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Compatibility of pesticides with bacterial bio-agents effective against *Xanthomonas axonopodis* PV. *Punicae* causing blight in pomegranate

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Abstract
The bacterial bio-agents *viz., Bacillus subtilis*-KK-9A (Bsp), *Brevibacillus borstelensis*-BK-6 (Bb), *Brevibacillus* sp-PM-2A (Bsp) and *Lysinibacillus xylanilyticus*- VK-6B (Lx) which were reported as effective against *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight of pomegranate were further confirmed for their efficacy. The experiment was conducted to understand the compatibility of these bio-agents among and with other agro-chemicals (fungicides, insecticides, bactericides, nematicides and disinfectant) routinely using in pomegranate feild. Among eight fungicides tested, three fungicides *viz.,* Bordeaux mixture, carbendazim and difenconazole and one dis-infectant bleaching powder were found compatible and able to recover the growth of bioagents on their poisoned medium in all the concentrations tested. The fungicides *viz.,* chlorothalonil, copper oxychloride, hexaconozole, propiconazole, and mancozeb found incompatible with all the four bio-agents. All the four insecticides tested *viz.,* dichlorovas, chlorpyrifos, imidachloprid and dimethoate and a nemeticide, phorate found compatible and allowed the bacterial bioagents to grow excellent (+++) on their poisoned medium at all concentrations. Whereas carbofuron found incompatible. Unfortunately there was no growth of bacterial antagonists was found in streptocyclin, K-cyclin and 2-bromo-2-nitropropane-1, 3-diol tested.

Keywords: compatibility, bacterial bio-agents, pesticides, pomegranate

Introduction
Pomegranate (*Punica granatum* L.) belongs to Lythraceae family is one of the oldest edible fruit known to humans and grows naturally in India, Afghanistan and Syria; it is a native of Iran. The fruit was domesticated around 2000 BC (Rana *et al.,* 2007) [9]. Although pomegranate is widely adapted across the world, the five major producers of pomegranate are India, Iran, China, USA and Turkey (Holland and Bar-Ya'akov 2014; Teixeira da Silva *et al*. 2013) [6, 11]. India gained importance in cultivation of pomegranate after 1986; thereafter, it steadily increased to become first in cultivation at the global level. Total area under pomegranate in India is 1.43 lakh ha with production 1773.66 MT and the average productivity is 10.3 MT /ha (Anon, 2015) [1]. Successful cultivation of pomegranate in recent years has met with different traumas such as pest and diseases. Among diseases bacterial blight caused by *Xanthomonas axonopodis* PV. *punicae* is a major threat. Since 2002, the disease has reached the alarming stage and hampering the Indian economy vis-à-vis export of quality fruits. The disease was once deemed as a minor disease has become a serious threat for pomegranate production resulting in severe yield loss both in terms of quality and yield. The use of chemicals is not only the solution for management of any diseases. Even though there are many bactericides which are available in market and their efficiencies are good but the major threat is the chemical residues in fruits.

Biological management of plant pathogen by antagonistic microorganisms is a potential non-chemical means (Harman, 1991) [5] and is known to be a cheap, effective and eco-friendly method for management of crop disease (Cook and Baker, 1983). The use of bio-control agent as an alternative to chemical pesticides is increasing rapidly in the present day agriculture due to the deleterious effect of chemical pesticides. Though the bio-agents were effective but their compatibility need to be known with other pesticides like fungicides, insecticides, bactericides and nematicides which are used routinely in pomegranate cultivation for controlling many pests and diseases. The information on compatibility will help (i) to decide whether pesticide molecule can be mixed with or not (ii) to reduce the cost incurred on individual spray (iii) to know deleterious effect of pesticide /disinfectant on survival and multiplication of bio-agents used. In this context current research has been focused on compatibility of effective bacterial bio-agents with commonly using agrochemicals in pomegranate field.

