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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Determination of Biological Control Agent Bacteria Against Crown Gall*

Kök Kanserine Karşı Biyolojik Mücadele Ajanı Bakterilerin Belirlenmesi

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Abstract

This study was carried out to determine new bioagent bacteria for *in vitro* and semi-*in vivo* biological control of crown gall disease [*Rhizobium radiobacter* (*Agrobacterium tumefaciens*)]. A total of 2012 potential bioagent bacterial strains belonging to different genera were tested *in vitro* against the pathogen. Microbial identification systems (MIS) diagnoses of bioagent bacterial strains found to be effective as a result of *in vitro* tests were supported by some conventional tests. Then, the strains' semi-*in vivo* biocontrol activities found to be effective were tested in carrot slices and squash fruits. Statistical analysis of the data was made according to the percentage of surface coverage in carrot slices and the number and size of tumors in squash fruits. Then, the most effective bioagent and pathogenic bacterial strain diagnoses were determined molecularly. According to the results; 106 bioagent bacterial strains (66 antibiosis; 40 hyperparasitic effects) were found to be effective against *R. radiobacter in vitro* conditions. It was determined that conventional test results of bioagent bacteria and MIS results supported each other. As a result of semi-*in vivo* biocontrol activity, it was determined that 8 bioagent bacterial strains out of 106 bioagent bacterial strains did not produce pectolytic activity, and 8 bioagent bacterial strains could be evaluated as a result of semi-*in vivo* test. The most effective strain suppressing the development of the pathogen in carrot slices and squash fruits was RK 1986 (carrot slices 1.78±0.47; squash fruits 0.26±0.04), followed by RK 570A (carrot slices 2.89±0.82; squash fruits 0.35+0.03) and RK 1074 (carrot slices 3.44±0.99; squash fruits 0.46±0.05) strains were followed. According to the results of molecular identification, the most effective bioagent bacterial strain (RK 1986) was *Bacillus mojavensis*, and the pathogenic bacteria strain (1B) was *R. radiobacter*.

Keywords: *Agrobacterium tumefaciens*, *Bacillus mojavensis,* Biological control, Crown gall, *Rhizobium radiobacter*

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Öz

Bu çalışma kök kanseri hastalığının [*Rhizobium radiobacter* (*Agrobacterium tumefaciens*)] *in vitro* ve yarı *in vivo* testlerle biyolojik mücadelesine yönelik yeni biyoajan bakterilerinin belirlenmesi amacıyla yapılmıştır. Farklı cinslere ait toplam 2012 adet potansiyel biyoajan bakteri straini patojene karşı *in vitro* koşullarda test edilmiştir. *In vitro* test sonucu etkili bulunan biyoajan bakteri strainlerinin mikrobiyal identifikasyon sistem tanıları (MIS) bazı konvansiyonel testler ile desteklenmiştir. Daha sonra etkili bulunan izolatların yarı *in vivo* biyokontrol aktiviteleri havuç dilimi ve kabak meyvesinde test edilmiştir. Havuç dilimlerinde yüzey kaplama yüzdesi kullanılırken, kabak meyvesinde ise ur sayı ve büyüklüğüne göre veriler elde edilmiş ve istatistiki analizi yapılmıştır. Daha sonra en etkili bulunan biyoajan ve patojen bakteri strainlerinin tanıları moleküler olarak belirlenmiştir. Elde edilen bulgulara göre; *R. radiobacter*'e karşı *in vitro* koşullarda 106 adet biyoajan bakteri straini (66 adedi antibiyozis etki; 40 adedi hiperparazitik etki) etkili bulunmuştur. Biyoajan bakterilerin konvansiyonel tanı test sonuçları ile mikrobiyal identifikasyon tanı sonuçlarının birbirini destekler nitelikte olduğu belirlenmiştir. Yarı *in vivo* biyokontrol aktivite test sonucu 106 adet biyoajan bakteri strainden 8 adetinin pektolitik aktivite oluşturmadığı belirlenmiş ve yarı *in vivo* test sonucu 8 adet biyoajan bakteri straini değerlendirilebilmiştir. Havuç dilimi ve kabak meyvesinde patojenin gelişimini baskılayan en etkili strainin RK 1986 (havuç dilimi 1.78±0.47; kabak meyvesi 0.26±0.04) olduğu, onu RK 570A (havuç dilimi 2.89±0.82; kabak meyvesi 0.35±0.03) ve RK 1074 (havuç dilimi 3.44±0.99; kabak meyvesi 0.46±0.05) strainlerinin takip ettiği tespit edilmiştir. Moleküler tanı sonuçlarına göre en etkili olan biyoajan bakteri straini (RK 1986) *Bacillus mojavensis*, patojen bakteri strainin ise (1B) *Rhizobium radiobacter* olduğu kaydedilmiştir.

