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Research Article

BIOCONTROL OF BACTERIAL DISEASES WITH BENEFICIAL BACTERIA IN LETTUCE

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ABSTRACT. In this study it was aimed to investigate the preventive effect of commercial *Bacillus subtilis* strain QST 713, endophytic bacteria isolated from healthy lettuce leaves, and the effect of application time of these treatments against *Pseudomonas cichorii* and *Pseudomonas viridiflava* infections on lettuce. *Bacillus subtilis* strain QST 713 was applied as recommended by the manufacturer. Thirty-two endophytic bacteria were isolated and the antagonistic effect of them was investigated by the disc diffusion method *in vitro*. The best promising strains were selected according to the antagonistic effect against the plant pathogenic bacteria *in vitro* and identified as *Pseudomonas gessardii* and *Bacillus mojavensis* by MALDI-TOF MS. *In vivo* tests were conducted on healthy lettuce plantlets. Statistical analysis revealed that commercial *Bacillus subtilis* strain QST 713 was an effective treatment against both pathogens at almost all application times. *Pseudomonas gessardii* and *Bacillus mojavensis* strains prevented *Pseudomonas viridiflava* infection at 0 and 24h prior application and decrease the infection at all application times. *Bacillus mojavensis* strain was found the most effective treatment at 24h prior application against *Pseudomonas cichorii* infection statistically.

Keywords: *Endophytic bacteria, Pseudomonas cichorii, Pseudomonas viridiflava, Bacillus subtilis QST 713*

INTRODUCTION

Lettuce (*Lactuca sativa*) is a one-year plant and leaves of the plant that are generally used as vegetables. Like all vegetables, lettuce is threatened by many plant pathogens. *Pseudomonas cichorii* (*Pc*) and *Pseudomonas viridiflava* (*Pv*) are important multi-host plant pathogenic bacteria of lettuce. *Pc* causes shiny dark brown, firm, necrotic spots that occur on the inner crop leaves as varnish spot and midrib rot of lettuce [1]. *Pv* is a non-fluorescence member of *P. syringae* group. Necrotic areas on the leaf, stem necrosis, and stem and root rot are the common symptoms of the pathogen on different host plants [2]. Both bacteria were reported on lettuce in Turkey previously [3, 4]. The yield loss can be economically important especially in wet seasons. There is not a successful or remarkable application to prevent or to cure the infection of both pathogens on lettuce.

Treatment with endophytic bacteria (EB), which is derived from plants but does not have any pathogenicity to the plant it is hosted in, is a new approach to combat plant pathogens in recent years. Endophytic bacteria can provide nutrients, promotes growth with the synthesis of plant growth regulators [5], protect plants from environmental stress factors with the synthesis of osmoprotectants and exopolysaccharides [6], supports the plant defense mechanism inducing systemic resistance (ISR) and inhibits some plant pathogens with antimicrobial metabolites [7]. As obtained from different parts of the plants, there are also known commercial formulations. *Bacillus subtilis* strain QST 713

(*Bs*) is one of the registered commercial *Bs* formulations used widely around the world as a bio-formulated pesticide.

The purposes of this study were to observe the preventive effect of commercial *Bs* strain QST 713 and two different EB which were isolated from healthy lettuce leaves in this study and the effect of application time of these beneficial bacteria against two important bacterial pathogens of lettuce, *Pc* and *Pv*.

MATERIAL AND METHODS

Isolation and identification of endophytic bacteria

Healthy lettuce leaves were surface disinfected in 1% NaOCl for 3 min and then rinsed at least three times with sterile distilled water (sdw). The disinfected leaves were dried on sterile filter papers and 3 cm diameters of the leaves were taken. The leaf pieces were ground in sterile extraction bags (Bioreba) which consist of 5ml 0.9% NaCl. The suspension was diluted to 10^4 and 50µl was streaked onto King's B medium [8] by a sterile loop. The Petri dishes were incubated at 28 °C in an incubator and after 48 h colonies were observed under binocular. Fluorescence single colonies were selected and re-streaked onto King's medium B for bacterial purification. The opaque, fuzzy white and irregular edges colonies were selected and re-streaked onto nutrient agar (NA). The selected bacteria were performed on *in vitro* to observe the antagonistic effect against pathogenic *Pc* and *Pv.*

Identification of Inhibitory Effect of Endophytic Bacteria in vitro

Pc. and *Pv.* strains were cultured on nutrient broth (NB) for 48h in a rotary shaker at 160 rpm at 28 °C. The bacterial suspension was transferred to microcentrifuge tubes as 2 ml and was centrifuged at 5 000 rpm for 5 minutes. The pellet was resuspended with 1ml 1X PBS (0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24g/L KH_2PO_4, H_2O, pH 7.4). The bacterial concentration was adjusted on 10^9 CFU/mL with a spectrophotometer at OD: 600 and 100 µl of bacterial suspension was streaked onto NA surface. After 30 min of streaking the pathogenic bacteria, sterile blank disks (Oxoid) were soaked into EB suspensions at 10^9 CFU/ml concentration individually and evenly spaced on the Petri dish surface. The Petri dishes were incubated at 28 °C for 48h. The inhibitory effect was evaluated by transparent zone occurrence and the diameter of the zone around the EB-soaked disks. The diameter of the transparent inhibition zone around the EB colony and the diameter of the EB colony were measured and the antagonistic index value was calculated by proportioning these values to each other [9]. Each endophytic bacterium was tested three times.