Material and Methods

Bacterial bio-agents

Four bacterial bio-agents *viz., Bacillus subtilis*-KK-9A, *Brevibacillus borstelensis*-BK-6, *Brevibacillus*sp-PM-2A and *Lysinibacillus xylanilyticus*- VK-6B which were previouslyidentified as effective biocontrol agents against *Xanthomonas axonopodis pv. punicae* (Ippikoppa, 2015) [8] used in this study. The cultures were recovered on AK. Agar No.2 (gelatin 6.0g, casein enzymic hydrolysate 4.0g, yeast extract: 3.0g, beef extract: 1.5 g, dextrose: 1.0g, manganous sulphate: 0.3g, agar: 15.0g, with pH maintained at 6.6±0.2, water 1 lit) the medium which was found supportive for their good growth. The inoculated plates were incubated at 30 °C for 48 h and the same were used in the further studies. The culture filtrate was prepared by growing the four bio-agents and consortium on nutrient broth for 72 hrs, in orbital shaker with 150 rpm at 30 ^{0}C, after that, it is subjected to centrifugation at 8,000 rpm for 20 min at 4 °C, the pellet and supernatant was separated and it was used as a culture filtrate.

Efficacy of bacterial bio-agents against *X.a.p* by agar well diffusion method

The efficacy of these bio-agents were reconfirmed by agar well diffusion method. The culture of *X.a.p* was added to nutrient broth then poured into Petri plates and allowed to solidify. The agar plate is punched asepectically with sterile cork borer and the well is filled with 50 µl of culture filtrate of bacterial bio-agents. Then it was incubated at 30 ^{0}C for 48 hrs.

Chemical pesticides

The pesticides which were routinely using in the pomegranate orchard were selected for testing the compatibleness with the effective four bacterial bio-agents mentioned above. The details of eighteen pesticide including fungicides, insecticides, bactericides, nematicides and their dosage used in the study are mentioned in the Table 1.

Procedure to test the compatibility among four bacterial bio-agents

All the four bacterial bio-agents and their combinations were multiplied in nutrient broth, by inoculating the well isolated antagonist colony separately in Erlenmeyer’s flask. The inoculated flask was incubated at temperature 30 ^{0}C for 48 hours at 150 rpm. Optical density of each bio-agent and their combinations was recorded in spectrometer at 600nm.

Procedure to test the compatibility of bio-agents with agrochemicals

Compatibility of bacterial biocontrol agents with different fungicides, bactericides, nematicides, insecticides and also one disinfectant mentioned in the Table 1 were tested by poison food technique.

Nutrient agar was the basal medium to which the calculated quantity of pesticides was mixed separately after sterilizing the medium to give required concentrations. In the sterilized Petri plates the poisoned medium was poured at 20 ml and allowed to solidify. Bacterial antagonists were placed by dipping three paper disks in bacterial culture broth on the medium and incubated at 30 °C. The medium without addition of fungicide served as control. Three replications were maintained for each treatment. Bacterial growths of antagonists were recorded after 48 h and compared with control plates.

Results and Discussion

In-vitro evaluation of bacterial bio-agents against *X.a.p*

Among all the four bio-agents and consortium recorded highest inhibition (9mm) followed by *Brevibacillus* sp (8mm) and least inhibition was observed in case of *Brevibacillus borstelensis* (6 mm) and similar results were obtained in the work of Ippikoppa (2015) [8].

Compatibility of bacterial bio-agents

The four bacterial bio-agents were tested for their compatibility in different combinations by using spectrophotometer at 600nm. The results obtained from this experiment are presented in table 2.