Anahtar Kelimeler: *Agrobacterium tumefaciens*, *Bacillus mojavensis,* Biyolojik mücadele, Kök kanseri, *Rhizobium radiobacter*

1. Introduction

Rhizobium radiobacter (*Agrobacterium tumefaciens*) is a soil-borne plant pathogen that is common worldwide, is among the top ten bacteria in plant bacterial diseases, and causes crown gall (EPPO, 2019). All virulent agent species have Tumor Inducing Plasmid (Ti), an extrachromosomal structure, except for DNA. Transferring the T-DNA region in the pathogen Ti plasmid to the plant genome, causes the formation of galls in the root and root collar of the plants (140 genera in more than 100 different plant families) (Agrios, 1997; Gupta et al., 2010), causing significant economic losses in seedling cultivation (Frikha-Gargouri et al., 2017).

In the control of crown gall disease, such as using production material without disease (Peluso et al., 2003), choosing tolerant rootstock (Goodman et al., 1993), avoiding injuring the plant (Vrain and Copeman, 1987), paying attention to the disinfection of pruning tools (Cazelles et al., 1991), not planting in heavy and moist soils (Gloyer, 1934), soil solarization and using chemical pesticides (Moore and Canfield, 1996; Gupta and Kamal, 2006). However, disadvantages such as intensive labor, low effect, high cost, and high phytotoxicity have made biological control, an alternative more environmentally friendly method, important (Kotan et al., 2009; Kotan and Tozlu, 2021). Although *R. radiobacter* K-84 and K-1026 are widely used in the biological control against the agent (Moore, 1988), the failure of K-84 and K-1026 in some economically important plants and the limited number of studies makes it necessary to determine different bacterial strains that can be alternative biological control agents showed (Khmel et al., 1998; Tolba and Soliman, 2013). In recent years, extensive research has been carried out to determine different bacterial strains that can be used against crown gall. Accordingly, it has been reported that bacterial genera such as *Pseudomonas*, *Bacillus*, *Brevibacillus*, *Lactobacillus*, *Curtobacterium*, and *Azospirillium* have strains that can control crown gall disease (Zhang et al., 1991; Farrand and Wang, 1992; Moore and Canfield, 1996; Khmel et al., 1998; Rhouma et al., 2004; Gupta and Kamal, 2006; Gupta et al., 2007; Hammami et al., 2008; Dandurishvili et al., 2010; Tolba and Soliman, 2013; Limanska et al., 2015; Abdallah et al., 2018; Bozkurt and Soylu, 2019).

In this study, it was aimed to detect new bioagents for the biological control of crown gall disease *in vitro* and to determine the ability of effective bioagents to suppress tumor formation in semi-*in vivo* conditions.

2. Materials and Methods

2.1. Preparation of microorganisms and cultures

R. radiobacter (1B) strain strain from a peach plant and tested for virulence was used as a pathogen strain (Tekiner Aydın and Kotan, 2022). As a potential bioagent bacterial strain, 2012 strains belonging to 99 bacterial species kept in the Recep Kotan Culture Collection of Atatürk University, Faculty of Agriculture, Department of Plant Protection were tested. Many of these strains have been tested against different plant diseases and pests before or are composed of strains to be used for the first time in this study. Diagnosis of potential bioagent strains has been done by different researchers in microbial identification systems (MIS) (MIDI, Sherlock Microbial Identification System version 4.5 inc., Newark, DE).

Bacterial inoculum was prepared according to Eastwell et al. (2006). Briefly, pure bacterial cultures were grown for 24 hours (h) by transferring them to 250 ml Nutrient Broth (NB). Bacterial cultures were cooled on ice for 30 minutes (min), then centrifuged at 6000 rpm for 15 min and washed 2 times with 0.85% NaCl (saline). Then, their density was adjusted to $1x10^8$ colony forming bacteria milliliter (cfu ml^{-1}) on a spectrophotometer (600 nm) with sterile distilled water (sdH_2O).

2.2. In vitro assay

1B pathogen strain was sown on the entire surface of the Petri dish (90 mm) containing Nutrient Agar (NA) with the help of a swab. Then, potential candidate bioagents were drawn as a single line in the middle of these plates with the help of a loop and the plates were incubated at 27 °C for 2-4 days. Antibiosis strains formed an inhibition zone (mm) and the width of this zone was measured. In those with hyperparasitism, the area covered by the bioagent strain (mm) was measured in the area where the pathogen strain developed. Measurements were taken from 3 different points in each petri dish for both interactions and 3 replications were made for each bioagent strain.

