

Allelopathic effects of rhizobacteria on *Fusarium* wilt and on the growth of citrus seedlings in Adjara, Georgia

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ABSTRACT

The allelopathic potential of rhizobacteria was investigated on root rot/wilt and on the growth of *Citrus reticulata* Blanco, *Citrus limon* Burmann and *Citrus sinensis* [L.] Osbeck seedlings. The highest means of incidence of disease and severity were recorded for lemon transplants (38.6 and 27.6.0 %, respectively) followed by mandarin transplants (34.5 and 23.8 %), while orange transplants revealed the lowest means (24.3 and 13.9 %). The most frequently fungi isolated from rotted roots of the citrus transplants were *Fusarium oxysporum*, *F. solani* and *F. proliferatum*. In pathogenicity tests, all the tested fungi were pathogenic to mandarin, lemon and orange transplants. The effect of plant growth promoting rhizobacteria (PGPR) individually and/or mixed when used as a soil drench treatment varied in reducing root rot/wilt incidence and severity under greenhouse conditions compared with control. The mixed culture of PGPR recorded the highly significant reduction in severity of root rot/wilt disease as compared to when the PGPR strains were used individually. On the other hand, the mixed culture of PGPR recorded the highest protection against infection with *F. oxysporum* (95-96 %) followed by *F. solani* (93-94 %) and *F. proliferatum* (89-91 %) in case of mandarin, lemon and orange transplants. Also, all treatments significantly increased plant height (cm), number of leaves transplanting⁻¹, leaf area (cm²), fresh and dry weights transplanting⁻¹(gm) compared with control treatment. This study indicated that soil drenched with PGPR strains can be used as a safe control measure of the disease in citrus transplants and as a stimulant of vegetative growth parameters.

Keywords: Allelopathy, *Fusarium oxysporum*, *F. solani*, *F. proliferatum*, rhizobacteria, mandarin, lemon, orange, transplants, PGPR

INTRODUCTION

Citrus are considered the most important economic fruit crops in the world and root rot and wilt diseases of citrus transplants primarily caused by *Fusarium* spp. are commonly found in soils of citrus orchards and of nurseries (15, 22,23,27,28). In Adjara, citrus is the highest cultivated fruit and several pathogens causing diseases in citrus result in serious losses in terms of quality and quantity. Root rot and wilt diseases of mandarin, lemon and orange transplants are primarily caused by *Fusarium oxysporum*, *F. solani*, *F. proliferatum* and other fungi.

In the recent past, the overuse of chemical pesticides has resulted in excessive pesticide residues in agricultural products, posing severe environmental and human health risks (31).

Allelopathy is a naturally occurring ecological phenomenon of interference among organisms by which one of them produces one or more chemical substances that have negative or positive influence on the growth and development of bacteria, fungi, or plants in their vicinity (14,20,24).

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Allelochemicals such as phenolic acids and terpenoid compounds (17,18) present in different parts of the plant, such as leaves, branches, or roots (4) are released into the environment. These are usually released directly into the aqueous phase of the soil, or volatile gaseous substances from aerial parts are released in the surrounding air (5). The allelochemical release is influenced by the soil, climatic conditions, and the plant itself (6).

The plant growth promoting rhizobacteria (PGPR) viz. *Azotobacter sp.*, *Bacillus cereus*, *B. megaterium* and *B. subtilis* produce biologically active compounds (antibiotics and toxic substances) that have antifungal activity, besides bioactive compounds, including plant growth hormones and regulators (11). *Bacillus subtilis* and *Agrobacterium radiobacter* when tested on lemon fruits significantly reduced disease severity (20). *A. radiobacter* specifically reduced severity of root rot/wilt diseases in apple and pear (25). PGPR can be a helpful in solubilization of mineral phosphates and other nutrients (10,13), enhance resistance to stress (1,5), stabilize soil aggregates and improve soil structure and organic matter content and retain more soil organic N and other nutrients in the plant soil system, thus reducing the need for chemical fertilizers for N and P supplementation (13).

