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Biological Control of *Coniella granati* Saccardo in Pomegranate

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Abstract *Coniella granati* Saccardo (Synonym *Pilidiella granati*) is a fungal pathogen that causes fruit brown rot, cankers on shoots and crown rot of pomegranate trees. Although cultural and chemical control is recommended against *C. granati*; cultural control is not enough and limited number of advisable fungicides used in chemical control against this pathogen. Therefore, alternative strategies are needed for this pathogen control. In this context, it was aimed to investigate the effect of some bacterial biocontrol agents against *C. granati* under *in vitro* conditions. Dual culture of eleven bacterial biocontrol agents [1 *Bacillus megaterium* (TV 3D), 3 *Bacillus substilis* (TV 6F, TV 17C, CP1), 1 *Bacillus cereus* (TV 85D), 1 *Paenibacillus polymxa* (TV 12E), 2 *Pseudomonas fluorescens* (MF 3, AR 9), 1 *Burkholderia cepacia* (BA 7) 1 *Pantoea agglomerans* (MF 1) and 1 *Bacillus thuringiensis* (BAB 420)] were tested for antagonistic properties against *C. granati*. Percent inhibition rate values changed from 11.90% to 66.67% in dual culture. *B. cereus* (TV 85D, 66.67%) was the most effective strains against *C. granati* respectively by *B. substilis* (TV 17C, 64.29%; TV 6F, 60.71%) in *in vitro*. As a result, promising results were obtained from these isolates in *in vitro* conditions. These isolates should be tested *in vivo* conditions for controlling the post-harvest decay of pomegranate fruits caused by *C. granati.*

Keywords Biological Control, Bioagent Bacteria, *Bacillus* sp., *Bacillus cereus*, *Coniella granati*

1. Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest tropical/subtropical fruit cultivated. In addition to its high nutritional value, it has gained more importance in recent years as it is an important source of antioxidants [1,2]. Pomegranate is a fruit produced in most countries in the world, because it can be grown in various climatic and soil conditions and stored for a long time [2]. However, many biotic and abiotic factors limit the production of this product. it is estimated that the annual loss rate due to biotic factors in plant production is 36.5% [3], and 1/3 of these losses are post-harvest losses [5].

Pomegranate fruit is susceptible to many fungal pathogens that cause pre-harvest and post-harvest losses. [6-10]. *Coniella granati* Sacc., one of these fungal pathogens. (sexual stage *Schizoparme versoniana*) is an important disease factor that threatens the whole world, leading to serious pre-harvest and post-harvest losses. Crown rot disease causes tree decline, blight on branches and post-harvest fruit decay. Presence of this disease has been identified in Brazil, Cyprus, Italy, Spain, Korea, Florida, Israel, Iran, China, North Carolina, Pakistan, Netherlands, Mexico, Greece, Turkey and the United States [11].

It is observed that the disease forms the first symptoms on pomegranate fruit as small, round and hard brown spots, and over time, the size of these spots grows, their color becomes darker, and eventually the whole fruit rots. Necrosis, which begins in the lower part of the root, causes wilting over time, and dieback occurs in young branches. The causal agent, which spends the winter in dead shoots, mummified fruits and pruning places, is transmitted to healthy plants through rain or irrigation water [9, 12-14].

Although cultural and chemical control is proposed against the agent, limited information is available on this subject. It is advised to avoid planting too close together, to perform regular pruning, to keep the number of stems small, since pomegranate plants constantly form new stems and to remove the infected fruits. In addition, the use of resistant varieties as a cultural measure is the best method of control, but the lack of study on the susceptibility/resistance of different genotypes and cultivars of pomegranate plants to the this disease

prevents an effective control [9]. Chemical control, which is an effective and practical method, is lacking because of limited number of fungicides (thiophanate methyl, tebuconazole and fludioxonil) and studies on new fungicides that will be effective against fruit decay [9, 15, 16]. In addition, due to the negative effects of fungicides on environment and human health, residues and lastingness problems, the search for alternative combat methods has become mandatory [17].