2.3. Identification of bioagent bacteria by conventional tests

Some conventional tests have been performed to support MIS diagnosis of bioagent strains found to be effective against 1B pathogen strain *in vitro*. These tests are; Potassium hydroxide (KOH) test (Moore et al., 2001), catalase test (Klement et al., 1990), oxidase test (Kovacs, 1956), starch hydrolysis (Klement et al., 1990), determination of nitrogen fixation of strains (Guerinot and Colwell, 1985), detection of phosphate solvent bacteria (Nautiyal, 1999), levan test, pectolytic activity test and hemolytic activity test (Lelliot and Stead, 1987).

2.4. Semi-in vivo biocontrol activity

The ability of bioagent strains to suppress tumor formation was tested in carrot slices and squash fruit since *in vitro* experiments have certain limitations that the efficacy of biocontrol activity may not be fully expressed *in vivo*. Briefly, carrot and squash fruits were subjected to surface sterilization. Sterilized carrots were cut into 0.5-0.8 cm thicknesses and placed in 90 mm diameter Petri dishes with moist blotting papers; sterile squash fruits were placed in transparent rectangular boxes (7 L) covered with moist blotting papers. Eastwell et al. (2006) pathogen/bioagent bacteria inoculum (1:1, v:v) prepared according to ($1x10^8$ cfu ml^{-1}) were inoculated on the surface of carrot slices and 100 µL in pits opened with toothpicks on squash fruits. Only pathogen bacteria were used as positive control and only bioagent bacteria as negative control. The study was performed in 3 replications for each bioagent. The evaluation was made 30 days after inoculation. Evaluation of carrot slices Limanska et al. (2015) + + + + cambial ring is completely covered with tumor; + + + covers 75% of the cambial ring; + + covers 50% of the cambial ring; + covers less than 25% of the cambial ring; - Evaluation was made, and evaluation was made in squash fruits by measuring the number and size of tumor according to Tolba and Soliman (2013).

2.5.Molecular identification of pathogen and bioagent bacteria

The comparative DNA sequencing method is one of the best genotypic methods in microbial diagnosis. Most commonly, strains are identified using the 16S rDNA gene region. DNA extraction was performed according to Lazo et al. (1987) for the molecular diagnosis of the bioagent and pathogen strain with the most effective semi-*in vivo* biocontrol activity test result. Then, universal primer pair (27F and 1492R) recognizing pathogen and bioagent bacterial strains was used and polymerase chain reactions (PCR) were performed. The reaction mix of PCR consisted of dH_2O 37.2 µl, 10X PCR buffer 5 µl, $MgCl_2$ 3 µl, dNTP mix 0.7 µl, forward primer 0.8 µl, reverse primer 0.8 µl, DNA 2 µl and Taq polymerase (250 U) 0.5 µl. PCR's cycle is 95 °C for 2 min (1 cycle); 94 °C 60 s, 53 °C 60 s, and 72 °C 90 s (35 cycles); Consisting of 10 min (1 cycle) at 72 °C. In order to perform sequence analysis of the PCR results, PCR products were purified with a commercially available PCR purification kit (Invitrogen), and the purified PCR samples were sequenced by obtaining sequence service from Refgen Biotechnology Company (Ankara, Türkiye).

3. Results

3.1. In vitro assay results

The antibiosis effect (mean inhibition zone) of the bioagent bacterial strains that were effective in the study and the hyperparasitic effect (mean spreading area) are given in *Table 1*. Out of 2012 potential bioagent bacterial strains belonging to 99 bacterial strains tested, 106 bioagent bacterial strains belonging to 27 bacterial strains were found to be effective. Of these 106 bioagent bacterial strains, 66 showed an antibiosis effect, while 40 showed a hyperparasitic effect. It was determined that the strain with the highest antibiosis effect in Petri trials was RK 1977 (37.5 mm), followed by RK 1978 (30 mm) and RK 1250 (24 mm). RK 1095 (3.5 mm) was determined to be the strain that formed the lowest mean inhibition zone. In Petri trials, the strain with the highest hyperparasitic effect was RK 593 (78.5 mm), followed by FDG 105 (70 mm), and FDG 137 (70 mm) strains. RK 1223 (12.5 mm) bioagent bacterial strain was also determined to be the strain with the lowest hyperparasitic effect.

3.2. Identification of bioagent bacteria by conventional tests results

The biochemical and cultural test results of 106 potential bioagent bacterial strains that were effective in Petri trials are given in *Table 1*. Thirty eight strains showed positive results, while sixty-eight strains gave negative results in the KOH test. According to the catalase test, all strains gave positive results. According to the starch hydrolysis test result; except for ten strains, all strains were able to hydrolyze starch. Thirty four strains of bacteria

planted in a nitrogen-free medium made strong nitrogen fixation, fifty- eight strains weak nitrogen fixation, while fourteen strains could not make nitrogen fixation at all. According to the scale used for the detection of phosphate solvent bacteria; Thirty two strains could not dissolve any phosphate (6:-), thirty one strains were the lowest (5:+), five strains (4:+ +), thirty strains (3:+ + +) and six strains (2:+ + + +) resolved phosphate. According to the Levan test result, nineteen strains gave a positive reaction, while eighty seven strains gave negative results. As a result of the oxidase test, twenty one strains gave positive results in the oxidase test, and eighty five bacterial strains gave negative results. According to the pectolytic activity test, except for eight strains, other bacterial strains caused pectolytic activity in potato slices. According to the hemolytic activity test, all strains showed hemolytic activity except three.

