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Tolerance of Pesticide and Antibiotics among Beneficial Soil Microbes Recovered from Contaminated Edible Crops Of Agriculture Soil of Banaskantha District (India)

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ABSTRACT
A total of 25 bacterial isolates were recovered from pesticide (Mancozeb) contaminated soil of a potato farm and were morphologically and biochemically characterized. Bacterial isolates produced indole-3-acetic acid (IAA), solubilized insoluble phosphate, secreted exopolysaccharide (EPS) and produced ammonia and cyanogenic compound (HCN). Selected isolates were tested for their tolerance ability against Mancozeb (bisdithiocarbamate – non-systematic fungicide) and 12 antibiotics. Among Pseudomonas, isolate PM3 showed maximum (1000 ppm) tolerance to mancozeb pesticide, while PM10 showed moderate and PM5, as well as PM1, had the lowest ability of tolerance towards 1000 ppm concentration of pesticide. The antibiotic sensitivity/resistance among all isolates varied considerably. As an example, Pseudomonas spp. It was susceptible to several antibiotics, and the inhibition zone differed between 11 mm (levofloxacin) to 22 mm (Amikacin). Also, isolate PM1 showed susceptibility towards 13mm (ofloxacin), 3 mm (gentamycin), 3 mm (amikacin), and 29 mm (gatifloxacin). PM10 showed resistance to ampicillin, co-trimaxazole, cefotaxime, piperacillin, chloramphenicol, tetracyclin, gentamycin and levofloxacin. The persistence of mancozeb in agriculture soil may contribute to an increase in multidrug resistance among soil microorganisms. In conclusion, plant growth-promoting substances releasing soil microorganisms comprising inherent properties of pesticide tolerance and antibiotics resistance may provide an attractive, agronomically feasible and long-term prospective alternative for pesticide degradation. However, more research is needed to enhance the tolerance and degradation of pesticide and antibiotic resistance among soil microorganisms in the future.
Keywords: *Mancozeb, Pesticide, Exopolysaccharide, Antibiotics.*

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INTRODUCTION

Pesticides are chemical substances or a mixture of substances used for preventing, destroying, repelling, or mitigating any pest, including insecticides, herbicides, fungicides and various other substances to control pests [1, 2] According to World Health Organization, as per the proposed classification of pesticides by hazard, the active components of pesticides (herbicides, fungicides, insecticides and rodenticides) are classified as extremely hazardous class 1A, highly hazardous class 1B, and moderately hazardous class 2 and slightly hazardous class 3 active components that's why excessive use of pesticide leads to accumulation of large amounts of pesticides residues in the soil affecting non-target species like humans, flora and fauna through the food chain [3, 4]. Mancozeb is a fungicide that is a coordination product of zinc ion and manganous ethylene-bis-dithiocarbamte) and is widely used for vine fruit and vegetable treatment, but it degrades more slowly in the plant than other dithiocarbamates. It is oxidized to ethylene-bis-isothiocyanate, ethylene thiuram monosulfide, ethylenethiourea, and ethylenediamine [5]. This fungicide was used to control diseases caused by pathogenic fungi in the agriculture sector like wheat, tomato, potato and grapevine [6] and as per the questionnaires to farmers of Banaskantha district mancozeb is widely

used in potatoes to control late blight disease [6]. Late blight is the most critical and frequent disease on potato caused by *Phytophthora infestans*. To fight against late blight, growers have at their disposal many fungicides. The active ingredients are multiple, but most of them have a single-site mode of action against the fungus, but only some ingredients have a multisite mode of action, like mancozeb which has been registered for 60 years with no known resistance and good efficacy against early and late blight diseases [7, 8]. In light of considerations, the current research was undertaken to (a) isolate the different groups of soil bacteria from pesticide (mancozeb) contaminated soil and their characterization using various biochemical treats, (b) evaluate the plant growth-promoting substances produced by bacterial isolates (c) tolerance ability of bacteria (d) assess the antibiotic sensitivity/resistance traits in selected bacterial isolates.

