 24–48 h and examined.

2.4.2. Nitrate reduction, catalase, oxidase and urease

For nitrate reduction activity, five drops of sulphanilic acid and a few drops of α-naphthylamine were added to bacteria inoculated trypticase nitrate broth tube and examined (Palleroni et al., 1984). For catalase test, 3% H_2O_2 solution was added inoculated NB broth and evolution of

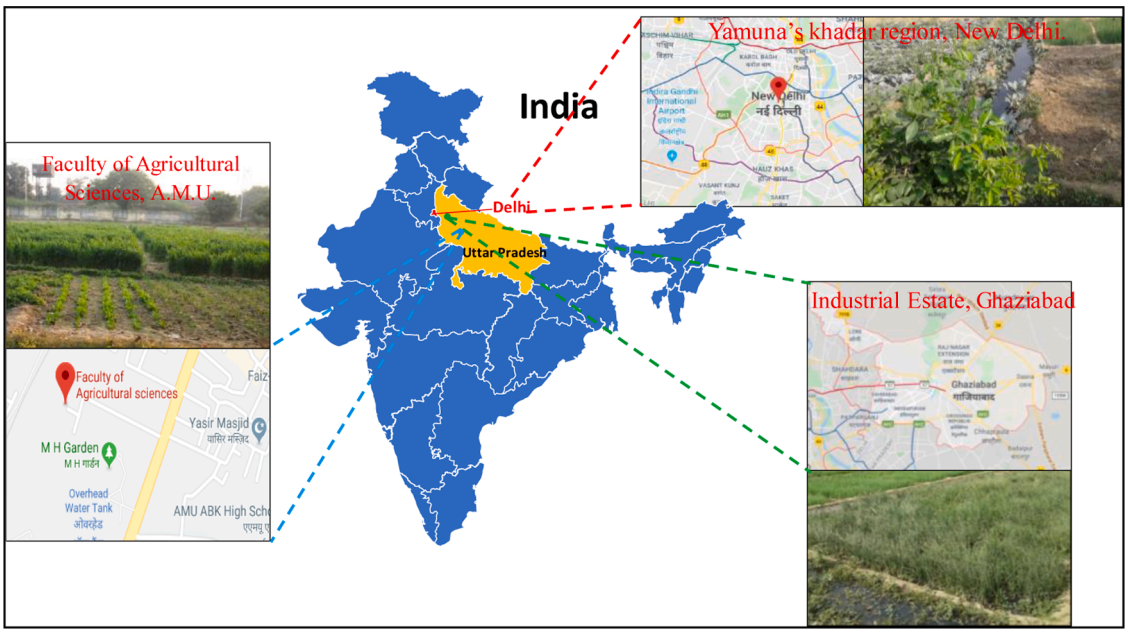

Fig. 1. Geographical representation of conventional and pesticide polluted sites.

O_2 in the form of bubbles was observed. Oxidase reaction was assessed by placing the oxidase discs on spread-inoculated (spot) NA agar plates. Likewise, urea broth was used for urease activity.

2.4.3. Starch and lipid hydrolysis and sugar fermentation

For starch and lipid hydrolysis, starch agar and tributyrin agar medium was used, respectively. A hydrolysis zone developed surrounding the bacterial growth appearing both on starch agar and tributyrin agar plates indicated a positive test (Church, 2016). Additionally, cultures were tested for carbohydrate (glucose, lactose, fructose and sucrose) utilization ability.

2.5. Assays for bacterial plant growth promoting activities

Plant Growth Promoting (PGP) traits of morphologically and biochemically screened rhizobacteria were estimated under *in vitro* conditions. The PGP activities such as indole-3-acetic acid (IAA), P-solubilization, production of siderophore, HCN, ammonia, ACC deaminase and exopolysaccharide (EPS) were determined.

2.5.1. Indole acetic acid production

Indole-3-acetic acid produced by rhizobacterial cultures were quantitatively assessed following the modified method of Brick et al. (1991) (See supplementary methods).

2.5.2. Qualitative and quantitative estimation of phosphate solubilization

To assess the P-solubilizing potential of bacterial isolates, they were cultured in PKV (both liquid and solid) medium (For detail, see supplementary method).

2.5.3. Production of siderophore

Secretion of siderophore by the isolated and screened rhizobacteria was determined qualitatively using $FeCl_3$ test (Atkin et al., 1970) and Chrome Azurol S (CAS) method (Alexander and Zuberer, 1991). Siderophore secreted by selected bacterial cultures were further assayed quantitatively by growing bacterial cultures in Modi medium and quantity of siderophore (SA and 2, 3-DHBA) was spectrophotometrically measured (Reeves et al., 1983).

2.5.4. Detection of cyanogenic compound (HCN) and ammonia

Production of HCN by rhizobacterial isolates was detected by method of Bakker and Schipper (1987). For detection of ammonia, selected rhizobacterial isolates were developed in peptone water medium and incubated at 28±2 °C for 3–4 days. After incubation, 1 mL of Nessler's reagent was added to each tube and yellow to orange color development indicating formation of ammonia was recorded (Dye, 1962).

2.5.5. ACC deaminase enzyme activity

ACC deaminase activity of bacterial isolates was quantitatively determined following the method of Honma and Shimomura (1978), later modified by Penrose and Glick (2003).

2.5.6. Exopolysaccharide (EPS) production

The exopolysaccharide (EPS) production was checked by the method of Mody et al. (1989) (See supplementary method).

2.6. Screening/selection of agrochemical tolerant soil bacteria

Isolates were subjected to a range of concentrations of 12 agrochemicals including four each from herbicides [glyphosate (GP), quizalofop, (QUIZ) atrazine (ATZ), butachlor (BUTA)], fungicides [kitazin (KTZ), metalaxyl (METL), hexaconazole (HEXA), carbendazim (CBZM) and insecticide [fipronil (FIP), imidacloprid (IMID), monocrotophos (MONO), thiamethoxam (THIA) (Table S2) to select pesticide tolerant bacterial isolates (Shahid et al., 2021a–c, Shahid and Khan, 2017, 2018, 2019). After sterility check, minimal salt agar (MSA) petriplates were supplemented with increasing rates (0–4000 μgmL^{-1}) of each herbicide, fungicide and insecticide and freshly grown bacterial cultures were spot (10^8 cell mL^{-1}) inoculated. The incubation of plates was done at 28±2 °C for a period of 2 days and colonies that survive at greatest pesticide concentrations of pesticides were selected. Each experiment was repeated three times.

2.7. Antibiotic sensitivity/resistance pattern of bacterial isolates

Sensitivity/resistance behavior of selected bacterial isolates against different antibiotics was established by technique of Bauer et al. (1966). (For detailed description, see supplementary methods). The potency of

all the used antibiotics has been given in (Table S3).

2.8. Data analysis and processing

All the experiments were performed three times ($n = 3$). The obtained three values calculated and represented as means± SD ($n = 3$). Also, results were statistically analysed using Duncan's multiple range test (DMRT).

3. Results and discussion

3.1. Physico-Chemical properties of rhizosphere soils

Soil samples collected from different vegetable rhizosphere displayed variable physical and chemical properties (Table 1). As an example, soils collected from *Vicia faba* (baqla) rhizosphere (S1) had: 8.9 pH value, EC = 0.995 mv cm^{-2},% (organic carbon) OC = 0.4%, total nitrogen (N) = 0.077 kg ha^{-1}, total P = 18.5 kg ha^{-1}, and K = 319.5 kg ha^{-1}. The content of sulfur (S) and boron (B) was recorded as 11.5 and 5.2 mg kg^{-1}, respectively. Similarly, trace elements were recorded as: zinc (Zn) = 1.14 5 mg kg^{-1}, iron (Fe) = 9.26 mg kg^{-1}, manganese (Mn) = 4.32 mg kg^{-1} and copper (Cu) = 0.54 mg kg^{-1}. The pH value of contaminated soils in general was found higher (pH 8.4 to 8.6) as compared to the conventional (7.0 to 7.6 pH) soil. Whereas, water holding capacity (WHC), macro and micro nutrients of contaminated soils (S2 and S3) was lower relative to conventional soils (S1). For instance, the WHC of S2 and S3 sites was found as 0.55 and 0.48 mL g^{-1} soil, respectively, compared to conventional soils (0.59–0.71 mL g^{-1} soil for chickpea) (Table 1).