In recent years, usability of microbial antagonists, which are friendly to the environment and human health, which may be an alternative to chemical control with no residue problem and no risk of lastingness, or the antibiotic, enzyme-like substances produced by them, to prevent plant diseases has been demonstrated by many studies [18, 19]. Bacteria within these microbial antagonists are used as an effective biological control agent against many fungal and bacterial diseases [20-26]. However, in the literature review, no biological control of bacterial origin against this pathogenic fungus has been encountered.

In this study, 11 bacteria [Bacillus megaterium (TV 3D), Bacillus substilis (TV 6F, TV 17C, CP 1), Bacillus cereus (TV 85D), Paenibacillus polymxa (TV 12E) and Pseudomonas fluorescens (MF 3 and AR 9), BA 7 (Burkholderia cepacia), MF 1 (Pantoea agglomerans) and BAB420 (Bacillus thrungiensis)] were tested in vitro against C. granati, which could be an alternative to the use of synthetic pesticides.

2. Materials and Methods

2.1. Pathogen Fungi and Bioagent Bacteria

Table 1. Bioagent bacteria, MIS results and isolated from

Bacteria	MIS results	Isolated from	References
TV 3D	*Bacillus megaterium*	Rye	[27]
TV 12E	*Paenibacillus polymyxa*	Wheat	[28]
AR 9	*Pseudomonas fluorescens*	Soil	[29]
MF 3	*Pseudomonas fluorescens*	Soil	[30]
TV 6F	*Bacillus substilis*	Wheat	[28]
TV 17C	*Bacillus substilis*	Raspberry	[27]
TV 85D	*Bacillus cereus*	Sugar beet	[28]
CP 1	*Bacillus subtilis*	*Ricania simulans*	[31]
BA 7	*Burkholderia cepacia*	*Soil*	[32]
MF 1	*Pantoea agglomerans*	Apple	In this study
BAB 420	*Bacillus thrungiensis*	*Ricania simulans*	In this study

Pathogen fungi was isolated from diseased pomegranate fruit taken from greengrocer in Erzurum/Turkey. Bioagent bacteria effective in our previous biological control studies were used in this study. A total of 11 bacteria isolates were obtained from the Department of Plant Protection, Plant Clinical Laboratory Microorganism Culture Collection, Faculty of Agriculture at Ataturk University, Turkey. The diagnosis according to fatty acid methyl esters result and host information of these bacterial isolates are given in Table 1.

2.2. Isolation and Identification Fungus

One more small pieces of tissue taken from diseased and healthy tissue and subjected to surface sterilization with 70% ethanol for 3 min, and rinsed with sterile distilled water and then dried on sterilized whatman paper. The pieces were placed on Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany) and petri plates were incubated at 25-27°C for 4 days in incubator. Afterwards, fungal hyphae plugs from the fruit tissues were transferred fresh PDA to obtain the pure culture. This fungus is maintained on PDA slant agar in the Department of Plant Protection, Plant Clinical Laboratory Microorganism Culture Collection, Faculty of Agriculture at Ataturk University, Turkey as strain ET 85.

2.3. Pathogenicity Test of Fungi

The pathogenicity of isolate ET 85 was tested on pomegranate fruit. The pomegranate fruits were washed under tap water and then surface sterilised with 70% ethanol, fruits washed twice by dipped in sterilised distilled water and then they were left on sterilized filter paper in laminar cabinet for remove excess water on the surface. Fungi isolate was developed for 4 days on PDA at 27^{o}C. The mycelial disks taken from the end portions of the pathogen fungi were placed in the wound area on the fuit surface and wound area wrapped with parafilm. Afterwards, the fruits were placed in transparent plastic boxes with damp sterile filter paper and they were stored at room temperature under a photoperiod of 12-h light and 12-h dark. After incubation for 5 days, decay on the fruit surface and fungal mycelial growth were determined as a positive pathogenicity test and re-isolation was done from the symptoms and Koch postulates were completed. Blank disc PDA was used as a negative control. Each treatment was applied to three replicates of 1 fruit in each experiment. The isolates were maintained on a slant agar which compound PDA for further use at 4°C in Atatürk University, Agriculture Faculty, Plant Protection Department, Plant Clinic Laboratory.