Table 1. Bioagent bacterial strains' antibiosis and hyperparasitic effects against 1B pathogen, and conventional test results

Strain	MIS	Antibiosis Effect		Conventional Tests								
		AIZ [1] (mm)		KOH[2]	K[3]	NH[4]	N[5]	P[6]	L[7]	O[8]	PA[9]	HA[10]
RK 1250	*Bacillus atrophaeus*	24±0.49	C	-	+	+	Z+	6	-	-	+	+
RK 1986	Not determined	21±0.08	D	-	+	+	Z+	2	-	+	-	+
FDG 48	*Bacillus sphaericus*	19±0.08	DE	-	+	+	Z+	5	-	+	+	+
RK 546	*Bacillus subtilis*	16.5±0.12	EF	-	+	+	K+	3	-	-	+	+
RK 1062	*Brevibacillus choshinensis*	16±0.34	GL	-	+	+	Z+	6	-	+	+	+
RK 554	*Bacillus atrophaeus*	15±0.0	FG	-	+	+	K+	3	-	-	+	+
RK 578B	*Brevibacillus choshinensis*	15±0.24	FG	+	+	+	-	4	+	+	+	-
RK 1077	*Achromobacter xylosoxidans*	15±0.41	FG	+	+	+	K+	6	-	+	+	+
RK 547	*Bacillus megaterium*	14.5±0.12	F-H	-	+	+	Z+	5	-	-	+	+
RK 576B	*Serretia fonticola*	14.5±0.29	FH	+	+	+	K+	3	+	-	-	-
RK 834	*Bacillus cereus*	14±0.08	F-I	-	+	+	Z+	3	-	-	+	+
RK 594	*Salmonella typhimurium*	14±0.33	F-I	+	+	+	K+	3	+	-	+	+
RK 1763	*Paenibacillus macerans*	13.5±0.12	G-J	-	+	+	Z+	2	-	-	+	+
RK 1224	*Pantoea agglomerans*	13±0.24	G-K	+	+	+	K+	2	+	-	-	+
R2/2	*Paenibacillus polymyxa*	12.5±0.20	G-L	-	+	+	Z+	3	-	-	+	+
FDG 98	*Yersinia pseudotuberculosis*	12.5±0.20	G-L	+	+	+	-	5	-	-	+	+
RK 578A	*Bacillus megaterium*	12.5±0.12	G-L	-	+	+	K+	5	-	-	+	+
RK 957	*Chryseobacterium meningosepticum*	12±0.33	H-M	+	+	+	-	4	+	+	-	+
RK 1071	*Stenotrophpmonas maltophilia*	12±0.24	H-M	-	+	+	Z+	3	-	-	+	+
RK 1088	*Vibrio hollisae*	12±0.24	H-M	+	+	+	Z+	6	-	+	+	+
RK 600	*Hafnia alvei*	11.5±0.12	I-N	+	+	-	Z+	3	+	-	+	+
RK 1064	*Pseudomonas stutzeri*	11.5±0.12	I-N	-	+	+	Z+	5	-	+	+	+
RK 1080	*Bacillus megaterium*	11.5±0.29	I-N	-	+	+	Z+	4	-	-	+	+
RK 844	*Bacillus megaterium*	11±0.08	J-O	-	+	+	Z+	3	-	-	+	+
RK 562	*Vibrio hollisae*	11±0.08	J-O	+	+	+	K+	3	+	+	+	+
RK 572B	*Stenotrophpmonas maltophilia*	11±0.08	J-O	-	+	+	Z+	5	-	-	+	+
RK 588	*Salmonella typhimurium*	11±0.08	J-O	+	+	+	K+	3	+	-	+	+
RK 1255	*Bacillus atrophaeus*	10.5±0.04	K-P	-	+	+	K+	3	-	-	-	+
FDG 27	*Brevibacillus brevis*	10±0.0	L-Q	-	+	+	Z+	3	-	+	+	+
RK 574A	*Bacillus cereus*	10±0.0	L-Q	-	+	+	Z+	3	-	-	+	+
RK 1031	*Alcaligenes faecalis*	10±0.0	L-Q	+	+	-	-	5	-	+	+	+
RK 1074	*Achromobacter xylosoxi*	10±0.0	L-Q	+	+	+	K+	6	-	+	-	+
RK 932	*Bacillus megaterium*	9.5±0.37	M-R	-	+	+	K+	4	-	-	+	+
RK 1257	*Bacillus atrophaeus*	9.5±0.12	M-R	-	+	+	K+	5	-	-	+	+
RK 561	*Bacillus subtilis*	9±0.08	N-S	-	+	+	Z+	3	-	-	+	+
RK 1061	*Pseudoalteromonas terraodonis*	9±0.08	N-S	-	+	+	K+	6	-	+	+	+