3.2. Isolation and biochemical characterization of bacterial isolates

Soil bacteria may invade the roots of plants and may induce beneficial, deleterious or neutral effects on overall growth of plants (Aly et al., 2017; Dudeja et al., 2012). Keeping in view the useful activities of soil microbes, numerous PGPR isolates were selected and assessed for their tolerance ability to different group of agrochemicals and antibiotics. In the present findings, a total of 45 rhizobacterial isolates involving 15 *Pseudomonas*, 15 *Azotobacter* and 15 P-solubilizers were tested. The PGPR belonging to genera *Burkholderia, Bacillus, Pseudomonas* and *Azotobacter* were chosen and assessed based on their capacity to endure agrochemicals and antibiotics. Similarly, *Burkholderia* (Teri et al., 2018), *Azotobacter* (Shirinbayan et al., 2019), *Pseudomonas* (Gamez et al., 2019), *Bacillus* (Ku et al., 2018) and other soil bacteria have been isolated from different rhizosphere sources.

3.3. Phenotypic and biochemical identification

In order to identify the unknown bacterial species, first and foremost step is the phenotypic and biochemical characterization. In this work, all ($N = 45$) rhizobacterial isolates recovered from different conventional and polluted rhizosphere soils were morphologically and biochemically characterized. Of the total isolates, 87.5% isolates were Gram negative rod shaped while 12.5% were Gram positive with small rods (Fig. 2). Similar to our study, Gram-ve and Gram+ve bacteria were isolated from glyphosate contaminated rhizosphere soil (Liu et al., 2018). Each rhizobacterial isolates displayed morphological and biochemical variables. In general, isolates were negative for indole, methyl red and urease tests while they indicated a favourable response to the use of citrate, mannitol, oxidase, catalase production, nitrate reduction and lipid hydrolysis. Among the total cultures, 15 isolates were positive for indole while 08 isolates were positive in methyl red test. Majority of rhizobacteria could produce catalase (67%), reduced nitrate (87%), hydrolysed starch (35%) and could use citrate (93.3%). However, 55% of the total rhizobacterial isolates showed positive Voges-Proskauer (VP) reaction. Moreover, 37.6% of total isolates showed the positive reaction towards the urease activity and 43% for oxidase activity. Among *Pseudomonas*, PS3 exhibited circular and smooth colonies and produced characteristic fluorescent green pigment (Fig. S1, panel A) when grown on King's B medium. This isolates could utilize citrate, hydrolyse lipid and was also positive for VP, catalase and nitrate reduction activity although it reacted negatively towards the indole reaction, MR, urease and starch hydrolysis (Table 2a). Among *Azotobacter* isolates, AZ2 produced irregular colonies with wavy margins and also revealed a dark brown pigmentation after 5–7 days of growth (Fig. S1, panel B) and a variable biochemical reaction (Table 2b). Phosphate solubilizing (PS) group producing a clear zone of solubilization (halo) around bacterial growth on PKV agar plates (Fig. S1, panel C) revealed a biological response that was varied (Table 2c). In accordance with the present study, microbiologists have isolated numerous *Pseudomonas* species like *P. fluorescens* (Manasa et al., 2017), *P. putida* (Wang et al., 2015), *P. azotoformans* (Nonakaran et al., 2015) and *Azotobacter* species including *A. chroococcum* (Chen et al., 2018), *A. vinelandii* (El-Badry et al., 2016) and *A. salinestris* (Chennappa et al., 2018) etc. from different rhizosphere soils of vegetables crops in conventional as well as contaminated environments. Similarly, phosphate solubilizing bacteria belonging to *B. cepacia* (You et al., 2020), *B. subtilis* (Ahmad et al., 2018), *B. megaterium* (Wyciszkiewicz et al., 2017), *Achromobacter* and *Serratia plymuthica* (Aroua et al., 2019) etc. isolated from different rhizosphere region have also been reported.

Table 1
Physico-chemical properties of conventional and pesticide contaminated rhizosphere soils.

Characteristics	S1 Cabbage	Wheat	Mustard	Chickpea	Balqa	S2 tomato	S3 chili
Physical factors							
pH	7.2	7.4	7.6	7.0	7.5	8.6	8.4
EC (mv/cm^2)	0.995	0.972	0.984	0.983	1.0	0.94	0.87
WHC (mL g^{-1} soil)	0.61	0.64	0.59	0.71	0.65	0.55	0.48
Chemical Composition							
Organic C (%)	0.4	0.72	0.90	0.66	0.58	0.31	0.37
P (kg/ha)	18	20.25	15.75	13.5	20.2	22	19.5
K (kg/ha)	319.5	196.6	269.5	198	23.04	215	145
Zn	1.14	1.08	1.14	1.19	1.3	1.5	0.87
Fe	9.26	9.14	9.14	9.71	9.6	10.2	7.0
Mn	4.32	4.05	4.16	4.0	4.05	3.1	2.8
Trace elements							
Cu	0.54	0.38	0.38	0.54	0.43	0.56	0.62

Physico-chemical characteristics of soils were determined by commercially available service provided by Krishi Vigyan Kendra (KVK), Aligarh. In this table, S1, S2 and S3 represents the soils collected from Faculty of Agricultural Sciences, A.M.U, Aligarh, Industrial estate Ghaziabad, and Khadar region of Yamuna river, New Delhi, respectively. EC = electrical conductivity, WHC = water holding capacity, C = Carbon, P = Phosphorous, K = Potassium, Zn = Zinc, Fe = Iron, Mn = Manganese and Cu = Copper.

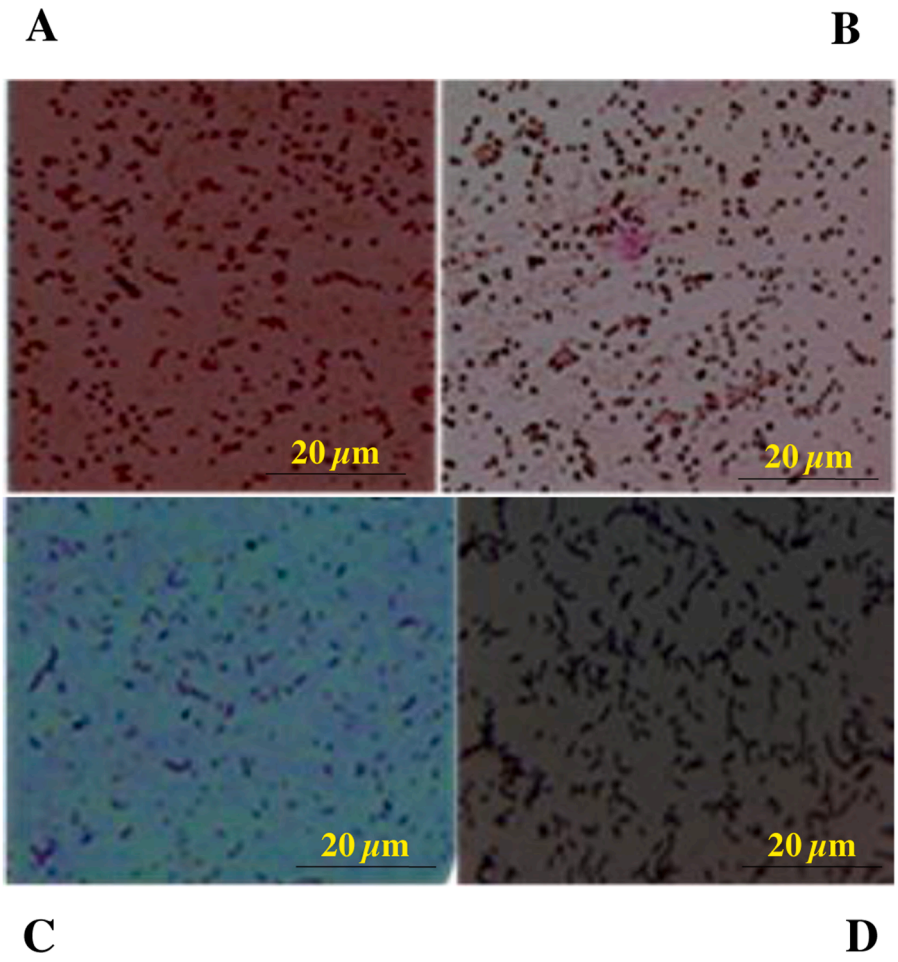

Fig. 2. Microscopic examination of stained bacterial isolates showing Gram negative (Gram -ve) (A and B) and Gram positive (Gram +ve) (C and D) rods-shaped cells.