2.4. Pathogen Fungus Molecular Identification

Molecular sequence was carried out to confirm the identify of the pathogen fungus. Genomic DNA was

extracted from mycelia using the protocol by Moller et al 1992. The rDNA internal transcribed spacer region of pathogen fungus was amplified using primers ITS1-ITS4 [34]. The amplified PCR product sample was sent to the Refgen Co. Ltd., Turkey for sequencing and the resulting sequence was submitted to Genbank.

2.5. Bioassay

Petri dishes (90 mm) containing 20 ml PDA were used in bioassay. Bioagent bacterial isolates were grown on Nutrient Broth (NB) at for 24 h to obtain fresh culture for *in vitro* dual culture method. Pathogen fungi isolate was grown for 5 days at 27°C on PDA. Bacterial suspensions were individually streaked with a sterile swap on the PDA plates as a circular inner edge of the plate and pathogen fungi was placed in the middle of the petri plates. The plates were wrapped with parafilm, and then were incubated at 27°C in incubator until fungal mycelia completely covered the control petri plates. As control, only mycelial disks of pathogenic fungi were placed in the middle of petri plates. Pathogen fungi radial growth was measured in mm. Three replicates of each treatment were made [35].

The percentage inhibition rate of pathogen fungi by bioagent bacterial isolates were calculated by using the formula [34].

$$\text{Inhibition (\%)} = (C-T)\times 100/(C-6)$$

C: the diameter of the pathogen colony of control group
T: the diameter of pathogen colony after treatments
6: the diameter of pathogen disk.

2.6. Statistical Analyses

All data in the present study were processed by JUMP 5.0.1 and the means were separated by LSMeans Student's tests. The statistical analyses of percentage values in relation to the fruit set were performed using transformed values.

3. Results

3.1. Isolation and Identification Fungus

Fungal hypha firstly developed in orange pigment in isolation. This orange pigment is from the fruit, not from the fungus. Afterwards, fungal colonies with white aerial mycelia and concentric rings of black pycnidia various sizes were observed when transferred to fresh PDA medium. Hyphae were septate and conidia were hyaline, one-celled, ellipsoid to fusiform (average 10.1–20.2×3.2–4.3 µm). Morphological character of *C. granati* (ET 85) isolate on PDA and pathogenicity test results were given in Fig 1.

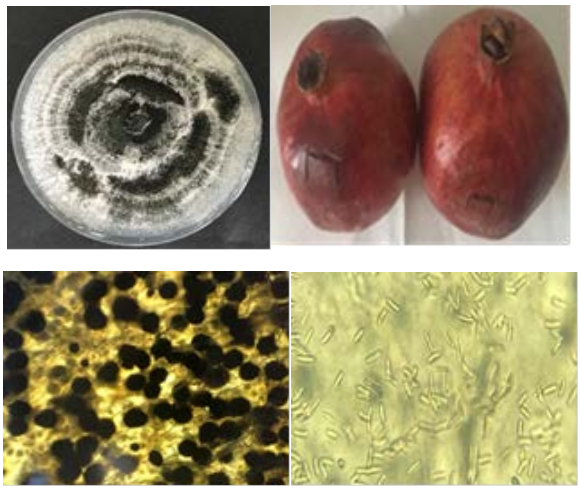

Figure 1. Morphological character and pathogenicity test result of *Coniella granati*

3.2. Pathogen Fungus Molecular Identification

Pathogen fungus sequence was done. All sequence was submitted to Genbank and registered with the 636bp Accesion number MH992151. Pathogen fungi sequence was compared with other ITS sequences by BLASTn analysis and 99% identity with those of *C. granati* from pomegranate from South Africa (KT279814), China (HQ166057) and Mexico (KX369239).