The present work was planned to survey and assess root rot/wilt of citrus, isolation, identification of pathogens and pathogenicity testing, to evaluate the effect of certain PGPR as a single treatment and/or in combination for controlling the disease as well as their effect on growth parameters of mandarin, lemon and orange in Adjara.

MATERIALS AND METHODS

Object of Study and material

The research work was carried out in the plant protection laboratory, greenhouse of Shota Gustaveli Batumi State University and in a citrus plantation in Khelvachauri and Kobuleti, district Adjara, Georgia in 2021 - 2022. Three citrus species viz., mandarin (*Citrus reticulata* Blanco), Lemon (*Citrus limon* Burmann) and Orange (*Citrus sinensis* [L.] Osbeck) were selected for this study.

Diseases survey

Percentages of diseased mandarin, lemon and orange transplants, showing symptoms of root rot and/or wilt diseases were recorded. Disease severity on transplants was assessed by the exhibited symptoms, typical of root rot and/or wilt diseases. Foliar symptoms, including dull, internally rolled or necrotic leaves, defoliated and death twigs, were evaluated on a scale of 0-4 (3), where: 0= transplants healthy, 1= from 0 to 25 % (milled symptoms), 2= from 26 to 50 % (intermediate symptoms), 3= from 51 to 75 % (severe symptoms), 4= more than 76 % diseased foliage (transplants nearly dead to dead).

Disease severity index (DSI) described (19) was adapted and calculated as follows:

$$DSI = \frac{\sum d}{d_{max} \times n} \times 100$$

Where:

d : Disease rating of each transplant,

d_{max} : Maximum disease rating, and n : Total number of transplants/samples examined in each replicate.

Diseased roots of mandarin, lemon and orange transplants showing yellowing, or wilt symptoms were collected and taken for fungal isolation. The root samples were thoroughly washed under running tap water then cut into small pieces (1 cm), and surface sterilized with dipping in 0.1 % mercuric chloride solution for 2 min, then washed several times with sterile

distilled water. The surface sterilized pieces were blotted dry on sterilized filter paper, and transferred individually to Petri dishes, each containing 20 ml potato dextrose agar (PDA) medium, incubated at 25 °C for 5-7 days and inspected for fungal growth. The developed fungal colonies were purified using hyphal tip or single spore techniques. The purified fungi were identified according to fungal morphological and microscopical characteristics (29). The obtained culture isolates were maintained on PDA slants in refrigerator at 5 °C for further study.

Pathogenicity tests

The pathogenic capability of the isolated fungi was carried out under greenhouse conditions in Batumi Shota Rustaveli State University. Plastic pots (40 cm in diam.) sterilized by dipping in 5 % formalin solution for 16 min. Soil was also sterilized with formalin solution (5 %), then covered with a polyethylene sheet for 7 days to retain the gas and left to dry for 2 weeks until all traces of formaldehyde disappeared. The sterilized pots were filled with sterilized soil (6 Kg/pot). The tested fungi were grown on autoclaved barley grain medium in 500 ml glasses. It was inoculated with discs (5 mm in diameter) taken from 7-day old cultures of each tested fungal isolate, then incubated at 27 ±1 °C for 15 days. The sterilized soil was individually infected with the tested fungi at the rate of 5 % of soil weight. The pots were irrigated regularly for three times a week before planting to ensure even distribution of the inoculated fungus in the soil. Four mandarin, lemon, and orange transplants (ten months old) were cultivated in each pot and six pots were used as replicates. Six pots containing uninfected soil were cultivated the same way of transplanting and used as control. Percentages of incidence and severity of disease were recorded after three months of planting in pots. Re-isolation was carried out from infected transplants showing disease symptoms and the isolated fungus was compared with the original culture used.