3.4. Evaluation of plant growth-promoting activities

3.4.1. Quantitative bioassay of indole-3-acetic acid

Indole-3-acetic acid synthesized by *Pseudomonas* (Table 3a), *Azotobacter* (Table 3b) and PSB (Table 3c) was variable when cultured in LB medium added with varying doses of tryptophan. In general, highest production of IAA by all rhizobacterial isolates occurred at 500 μg mL^{-1} of tryptophan (Trp). For instance, among *Pseudomonas* isolates, PS3 produced maximum amount of IAA (92.5 ± 7.2 μg mL^{-1}) at 500 μg mL^{-1} of tryptophan. When comparing the IAA mean values, produced by all *Pseudomonas* strains at all tryptophan concentrations, the pattern of increase in IAA followed order: (45 μg mL^{-1}) >400T (36 μg mL^{-1}) >200T (29.45 μg mL^{-1}) >100T (22 μg mL^{-1}) >0T (16.46 μg mL^{-1}) (Table 3a). Similarly, among all *Azotobacter* strains, AZ6 displayed the maximum production of 82.5 ± 7.2, 78.3 ± 6.2, 68.8 ± 5.2, 50.8 ± 3.7 and 35.3 ± 1.5 μgmL^{-1} indole-3-acetic acid at 500, 400, 200, 100 and 0 μg mL^{-1} of tryptophan, respectively (Table 3b). When evaluating the impact of tryptophan on *Azotobacter* strains, IAA production was increased by 64, 49, 48 and 30% at 500, 400, 200 and 100 μg Trp mL^{-1}, respectively, compared to control. Of the 15 PSB isolates, strain PSB1 produced maximum amount (114±6 μg mL^{-1}) at 500 μg Trp mL^{-1} and it was 63% greater than those recorded at 0 μg T mL^{-1}. While comparing the impact of different doses of tryptophan on IAA produced by all PSB strains, 500 μg T mL^{-1} had maximum inducible effect on IAA (mean value 36.6 μg mL^{-1}) which was followed by 400 (29.3 μg mL^{-1}), 200 (23.3 μg mL^{-1}) 100 (18.9 μg mL^{-1}) and 0 (17.5 μg mL^{-1}) (Table 3c). The enhancement in bacterial synthesis of IAA with increasing concentration of tryptophan (Trp) is not shocking because Trp-is main precursor of indole-3-acetic acid production and considered to be principal precursor to IAA development in microorganisms and plants as well (Casanova-Sáez et al., 2021). Trp-induced indole-3-acetic acid biosynthetic pathways in bacteria have been discovered (Estenson et al., 2018). Different forms of plant growth regulators, such as phytohormones, including auxins (Ghosh et al., 2019b), cytokinins (Zafar-Ul-Hye et al., 2019), gibberellins (Patten and Glick, 1996), ethylene (Novo et al., 2018) and abscisic acids (Estenson et al., 2018) can be produced by soil microorganisms in diverse environments. Phytohormones are essential for a plant's general development and expansion (Patle et al., 2018). IAA secretion is highly significant among phytohormone, owing to its function in cell elongation and morphogenesis of root, growth and plants physiology even in stressed environment (Parvin et al., 2018). In this investigation, raising the quantities of the inducer molecule (tryptophan) greatly enhanced the synthesis of IAA by bacterial strains. Similarly, *Burkholderia cepacia* UPMB3 (Sharma et al., 2016) and other plant useful rhizobacteria cultured in Luria broth amended with inducer molecules excreted a considerable quantity of indole-3-acetic acid.

3.4.2. Quantitative analysis of ACC deaminase activity

When cultivated on DF salt medium supplemented with 3 mM ACC instead of ammonium sulfate, the rhizobacterial isolates displayed varied ACC deaminase (ACCD) activity. Among all isolates, 7 each of *Pseudomonas* and *Azotobacter* and 8 PSB isolates showed positive reaction for ACCD. Further, ACC deaminase activity was quantified in liquid broth. Among *Pseudomonas* isolates, ACC deaminase activity ranged between 9.5 ± 0.4 μM α- ketobutyrate mg^{-1} protein $hour^{-1}$ (PS1) to 33.5 ± 1.5 μM α-ketobutyrate mg^{-1} protein $hour^{-1}$ (PS3) (Table 3a).

Table 2a
Morphological, microbiological and biochemical characteristics of Gram-negative *Pseudomonas* isolates isolated from various rhizosphere soils.

CD	Colony features			Biochemical Reactions													
	color	Shape	Margin	IMVIC test				Catalase	Urease	Oxidase	NR	Hydrolysis		Carbohydrate utilization			
				Indole	MR	VP	CU					Lipid	Starch	Glucose Acid/Gas	Fructose Acid/Gas	Lactose Acid/Gas	Sucrose Acid/Gas
PS1	Dull yellow	Circular	Smooth	–	–	+	+	+	–	+	+	+	+	+/-	+/-	+/-	+/-
PS2	Dull green	Circular	Smooth	–	–	+	+	+	–	–	+	+	–	+/-	+/-	+/-	+/-
PS3	Fluorescent	Circular	Smooth	–	–	+	+	+	–	–	+	+	–	+/-	+/-	+/-	+/-
PS4	Green	Circular	Smooth	+	–	+	+	+	–	–	+	+	–	+/-	+/-	+/-	+/-
PS5	Yellowish	Circular	Smooth	–	–	+	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
PS6	Fluorescent	Circular	Smooth	–	–	–	+	+	–	+	+	+	+	+/-	+/-	+/-	+/-
PS7	Yellowish	Circular	Smooth	+	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
PS8	Yellowish	Circular	Smooth	–	–	+	+	+	–	–	+	+	–	+/-	+/-	+/-	+/-
PS9	Yellowish	Circular	Smooth	–	–	+	+	+	–	+	+	+	–	+/-	+/-	+/-	+/-
PS10	Green	Circular	Smooth	–	+	+	+	+	+	–	+	+	–	+/-	+/-	+/-	+/-
PS11	Fluorescent	Circular	Smooth	–	–	+	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
PS12	Green	Circular	Smooth	–	–	+	+	+	–	–	+	+	–	+/-	+/-	+/-	+/-
PS13	Green	Circular	Smooth	–	–	+	+	+	–	–	+	+	–	+/-	+/-	+/-	+/-
PS14	Fluorescent	Circular	Smooth	+	–	+	+	+	–	–	+	+	–	+/-	+/-	+/-	+/-
PS15	Green	Circular	Smooth	–	–	+	+	+	–	+	+	+	+	+/-	+/-	+/-	+/-

In this and succeeding tables CD = culture designation, CU = citrate utilization, MR= methyl red, VP= Voges-Proskauer and NR = nitrate reduction. Symbols '+' and '-' indicates positive and negative reactions, respectively.

Table 2b
Morphological, microbiological and biochemical characteristics of Gram negative *Azotobacter* isolates isolated from various rhizosphere soil.

CD	Colony features			Biochemical Reactions													
	color	Shape	Margin	IMVIC test				Catalase	Urease	Oxidase	NR	Hydrolysis		Carbohydrate utilization			
				Indole	MR	VP	CU					Lipid	Starch	Glucose Acid/Gas	Fructose Acid/Gas	Lactose Acid/Gas	Sucrose Acid/Gas
AZ1	Yellow	Rods	Irregular	–	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ2	Brown	Rods	Irregular	+	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ3	Yellow	Rods	Entire	–	–	–	–	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ4	Yellow	Rods	Irregular	–	–	–	+	+	+	–	+	+	+	+/-	+/-	+/-	+/-
AZ5	White	Rods	Irregular	–	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ6	Brown	Rods	Irregular	+	+	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ7	Yellow	rods	Entire	–	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ8	Yellow	rods	Irregular	–	–	–	–	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ9	Yellow	rods	Irregular	–	–	–	–	+	–	–	+	+	–	+/-	+/-	+/-	+/-
AZ10	Yellow	rods	Irregular	–	–	–	+	+	–	–	+	+	–	+/-	+/-	+/-	+/-
AZ11	Brown	rods	Irregular	–	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ12	Brown	rods	Irregular	+	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ13	Brown	rods	Irregular	–	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ14	Creamy	rods	Entire	–	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-
AZ15	Creamy	rods	Entire	–	–	–	+	+	–	–	+	+	+	+/-	+/-	+/-	+/-

Table 2c
Morphological, microbiological and biochemical characteristics of Gram –ve/+ve phosphate solubilizer isolated from various rhizosphere soil.