Among four bio-agents, *Brevibacillus borstelensis* recorded highest OD value (1.94) with more turbid growth followed by *Brevibacillus* sp with OD values of 1.84, *Lysinibacillus xylanilyticus* of 1.68. Least growth was observed in case of *Brevibacillus borstelensis* with the OD value of 1.59. However their combinations recorded in more OD values compared to organisms grown individually. In double combinations, *Brevibacillus borstelensis*+ *Bacillus subtilis* which recorded highest OD value of 2.86 followed by *Lysinibacillus xylanilyticus* + *Brevibacillus* sp recorded 2.69. However in triple combinations *Brevibacillus borstelensis*+ *Brevibacillus* sp + *Bacillus subtilis* recorded highest OD value of 3.36 followed by *Brevibacillus* sp+ *Lysinibacillus xylanilyticus* + *Bacillus subtilis* which recorded 3.20.

Finally the combination of all four bio-agents recorded highest OD value (3.82) compared to other combinations.

Compatibility of bio-agents with pomegranate

The above study was conducted to know the compatibility of *Bacillus subtilis*-KK-9A, *Brevibacillus borstelensis*-BK-6, *Brevibacillus* sp-PM-2Aand *Lysinibacillus xylanilyticus*- VK-6B with commonly using agrochemicals in pomegranate orchard at different concentrations. The results obtained from this experiment are presented in table 3-6 and depicted in the plate 1.

Among eight fungicides tested three fungicides *viz.*, Bordeaux mixture, carbendazim, and difenconazole were found compatible and able to observe the growth of bio-agents on poisoned medium in all the concentrations. Bordeaux mixture (0.25%, 0.5%, 1.0 %) and carbendazim 50 WP (1.0 g/lit, 2.0 g/lit, 2.5g /lit) recorded very good growth (++) when compared to difenconazole 25 EC (0.5ml/lit, 1ml/lit, 2ml/lit) which recorded good growth (+).

The fungicides *viz.*, chlorothalonil 75WP (1g/lit 2.0g/lit, 3.0g/lit), hexaconozole 5EC (0.5ml/lit, 1ml/lit, 1.5ml/lit), propiconazole 25 % EC (0.5ml/lit, 1ml/lit, 2ml/lit) and mancozeb 75 WP (1.0 g/lit, 2.0 g/lit, 4.0 g /lit) were found incompatible with all the four bio-agents evaluated as No growth of these four bio-agents on poisoned medium was even at their lower concentrations. The disinfecting chemical, bleaching powder found to support very good growth (++) of the four bio-agents in all the three concentration (250 ppm, 500 ppm, 750 ppm) tested.

All the four insecticides *viz.*, dichlorovas (0.25per cent, 0.50 per cent, 0.75per cent), chlorpyrifos (1.5g/lit 2.0g/lit, 2.5g/lit), imidachloprid (0.15ml/lit, 0.3ml/lit, 0.45ml/lit) and dimethoate 30 per cent EC (0.5ml/lit, 1.0 ml/lit, 2.0 ml/lit) found compatible with all bio-agents and allowed them to grow excellent (+++) at all concentrations. However a nematicide carbofuron found incompatible with all the four bio-agents tested and not allowed them to grow on its poisoned medium. Whereas phorate found compatible and able to observe the very good growth (++) of all the four bacterial bio-agents.

Unfortunately, all four bio-agents were found sensitive and incompatible with the antibiotics/bactericides tested *viz.*, streptocyclin, K-cyclin and 2-bromo-2-nitropropane-1, 3-diol and unable to record the growth of any bacterial bio-agents tested even at lower concentration (100 ppm).

The bactericides were found sensitive /incompatible since bactericides having the mode of action targeting on prokaryotic organisms and the bio-agents used in present study were also belong to same group. The bio-agent used might not have developed resistance over the tested antibiotics still. However some of insecticides, nemeticides and fungicides found compatible since their targets were of different group of organisms.