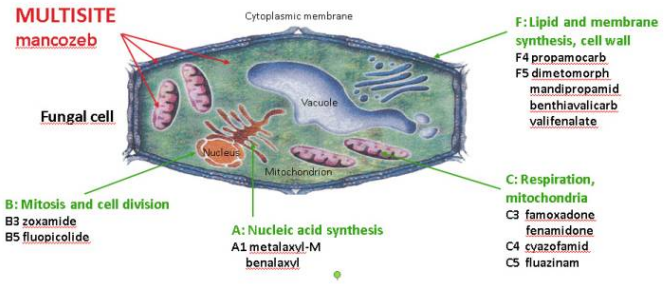

Figure 1: Mode of action of fungicide diseases (Serge, D., & Daniele, R., 2015).

MATERIAL AND METHODS

Collection of soil samples

Site of collection

Ten villages of Banaskantha district formed areas of study. Information regarding the crop grown, agrochemical, herbicides and pesticides that are applied in the farm, the span of uses and their consequences on the form of crop cultivated is gathered from the farmers of villages [9].

Collection of soil samples

For the purpose of this study soil samples were collected from the potato growing areas of different villages of Banaskantha district, Gujarat. Soil samples collected from the study areas in February 2020 by proper sampling method with the help of sampling equipment's like soil auger, bucket, plastic bags etc. by making four quarters and remove two part, then further mixed those two parts, make four parts and remove opposite part. This procedure was carried out by 3-4 times and final samples were allowed to air dried, grounded and sieved through a mesh with the grain size of 2 mm. samples were packed in air tight plastic bags coded 1 -10 for 10 samples and then transported to laboratory and refrigerate it till further processing. At the time of sample collection soil samples were collected from the upper part as well as from the 0 to 15 cm (Karishma, B., & Prasad, S. H. 2016) depth with the help of spatula. Temperature was 30°C to 32°C at the time of sample collection, which was measured by thermometer, pH was also measured by pH meter as well as pH stripe at the time of sample collection [10-11].

Chemicals and culture media

Mancozeb (Rediniol 75%, powder), purchased from Palanpur, Banaskantha, Gujarat; concentrated stock solutions of Mancozeb (100 ppm/L) was prepared in dimethyl sulfoxide. Methanol and acetonitrile (HPLC grade), hexene, ethyl acetate were purchased from HiMedia Laboratory, Gujarat. Luria-Bertani medium contained: tryptone 10.0g/L, yeast extract 5.0g/L, NaCl 10.0g/L and pH 7.0 was used for isolation of bacteria and the Mineral salt medium (MSM) contained (g/L) $(NH_4)_2SO_4$ 2.0, K_2HPO_4 1.5, KH_2PO_4 0.5, NaCl 1.0, $MgSO_4$ 0.2, pH 7.0. All mediums were sterilized by autoclaving at 121.3 °C for 30 min [13].

Physico-chemical analysis of soil samples

The samples collected from the different villages of Gujarat, were analysed to determine their physicochemical characteristics. The pH of the soil samples was determined using L1 glass electrode pH meter as well as with the help of pH strip. Furthermore, conductivity measurement was done by the conductivity meter (Soni, M. *et al*., 2016). Samples were analysed for Organic carbon and available nutrients like potassium, phosphorous, iron, manganese, zinc and copper [14].

Isolation of Mancozeb tolerant bacteria by enrichment culture

For the enrichment process Minimal Salt Medium (MSM) was used, from the ten soil samples 10 gram of soil was added to 250 ml Erlenmeyer flask containing 100 ml MSM media supplemented with 100 ppm Mancozeb as a sole source of carbon. All the above flasks were incubated at 28±2 ^{0}C for on shaking condition for 7days on (1st enrichment). From every flask, 5 ml was reinoculated to the flask with same medium

condition aseptically and future incubated at the same temperature for 7 days on shaking condition (2nd enrichment). Then form every flask a loopful of culture was streaked on sterile MSM agar media containing plate supplemented with 100 ppm Mancozeb as a sole source of carbon and incubated at 28±2 ^{0}C for 7 days. The well isolated colonies were grown on nutrient agar plate and purification was carried out on nutrient agar slant [15].

Morphology and biochemical identification

All the isolates were cultured on nutrient medium supplemented with 100 ppm mancozeb to observe their colony morphology and microscopic observation was carried out for gram staining, size and shape. Biochemical test like Carbohydrate fermentation test, MR-VP test, citrate utilization, triple sugar iron, catalase and oxidase test were also carried out according to the methods described in "Mannual of Methods for General Bacteriology [16, 17].