CD	Colony features			Biochemical Reactions									Carbohydrate utilization			
	color	Shape	Margin	IMVIC test				Catalase	Urease	Oxidase	NR	Hydrolysis		Glucose	Fructose	Lactose	Sucrose
				Indole	MR	VP	CU					Lipid	Starch	Acid/Gas	Acid/Gas	Acid/Gas	Acid/Gas
PSB1	White	Irregular	-	+	+	-	+	-	+	+	+	+	+	+/-	-/-	+/-	+/-
PSB2	White	Entire	-	+	+	+	+	-	+	+	-	+	+	+/-	+/+	+/-	+/-
PSB3	Yellow	Entire	-	+	+	+	+	-	+	+	+	+	+	+/-	+/+	+/-	+/-
PSB4	White	Irregular	-	-	-	+	+	-	+	+	+	+	+	+/-	+/+	+/-	+/-
PSB5	White	Irregular	-	-	-	+	+	-	+	+	-	+	+	+/-	+/+	+/-	+/-
PSB6	Orange	Irregular	-	+	-	+	+	-	+	+	-	+	+	+/-	+/+	+/-	+/-
PSB7	Yellow	Entire	-	+	-	+	+	-	+	+	+	+	+	+/-	+/+	+/-	+/-
PSB8	White	Entire	-	+	-	-	+	-	+	+	+	+	+	+/-	+/+	+/-	+/-
PSB9	White	Entire	-	-	+	+	+	-	+	+	+	+	+	+/-	+/+	+/-	+/-
PSB10	Light pink	Entire	-	-	+	+	+	-	+	+	-	+	+	+/-	+/+	+/-	+/-
PSB11	White	Irregular	-	-	+	+	+	-	+	+	+	+	+	+/-	+/+	+/-	+/-
PSB12	White	Irregular	-	-	-	-	+	-	+	+	-	+	+	+/-	+/+	+/-	+/-
PSB13	Yellow	Entire	-	+	-	+	+	-	+	+	+	+	+	+/-	+/+	+/-	+/-
PSB14	White	Entire	-	+	-	+	+	-	+	+	-	+	+	+/-	+/+	+/-	+/-
PSB15	White	Entire	-	+	-	-	+	-	+	+	+	+	+	+/-	+/+	+/-	+/-

Among ACC deaminase positive *Azotobacter* ($N = 7$) and PSB ($N = 8$) strains, AZ6 and PSB1 showed maximum production of 22±2.1 and 69.3 ± 5.8 α-ketobutyrate mg^{-1} protein $hour^{-1}$, respectively (Table 3b). Many rhizobacteria produce ACC deaminase, a biochemical characteristic that reduces exceptionally high levels of ethylene and therefore improves the performance of plants growing under unfavorable conditions. The release of ACCD enzymes by bacterial strains, though even under harsh environment is an agronomically advantageous trait for increasing the yield of pesticide-stressed crops. Because of this inherent characteristic, ACC deaminase synthesis is a fascinating and promising option for crop development. Similarly to this, ACC deaminase producing rhizobacterial strains such as *Bacillus* (Gowtham et al., 2020) and *Pseudomonas* (Gao et al., 2020) have been reported.

3.4.3. Siderophore production

Siderophore producing ability of strains assessed (both qualitatively and quantitatively) by CAS agar plates and ethyl acetate (EA) extraction method differed considerably. An orange-coloured zone (halo) produced on CAS agar plates indicated positive reactions by test bacterial isolates. Among bacterial strains, *Pseudomonas* (60%), *Azotobacter* (46%) and PSB (60%) showed siderophore activity on CAS agar plates after 3–4 days of growth. The intensity and zone size produced on CAS agar plates, however, varied among strain to strain within genera. The orange halo on CAS agar plate showed by positive *Pseudomonas* isolates ($N = 9$) ranged between 11 mm (PS18) to 24 mm (PS3) (Table 3a). Among *Azotobacter*, largest size (18 mm) on plates of CAS agar was developed by AZ6 (Table 3b). Of the total PSB isolates, the maximum (16 mm) and minimum (12 mm) halo size was shown by PSB1 and PSB9, respectively (Table 3c). The different forms of siderophore like SA and 2, 3- DHBA developed by each rhizobacterial isolates was variable. Among all siderophore positive *Pseudomonas* strains, maximum quantity of SA (24.6 ± 1.6 μg mL^{-1}) and DHBA (13.9 ± 1.5 μg mL^{-1}) was produced by strain PS3 whereas, SA (11±1.0 μg mL^{-1}) and DHBA (5.3 ± 0.5 μg mL^{-1}) was released minimally by strain PS12. A total of 7 *Azotobacter* isolates showed siderophore activity; the maximum being produced by strain AZ6. The minimum quantity of SA (15.3 ± 3.0 μg mL^{-1}) was observed for strain AZ10 whereas; DHBA (5.2 ± 0.3 μg mL^{-1}) was minimally secreted by strain AZ5. While comparing the release of SA and DHBA by siderophore positive *Azotobacter* strains, the amount of SA (mean value 26.7 μg mL^{-1}) was 61% greater than DHBA (mean value 10.4 μg mL^{-1}). Among PSB isolates, PSB1 maximally produced both SA (39.3 ± 2.6 μg mL^{-1}) and DHBA (26.2 ± 2.0 μg mL^{-1}) while, strain PSB8 secreted the lowest amount of SA (17.6 ± 1.7μg mL^{-1}) and DHBA (8.7 ± 0.4 μg mL^{-1}) (Table 3c). Fe is mostly found as insoluble hydroxide and oxyhydroxide in aerobic conditions, making it inaccessible to microbial populations. As a result, siderophore (an iron chelating compound) are synthesized under Fe starved condition by microbial communities. This is useful because strains that produce siderophore might be employed in phytopathogens bio management (Kumar et al., 2017). Siderophore (*e.g.* Pyoverdine) synthesised by the supply of microbial colonies when plants are cultivated in an iron-deficient environment, they gain Fe (Nagata, 2017). Considerable amount of siderophore synthesized by rhizobacterial strains tested in this study suggest that if such strains are applied in agricultural systems as bio-inoculants, they are likely to augment the performance of crops by restricting the soil borne phytopathogens.

3.4.5. EPS production

Among soil isolates ($N = 45$), 6 *Azotobacter* sp. and 5 PSB isolates showed the EPS production activity. However, none of the *Pseudomonas* sp. showed the production of EPS. The other important feature which ultimately influences plants' growth is the secretion of exopolysaccharides (EPS) by rhizobacteria. Exopolysaccharides is a key polymer that protects microorganisms against unwanted conditions (Gafri et al., 2019). Recognizing the importance of EPS in the biological nitrogen fixation (BNF), soil aggregation and protection from harsher environment, under *in vitro* conditions, rhizobacterial isolates were tested for

Table 3a
Plant growth promoting substances secreted by *Pseudomonas* strains recovered from various rhizosphere soils.

