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Bhawna Dipta
Division of Plant Protection, ICAR-Central Potato Research Institute, Bemloe, Shimla, Himachal Pradesh, India

Rajesh Kaushal
Department of Soil Science and Water Management, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Correspondence
Bhawna Dipta
Division of Plant Protection, ICAR-Central Potato Research Institute, Bemloe, Shimla, Himachal Pradesh, India

Assessment of antibiotic and fungicide resistance by indigenous *Bacillus* strains of cauliflower (*Brassica oleracea* var. *botrytis* L.)

Bhawna Dipta and Rajesh Kaushal

Abstract
The chemical inputs are being used to increase yields, control pathogens, pests, and weeds, but their imbalanced and indiscriminate use over the years resulted in an accumulation of toxic chemical substances in the soil, depletion of organic carbon content and reduction in native micro-flora and fauna, hence lowered the crop productivity. Further the escalating prices of chemical inputs making them dearer to the reach of small and marginal farmers. To reduce these deleterious effects, an efficient alternative method is the application of plant growth promoting rhizobacteria (PGPR). PGPR colonize plant root interiors, migrate to the different plant parts exclusively in the intercellular space and exert beneficial effects on plant growth and development through a wide variety of mechanisms. So, the aim of the present study was to investigate the influence of the four indigenous *Bacillus* strains viz., *Bacillus pumilus* MK_5, *Bacillus sp*. MK_7, *Bacillus sp.* MK_9 and *Bacillus safensis* VG_1 on antibiotic and fungicide susceptibility. These bacterial cultures were subjected to antibiotic resistance by disc diffusion method. Antibiotics tested were amoxicillin (30 mcg), ampicillin (10 mcg), bacitracin (0.05 mcg), erythromycin (5 mcg), gentamycin 50 mcg), kanamycin (5 mcg) and tetracycline (30 mcg). Further, fungicide resistance to bacterial cultures was performed by well plate assay. Commercial formulation of fungicides were taken at the concentration used under field condition for two major fungicides i.e. mancozeb (0.12, 0.25, 0.37%) and carbendazim (0.05, 0.10, 0.15, 0.20%). Results revealed that all the bacterial strains were sensitive to amoxicillin, ampicillin, erythromycin, gentamycin, kanamycin and tetracycline but showed resistance to bacitracin. Further it was observed that all bacterial isolates showed tolerance to low concentrations of fungicides.

Keywords: Ampicillin, Carbendazim, cauliflower, pesticides, plant growth promoting Rhizobacteria

Introduction
The demand of chemical fertilizers for crop production has increased tremendously due to the release of several high yielding and nutrient demanding varieties of crops. The indiscriminate use of chemical fertilizers has resulted not only in the deterioration of soil health but also has led to some major environmental problems. In order to sustain the fertility of soil without heavy dependence on chemical inputs, the new approaches consisting of beneficial rhizospheric microorganisms especially plant growth promoting rhizobacteria (PGPR) have been recognized by different researchers all over the world (Mandyal *et al*., 2012; Bhardwaj *et al*., 2017) [6, 2]. The possible reasons for plant growth by these PGPR are through various direct and indirect mechanisms like associative nitrogen fixation, lowering the ethylene levels, production of phytohormones such as auxins and cytokinins, solubilization of nutrients such as phosphorus (Saleem *et al*., 2007) [9]. PGPR mainly includes the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkhoideria*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium* and *Serratia* (Tilak *et al*., 2005; Egamberdiyeva, 2007) [12, 4]. The present study was therefore, undertaken to check the resistance pattern of plant growth promoting rhizobacteria to antibiotics and fungicides.

Materials and Methods
The present investigations were conducted in the Microbiology section, Department of Basic Sciences at Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India.

Procurement of bacterial cultures

Four indigenous *Bacillus* strains viz., *Bacillus pumilus* MK_5, *Bacillus sp*. MK_7, *Bacillus sp.* MK_9 and *Bacillus safensis* VG_1 of cauliflower were procured from culture collection centre of Microbiology section, Department of Basic Sciences, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India. *Bacillus* strains were stored at 4 °C for further experimentation work

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Preparation of cell-free culture extract

All *Bacillus* strains were initially revived from glycerol stocks maintained at -80 °C on Nutrient agar at 35±2 ºC. Single colonies from each *Bacillus* strains were first grown in 5 ml Luria Bertani medium in test tubes at 35±2 ºC in a shaker incubator. One millilitre of each *Bacillus* strains was added to 50 ml Luria Bertani medium contained in a 250 ml flask and incubated at 35±2 ºC in a shaker incubator for 24 h. At this point, all cultures grew to early stationary phase. Cells were pelleted by centrifugation at 5,000 *g* for 10 min at room temperature. Supernatant was stored at 4 °C for further use.