Table 1. Bioagent bacterial strains' antibiosis and hyperparasitic effects against 1B pathogen, and conventional test results (continued)

Strain	MIS	Antibiosis Effect		Conventional Tests								
		AIZ[1] (mm)		KOH[2]	K[3]	NH[4]	N[5]	P[6]	L[7]	O[8]	PA[9]	HA[10]
RK 581	*Salmonella typhimurium*	8.5±0.12	O-T	+	+	+	K+	2	+	-	+	+
RK 590	*Salmonella typhimurium*	8.5±0.12	O-T	+	+	+	K+	2	+	-	+	+
RK 602	*Salmonella typhimurium*	8.5±0.12	O-T	+	+	+	Z+	3	+	-	+	+
RK 1253	*Bacillus atrophaeus*	8±0.16	P-T	-	+	+	Z+	5	-	-	+	+
RK 1273	*Peanibacillus macerans*	8±0.16	P-T	-	+	+	Z+	3	-	-	+	+
RK 37	*Curtobacterium flaccumfaciens*	7.5±0.20	Q-U	-	+	+	K+	3	-	-	+	+
RK 1050	*Bacillus megaterium*	7.5±0.12	Q-U	-	+	+	Z+	5	-	-	+	+
RK 1078	*Bacillus subtilis*	7.5±0.20	Q-U	-	+	-	Z+	5	-	-	+	+
RK 605	*Brevibacillus choshinensis*	7±0.16	R-V	+	+	+	K+	2	+	+	+	+
RK 985	*Stenotrophpmonas maltophilia*	7±0.16	R-V	+	+	+	Z+	3	-	-	+	+
RK 1086	*Aeromonas salmonicida*	7±0.24	R-V	+	+	+	-	6	-	+	-	+
RK 1252	*Bacillus atrophaeus*	7±0.08	R-V	-	+	+	K+	5	-	-	+	+
RK 1022	*Bacillus-GC group*	6.5±0.12	S-W	-	+	+	Z+	6	-	-	+	+
RK 1104	*Bacillus megaterium*	6.5±0.12	S-W	-	+	+	Z+	5	-	-	+	+
RK 1239	*Bacillus subtilis*	6.5±0.12	S-W	-	+	+	Z+	5	-	-	+	+
RK 506	*Bacillus subtilis*	6±0.16	T-X	-	+	+	Z+	3	-	-	+	+
RK 601	*Serretia fonticola*	6±0.08	T-X	+	+	+	Z+	5	+	-	+	+
RK 877	*Aeromonas salmonicida*	5±0.00	U-X	+	+	-	-	6	-	+	+	+
RK 1274	*Peanibacillus macerans*	4.5±0.04	V-X	-	+	+	Z+	5	-	-	+	+
RK 1275	*Bacillus coagulans*	4.5±0.12	V-X	-	+	+	Z+	3	-	-	+	+
RK 981	*Zobellia uliginosa*	4±0.08	WX	-	+	+	K+	3	-	-	+	+
RK 1058	*Arthrobacter agilis*	4±0.16	WX	-	+	+	Z+	6	-	+	+	+
RK 1095	*Paenibacillus larvae*	3.5±0.12	X	-	+	+	Z+	5	-	-	+	+
FDG 97	Not determined	12.5±0.20	G-L	+	+	+	K+	6	-	-	+	+
RK 1238	Not determined	8±0.0	P-T	-	+	-	Z+	5	-	-	+	+
RK 1752	Not determined	8±0.16	P-T	-	+	+	Z+	3	-	-	+	+
RK 999	Not determined	7.5±0.20	Q-U	-	+	+	Z+	4	-	-	+	+
RK 952	Not determined	6.5±0.12	S-W	+	+	+	Z+	6	-	-	+	+
RK 1977	Not determined	37.5±0.20	A	-	+	+	Z+	5	-	+	+	+
RK 1978	Not determined	30±0.82	B	-	+	+	Z+	3	-	-	+	+
Strain	**MIS**	**Hyperparasitic Effect**		**Conventional Tests**								
		ASA [11] (mm)		**KOH[2]**	**K[3]**	**NH[4]**	**N[5]**	**P[6]**	**L[7]**	**O[8]**	**PA[9]**	**HA[10]**
RK 593	*Bacillus cereus*	78.5±0.69	A	-	+	+	K+	6	-	-	+	+
FDG 105	*Bacillus mycoides*	70±1.22	AB	-	+	+	Z+	3	-	-	+	+
FDG 137	*Bacillus cereus*	70±0.82	AB	-	+	+	K+	6	-	-	+	+
RK 587	*Photorhabdus luminescens*	70±0.82	AB	+	+	+	Z+	5	-	-	+	+
RK 504	*Bacillus megaterium*	65±1.22	BC	-	+	+	K+	5	-	-	+	+
FDP 8	*Bacillus thuringiensis*	65±1.22	BC	-	+	+	K+	6	-	-	+	+
RK 38	*Sphingomonas capsulata*	65±0.10	BC	+	+	+	Z+	6	-	-	+	+
RK 1786	*Photorabdus luminescens*	65±0.41	BC	+	+	+	K+	3	-	-	-	+
RK 23	*Bacillus mycoides*	63±0.65	B-D	-	+	+	Z+	5	-	-	+	+
RK 75	*Bacillus cereus*	62.5±0.61	B-D	+	+	+	Z+	6	-	-	+	+
FDG 110	*Bacillus mycoides*	62.5±0.20	B -D	-	+	+	K+	5	-	-	+	+
RK 159	*Plesiomonas shigelloides*	56±0.33	C-F	-	+	+	K+	6	-	-	+	+
RK 574B	*Stenotrophpmonas maltophilia*	53.5±0.12	D-G	-	+	+	Z+	5	-	-	+	+
RK 301	*Bacillus cereus*	52.5±0.20	D-G	+	+	+	K+	5	-	-	+	+
RK 142	*Pseudomonas putida*	48±2.29	F-H	-	+	+	K+	3	-	-	+	+
RK 1395	*Serretia odorifera*	43±0.57	G-J	-	+	-	Z+	6	-	-	+	+
RK 52	*Pseudomonas syringae*	40±1.22	H-L	+	+	+	K+	5	-	-	+	+
RK 1079	*Pseudomonas agarici*	40±1.22	H-L	-	+	+	-	6	+	+	+	+
PM 18	*Pseudomonas chlororaphis*	36±1.96	I-M	+	+	+	K+	5	-	-	+	+
RK 1364	*Varivorax paradoxus*	32.5±1.02	J-N	-	+	-	-	6	-	-	-	+
RK 1094	*Vibrio hollisae*	30±0.41	K-O	+	+	+	Z+	5	-	+	+	+