Shukla (2011) [10] reported the incompatibility of carbofuron (0.05%) and bavistin (0.1%) with *Trichoderma viridae.* Whreas Bheemarya *et. al.*, 2012, reported compatibleness of mancozeb and metalaxyl and metalaxyl+ mancozeb and incompatibleness of propicanozole, captan with *Trichoderma* sp. however imidachloprid showed compatibility with isolates of *Trichoderma harizianum.*

Similarly the experiment conducted by Devi and Prakasam (2013) [4] to know the compatibility of azoxystrobin 25 SC with biocontrol agents by poisoned food technique as well as turbidity method and with insecticides by emulsion stability test. The biological compatibility was done under glass house conditions to find the per cent injury. The compatibility showed that *B. subtilis* was compatible with azoxystrobin 25 SC at 5, 10, 50, 100 and 250 ppm concentration.

Leha and Venkataraman (2001) [7] reported that carbendazim was very much compatible with *Pseudomonas fluorescens*. Significantly higher bacterial population was obtained in 100 ppm carbendazim amended King's B medium (KBM) than in 500 ppm amended KBM alone.

Conclusion

All the four bio-agents *viz.*, *Bacillus subtilis*-KK-9A, *Brevibacillus borstelensis*-BK-6, *Brevibacillus* sp-PM-2A and *Lysinibacillus xylanilyticus*- VK-6B were found compatible with each other and recorded no depletion in their growth. Thereby these four bio-agents can be utilized for preparation of consortium and enhancing their efficacy against plant pathogen.

The compatibility of bio-agents with commonly using agro-chemicals in pomegranate orchard revealed that, three fungicide *viz.* (Bordeaux mixture, carbendazim and difenconazole) one disinfectant (bleaching powder), four insecticides (dichlorovas, chlorpyrifos, imidachloprid and dimethoate) and nemeticide (phorate) were found compatible whereas, five fungicides (chlorothalonil, copper oxychloride, hexaconozole, mancozeb and propicanazole), one nematicide (carbofuran 3G) and three bactericides/antibiotics (streptocyclin, K-cyclin and 2-bromo-2-nitropropane-1,3-diol) were found incompatible with all the bio-agents tested. Therefore these four bio-agents can be used for application along with comapatible pesticides and should be cautious with the incompatible pesticides.

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Table 1: Treatments used in compatibility of bio-agents with commonly using agrochemicals in pomegranate

Treatments	Chemicals	Trade name	Concentration		
Fungicides					
T_1	Bordeaux mixture	Bordo	0.25%	0.5%	1%
T_2	Carbendazim 50 WP	Bavistin	1g/lit	2g/lit	2.5g/lit
T_3	Chlorothalonil 75 WP	Kavach	1.0g/lit	2.0g/lit	3.0g/lit
T_4	Copper oxychloride 50 WP	Blitox	1g/lit	2.0g/lit	3g/lit
T_5	Difenconazole 25 EC	Score	0.5ml/lit	1ml/lit	2ml/lit
T_6	Hexaconozole 5 EC	Contaf	0.5ml/lit	1ml/lit	1.5ml/lit
T_7	Mancozeb75 WP	Indofil M-45	1g/lit	2g/ml	4g/lit
T_8	Propiconazole 25% EC	Tilt	0.5ml/lit	1ml/lit	2ml/lit
Bactericides					
T_9	2-bromo-2-nitropropane-1,3-diol	Bronopol	0.25/lit	0.5g/lit	0.75g/lit
T_{10}	K-cyclin	-	250ppm	500ppm	750ppm
T_{11}	Streptomycin sulphate90%+Tetracycline hydrochloride+10%	Streptocyclin	100ppm	250ppm	500ppm
Insecticides					
T_{12}	Chlorpyrifos 20% EC	Predator	1.5ml/lit	2ml/lit	2.5ml/lit
T_{13}	Dichlorovas 76% EC	Nuvan	0.25%	0.5%	0.1%
T_{14}	Dimethoate 30% EC	Rogor	0.5ml/lit	1m/llit	2ml/lit
T_{15}	Imidachloprid 70%WG	Admire	0.15ml/lit	0.3ml/lit	0.45ml/lit
T_{14}	Dimethoate 30% EC	Rogor	0.5ml/lit	1m/llit	2ml/lit
T_{15}	Imidachloprid 70%WG	Admire	0.15ml/lit	0.3ml/lit	0.45ml/lit
Nematicides					
T_{16}	Phorate 10G	Thimate	250ppm	500ppm	750ppm
T_{17}	Carbofuron 3G	Furadan	250ppm	500ppm	750ppm
Disinfectant					
T_{18}	Bleaching powder	-	50g/pit	100g/pit	200g/pit
T_{19}	Control	-	-	-	-