Determination of antibiotic resistance

To determine resistance to antibiotics, all four *Bacillus* strains were tested for their sensitivity to seven antibiotics. The resistance to antibiotics was tested by disc diffusion method as described by Bauer *et al*. (1966) [1]. The antibiotic discs of known potency were spotted over the pre poured nutrient agar plates spreaded with 0.1 ml of the *Bacillus* strains to be tested. The plates were incubated at 35±2 ºC. After 24 h of incubation, the plates were examined, and the diameters of the zones of complete inhibition to the nearest whole millimetre were measured. The zone diameter for individual antimicrobial agents was then translated into sensitive and resistant categories**.** The antibiotics tested were amoxicillin (30 mcg), ampicillin (10 mcg), bacitracin (0.05 mcg), erythromycin (5 mcg), gentamycin (50 mcg), kanamycin (5 mcg) and tetracycline (30 mcg).

Determination of fungicide resistance

Fungicide resistance of *Bacillus* strains was determined by well plate assay method (Magaldi *et al*., 2004) [5]. In this method, 0.1 ml of the bacterial culture was spreaded over the pre poured nutrient agar plates and then wells were made with the help of borer. Then different concentrations of the fungicides to be tested were poured into the specified well. The plates were incubated at 35±2 ºC for 72 h and examined for growth. Fungicides tested were mancozeb (0.12, 0.25, 0.37%) and carbendazim (0.05, 0.10, 0.20%).

Results and Discussion

Antibiotic resistance of plant growth promoting rhizobacteria

Antibiotics are chemically heterogeneous group of organic, low-molecular weight compounds produced by microorganisms that are deleterious to the growth or other metabolic activities of other microorganisms. Secretion of antibiotic production by many microorganisms also results in the suppression of plant pathogen activities (Raaijmakers *et al*., 2002) [8]. Antibiotic sensitivity/resistance assay revealed that all *Bacillus* strains were sensitive to amoxicillin, ampicillin, erythromycin, gentamycin, kanamycin and tetracycline. It was interested to note that none of the *Bacillus* strains were inhibited by bacitracin. Maximum zone of inhibition (2.87 mm) was observed in MK_7 isolate for amoxicillin followed by tetracycline. Minimum zone of inhibition (1.05 mm) was shown by MK_5 isolate for ampicillin. The effect of different antibiotics used is depicted in Table 1.

Table 1: Tolerance of PGPR to different antibiotics

***Bacillus* strains**	**Amoxycillin (30 mcg)**	**Ampicillin (10 mcg)**	**Bacitracin (0.05 mcg)**	**Erythromycin (5 mcg)**	**Gentamycin (50 mcg)**	**Kanamycin (5 mcg)**	**Tetracycline (30 mcg)**
Bacillus pumilus MK_5	-	-	+	-	-	-	-
Bacillus sp. MK_7	-	-	+	-	-	-	-
Bacillus sp. MK_9	-	-	+	-	-	-	-
Bacillus safensis VG_1	-	-	+	-	-	-	-

(+) indicates bacteria is resistant to antibiotic at specific concentration

(-) indicates bacteria is sensitive to antibiotic at specific concentration

Fungicide resistance of plant growth promoting rhizobacteria

Fungicides are used for controlling plant diseases caused by pathogenic fungi, but intensive use of fungicides increases environmental pollution, health hazards and sometimes induces phytotoxicity (Polavarapu, 2000) [7]. To reduce the deleterious effect of fungicides, an efficient alternative method is the use of PGPR. The results revealed that all bacterial isolates showed tolerance to low concentration of commonly used fungicide mancozeb and carbendazim for seed treatment and control of stalk rot and root rot disease of cauliflower. The effect of different concentrations of mancozeb and carbendazim on all the four *Bacillus* strains is presented in Table 2.

Table 2: Tolerance of PGPR to different fungicides

***Bacillus* strains**	**Mancozeb**			**Carbendazim**			
	0.12%	**0.25%**	**0.37%**	**0.05%**	**0.10%**	**0.15%**	**0.20%**
Bacillus pumilus MK_5	-	-	-	+	-	-	-
Bacillus sp. MK_7	-	-	-	+	-	-	-
Bacillus sp. MK_9	-	-	-	+	-	-	-
Bacillus safensis VG_1	-	-	-	+	-	-	-

(+) indicates bacteria is resistant to fungicide at specific concentration

(-) indicates bacteria is sensitive to fungicide at specific concentration

The variation in the resistance to many tested antibiotics and fungicides may possibly be due to the differences in growth conditions and exposure of PGPR to stress conditions or toxic substance as well as presence or absence of resistance mechanisms that could be encoded either by chromosome and/or R-plasmid (Spain and Alm, 2003) [11]. Resistance to antibiotics and fungicides is acquired by a change in the genetic make up of microorganisms which can occur by either a genetic mutation or by transfer of antibiotic/fungicide resistant genes between organisms in the environment (Spain and Alm, 2003) [11]. Clustering of genes on a plasmid, are beneficial to the survival of that organism and its species because those genes are more likely to be transferred together in the event of conjugation. Subsequently, in an environment with multiple stresses, for example antibiotics, it would be more ecologically favorable in terms of survival for a bacterium to acquire resistance. Antibiotic resistance has been reported by Wani and Khan (2013) [15] and Wani and Irene (2014) [14]. It has been suggested that antibiotic resistant microorganisms will adapt faster by the spread of R-factors than by mutation and natural selection (Silver and Misra, 1988) [10]. Similarly, fungicide resistance has been reported by Vohra (2007) [13] and Cox *et al*. (2009) [3].

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