PGPR and Inoculum Preparation

Four PGPR obtained from Bioagro (Center of Biological Protection of plant, Ltd Georgia, Tbilisi, 0017, orkhevi, tvarchrelidze street 6). *Agrobacterium radiobacter* (isolate AR1), *Bacillus cereus* (isolate BC M2), *Bacillus megaterium* (isolate Bm M3) and *Bacillus pumilus* (isolate BP M4), were used in this study. Inoculum was produced as described by Landa et al., (15). Bacterial concentration in suspension was adjusted to proximately 5×10^8 cells ml⁻¹ by measuring absorbance at 600 nm in a spectrophotometer and using standard curves for each bacterial isolate. Effect of PGPR as individual culture and as a mixed culture of *A. radiobacter*, *B. cereus*, *B. megaterium* and *B. pumilus* to control root rot and wilt diseases in vivo was evaluated on transplants of mandarin, lemon and orange. This experiment was carried out on healthy looking plants of mandarin, lemon, and orange under pot experiments. Six pots of each treatment were used as replicates containing sterilized soil previously infected with inoculum of each fungus and drenched with each tested PGPR (250 ml per pot), 7 days later soil infection. Four mandarin, lemon and orange transplants (ten-months old) were cultivated in each pot during 15th February, 2022. Six pots contained uninfected soil and were cultivated in the same way of transplanting and used as a control. After three months, disease severity index (DSI) and efficacy values were calculated according to the following formula:

$$DSI \% = \frac{\sum d}{d_{\max} \times n} \times 100$$

Where: d : Disease rating of each transplant, d_{max} the maximum disease rating and n : Total number of transplants/samples examined in each replicate. In the end of the experiment, vegetative growth parameters i.e. plant height (cm), number of leaf plant⁻¹, leaf area (cm²) according to (4) as well as fresh and dry weights (gm plant⁻¹) were recorded.

RESULTS AND DISCUSSION

Survey of root rot and wilt diseases: Long-term observations have shown that typical symptoms of root rot and wilt disease on mandarin, lemon and orange transplants were observed in two municipalities of Adjara (Khelvachauri, Kobuleti) and six villages (Adliya, Akhashen, Sameba, Benze, Makhinjauri, Chakvi). Data in Table (1) indicate that incidence and severity of root rot and wilt complex differed in the tested citrus in different inspected locations in the Adjara.

Table 1. Occurrence of root rot/wilt disease complex of citrus transplants in nurseries and new orchards of the Adjara (2021-2022)

Locations	Mandarin		Lemon		Orange	
	Disease incidence	Disease severity %	Disease incidence	Disease severity %	Disease incidence	Disease severity %
Adliya	30.8	19.8	38.5	28.8	24.6	12.4
Akhashen	36.3	25.6	37.2	26.1	23.3	13.3
Benze	37.8	28.7	33.6	23.4	23.6	13.5
Chakvi	44.3	32.8	49.5	38.5	27.8	17.1
Makhinjauri	30.2	18.3	36.5	25.2	25.2	14.6
Sameba	28.7	17.7	36.3	23.7	21.4	12.7
Mean	34.5	23.8	38.6	27.6	24.3	13.9

Disease incidence and severity of root rot and wilt of mandarin transplants ranged from 28.7 to 44.3 % and 17.7 to 32.8 %, respectively, while, disease incidence and severity of lemon transplants ranged from 36.5 to 49.5 % and 23.7 to 38.5 %, respectively. The disease incidence and severity of orange transplants ranged from 21.4 to 27.8 % and 12.7 to 17.1 %, respectively. Generally, the disease incidence and severity differed at the six inspected locations, where the highest disease incidence and severity were recorded in Chakvi and the lowest disease incidence and severity recorded in Sameba in the tested citrus crops. On the other hand, disease incidence and severity differed with different citrus crops. The highest means of disease incidence and severity were recorded for lemon transplants (38.6 and 27.6 %, respectively) followed by mandarin transplants (34.5 and 23.8 %), while, orange transplants revealed the lowest means (24.3 and 13.9 %). These results are consistent with the observations and data presented by earlier workers (5,6,9,12,24).

Isolation, identification of pathogens and pathogenicity tests

Three main isolates causing root rot/ wilt in citrus crops were identified as *F. oxysporum*, *F. solani* and *F. proliferatum*. Pathogenicity test of these fungi on mandarin, lemon and orange transplants confirmed their pathogenicity to these crops. All the pathogenic fungi isolates exhibited different degrees of incidence (Fig. 1) and severity (Fig. 2).