Table 2: Compatibility among four bacterial bio-agents

Sl No.	Bio-agents and their combinations	OD values at 600nm
1.	*Brevibacillus* sp-SM-1A	1.84
2.	*Lysinibacillus xylanilyticus*-VK-6B	1.68
3.	*Brevibacillus borstelensis*-BK-6	1.94
4.	*Bacillus subtilis*- KK-9A	1.59
5.	*Brevibacillus* sp-SM-1A+*Lysinibacillus xylanilyticus*-VK-6B	2.09
6.	*Brevibacillus* sp-SM-1A + *Brevibacillus borstelensis*-BK-6	2.46
7.	*Brevibacillus* sp-SM-1A + *Bacillus subtils*-KK-9A	2.63
8.	*Lysinibacillus xylanilyticus*-VK-6B + *Brevibacillus borstelensis*-BK-6	2.27
9.	*Lysinibacillus xylanilyticus*-VK-6B + *Brevibacillus* sp-SM-1A	2.69
10.	*Brevibacillus borstelensis*-BK-6+ *Bacillus subtils* -KK-9A	2.86
11.	*Brevibacillus* sp-SM-1A + *Lysinibacillus xylanilyticus*-VK-6B + *Brevibacillus borstelensis*-BK-6	3.05
12.	*Brevibacillus* sp-SM-1A + *Lysinibacillus xylanilyticus*-VK-6B + + *Bacillus subtilis* -KK-9A	3.20
13.	*Lysinibacillus xylanilyticus*-VK-6B + *Brevibacillus borstelensis*-BK-6+ *Bacillus subtils*-KK-9A	3.01
14.	*Brevibacillus borstelensis*-BK-6+ *Bacillus subtils*-KK-9A +*Brevibacillus* sp-SM-1A	3.36
15.	*Brevibacillus* sp-SM-1A + *Lysinibacillus xylanilyticus*- + *Brevibacillus borstelensis*-BK-6+ *Bacillus subtilsi*-KK-9A	3.42
16	Control - Broth	0.00

Table 3: Sensitivity of bacterial antagonists against commonly using Fungicide in pomegranate

Treatments		Concentrations	*Brevibacillussp.*	*Lysinibacillus xylanilyticus*	*Brevibacillusborstelensis*	*Bacillus subtilis*
T_1	Bordeaux mixture (Bordo)	0.25 %	++	++	++	++
		0.5 %	++	++	++	++
		1.0 %	++	++	++	++
T_2	Carbendazim 50WP (Bavistin)	1.0 g/lit	++	++	++	++
		2.0 g/lit	++	++	++	++
		2.5 g/lit	++	++	++	++
T_3	Chlorothalonil 75WP (Kavach)	1.0 g/lit	-	-	-	-
		2.0 g/lit	-	-	-	-
		3.0 g/lit	-	-	-	-
T_4	Copper oxychloride 50WP (Blitox)	1.0 g/lit	-	-	-	-
		2.0 g/lit	-	-	-	-
		3.0 g/lit	-	-	-	-
T_5	Difenconazole 25 EC (Score)	0.5ml/lit	+	+	+	+
		1 ml/lit	+	+	+	+
		2 ml/lit	+	+	+	+
T_6	Hexaconozole 5 EC (Contaf)	0.5ml/lit	-	-	-	-
		1 ml/lit	-	-	-	-
		1.5 ml/lit	-	-	-	-
T_7	Mancozeb 75 WP(Indofil M-45)	1.0 g/lit	-	-	-	-
		2-3 g/lit	-	-	-	-
		4.0 g/lit	-	-	-	-
T_8	Propiconazole 25% EC (Tilt)	0.5ml/lit	-	-	-	-
		1 ml/lit	-	-	-	-
		2 ml/lit	-	-	-	-
T_9	Bleaching powder	250 ppm	++	++	++	++
		500 ppm	++	++	++	++
		750 ppm	++	++	++	++
T_{10}	Control	-	+++	+++	+++	+++