Assay for bacterial plant growth promoting activities

Plant growth promoting traits of isolated bacteria were determined in in vitro condition. The traits of PGPR such as indole-acetic acid production, phosphorous solubilization, HCN production, ammonia production and exopolysaccharide production were determined [18].

Indole acetic acid production

For evaluation of isolates for IAA production, the nutrient broth (Peptone 5.0 g, Beef extract 3.0 g, per liter of distilled water with 7.0 pH) was prepared. Loopful of culture was inoculated in 25 ml nutrient broth and incubated at 28^{o}C for 24 hrs. on rotary shaker. The broth was centrifuged at 10000 rpm for 15 min. Two ml of supernatant was taken and added 2-3 drops of orthophosphoric acid and 4ml of freshly prepared sollkouski's reagent (0.5 M FeCl3 ml and 35% perchloric acid 50 ml) was added to the aliquot (Tsegaye, Z. *et al*., 2019). The samples were incubated for 25 minutes at room temperature. Pink colour was observed and recorded optical density at 530 nm. [19].

Phosphate solubilization

All bacterial isolates were used to check phosphate solubilization activity by using Pikovaskaya (PVK) agar, containing tricalcium phosphate (0.5%). [20]. A 5-mm diameter agar plug of bacteria grown nutrient broth was inoculated to PVK agar plates and incubated at 28±0.2^o C for 7 days. The visibility of clear zone around the agar plug showed positive phosphate solubilizing activity [21]. Phosphate solubilization was analysed by computing the Solubilization Index (SI) which is the ratio of total diameter (colony+halo) to colony diameter. The effect of Mancozeb on the phosphate solubilization ability of the bacteria was determined by supplementing Pikovskaya agar plates with 100 ppm Mancozeb [22].

Detection of cyanogenic compound (HCN) and ammonia

All the bacterial isolates screened for the production of hydrogen cyanide using Nutrient broth which was amended with 4.4 g glycine/L and bacteria were streaked on modified agar plate. A Whatman Filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution [23] was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28±0.2^o C for 4 days. Development of orange to red colour indicated HCN production [2].

According to the method of Ahmed *et al*., 2008, the qualitative ammonia production was analyzed in peptone water by bacterial isolates. Freshly grown cultures of bacterial isolates were inoculated in 10 mL peptone water in each tube and incubated for 48-72 hr. at 28±0.2^o C. Nessler's reagent (0.5 mL) was added in each tube. Development of a brown to yellow color was a positive test for ammonia production (Kifle, M. H., & Laing, M. D. 2016).

Exopolysaccharide (EPS) production

For the determination of exopolysaccharides (EPS), the isolated bacteria were grown in 100 ml basal medium with 5% sucrose supplemented with 100 ppm mancozeb and incubated for 5 days at 28±0.2^o C on rotary shaker at 100 rpm. Culture broth was rotated (5,433 g) for 30 min and EPS was extracted by adding three volumes of chilled acetone to one volume of supernatant. The precipitated EPS was washed repeatedly three times alternatively with distilled water and chilled acetone and transferred to a filter paper and weighed after drying overnight [2].

Antibiotic sensitivity/resistance pattern of bacterial isolates

Sensitivity / resistance of selected bacterial isolates against different antibiotics was done by the method of Bauer, A. W. (1966).

RESULTS AND DISCUSSION

Physico-Chemical properties of Agriculture soil

Soil samples collected from different villages of potato farm displayed variable in physical and chemical properties (Table 1). As an example, PM1 Soil sample had 7.72 pH value, EC 0.17 dsm-1, 0.331 - % organic carbon (OC), the content of phosphorous (P_2O_5) and potassium (K_2O) = 42.95 and 248.6 kg/ha respectively.

Similarly trace elements were also recorded as Zinc (Zn) = 0.50 ppm, Manganese (Mn) = 7.26 ppm, iron (Fe) = 4.50 ppm and copper (Cu) = 0.32 ppm indicate the normal soil characteristics. Now, the differences in texture can affect many other physical and chemical properties of the soil. Soil texture plays a prominent role in soil production. Soils with predominantly large particles tend to drain quickly and have lower fertility. Very fine texture soils may be poorly drained, tend to become waterlogged, and are therefore not well-suited for agriculture. As we can observed from the table, percentage sand particles varied from 83.12 to 84.52. Percentage slit particles varied from 7.80 to 10.20 and Percentage clay particles varied from 5.28 to 8.36

Isolation of bacteria from pesticide contaminated soil

In the present study twenty-five bacterial isolates were obtained from different soil samples of potato farm following by enrichment culture technique. Keeping in view the useful activities of soil microbes, numerous PGPR isolates were selected and assessed for their tolerance ability to pesticide and antibiotics [2]. According to the study of Alam. S *et al*., (2018) their isolates could tolerate as maximum concentration of Mancozeb 75 WP (20000 µg/ml), carbendazim 50 WP (50000 µg/ml), chlorpyrifos 20 EC (2000 µg/ml) and fenvalerate 20 EC (2000 µg/ml) respectively [1].