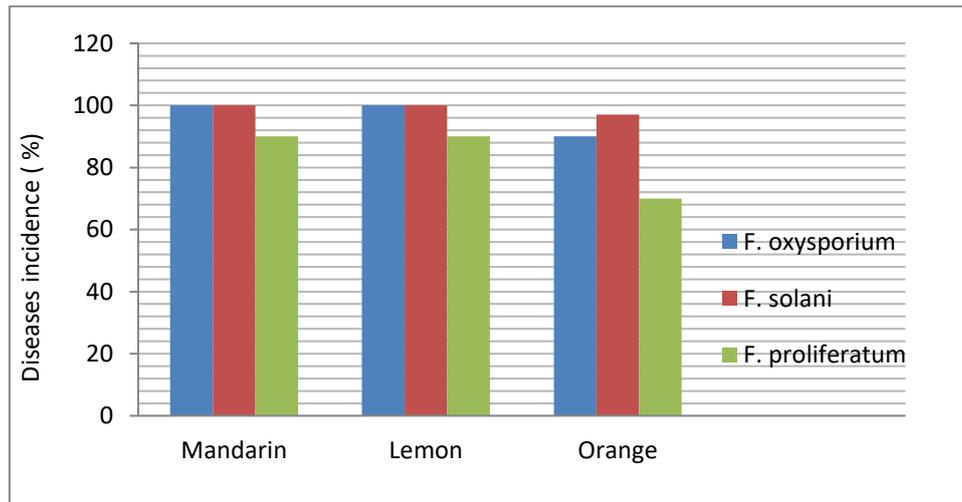


Figure 1. Incidence of fungal isolate of citrus transplants under greenhouse conditions in Adjara (2021-2022)

In case of mandarin transplants, *F. oxysporium* and *F. solani* caused the highest root rot/wilt incidence and severity, whereas they caused 100, 100 % root rot/wilt incidence and 100, 91.80 % root rot/wilt severity, respectively. Similar results were obtained in the case of lemons. On the other hand, orange transplants affected with *F. solani* than *F. oxysporium* or *F. proliferatum*, showed 96.70 % root rot/wilt incidence and 92.40 % severity.

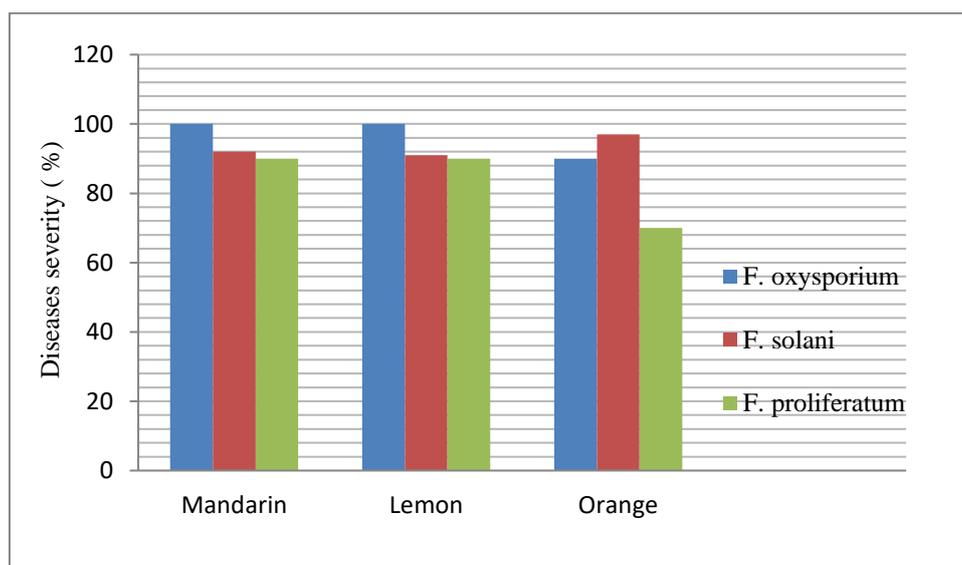


Figure 2. Root rot/wilt severity of citrus transplants under greenhouse conditions in Adjara (2021-2022)