"+++" - Excellent growth (>20mm) ;; "++" - Very good growth (10-20 mm) "+" - good growth (0-10mm);; "-" -No growt(0.00 mm)

Table 4: Sensitivity of bacterial antagonists against commonly using insecticides using in pomegranate

Treatments	Concentration		*Brevibacillussp.*	*Lysinibacillusxylanilyticus*	*Brevibacillusborstelensis*	*Bacillus subtilis*
T_1	Chlorpyrifos 20%EC (Predator)	1.5 g/lit	+++	+++	+++	+++
		2.0 g/lit	+++	+++	+++	+++
		2.5 g/lit	+++	+++	+++	+++
T_2	Dichlorovas 76%EC (Nuvan)	0.25%	+++	+++	+++	+++
		0.5 %	+++	+++	+++	+++
		0.1 %	+++	+++	+++	+++
T_3	Dimethoate 30%EC (Rogor)	0.5ml/lit	+++	+++	+++	+++
		1 ml/lit	+++	+++	+++	+++
		2 ml/lit	+++	+++	+++	+++
T_4	Imidachloprid 70%WG (Admire)	0.15 ml/lit	+++	+++	+++	+++
		0.3 ml/lit	+++	+++	+++	+++
		0.45 ml/lit	+++	+++	+++	+++
T_5	Control	-	+++	+++	+++	+++

"+++" - Excellent growth (>20mm)

Table 5: Sensitivity of bacterial antagonists against commonly using bactericides in pomegranate

Treatments		Concentration	*Brevibacillus* sp.	*Lysinibacillus xylanilyticus*	*Brevibacillusborstelensis*	*Bacillus subtilis*
T_1	2-bromo-2-nitropropane-1,3-diol (Bronopol)	0.25 g/lit	-	-	-	-
		0.5 g/lit	-	-	-	-
		0.75 g/lit	-	-	-	-
T_2	K-cyclin	250 ppm	-	-	-	-
		500 ppm	-	-	-	-
		750 ppm	-	-	-	-
T_3	Streptomycin sulphate90%+ Tetracycline hydrochloride 10% (Streptocyclin)	100 ppm	-	-	-	-
		250 ppm	-	-	-	-
		500 ppm	-	-	-	-
T_4	Control	-	+++	+++	+++	+++

"+++" - Excellent growth (>20mm); "-"-No growth (0.00 mm)

Table 6: Sensitivity of bacterial antagonists against commonly using nematicides in pomegranate

Treatments		Concentration	*Brevibacillus*sp.	*Lysinibacillus xylanilyticus*	*Brevibacillus borstelensis*	*Bacillus subtilis*
T_1	Carbofuron (Furadan) 3G	250 ppm	-	-	-	-
		500 ppm	-	-	-	-
		750 ppm	-	-	-	-
T_2	Phorate (Thimate)	250 ppm	++	++	++	++
		500 ppm	++	++	++	++
		750 ppm	++	++	++	++
T_3	Control	-	+++	+++	+++	+++

"+++" - Excellent growth (>20mm); "++" - Very good growth (10-20 mm)