Strains	Plant growth promoting (PGP) substances Indole-3-acetic acid production (μgmL^{-1})					Siderophore		SA (μgmL^{-1})	2, 3-DHBA (μgmL^{-1})	EPS (μgmL^{-1})	ACC deaminase activity (μM α-ketobutyrate mg^{-1} protein hour^{-1})	NH_3	HCN
	0T*	100T*	200T*	400T*	500T*	$FeCl_3$test	Halo size (mm)						
PS1	9.3^{f} ± 0.2	31.7^{b} ± 2.3	40.4^{b} ± 2.6	52.6^{b} ± 3.2	68.5^{b} ± 5.2	++	–	–	–	–	9.5 ^{e} ± 0.4	++	–
PS2	16.4^{c} ± 1.0	18.6^{e} ± 1.3	22.8^{e} ± 1.5	27.5^{f} ± 1.8	38.6^{e} ± 2.2	++	–	–	–	–	–	++	–
PS3	23.3^{b} ± 1.5	36.8 ^{a} ± 2.0	79.8^{a} ± 5.2	82.3^{a} ± 6.2	92.5^{a} ± 7.2	++	24.0^{a} ± 1.0	24.6 ^{a} ± 1.6	13.9^{a} ± 1.5	–	33.5^{a} ± 1.5	+++	++
PS4	–	–	–	–	–	++	–	–	–	–	19.1^{c} ± 1.6	++	++
PS5	–	–	–	–	–	++	14.0^{c} ± 1.0	13.0^{d} ± 1.0	6.0^{c} ± 1.0	–	–	++	++
PS6	17.1^{c} ± 1.3	19.7^{e} ± 1.4	20.6^{f} ± 1.8	26.2^{f} ± 2.3	33.1 ^{f} ± 3.2	++	–	–	–	–	–	++	++
PS7	12.8^{e} ± 1.1	18.2^{e} ± 1.6	21.5^{f} ± 1.5	28.2^{f} ± 2.2	34.2 ^{f} ± 2.0	++	–	–	–	–	20.1^{c} ± 1.3	++	–
PS8	15.5^{d} ± 1.0	19.6^{e} ± 1.3	23.8^{e} ± 1.5	29.5^{f} ± 2.0	39.7^{e} ± 3.0	++	17.0^{b} ± 2.5	15.6^{c} ± 1.0	6.3^{c} ± 0.5	–	–	++	–
PS9	17.9^{c} ± 1.0	22.3^{d} ± 1.5	32.4^{c} ± 2.1	39.3^{d} ± 2.6	54.2^{c} ± 4.0	++	–	–	–	–	–	++	–
PS10	–	–	–	–	–	++	15.0^{c} ± 0.6	22.0^{b} ± 1.0	11.0^{b} ± 1.0	–	–	++	–
PS11	17.1^{c} ± 0.6	19.5^{e} ± 0.9	30.4^{d} ± 1.3	41.2^{d} ± 1.9	48.3^{d} ± 3.4	++	–	–	–	–	–	++	–
PS12	18.8^{c} ± 1.2	25.1^{c} ± 1.5	32.0^{c} ± 1.9	39.4^{d} ± 2.6	45.2^{d} ± 3.5	++	12.0^{d} ± 0.5	11.0^{e} ± 1.0	5.3^{d} ± 0.5	–	16.3 ^{d} ± 2.1	++	–
PS13	23.7^{b} ± 1.9	25.5^{c} ± 2.3	33.6^{c} ± 2.6	36.4^{e} ± 3.1	45.2^{d} ± 4.2	++	–	–	–	–	22.0^{b} ± 2.1	++	–
PS14	28.2^{a} ± 2.0	32.4^{b} ± 3.0	42.0^{b} ± 3.5	48.0^{c} ± 3.6	51.2^{c} ± 4.0	++	17.0^{b} ± 2.0	15.0^{c} ± 1	6.0 ^{c} ± 1.0	–	17.5 ^{d} ± 1.9	++	–
PS15	–	–	–	–	–	++	13.0^{d} ± 0.5	12.3^{d} ± 2	5.3^{d} ± 0.5	–	–	++	–

Values in this and subsequent tables indicate mean ± S.D. of three independent replicates. Means followed by alphabets a, b, c, d and e etc. are significantly different from each other according to Duncuan's multiple range (DMRT) test. Here, IAA = indole-3-acetic acid, CAS = Chrome Azurol S agar, SA = Salicylic acid, DHBA = 2, 3 Dihydroxybenzoic acid, ACC = 1-Amino cyclopropane 1-carboxylate, NH_3; = Ammonia, HCN = Hydrogen cyanide and *T* = Tryptophan concentration (μg mL^{-1}), symbols '+' and '–' indicate positive and negative reactions, respectively.

their capacity to produce EPS. Moreover, by synthesizing superfluous amounts of EPS, bacterial strains could safeguard itself from poisonous/noxious effect of contaminants such as pesticides by masking the effect of toxic (Ghosh et al., 2019a). As a result, there has been a surge in interest in finding EPS-producing organisms in recent years. Under controlled/stressed environmental conditions, numerous species of soil isolates recovered from different rhizosphere sources are reported to produce exopolysacchrides (Syed et al., 2021; Khan and Bano, 2019; Naseem et al., 2018; Shahid and Khan, 2017).

3.4.6. *Detection of ammonia and hydrogen cyanide*

The isolated bacterial strains were checked further to assess the production of NH_3 and cyanogenic compounds (HCN) by growing them in peptone water and HCN induction medium, respectively. All rhizobacterial isolates (N = 45) were positive to NH_3 whereas only 16% strains were positive to HCN. Cyanogenic chemicals such as HCN, which may be made directly from glycine, and cyanogenic glycosides are examples of microbial metabolites, are also secreted by several microorganisms (Rijavec and Lapanje, 2017). Another bacterial metabolite is ammonia produced via amino acid degradation and ammunition, hydrolyte urea-mediated degradation and amino acid decarboxylation. In addition, many rhizobacterial strains contain ammonia conveyors inside their cells that are supposed to be involved in NH_4^+ re-absorption due to bacterial membrane NH_3 diffusion (Patriarca et al., 2002). The synthesis of HCN and ammonia is shown to be a common feature among PGPR strains such as *Kosakonia sacchari* (Shahid et al., 2021b), *Enterobacter* sp. (Ahmed et al., 2021); *Pseudomonas* sp. (Khan et al., 2020) and *Burkholderia cepacia* (Shahid et al., 2018) etc.

3.4.4. *Phosphate solubilization*

The phosphate solubilizing (PS) activity tested both qualitatively and quantitatively varied considerably among rhizobacterial isolates. Around the bacterial growth on TCP supplemented PKV and plates, a clear halo (zone of solubilization) was developed by rhizobacterial isolates. Size of halo ranged between 3 mm (PSB3 and PSB12) to 11 mm (PSB8). The colony size was linked positively (r^2=0.57) with halo size (Fig S4 a). Keeping in view the halo size and diameter of colony and S.I and solubilization efficiency (S.E.) was calculated (Table 4). The solubilization index (SI) value varied between 1.83 (PSB7) to 3.2 (PSB8) and S.E. value differed between 60 (PSB12) to 220 (PSB8). After measuring the PS activity on solid PKV medium, phosphate solubilization activity (PSA) was determined in PKV broth. In general, the quantum of P solubilised varied between 37.3 μg mL^{-1} (PSB15) to 117.9 μg mL^{-1} (PSB8). In liquid culture medium, strain PSB1 exhibited the maximum solubilization of TCP (102.7 μg mL^{-1}). The size of bacterial colony showed a positive correlation (r^2=0.83) (Fig S4 b) with PSA recorded in case of solid PKV and total diameter was positively correlated (r^2=0.9) (Fig S4 c) with P-solubilization in liquid culture medium. The P-zone was positively correlated (r^2=0.74) (Fig S4 d) with P-solubilised in liquid culture media. P is second largest plant nutrient among many nutrients, the lack of which limits development of plants severely (Rahman et al., 2017). And so, phosphatic fertilizers are administered from external sources to bypass P shortage and allow the plants to properly develop. Phosphate solubilizing bacteria which cover many genera, have offered a number of alternatives for costly synthetic P fertilizers. The PSA of rhizobacteria has been discovered in several researches owing to the production of low molecular weight organic acids (Khan et al., 2007). Like this, numerous soil bacterial species are reported to exhibit the ability of phosphate solubilization. For instance,

Table 3b
Plant growth promoting substances secreted by *Azotobacter* isolates recovered from different rhizosphere.