Effect of PGPR on Severity of root rot/wilt and Growth of transplants in vivo

All the PGPR strains tested showed reduced severity of root rot/wilt diseases in mandarin, lemon, and orange transplants when experimentally infected by *F. oxysporum*, *F. solani* and *F. proliferatum* were treated with PGPR as individual strains or as mixed culture as a soil drench method in pots (Fig. 3).

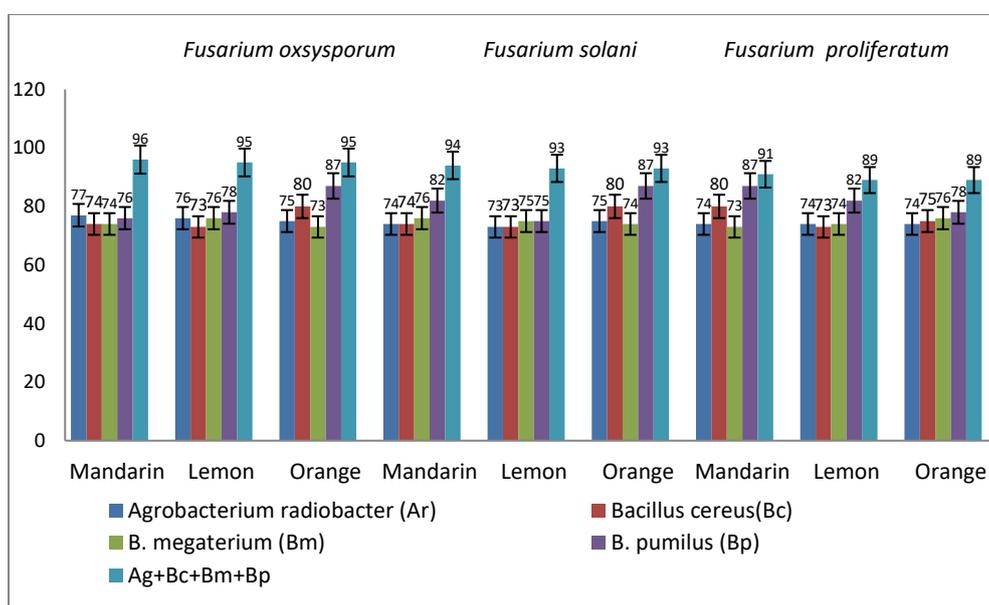


Figure 3. Effects of tested PGPR strains on disease severity caused by pathogenic fungi

The results showed varied efficiency of the tested PGPR in controlling these diseases. The mixed culture of PGPR recorded the most significant reduction in severity of root rot/wilt disease as compared to when the PGPR strains were used individually. On the other hand, the mixed of PGPR recorded the highest protection against infection with *F. oxysporum* (95-96 %) followed by *F. solani* (93-94 %) and *F. proliferatum* (89-91 %) in case of mandarin, lemon and orange transplants.

Vegetative growth parameters: Effects of PGPR strains individually and/or as mixed culture on some growth parameters of mandarin, lemon and orange transplants under artificial infections with *F. oxysporum*, *F. solani*, *F. proliferatum* in pot culture conditions were studied. The obtained data revealed low values of growth parameters, plant height (cm), number of leaf plant⁻¹, leaf area (cm²), fresh and dry weights (gm transplant⁻¹) in the control treatment compared with other treatments. All tested growth parameters of citrus transplants were significantly increased with the mixed inoculation of PGPR strains compared with the individual one. Compared to untreated transplants (control) in mandarin (infected with *Fusarium oxysporum*) plant height increased by 13.99 cm, number of plant leaves by 21.0, leaf area by 12.53 cm², fresh weight by 9.13 gm plant⁻¹ and dry weight by 9.13 gm plant⁻¹ with mixed inoculation of PGPR strains (Table 2).