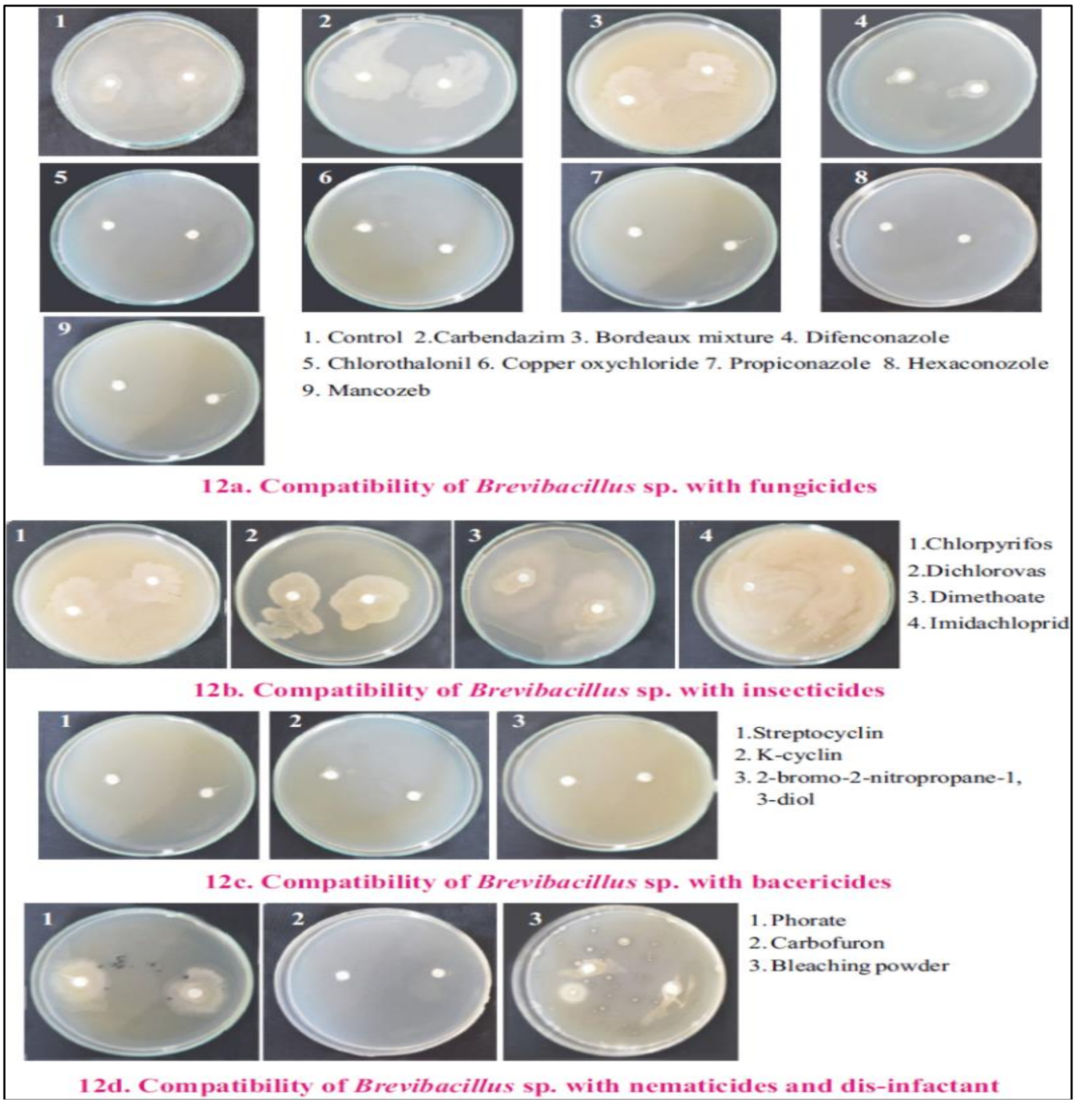

Fig 1: Compatibility of *Brevibacillus* sp. with commonly using agro-chemicals in pomegranate

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