Strains	Plant growth promoting (PGP) substances Indole-3-acetic acid production (μgmL^{-1})					Siderophore							
	0T*	100T*	200T*	400T*	500T*	$FeCl_3$Test	Halo size (mm)	SA (μgmL^{-1})	2, 3-DHBA (μgmL^{-1})	EPS (μgmL^{-1})	ACC deaminase activity (μM α-ketobutyrate mg^{-1} protein hour^{-1})	NH_3	HCN
AZ1	12.3^{f} ± 2.3	16.6 ^{e} ± 3.2	20.2 ^{d} ± 5.2	26.2 ^{f} ± 5.6	41.0^{d} ± 6.0	–	–	–	–	–	11.2^{f} ± 0.5	++	–
AZ2	23.3^{c} ± 1.5	26.8^{c} ± 2.0	35.8^{b} ± 5.2	42.3^{b} ± 6.2	52.5^{b} ± 7.2	+	12.0^{d} ± 0.5	30.0^{b} ± 3.9	9.3^{c} ± 0.7	128^{a} ± 8.6	15.3^{e} ± 1.1	+++	+
AZ3	16.4^{e} ± 1.0	18.6 ^{e} ± 1.3	20.8^{d} ± 1.5	27.5^{e} ± 1.8	38.6^{e} ± 2.2	–	–	–	–	–	–	+	–
AZ4	6.3 *g* ± 1.5	11.8 ^{g} ± 2.0	19.8 ^{d} ± 5.2	25.3^{f} ± 6.2	29.5^{f} ± 7.2	–	–	–	–	78.1^{d} ± 5.4	–	+	–
AZ5	–	–	–	–	–	+	14.0^{c} ± 0.5	32.7^{b} ± 3.2	5.2^{d} ± 0.3	–	–	++	+
AZ6	35.3^{a}± 1.5	50.8^{a} ± 3.7	68.8^{a} ± 5.2	78.3^{a} ± 6.2	82.5^{a} ± 7.2	+	18.0^{a} ± 1.0	40.8^{a} ± 3.2	15.5^{a} ± 2.6	120^{b} ± 7.0	46.0^{a} ± 1.4	+++	+
AZ7	17.1^{d} ± 1.3	19.7^{e} ± 1.4	30.6^{c} ± 1.8	36.2^{d} ± 2.3	43.1^{d} ± 3.2	–	–	–	–	–	–	++	–
AZ8	–	–	–	–	–	+	10.0^{e} ± 0.5	22.4^{c} ± 2.6	10.3^{c} ± 0.5	65.8^{e} ± 5.0	42.0^{b} ± 4.2	+	+
AZ9	15.5^{e} ± 1.0	23.6 ^{d} ± 1.3	29.8^{c} ± 1.5	39.5^{c} ± 2.0	49.7^{c} ± 3.6	–	–	–	–	–	32.1^{c} ± 2.4	+	–
AZ9	15.5^{e} ± 1.0	23.6 ^{d} ± 1.3	29.8 ^{c} ± 1.5	39.5^{c} ± 2.0	49.7^{c} ± 3.6	+	–	–	–	–	32.1^{c} ± 2.4	+	–
AZ10	27.9^{b} ± 1.0	32.3^{b} ± 1.5	36.4^{b} ± 2.1	39.3^{c} ± 2.6	44.2^{d} ± 4.4	+	15.0^{b} ± 0.5	15.3^{d} ± 3.0	9.3^{c} ± 1.1	98.2^{c} ± 3.5	42.1^{b} ± 3.2	++	+
AZ11	–	–	–	–	–	+	16.0^{b} ± 2.5	23.6^{c} ± 1.5	13.0^{b} ± 2.0	–	–	++	+
AZ12	6.1 *g* ± 0.6	11.5 *g* ± 0.9	13.4^{e} ± 1.3	19.2 *g* ± 1.9	28.3^{f} ± 3.4	+	–	–	–	56.4^{f} ± 3.2	–	++	–
AZ13	12.8 ^{f} ± 1.2	15.1^{f} ± 1.5	22 ^{d} ± 1.9	29.4^{e} ± 2.6	35.2^{e} ± 3.5	+	–	–	–	–	–	++	–
AZ14	–	–	–	–	–	+	15.0^{b} ± 0.6	22.3^{c} ± 1.5	10.3^{c} ± 0.5	–	18.1^{d} ± 1.5	++	–
AZ15	13.2 ^{f} ± 1.5	17.4^{e} ± 2.0	24.0^{cd} ± 2.5	33^{d} ± 2.6	36.3^{e} ± 2.3	+	–	–	–	–	–	++	–

Values in this and subsequent tables indicate mean ± S.D. of three independent replicates. Means followed by alphabets a, b, c, d and e etc. are significantly different from each other according to Duncuan's multiple range (DMRT) test. Here, IAA = indole-3-acetic acid, CAS = Chrome Azurol S agar, SA = Salicylic acid, DHBA = 2, 3 Dihydroxybenzoic acid, ACC = 1-Amino cyclopropane 1-carboxylate, NH_3; = Ammonia, HCN = Hydrogen cyanide and *T* = Tryptophan concentration (μg mL^{-1}), symbols '+' and '–' indicate positive and negative reactions, respectively.

PGPR strains *Burkholderia anthina* and *P. agglomerans* have shown PSA when developed in PKV medium (Walpola et al., 2013).

3.5. Agrochemicals tolerance among bacterial isolates

Here, overall 45 rhizobacterial isolates affiliated to different functional groups were exposed to varying doses of 12 pesticides including four each of herbicides (GP, QUIZ, ATZ and BUTA), fungicides (KITZ, HEXA, METL and CBZM) and insecticides (FIP, MONO, IMID and THIA) supplemented to minimal salt agar (MSA) medium to find pesticide tolerant bacterial isolates. Mostly, rhizobacteria displayed varying levels of resistance to several pesticides. Among *Pseudomonas*, strain PS3 tolerated a significantly higher level (3200 μg mL^{-1}) of KTZ, HEXA, METL and THIA of the 12 test pesticides when cultured on MSA plates (Fig. S2) treated separately with varying concentrations of all pesticides (Table S4). Among all *Pseudomonas* isolates, PS1 showed the maximum (mean value = 2183 μg mL^{-1}) tolerance to all pesticides followed by PS2 (mean value = 2050 μg mL^{-1}) and PS3 (mean value = 1983 μg mL^{-1}) (Table S4). Similarly, when evaluating the mean of all agrochemicals, METL showed the lower toxicity, whereas CBZM was marked as higher toxic agrochemical to *Pseudomonas* spp. Similarly, among all *Azotobacter* isolates, AZ12 showed the maximum tolerance (mean value=1766 μg mL^{-1}) while AZ7 represent the lower (mean value=950 μg mL^{-1}) to agrochemicals (Table S4). While assessing the impact of all 12 agrochemicals to *Azotobacter* spp., the sensitivity followed the order (mean value): HEXA (3146 μg mL^{-1})> METL (2720 μg mL^{-1}) > THIA (1946 μg mL^{-1}) > KITZ (1426 μg mL^{-1}) > GP (1386 μg mL^{-1}) > BUTA (1280 μg mL^{-1}) > QUIZ (1093 μg mL^{-1}) > MONO (973 μg mL^{-1}) > FIP (773 μg mL^{-1}) > IMID (666 μg mL^{-1}) > ATZ (573 μg mL^{-1}) > CBZM (153.3 μg mL^{-1}) (Table S4). Moreover, the tolerance level among phosphate solubilizing bacteria (PSB) isolates varied both with concentrations and species of pesticides. For instance, PSB1 tolerated 3200, 3200, 1600, 200, 3200, 400, 800, 2400, 1600, 800, 1200 and 2400 μg mL^{-1} to KTZ, HEXA, METL, CBZM, GP, QUIZ, ATZ, BUTA, FIP, MONO, IMID and THIA, respectively. All the PSB isolates ($N = 15$) tolerated the KTZ up to 3200 (μg mL^{-1}) and 2400 μg mL^{-1} to QUIZ. While comparing the tolerance of 12 pesticides to PSB isolates, the maximum and (mean value = 1970 μg mL^{-1}) minimum (mean value = 1308 μg mL^{-1}) tolerance to all pesticides was represented by PSB8 and PSB13, respectively (Table S4).

Considering the importance of soil microflora, they were exposed to variable concentrations of pesticides to find pesticide tolerant rhizobacterial isolates for their decisive application as bio-inoculants to augment the crop production in pesticide stressed condition. Pesticide tolerance levels of PGPR strains such as *Pseudomonas* (da Silva et al., 2021), *Azotobacter* (Chennappa et al., 2014) and phosphate solubilizers (Rani et al., 2018) however, were relatively high and varied considerably among rhizobacterial isolates. The capacity to develop even at higher rates of pesticides is however, a unique property among microorganisms including N_2 fixers and phosphate solubilizers which may either be due to constitutive or induced mechanisms (Kirubakaran et al., 2019). Since the medium we utilized to identify pesticides tolerant rhizobacterial isolates in our research had no carbon and nitrogen source in addition to pesticides, it is persuasively supposed that

Table 3c
Plant growth promoting substances secreted by phosphate solubilizing (PSB) isolates recovered from various rhizospheres.