Table 1. Bioagent bacterial strains' antibiosis and hyperparasitic effects against 1B pathogen, and conventional test results (continued)

Strain	MIS	Hyperparasitic Effect		Conventional Tests								
		ASA [11] (mm)		KOH[2]	K[3]	NH[4]	N[5]	P[6]	L[7]	O[8]	PA[9]	HA[10]
RK 1066	*Dunganella zoogloeoides*	29±0.49	L-P	-	+	-	-	6	-	-	+	+
RK 993	*Brevibacillus centrophor*	28±1.63	M-P	+	+	+	Z+	5	-	-	+	+
RK 1338	*Bacillus cereus*	26±1.14	M-P	-	+	+	Z+	6	-	-	+	+
RK 570A	*Bacillus cereus*	25±0.82	MQ	-	+	+	K+	3	-	-	-	+
RK 54	*Pseudomonas viridiflava*	23.5±1.35	N-R	-	+	+	Z+	5	-	-	+	+
RK 1433	*Paenibacillus macerans*	23±0.98	N-R	-	+	+	Z+	3	-	-	+	+
RK 1036	*Bacillus megaterium*	19±1.14	O-R	-	+	-	Z+	5	-	-	+	+
RK 1223	*Brevibacterium epidermidis*	12.5±0.45	R	-	+	+	K+	6	+	-	+	+
RK 1429	Not determined	70±1.63	AB	-	+	-	Z+	3	+	-	+	+
IA 1	Not determined	67.5±0.61	AB	-	+	+	Z+	6	-	-	+	+
RK 1417	Not determined	65±2.04	BC	-	+	+	Z+	6	-	-	+	+
RK 1416	Not determined	61±1.14	B-E	+	+	+	K+	6	-	-	+	+
RK 1305	Not determined	60±0.41	B-E	-	+	+	K+	6	-	-	+	+
RK 1414	Not determined	50.5±1.27	E-H	-	+	+	K+	6	-	-	+	+
RK 1413	Not determined	46.5±0.86	F-I	-	+	+	K+	6	-	-	+	+
RK 1407	Not determined	40.5±2.0	H-K	-	+	+	Z+	6	-	-	+	+
RK 1458	Not determined	20±0.24	O-R	+	+	+	K+	5	+	-	+	-
RK 1278	Not determined	18±0.98	P-R	+	+	+	Z+	6	-	-	+	+
RK 1102	Not determined	14±0.65	QR	-	+	+	Z+	5	-	-	+	+