Screening of pesticide tolerating bacteria

Among twenty-five bacterial isolates isolated from potato farm contaminated with mancozeb pesticide, eight isolates showed tolerance against the pesticide. the present study said that isolate no. PM3 and PM10 were higher tolerance (1000 ppm mancozeb), PM1, PM5 and PM7 had shown medium tolerance while isolates PM8, PM9 and PM11 had shown poor tolerance against 1000 ppm concentration of mancozeb (Figure 2).

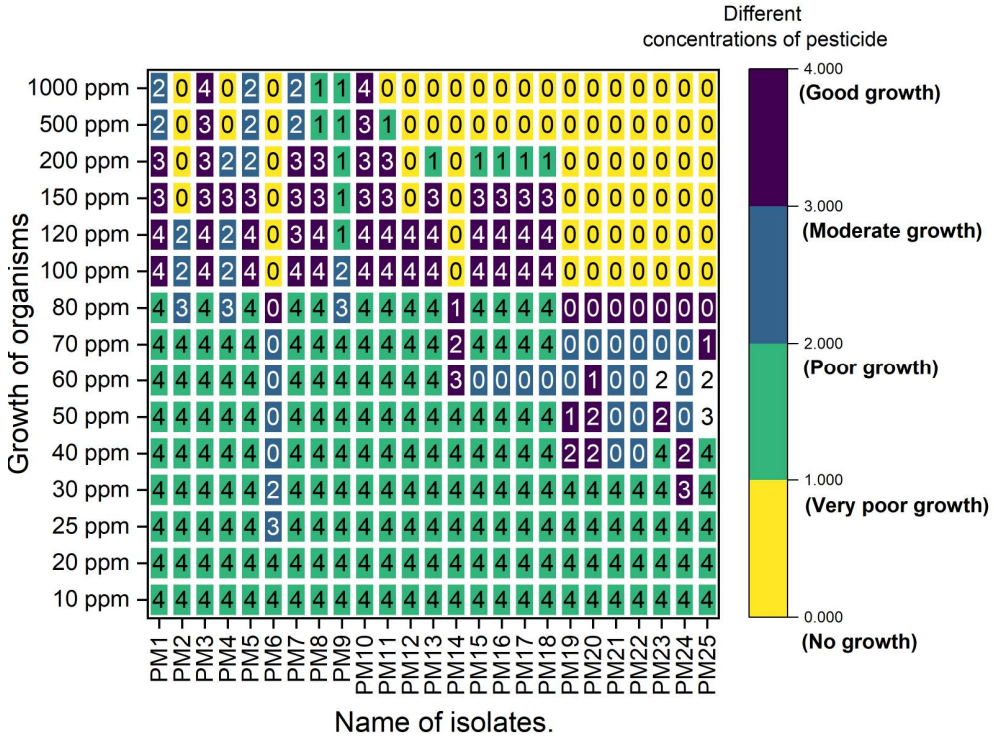

Figure 2: Growth of bacterial isolates at different concentration of Mancozeb

Biochemical characterization of pesticide Tolerant bacterial isolates

In order to identify the unknown bacterial species, first and foremost step is the phenotypic and biochemical characterization. In present study, biochemical characterization of pesticide tolerant isolates was carried out by Hi Assorted™ biochemical test kit (KB009A). biochemical test kit was used for screening of pathogenic/non-pathogenic organisms from environmental samples and other relevant samples. The kit provides the complete list of organisms that can be identified with this system is given in the identification index provided with the kit. Mostly gram-negative bacteria found in isolation. *Pseudomonas* gave positive citrate utilization and glucose while H_2S production showing negative.