3.3. Bioassay

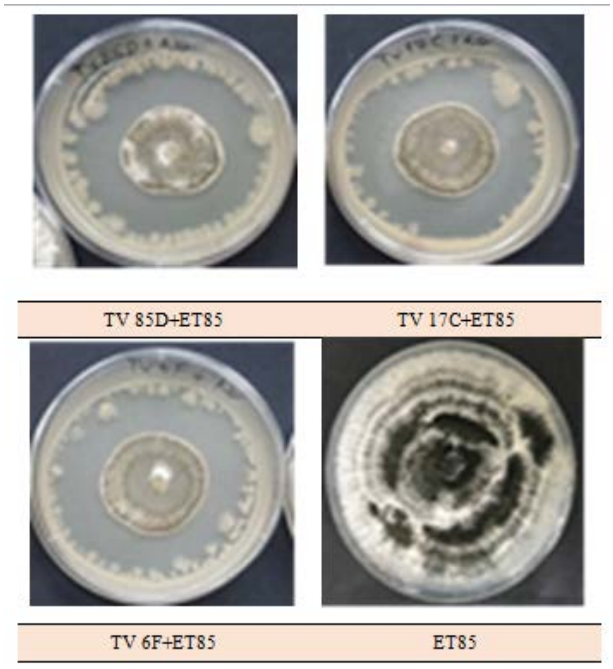

Figure 2. The three most effective bioagent bacteria in the dual culture test

All bacterial isolates showed more or less antifungal activity against the pathogen compared with the control,

according to the results of biocontrol activity *in vitro* in this study, The radial growth of the pathogenic fungus ranged from in dual culture. *In vitro* inhibition test results of the most effective bioagent bacteria isolates were shown in Fig. 2.

In vitro inhibition test results of bioagent bacteria isolates were tested against *C. granati* were shown in Table 2. The percent inhibition rate in the control was statistically different from all other tested bacteria (Table 2). Percentage inhibition rate values were changed between 11.9-66.67%. The highest percent inhibition rate was observed in TV85D (66.67%), followed by isolate TV 17C (64.29%) and TV6F (60.71%). The lowest percent inhibition rate was observed in BAB 420, AR 9 and TV 3D isolates (11.9%).

Table 2. The percentage inhibition rate of *Coniella granati* in dual culture

Bacteria	**PIRG***	
TV 85D	66.67	A
TV 17C	64.29	A
TV 6F	60.71	B
CP 1	46.43	C
MF 3	26.19	D
MF 1	23.81	D
BA 7	19.64	E
TV 12E	17.26	E
BAB 420	11.90	F
AR 9	11.90	F
TV 3D	11.90	F
Control	0.00	G
LSD	3.17	
CV	0.06	

*There is no statistically significant difference between values expressed in the same column with similar letters (P<0.01).

4. Discussion

C. granati is an important fungal agent that causes pre- and post-harvest fruit decay and poses a new threat to the rapidly growing pomegranate industry in many parts of the World [11]. Post-harvest disease management for pomegranate fruit remains an important problem [37]. In the control against disease, cultural measures and chemical control are recommended. However, while cultural measures provide limited protection, limited information on chemical control and its disadvantages necessitates the use of alternative control methods [8]. For this reason, the biological control method, which is among alternative combat methods and friendly to the environment and human health with no risk of lastingness and residue problem, is an effective and important control method [9].

The species belonging to the genera *Bacillus, Pseudomonas* and *Burkholderia* have been used by different researchers as a bioagent in biological control [10, 19, 31, 38]. Since *Bacillus* group bacteria, among these bacteria, have a number of advantages over other bacteria (such as endospore formation, wide spectrum activity of their antibiotics), it is emphasized that they have the potential to be used against pathogens [39]. It has been noted that bacteria from the genus *Pseudomonas* lives in the soil, encourage plant growth and bioremediation, and that due to their easy colonization, competitive ability and wide antimicrobial spectra they have an important place among bioagents [40].