Table 2. Effects of tested PGPR strains on growth parameters mandarin transplants

Treatments	Pathogen	Plant height (cm)	Number of leaf Plant ⁻¹	Leaf area (cm ²)	Fresh weight (g plant ⁻¹)	Dry weight (g plant ⁻¹)
<i>Agrobacterium radiobacter</i> (Ar)	<i>Fusarium oxysporum</i>	17.12	19.68	14.27	5.89	2.20
<i>Bacillus cereus</i> (Bc)		15.78	18.77	13.24	5.43	2.12
<i>B. megaterium</i> (Bm)		16.14	15.45	15.31	4.37	1.82
<i>B.pumilus</i> (Bp)		21.12	22.66	16.81	6.56	2.56
Ag+Bc+Bm+Bp		25.87	28.13	18.65	10.78	3.87
Control		11.88	6.78	6.12	1.65	0.47
<i>Agrobacterium radiobacter</i> (Ar)	<i>F. solani</i>	20.12	20.23	14.12	5.64	2.13
<i>Bacillus cereus</i> (Bc)		15.87	16.55	13.78	3.67	1.42
<i>B. megaterium</i> (Bm)		18.33	17.89	13.67	4.68	1.90
<i>B.pumilus</i> (Bp)		21.56	20.78	15.90	6.23	2.54
Ag+Bc+Bm+Bp		25.87	27.56	17.78	9.12	3.12
Control		5.56	5.80	10.66	1.54	0.48
<i>Agrobacterium radiobacter</i> (Ar)	<i>F. proliferatum</i>	17.88	15.70	12.92	4.65	1.61
<i>Bacillus cereus</i> (Bc)		14.88	12.55	12.64	3.93	1.35
<i>B. megaterium</i> (Bm)		18.23	16.89	13.68	5.17	1.88
<i>B.pumilus</i> (Bp)		19.78	18.77	15.45	5.90	2.21
Ag+Bc+Bm+Bp		22.56	21.45	16.56	7.66	2.68
Control		5.14	4.23	1.76	1.22	0.42

Compared with mandarin and other pathogens, all the growth parameters studied for lemon transplants were significantly increased by mixed inoculation with PGPR strains (Table 3). Practically similar results were obtained in the case of orange and other pathogens (Table 4).

Table 3. Effects of tested PGPR strains on growth parameters of lemon transplants

Treatments	Pathogen	Plant height (cm)	Number of leaf Plant ⁻¹	Leaf area (cm ²)	Fresh weight (g plant ⁻¹)	Dry weight (g plant ⁻¹)
<i>Agrobacterium radiobacter</i> (Ar)	<i>Fusarium oxysporum</i>	29.62	26.67	26.72	21.55	7.20
<i>Bacillus cereus</i> (Bc)		27.75	22.32	23.98	19.85	6.95
<i>B. megaterium</i> (Bm)		28.19	23.44	27.76	21.86	6.88
<i>B.pumilus</i> (Bp)		33.27	24.43	29.86	22.97	7.34
Ag+Bc+Bm+Bp		37.38	30.78	33.88	32.98	10.57
Control		13.48	9.68	10.88	6.75	2.20
<i>Agrobacterium radiobacter</i> (Ar)	<i>F. solani</i>	29.77	19.89	25.35	16.15	5.67
<i>Bacillus cereus</i> (Bc)		25.65	17.23	23.88	15.56	5.60
<i>B. megaterium</i> (Bm)		26.45	19.54	27.76	16.87	5.78
<i>B.pumilus</i> (Bp)		32.33	20.67	28.94	24.21	8.25
Ag+Bc+Bm+Bp		33.34	25.33	30.37	28.69	9.79
Control		9.67	8.12	10.26	3.96	1.27
<i>Agrobacterium radiobacter</i> (Ar)	<i>F. proliferatum</i>	24.78	22.88	22.87	19.77	6.82
<i>Bacillus cereus</i> (Bc)		26.34	23.21	23.00	20.87	6.78
<i>B. megaterium</i> (Bm)		28.54	24.88	26.54	23.32	7.86
<i>B.pumilus</i> (Bp)		29.76	25.58	27.32	26.54	8.88
Ag+Bc+Bm+Bp		38.44	32.45	30.12	30.66	10.66
Control		12.33	4.76	11.60	1.20	0.48