Evaluation of Plant growth promoting activities

Quantitative estimation of bacterial isolates for indole acetic acid production

In present study almost all bacterial isolates showed light to high production of indole acetic acid while the isolate PM3 and PM10 showed high production of indole acetic acid and other isolates appeared poor to medium indole acetic acid producer (Figure 3).

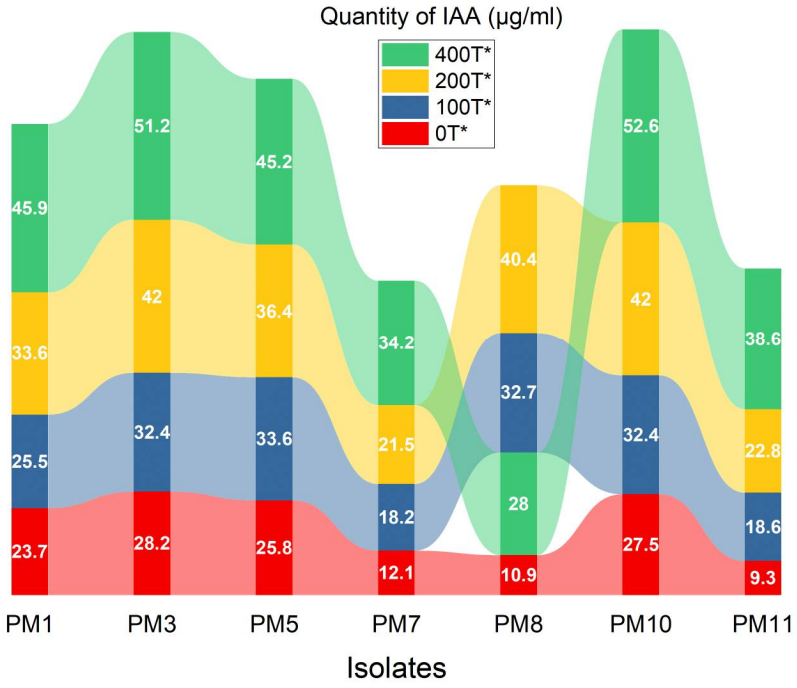

Figure 3: Indole acetic acid production by selected bacterial isolates

phosphate solubilization, HCN production and Ammonia production

The phosphate solubilization activity was tested by qualitative method. Around the bacterial growth on TCP (Tri-Calcium phosphate) supplemented with PKV and plates a cleat zone of solubilization was developed by selected bacterial isolates. All eight selected bacterial isolates give positive result of phosphate solubilization by producing clear zone of solubilization surrounding the colony. The isolated bacterial strains were checked further to assess the production of ammonia and cyanogenic compound all selected bacterial isolates were able to produce ammonia while HCN produced only by PM1, PM3, PM5 and PM10.

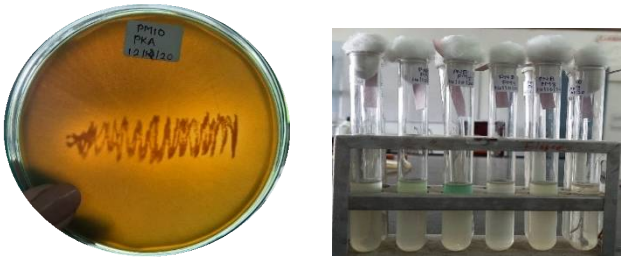

Figure 4: Phosphate solubilization and ammonia production

EPS production

Among soil isolates (N=25), only one bacterial isolates (*Rhizobium* spp) showed the EPS production activity. Howevere, none of the *Pseudomonas* spp. showed the EPS production. Exopolysaccharide is a key polymer that protects microorganisms against unwanted conditions. Recognizing the importance of EPS in the biological nitrogen fixation (BNF), soil aggregation and protection from harsher environment, under in vitro conditions.

Antibiotic sensitivity/resistance profile of bacterial isolates

The antibiotics present in the disc and abbreviations.