Strains	Plant growth promoting (PGP) substances IAA (μgmL^{-1}) 0T*	100T*	200T*	400T*	500T*	Siderophore $FeCl_3$test	Halo size (mm)	SA (μgmL^{-1})	2, 3-DHBA (μgmL^{-1})	EPS (μgmL^{-1})	ACC deaminase activity (μM α-ketobutyrate mg^{-1} protein hour^{-1})	NH_3	HCN
PSB1	42.3^{a} ± 2.3	81.6^{a} ± 3.2	88.2^{a} ± 5.2	96.2^{a} ± 5.6	114^{a} ± 6.0	++	16.3^{a} ± 0.5	39.3^{a} ± 2.6	26.2^{a} ± 2.0	29.4^{a} ± 0.7	69.3^{a} ± 5.8	++	+
PSB2	–	–	–	–	–	++	15.0^{b} ± 1.0	33.7^{b} ± 1.5	18.7^{c} ± 0.6	–	42.1^{b} ± 4.3	++	–
PSB3	26.4^{c} ± 1.0	28.6^{d} ± 1.3	32.8^{d} ± 1.5	37.5^{f} ± 1.8	48.6^{e} ± 2.2	++	–	–	–	–	36.0^{c} ± 2.5	++	–
PSB4	13.3^{f} ± 1.5	16.8 *g* ± 2.0	29.8^{e} ± 5.2	42.3^{f} ± 6.2	72.5^{b} ± 7.2	++	13.0^{c} ± 1.0	27.4^{c} ± 1.6	15.6^{d} ± 1.5	–	–	++	–
PSB5	–	–	–	–	–	++	–	–	–	–	–	++	–
PSB6	15.3^{e} ± 1.5	19.8^{f} ± 2.0	28.8^{e} ± 5.2	38.3^{f} ± 6.2	62.5^{c} ± 7.2	++	16.0^{a} ± 2.5	32.6^{b} ± 2.0	14.3^{d} ± 2.0	–	44.0^{b} ± 3.6	++	+
PSB7	27.1^{c} ± 1.3	39.7^{b} ± 1.4	50.6^{b} ± 1.8	66.2^{b} ± 2.3	73.1^{b} ± 3.2	++	–	–	–	15.0^{d} ± 2.3	29.4^{d} ± 2.6	++	–
PSB8	–	–	–	–	–	++	13.0^{c} ± 1.0	17.6^{f} ± 1.7	8.7^{f} ± 0.4	–	37.2^{c} ± 4.1	++	+
PSB9	25.5^{c} ± 1.0	39.6^{b} ± 1.3	43.8^{c} ± 1.5	49.5^{e} ± 2.0	59.7^{c} ± 3.0	++	12.0^{d} ± 0.5	21.6^{e} ± 1.5	10.3^{e} ± 0.5	–	–	++	+
PSB10	37.9^{b} ± 1.0	42.3^{b} ± 1.5	52.4^{b} ± 2.1	59.3^{c} ± 2.6	64.2^{c} ± 4.0	++	–	–	–	–	–	++	–
PSB11	–	–	–	–	–	++	15.0^{b} ± 1.0	25.3^{c} ± 1.5	21.3^{b} ± 2.0	22.0 ^{c} ± 1.5	23.5^{e} ± 1.2	++	+
PSB12	16.1^{e} ± 0.6	21.5^{e} ± 0.9	33.4^{d} ± 1.3	51.2^{d} ± 1.9	58.3^{c} ± 3.4	++	13.0^{c} ± 1.2	23.3^{d} ± 0.5	16.0^{d} ± 1.0	25.6^{b} ± 2.3	–	++	+
PSB13	22.8^{d} ± 1.2	35.1^{c} ± 1.5	42.0^{c} ± 1.9	59.4^{c} ± 2.6	75.2^{b} ± 3.5	++	–	–	–	20.0^{c} ± 1.0	–	++	–
PSB14	13.7^{f} ± 1.9	15.5 *g* ± 2.3	20.0^{f} ± 2.6	35.1 *g* ± 3.1	49.2^{e} ± 4.2	++	14.0^{b} ± 0.5	23.6^{d} ± 2.5	14.3^{d} ± 0.5	–	18.2^{f} ± 2.4	++	–
PSB15	23.2^{d} ± 2.0	37.4^{c} ± 3.0	44.0^{c} ± 3.5	53.0^{d} ± 3.6	56.3^{d} ± 4.0	++	–	–	–	–	16.2^{f} ± 1.8	++	–

Values in this and subsequent tables indicate mean ± S.D. of three independent replicates. Means followed by alphabets a, b, c, d and e etc. are significantly different from each other according to Duncuan's multiple range (DMRT) test. Here, IAA = indole-3-acetic acid, CAS = Chrome Azurol S agar, SA = Salicylic acid, DHBA = 2, 3 Dihydroxybenzoic acid, ACC = 1-Amino cyclopropane 1-carboxylate, NH_3; = Ammonia, HCN = Hydrogen cyanide and *T* = Tryptophan concentration (μg mL^{-1}), symbols '+' and '–' indicate positive and negative reactions, respectively.

Table 4
Tri-calcium phosphate (TCP) solubilizing activity of phosphate solubilizing bacteria (PSB) grown both in liquid and on solid PKV agar medium.

Bacterial strains	Phosphate solubilised Diameter (mm) Colony	Zone	Total	Days of incubation	Solubilisation index (S.I)	Solubilisationefficiency (SE)	Liquid medium (μg mL^{-1})
PSB1	6	9	15	6	2.5	150	102.7
PSB2	4	5	9	6	2.25	125	63.4
PSB3	4	3	7	6	1.75	75	47.7
PSB4	4	5	9	6	2.25	125	65.5
PSB5	4	5	9	6	2.25	125	50.6
PSB6	4	5	9	6	2.25	125	52.7
PSB7	6	5	11	6	1.83	83.3	64.9
PSB8	5	11	16	6	3.2	220	117.9
PSB9	4	7	11	6	2.75	175	48.8
PSB10	6	7	13	6	2.16	116.6	45.5
PSB11	4	8	12	6	3.0	200	53.8
PSB12	5	3	8	6	1.6	60	36.3
PSB13	4	5	9	6	2.25	125	65.2
PSB14	6	7	13	6	2.16	116.6	57.4
PSB15	4	5	9	6	2.25	125	37.3

Values are mean of three replicate ($n = 3$).

rhizobacteria may possibly utilized the pesticides as a single source of energy through biodegradation. The utilization of agrochemicals as carbon and energy sources by rhizobacterial isolates, probably through partial transformation events that can occur with several pesticide chemical families (Briceño et al., 2020). This characteristic of a larger tolerance threshold is significant for a variety of reasons—(i) pesticide tolerant isolates can flourish in a pesticide-stressed environment and (ii) if applied as inoculants in the presence of pesticide stress, can augment the growth performance of crop plants. However, as far as the tolerance limit of these bacterial strains to pesticides *vs* those previously reported is concerned, there may be diversity in their capacity to handle toxicity. Such discrepancies are linked to changes in culture media, growth circumstances, pesticide toxicity severity, and plant genotypes. Furthermore, the research on compatibility of these microorganisms with agrochemicals is debatable. Shifts in growing circumstances, such as pH changes produced by acid or alkali generation, variations in test methodologies, and specific factors, may have resulted in changes in pesticide tolerance or susceptibility. As a result, it's impossible to generalize the

elements that influence toxicity or tolerance of agrochemicals. Another probable cause for the bacterium's resilience to greater agrochemical concentrations is the poor solubility of tested chemicals, and therefore their unavailability to the targeted bacteria. According to several research, certain pesticides, even at extremely high doses, may have no effect on the development and survival of these bacteria (Wesley et al., 2017). Contrarily, some chemicals pesticides may induce toxicity to beneficial rhizospheric microorganisms even at extremely low doses (Shahid et al., 2021d). Likewise our study, *Bacillus aryabhattai* strain MoB09, recovered from agricultural soils contaminated with paraquat herbicide, utilized herbicide as carbon source under controlled condition and grew well (Inthama et al., 2021). In addition, a similar study conducted by Aroua et al. (2019) reported that, *SinoRhizobium meliloti* isolated from pesticide contaminated agricultural soils tolerated a maximum level of prosper (10 mg L^{-1}), copper oxychloride (12 mg L^{-1}), Fungastop (6 mL L^{-1}), Nimbecidine (7.5 mL L^{-1}) and maneb (25 mL L^{-1}).