1: AIZ: Average inhibition zone (mm), 2: KOH: potassium hydroxide test (+: positive, -: negative), 3: K: catalase test (+: positive, -: negative), 4: NH: Starch hydrolysis (+: positive, -: negative), 5: N: Nitrogen fixation (K: strongly positive, Z: weak positive, -: negative), 6: P: Phosphate solubilization (1: (+++++), 2: (++++), 3: (+++), 4: (++), 5: (+) 6: (-) Phosphate test scale values), 7: L: Levan test (+: pozitif, -: negatif), 8: O: Oxidase testi (+: positive, -: negative), 9: PA: Pectolytic activity (+: positive, -: negative), 10: HA: Hemolytic activity (+: positive -: negative), 11: ASA: Average spreading areas (mm)

3.3. Semi-in vivo biocontrol activity tests results

It was determined that ninety eight of one hundred and six bioagent bacterial strains were found to be effectively caused pectolytic activity in carrot slices and squash fruit. As a result of the semi-*in vivo* biocontrol activity test, eight bioagent strains that did not show pectolytic activity could be evaluated. The statistical analysis results of the bioagents used in the study on the pathogen inhibition of gall formation in carrot slices and squash fruit are given in *Table 2*.

***Table 2*. Semi-*in vivo* biocontrol activity test results**

Application	Carrot Slices Test		Squash Fruit Test	
1B+ RK 1986	1.78±0.47	E	0.26±0.04	H
1B+ RK 570A	2.89±0.82	D	0.35±0.03	G
1B+ RK 1074	3.44±0.99	CD	0.46±0.05	F
1B+ RK 1086	3.78±0.63	B-D	0.59±0.03	E
1B+ RK 1224	3.89±0.92	B-D	0.68±0.03	E
1B+ RK 957	4.12±0.31	BC	0.82±0.05	D
1B+ RK 1364	4.56±0.68	AB	0.98±0.05	C
1B+ RK 1786	4.78±0.63	AB	1.16±0.07	B
1B	5.44±0.83	A	1.61±0.28	A
CV:	0.29		0.12	
LSD:	1.08		0.09	

It was observed that the most effective strain suppressing tumor formation in carrot slices and pumpkin fruit was RK 1986, followed by RK 570A. The image of RK 1986, the most effective strain, is given in *Figure 1*.

3.4. Molecular identification test results of pathogen and bioagent bacteria

DNAs of pathogen and bioagent bacterial strains were purified by PCR amplification and sequence analysis of the purified products was performed. The obtained sequences were compared with the sequence analyses in the GenBank and identified at the species level. The most effective bioagent strain RK 1986 (Genbank Number:

MN967303) was determined to be *Bacillus mojavensis* and 1B pathogen strain (Genbank Number: MN967438) *Rhizobium radiobacter* as a result of a semi-*in vivo* test.

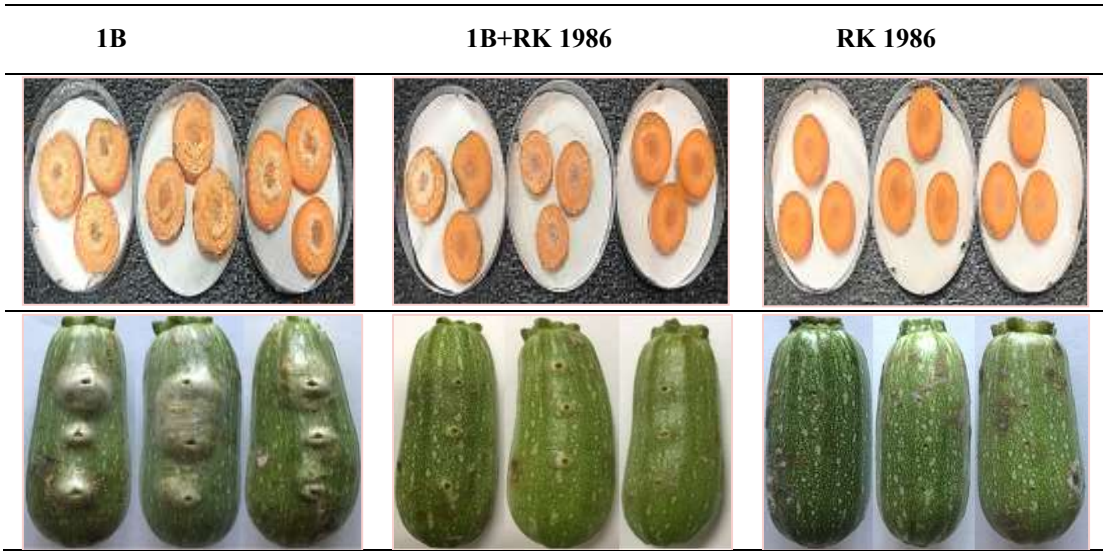

Figure 1. The most effective bioagent bacteria RK 1986 strain view on carrot slices and squash fruits

4. Discussion

The fact that the crown gall disease has spread all over the world has an effective mechanism against the plant defense system and Ti plasmid has made it difficult to control the disease. Biological control, which is an alternative control method, has gained importance due to the inadequacy of the control against the agent (Frikha-Gargouri et al., 2017).