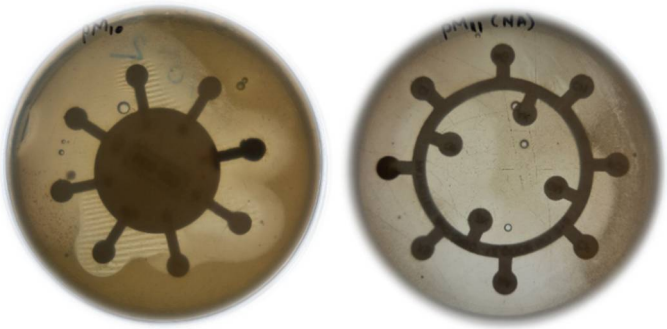

Figure 5: Antibiotic sensitivity/resistance

Table 1 Physico-chemical properties of pesticide contaminated soil samples

Chemical Properties of Soil									
Sample No.	**EC (dSm^{-1})**	**pH (1:2.5)**	**Organic Carbon (%)**	**Available Nutrients (Kg/ha)**		**Available Nutrients (ppm)**			
				P_2O_5	**K_2O**	**Fe**	**Mn**	**Zn**	**Cu**
PM1	0.17	7.72	0.311	42.95	248.6	4.50	7.26	0.52	0.32
PM2	0.15	7.90	0.233	36.02	194.9	5.56	7.82	0.56	0.52
PM3	0.18	7.45	0.207	33.25	197.6	4.76	6.12	0.48	0.64
PM4	0.20	7.85	0.246	37.41	264.8	4.20	10.56	0.68	0.74
PM5	0.19	7.39	0.220	52.65	384.4	4.82	8.00	0.66	0.58
PM6	0.16	7.50	0.337	49.10	215.0	5.90	9.84	0.74	0.60
PM7	0.13	7.70	0.220	42.95	208.3	6.36	7.60	0.64	0.62
PM8	0.12	7.55	0.350	33.25	338.7	5.24	10.78	0.46	0.54
PM9	0.16	7.80	0.363	39.10	232.5	5.74	7.60	0.48	0.58
PM10	0.14	7.70	0.233	52.65	223.1	7.08	5.54	0.56	0.70
Mean	**0.16**	**7.66**	**0.27**	**41.93**	**250.79**	**5.42**	**8.11**	**0.58**	**0.58**
Standard Deviation (SD)	**0.03**	**0.17**	**0.06**	**7.43**	**63.18**	**0.89**	**1.76**	**0.1**	**0.11**

Physical Properties of Soil				
Sample No.	**Sand (%)**	**Slit (%)**	**Clay (%)**	**Textural Class**
PM1	83.52	8.12	8.36	Loamy Sand
PM2	83.77	9.14	7.09	Loamy Sand
PM3	84.14	8.70	7.16	Loamy Sand
PM4	83.14	8.60	8.26	Loamy Sand
PM5	83.66	9.40	6.94	Loamy Sand
PM6	84.80	7.80	7.40	Loamy Sand
PM7	83.12	8.52	7.36	Loamy Sand
PM8	84.14	8.96	6.90	Loamy Sand
PM9	84.00	9.00	7.00	Loamy Sand
PM10	84.52	10.20	5.28	Loamy Sand
Mean	83.88	8.84	7.18	
Standard Deviation (SD)	0.55	0.67	0.85	

Table 2 morphological characteristics of bacterial isolates on MSM medium supplemented with 100 ppm Mancozeb

Isolates.	Size	Shape	Margin	Elevation	Surface texture	Consistency	Opacity	Pigment production	Gram reaction
PM1	B	E	G	J	N	P	T	V	Gram positive coccobacilli
PM2	A	E	G	J	N	P	Translucent	Light yellow	Gram positive cocci
PM3	C	E	G	K	N	Q	T	X	Gram positive Cocco bacilli
PM4	D	E	G	K	N	R	U	White	
PM5	B	E	G	L	O	Q	T	Z	Actinomycetes
PM6	A	E	G	L	N	P	U	Dew drop	Gram negative short rod
PM7	D	F	G	M	Sticky with agar, wavy	Q	T	X	Gram positive cocci
PM8	C	E	G	K	N	S	T	Y	Gram positive bacilli
PM9	D	E	H	M	O	Q	T	Z	Actinomycetes
PM10	C	E	G	L	N	S	T	Y	Gram negative short rod
PM11	B	E	G	L	N	S	U	V	Gram negative short rod
PM12	B	E	G	L	N	S	T	yellow	Short rod
PM13	C	E	G	L	N	S	U	Off white	Short rod
PM14	B	E	G	K	N	R	U	White	Short rod
PM15	B	E	G	M	N	S	U	Light brown	Short rod
PM16	D	F	I	M	N	S	T	Y	Gram positive big rod
PM17	D	E	H	M	O	Q	T	Z	Actinomycetes
PM18	B	E	G	M	N	S	U	Light brown	Short rod
PM19	B	E	G	M	N	S	U	Y	Short rod
PM20	B	E	G	M	N	U	Translucent	V	Short rod
PM21	B	E	G	M	N	S	T	Off white	Short rod
PM22	D	E	H	M	O	Q	T	Z	Actinomycetes
PM23	B	E	G	M	N	S	U	Light brown	Short rod
PM24	B	E	G	M	N	S	U	Light brown	Short rod
PM25	D	F	I	M	N	S	T	Y	Gram positive big rod