The emergence of novel pesticide breakdown pathways, as well as genetic mutation, may result in greater multi-drug resistance (MDR) among soil bacteria as a result of pesticide application and persistence (Al-Waili et al., 2012). As a mechanism of cross resistance, pesticide resistances acquired in soil flora contribute to drug resistance (Huete--Soto et al., 2017). As a result, continual pesticide exposure would put constant pressure on the genes, resulting in drug resistance. The production of slime materials (glycocalyx) or biofilms by microbes is the fundamental mechanism of resistance. The decreases in porin proteins, which may let molecules pass through the cell membrane more easily, appears to contribute to the resistance (Pan-Hou et al., 1981). Even a minor degree of pesticide application/accumulation in the agricultural field might impose selective pressure on bacterial selection. By deleting the plasmid from the chosen isolates, bacteria gradually acquire cross resistance to antibiotics and lose their capacity to grow/use pesticides. Returning the plasmid to the organisms restored drug resistance as well as pesticide degradation capacity.

3.6. Antibiotic sensitivity/resistance profile of bacterial isolates

It is widely assumed that rhizobacteria with numerous stress resistance genes are suited to a wide range of environmental conditions. As a result, many researchers are becoming increasingly interested in screening resistant rhizobacteria for broad adaptability. For instance, numerous species of *Bacillus* and *Pseudomonas* have been utilized as microbial agents and have the ability to promote plant development (Santoyo et al., 2012). However, many of the antibiotic resistance genes (ARGs) found in soil are thought to be rather prevalent in these two taxa (Wellington et al., 2013). Considering theses, sensitivity/resistance behavior of recovered isolates *viz., Pseudomonas, Azotobacter* and PSB isolates to different antibiotics was assessed (Fig. S3). The antibiotic sensitivity/resistance ability of selected rhizobacterial isolates toward different antibiotics was variable. All selected rhizobacterial isolates displayed an inconsistent response to a broad range of antibiotics and multiple resistances to numerous antibiotics tested in this study. As an example, among isolates, *Pseudomonas* species was susceptible to numerous antibiotics and inhibition zone differed from 10 mm (PS1, PS2, PS4, PS8, PS10 and PS13) against polymyxin B to 34 mm (PS8) nalidixic acid. The *Pseudomonas* isolate PS2 was resistant to erythromycin (15 μg $disk^{-1}$), ciprofloxacin (5 μg $disk^{-1}$), methicillin (10 μg $disk^{-1}$) novobiocin (5 μg $disk^{-1}$) and penicillin (2 μg $disk^{-1}$) (Table S5). The percentage of resistance to multiple antibiotics among *Azotobacter* strains differed between 7 (AZ4, AZ5 and AZ8) to 33% (AZ10) (Table S5). Drug resistance mutants are fairly prevalent in areas where pesticides or antibiotics are used indiscriminately et al., (Džidić et al., 2008). Drug resistance is fairly prevalent because microorganisms in the environment may spread resistance genes both vertically and horizontally (Davison, 1999). The phosphate-solubilizing bacterial isolates also showed a similar sensitivity pattern to several antibiotics, with the zone of inhibition varying from 10 to 40 mm. The PSB9 strain was antibiotic-resistant in the majority of cases. The percent resistance to numerous antibiotics amongst the PSB isolates differed between 20% (PSB1 and PSB) to 47% (PSB11 and PSB12) (Table S5). While, calculating the resistance percentage of bacterial strains to different antibiotics, phosphate solubilizing bacterial isolates among recovered rhizobacteria showed the maximum resistant to multiple antibiotics compared to *Pseudomonas* and *Azotobacter* isolates (Table 5). The main reason behind this is the huge population size of bacilli in soil, many bacteria, including *Bacillus*, are resistant to different antibiotics, most likely by horizontal gene transfer (HGT) (De la Cruz and Davies, 2020). While calculating the correlation, a variable pattern was recorded between pesticides tolerance and antibiotic resistance among soil isolates. For instance, *Pseudomonas* isolates showed the positive correlation (r^2=0.77) between kitazin tolerance and antibiotic resistance/sensitivity (Fig S5 a), whereas *Azotobacter* (r^2=0.625) (Fig S5 b), and PSB (r^2=0.325) (Fig S5 c) isolates exhibited moderately positive correlations. The multiple antibiotic resistances displayed for example by *Pseudomonas* might possibly be due to alterations in their chemical, biological or genetic makeup. In accordance with our findings, various workers have been reported the antibiotic sensitivity/resistance behavior of PGPR isolates (Bettencourt, 2016). Interestingly, in the present finding, isolates PSBB1 was found maximally resistant to both pesticides and various antibiotics. Similarly, Asmiran et al. (2018) in an observation found pesticides and tetracycline resistant *Azotobacter* isolated from paddy rhizosphere. In a research, Popowska et al. (2010) looked at the impact of antibiotics (tetracycline and streptomycin) on soil microbes from three different soil habitats: forest soil, agriculture soil, and compost soil. Among them, *Bacillus vesicularis, Burkholderia cepacia, Pasteurella multocida* and *Rhizobium radiobacter* showed highest resistance to tetracycline while soils with high concentrations of streptomycin (5 mg kg^{-1}), had *Sphigomonas multivorum, B. cepacia*, and *R. radiobacter* among other soil microbes. The bacterial isolates exhibiting both agrochemicals (pesticides) and antibiotics tolerance/resistance have been found to adapt faster the contaminated environment due to the presence of R-factors and not by the mutation and natural selection (Wani and Khan, 2014). However, the deviation in resistance pattern to various tested antibiotics as detected/observed here may probably be due to the variations in growth conditions, intrinsic property of cells. The presence or absence of resistance mechanisms encoded by chromosome and/or R-plasmid, as well as the exposure of PGPR to stress conditions or toxic substances (Dipta and Kaushal, 2018). Bacteria/rhizobacteria can potentially cause multidrug resistance (MDR) by making more copies of target molecules, causing the prior concentration of antibiotics to be insufficient for metabolic process. Simply transferring transposable elements/plasmids stretched out cytoplasm, resulting in MDR in many different bacteria that have been used to the soil environment. Due to a gene carried on self-transmissible genes that may move between plasmid and chromosomal walk-up, multidrug resistant populations were rather widespread among pesticide degrading soil flora (Rangasamy et al., 2017).

4. Conclusion

Pesticide tolerance and multiple antibiotics resistance is however, most likely to improve the abilities of bacterial isolates to persist in polluted soil environment which might prove advantageous to maintain antibiotic resistance genes by increasing environmental selection pressure. In addition, the antibiotic resistance in pesticide-enriched environment might be utilized as a flag to identify pesticide tolerant microorganisms. In this work, selected rhizobacterial isolates showed the intrinsic properties of pesticides tolerance, multiple antibiotic resistances and synthesized multifarious PGP substances. Therefore, these interesting features make them (rhizobacteria) an attractive, agronomically feasible, and long-term prospective alternative for crop production. Furthermore, it may be deduced from the foregoing data that

Table 5
Number of bacterial isolates exhibiting resistance to multiple antibiotics and percentage resistance.

Antibiotics	Number of rhizobacterial strains resistant to antibiotics			Resistance percentage		
	Pseudomonas ($n = 15$)	*Azotobacter* ($n = 15$)	PSB ($n = 15$)	*Pseudomonas*	*Azotobacter*	PSB
Nalidixic acid (NA)	0	1	3	0%	6.6%	20%
Nitrofurantoin	0	2	9	0%	13.3%	60%
Rifampicin	0	1	11	0%	6.6%	73.3%
Erythromycin	4	2	11	26.7%	13.3%	73.3%
Streptomycin	0	0	0	0%	0%	0%
Ciprofloxacin	11	10	0	73.3%	66.6%	0%
Doxycycline	0	1	9	0%	6.6%	60%
Polymyxin B	0	0	1	0%	0%	6.6%
Norfloxacin	0	0	0	0%	0%	0%
Methicillin	15	15	11	100%	100%	73.3%
Novobiocin	1	2	3	6.6	13.3%	20%
Tetracycline	0	1	8	0%	6.6%	53.3%
Chloramphenicol	0	0	4	0%	0%	26.6%
Penicillin	4	5	10	26.6%	33.3%	66.6%
Kanamycin	0	0	0	0%	0%	0%

microorganisms evolve continually under the influence of their habitat. However, the conclusions presented in this work are based on laboratory tests, and more research is required to validate this data in a real-world scenario (field experiments). Furthermore, to establish the molecular mechanisms behind the development of antibiotic resistance and pesticide tolerance among rhizobacteria, more research is needed.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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Supplementary materials

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