In this study, 11 bacterial bioagents belonging to the genera *Bacillus, Paenibacillus, Pseudomonas* and *Burkholeria*, which were previously used as biological warfare agents in different studies were used. All of the bacterial bioagents tested *in vitro* against *C. granati* isolate were more or less effective on the development of the pathogen, except for the control. In this study, the most promising results were obtained, and *B. cereus* (TV 85D) and *B. substilis* (TV 17C) species were successfully used in the control against pests and diseases, which has also been recorded by different researchers [26,31,41-43].

Tozlu et al. (2016), tested two isolates of *B. substilis in vitro* and *in vivo* against *Sclerotinia sclerotiorum* and determined that TV 17C isolates were highly effective in preventing the disease. Again, Tozlu et al. (2017) and Tozlu et al. (2018c) noted that the same isolate yielded effective results against *Penicillium digitatum* and *Alternaria alternata*, respectively. These results indicate that TV 17C is also effective on other pathogens. In addition, Tozlu et al. (2018c) showed that this isolate inhibits the development of the pathogen by degrading the chitin found in the cell wall of the pathogen with glucanase and protease enzymes. In this study, the fact that TV 17C at 64.29% *in vitro* conditions prevented the development of *C. granati* showed that this bacterial isolate can be used as an important biological warfare agent.

Tekiner et al. (2018) tested the TV 85D bacterial isolate against 3 *B. cinerea* and 1 *A. alternata in vitro* and found that it prevented the development of *A. alternat* at 64.29% and *B. cinerea* at 64.29-80.36%.

Jiang et al. (2017) revealed that *B. cereus* AR156 isolate promotes plant growth, induces systemic resistance mechanism and protects tomato plants against bacterial wilt caused by *Ralstonia solanacearum* and root-knot nematode *Meloidogyne incognita*. In this study also, TV 85D (*B. cereus*), the most effective isolate, has been shown to inhibit the development of pathogenic fungi *in vitro* conditions as well.

In a study conducted by Kotan et al. (2014), it was determined that TV 85D, TV 17C and TV 6F bacterial isolates could be used both in agriculture as biopesticides and bio-fertilizer. In another study, it was found that, the

application of TV 17C bacteria in the cauliflower plant increased plant growth parameters such as wet and dry shoot weight, root diameter and root length, wet and dry root weight, plant height, leaf surface area and chlorophyll content [27]. Thus, it was noted that this bacteriological isolate has both regulatory effects on plant development and prevents from diseases.

Al-Hussini et al. (2018) tested that *B.cereus* (D1/17) used against *Pythium aphanidermatum* (damping-off on tomato) and they observed that according to control 27% prevent the disease. Also they revealed that this bioagent bacteria is an plant growth promoting bacteria, induced the maximum shoot length and seedling vigor.

Awan and Shoaib (2019) explored that *in vivo* biocontrol efficacy of *B. subtilis* alone and in combination with plant nutrients [NPK, Zn (1X & 2X), Mg and B] against early blight disease of tomato (*A. solani*). They revealed that foliar application of *B. subtilis* alone and in combination with the plant nutrients managed early blight disease significantly by 67–83%, while improved plant growth attributes by 20–77%. *B. subtilis* and plant nutrients helped the tomato plant to fight off the plant hacker by up-regulating the production of total phenolic contents and defensive enzymes. This and other studies shows that *Bacillus* sp. effectively managed the diseases.

5. Conclusions

In conclusion, this study is important in terms of identification of bacteria that can be used as a bio-agent against *C. granati*, which causes significant yield losses in pomegranate. In this study, it was determined that bacterial isolates belonging to *B. cereus* and *B. subtilis* were effective on the development of the disease causing agent *in vitro* conditions. It is likely that this effect may vary in storage conditions with different values of temperature and humidity. It is of great importance to conduct studies in order to determine the effectiveness of the formulations of these two bacteria species, which are effective against fruit decay, together or separately in the storage conditions.

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