K-84 and K-1026 strains have been used effectively as biological control agents against crown gall for ~40 years. However, since these bioagents are ineffective against some *R. radiobacter* strains, there is a need to identify new biological control agents. In recent years, it has been determined by different researchers that bioagents of different genera (*Bacillus*, *Curtobacterium*, *Pseudomonas*, *Brevibacillus*, *Paenibacillus*, *Serretia*, *Stenotrophomonas*) have been successfully used against crown gall (Dandurishvili et al., 2010; Gupta et al., 2010; Tolba and Soliman, 2013). The result of this study has demonstrated that the obtained data show parallelism with the bacterial species found to be effective by other researchers, and it has been determined that the development of the agent is suppressed *in vitro* and semi-*in vivo* conditions. It has been determined that the most effective bioagent strains belong to the genus *Bacillus* and it has been reported that species belonging to this genus are used effectively against a wide variety of plant diseases (Commare et al., 2002; Kim et al., 2003; Tozlu et al., 2018; Tekiner et al., 2019; Tekiner et al., 2020). This situation is due to ubiquitous, high colonization capabilities, ability to form endospores, and production of lytic enzyme-lipopeptide-antibiotic of *Bacillus* genus (Frandberg and Schnurer, 1994; Jiang et al., 2001; Parke and Gurian-Sherman, 2001; Zhang and Dou, 2002; Schallmey et al., 2004; Hardoim et al., 2008; Kotan et al., 2009; Tiwari and Thakur, 2014). It was determined that it inhibited the development of the pathogen at different levels among strains belonging to the same species in *in vitro* test results.

Researchers have reported that it may be caused by features such as whether the strains are epiphytic or endophytic, their biochemical structures, and their genetic diversity (Araujo et al., 2005; Aktan, 2018). MIS diagnoses of effective bioagent bacteria were supported by conventional test results. Bioagent bacteria directly prevent the development of plant diseases; They use mechanisms such as fixing nitrogen, dissolving phosphate, producing siderophores, competing for carbon sources, and promoting plant hormones (Kotan et al., 2009). In addition to suppressing the development of the disease, some of the effective strains within the scope of the study were found to fixation nitrogen and dissolved phosphate. Different researchers have reported that strains with these characteristics can increase plant growth as well as suppress the development of the disease (De Freitas et al., 1997; Gokce and Kotan, 2016; Mohammadi, 2018; Banerjee et al., 2018).

The strains that were effective as a result of the *in vitro* test were found to be low or highly effective. However, since *in vitro* tests have certain limitations, it is important to support *in vitro* tests with *in vivo* tests (Inam-ul-Haq et al., 2003). For this reason, strains that were effective *in vitro* were tested on carrot and squash fruits. In general, it was determined that the tested bioagent strains were effective semi-*in vivo* and significantly reduced tumor formation. There are other studies in which *in vitro* and *in vivo* tests give consistent results in studies on the biological control of crown gall disease. However, it has been reported in different studies that such a correlation may not always be the case (Gupta et al., 2010; Tolba and Soliman, 2013; Limanska et al., 2015; Bozkurt and Soylu, 2019). In this study, it was determined that the strains that were low effective *in vitro* were found to be highly effective in semi-*in vivo* studies.

5. Conclusions

As a result, the most effective bioagent strain RK 1986 (*B. mojavensis*; antibiosis effect) was a promising candidate in preventing crown gall and was found to be effective against crown gall for the first time. In addition to being the second study in our country for the biological control of crown gall, it is the first study in which the squash fruit test was used. It has been determined that the use of squash fruit is more advantageous than the carrot slice test since it causes symptoms earlier. In this respect, this study is a guide to research. As a future study, it is important to contribute to the literature to determine the disease prevention potentials and mechanisms of action of bioagent strains by testing the effective bioagents on seedlings under greenhouse conditions.

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Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: Tekiner Aydın, N., Kotan, R.; Design: Tekiner Aydın, N.; Data Collection or Processing: Tekiner Aydın, N.; Statistical Analyses: Tekiner Aydın, N.; Literature Search: Tekiner Aydın, N.; Writing, Review and Editing: Tekiner Aydın, N., Kotan, R.

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