Table 4. Effects of tested PGPR strains on growth parameters orange transplants

Treatments	Pathogen	Plant height (cm)	Number of leaf Plant ⁻¹	Leaf area (cm ²)	Fresh weight (g plant ⁻¹)	Dry weight (g plant ⁻¹)
<i>Agrobacterium radiobacter</i> (Ar)	<i>Fusarium oxysporum</i>	31.32	31.42	3.91	23.90	11.42
<i>Bacillus cereus</i> (Bc)		29.78	38.12	4.22	29.12	14.68
<i>B. megaterium</i> (Bm)		29.45	24.76	4.23	21.23	10.98
<i>B.pumilus</i> (Bp)		34.45	34.14	4.21	28.81	14.54
Ag+Bc+Bm+Bp		38.68	38.12	4.51	40.58	19.98
Control		14.70	10.77	3.30	6.86	3.82
<i>Agrobacterium radiobacter</i> (Ar)	<i>F. solani</i>	19.75	19.56	13.79	5.65	2.13
<i>Bacillus cereus</i> (Bc)		15.73	16.57	13.57	3.56	1.34
<i>B. megaterium</i> (Bm)		18.37	17.87	13.58	4.67	1.76
<i>B.pumilus</i> (Bp)		21.38	20.83	15.92	6.14	2.37
Ag+Bc+Bm+Bp		25.71	27.42	17.74	9.03	3.06
Control		5.34	5.75	10.60	1.28	0.48
<i>Agrobacterium radiobacter</i> (Ar)	<i>F. proliferatum</i>	17.87	15.72	12.91	4.53	1.61
<i>Bacillus cereus</i> (Bc)		14.75	12.44	12.58	3.90	1.38
<i>B. megaterium</i> (Bm)		18.12	16.76	13.52	5.12	1.84
<i>B.pumilus</i> (Bp)		19.73	18.71	15.28	5.90	2.20
Ag+Bc+Bm+Bp		22.42	21.42	16.39	7.32	2.62
Control		5.11	4.34	1.40	1.14	0.43

Studies have shown that rhizobacteria stimulated plant growth and effective fertilizers through increased cell division, as well as optimized uptake of nutrients and water as well stimulating as soil microorganisms playing role in reducing root rot and wilt diseases. Almost the same results were obtained by different researchers (1,3,10,29).

CONCLUSIONS

This study confirms the existence of allelopathic relationships capable of inhibiting the infective pathogen activity and stimulating plant growth activity in vivo conditions.

All the plants treated by PGPR as individual strains or as mixed culture showed reduced severity of root rot/wilt diseases in mandarin, lemon, and orange transplants experimentally infected by *F. oxysporum*, *F. solani* and *F. proliferatum*. The mixed culture of PGPR recorded the highly significant reduction in severity of root rot/wilt disease as compared to when the PGPR strains were used individually. On the other hand, the mixed culture of PGPR recorded the highest protection against infection with *F. oxysporum* (95-96 %) followed by *F. solani* (93-94 %) and *F. proliferatum* (89-91 %) in case of mandarin, lemon and orange transplants. Also, all treatments significantly increased plant height (cm), number of leaves after transplanting⁻¹, leaf area (cm²), fresh and dry weights transplanting⁻¹(gm) compared with control treatment. The soil drench with PGPR strains can be used as a safe control measure of the diseases in mandarin, lemon and orange transplants and as a stimulant of vegetative growth parameters.

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DECLARATION

Kanchaveli, Sh. S. designed and planned experiment. Chkhubadze G. Conducted experiment in greenhouse and plantation. Shainidze O. T. conducted experiments in the laboratory and wrote article.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct.

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