***A : Very Small, B : Small, C : Medium, D : Big, E : Round, F : Irregular, G : Entire, H : Wavy, I : uneven, J : Slightly convex, K : Raised, L : Convex, M : Flat, N : Smooth, O : Rough, P : Butyrous, Q : Dry, R : Mucoid, shiny, S : Moist, T : Opaque, U : Translucent, V : Brown, X : Creamish, Y : Dull white, Z : Chalky white

Table 3 Biochemical characterization of bacterial isolates

Sr No.	Name of the Test	PM1	PM3	PM5	PM7	PM8	PM10	PM11
1	Simmons Citrate Agar test	Positive	Positive	Positive	Positive	Positive	Positive	Positive
2	Catalase Test	Negative	Negative	Negative	Positive	Negative	Positive	Negative
3	Dehydrogenase test	Positive	Positive	Negative	Negative	Negative	Positive	Positive
4	TSI test	Upper part yellow, H2S -ve	Upper part yellow/red, H2S -ve	Upper part yellow, H2S -ve, butt red	Upper part yellow, H2S -ve, butt red	Upper part yellow, H2S -ve, butt red	Upper part yellow, H2S -ve, butt red	Upper part yellow, H2S -ve, butt red
5	Ammonia production	Positive	Positive	Positive	Positive	Positive	Positive	Positive
6	Nitrate reduction	Negative	Negative	Positive	Negative	negative	Negative	Negative
7	Starch Hydrolysis test	Negative	Negative	Negative	Negative	Negative	Positive	Negative

Table 4 phosphate solubilization, HCN production and Ammonia production by selected bacterial isolates

Isolates code	Phosphate solubilization activity	Ammonia production	Hydrogen cyanide (HCN) production (After 48 hrs)
	Qualitative		
PM1	+	+	+++
PM3	+	+	+++
PM5	+	+	+++
PM7	+	+	-ve
PM8	+	+	-ve
PM9	+	+	-ve
PM10	+	+	+
PM11	+	+	-ve

Table 5 Antibiotic sensitivity/resistance profile of bacterial isolates

Antibiotic	Strength	PM1	PM3	PM5	PM7	PM8	PM9	PM10	PM11
AS	20 mcg	R	R	R	R	R	R	R	R
BA	25 mcg	R	R	R	R	R	R	R	R
CF	30 mcg	R	R	R	R	R	R	R	R
PC	100 mcg	R	R	R	R	R	R	R	R
CH	30 mcg	R	R	R	R	R	R	R	R
RC	5 mcg	R	R	S	R	S	R	S	R
CI	30 mcg	R	R	R	R	R	R	R	R
TE	30 mcg	R	R	R	R	R	R	R	R
ZN	5 mcg	S	R	S	R	S	R	R	R
GM	10 mcg	S	S	R	R	R	R	R	R
AK	30 mcg	S	R	R	S	R	R	R	R
GF	10 mcg	S	R	S	S	S	R	R	S

CONCLUSION

The ability of bacterial isolates to survive in a contaminated soil environment may be improved by pesticide tolerance and multiple antibiotic resistance, which may help to keep antibiotic resistance genes by raising environmental selection pressure. Additionally, antibiotic resistance in pesticide-rich environments may be used as a flag to find pesticide-tolerant microbes. In this study, bacterial strains were isolated from the agricultural soil collected from a potato farm. Results indicate the role of bacterial strain in tolerating pesticides at 1000 ppm concentration of pesticide. The isolates that had the ability to tolerate pesticide at 1000 ppm were gram-negative *Pseudomonas* spp. and *Rhizobium* spp. Therefore, strains of selected bacterial isolates demonstrated inherent pesticide tolerance, numerous antibiotic resistances, and the ability to synthesize a wide variety of plant growth-promoting compounds.

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