EB that had an inhibitory effect *in vitro* screening on *Pc* and *Pv* were identified with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics GmbH, Bremen, Germany) and were analyzed as described by Pavlovic et al [10]. For identification, the bacteria were grown as mentioned below and 18h culture was used as instructed.

Molecular identification by 16S rRNA sequence analysis

Genomic DNA of the selected EB strains which had an inhibitory effect *in vitro* screening on *Pc* and *Pv* was isolated by a EURX GeneMATRIX DNA purification kit

according to the manufacturer's instructions. The concentration of the extracted DNA was measured with a nanodrop (Nano2000, Thermo Fisher), and 20 ng of genomic DNA was used as a template for polymerase chain reaction (PCR). The 16S rRNA gene was amplified for the identification of the strains. The universal 16S rRNA primer pair 63f/1387r [11] was used for PCR. PCR was performed with GoTaq flexi master mix (Promega, Madison, WI), 0.2 µM each primer, and 10 µl of dH_2O, with a final volume of 25µL. The PCRs were conducted with the following steps: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec; and a final extension at 72 °C for 10 min. Aliquots of 5 mL from the PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized under ultraviolet (UV) light after staining the gel with ethidium bromide. 16S rRNA PCR products of selected strains were sequenced from both directions with the primer pair 63f/1396r. DNA sequences were trimmed manually based on the pick quality and aligned, and a consensus sequence was obtained by using MEGA version X. The obtained sequences were blasted and deposited in The National Center for Biotechnology Information (NCBI).

Application of Bs strain QST713 and endophytic bacteria to lettuce

Preventive effect of *Bs* strain QST 713 and selected EB were tested on Romain type lettuce (*Lactuca sativa* var. Romaine) variety Sangio. *Bs* strain QST 713 were mixed in water at the highest labeled rates recommended by the manufacturers. The experiment was conducted in a completely randomized block design and three replicates were used for each treatment. Healthy, 5 weeks old lettuce plantlets were used in the experiment. Selected EB were grown from pure cultures in NB in a rotary shaker at 160 rpm at 28 °C for 24h. The bacterial suspension was prepared as mentioned below. All suspensions were sprayed to the lettuce plantlets by a hand spray and the foliar parts of each plantlet were covered with suspension. The experiment was repeated twice with the same number of repetitions.

Pathogenic *Pc* (G5) and *Pv* (P 5.1) strains were used as inocula which were previously isolated from lettuce. The pathogenicity of the selected strains was recorded as moderate on lettuce from the previous studies (Canik Orel, unpublished). The pathogenic *Pc* and *Pv* inocula were also prepared as mentioned below and were sprayed onto plantlets as 10^6 CFU/ml at 0, 24, 48, 72, and 96 h after foliar application. After the pathogen inoculation, inoculated plantlets were covered with a thin, transparent polyethylene layer, to keep moisture in, which were removed after 24 h. Plantlets were kept in a controlled climate room at 26°C, 50% humidity, and 14h light/10h dark conditions. The results were evaluated 14 days after inoculation (dai). Nine plantlets were used for each application. Sdw only sprayed plantlets were used as healthy control and pathogen only sprayed plantlets were used as infected control. Disease severity was assessed according to a qualitative rating scale as described by Thirthamallappa Lohithaswa [12] with some modifications on a 0-to-4 scale, in which 0: Symptomless, 1: <10% of the leaf area, 2: 10-25 % of the leaf area, 3: 26-49 % of the leaf area, 4: 50-100 % of the leaf area.

Statistical analysis

Obtained results were analyzed statistically by variance analysis (ANOVA) and Tukey's t-test at $P \leq 0.05$ by using the Minitab Statistic Software Version 18.0 (Minitab Inc.).

RESULTS AND DISCUSSION

Isolation and identification of endophytic bacteria

Totally, 32 endophytic bacteria were obtained after isolation from the healthy lettuce leaves (Table 1). Eleven of the isolated bacteria were fluorescence pseudomonads. MALDI-TOF analysis result showed that the most common strains were *Serratia liquifaciens* and *Pseudomonas gessardii* (Table 1).

Table 1. *MALDI-TOF MS analysis of endophytic bacteria obtained from healthy lettuce leaves and the antagonistic index value of the strains against pathogenic Pv and Pc*

MALDI TOF MS identification result	**Strain no**	**Antagonistic index value**	
		P.cichorii	***P. viridiflava***
Serratia liquefaciens	Lt 1	1.2	2.2
	Lt 4	2.2	4
	Lt 5	2.3	6.2
	Lt 6	1.1	-
	Lt 8	-	-
	Lt 9	-	-
	Lt 10	1.1	2
	Lt 12	1.4	4.6
Serratia proteamaculans	Lt 2	1.3	-
	Lt 3	1.4	1.6
	Lt 7	2.3	8
	Lt 11	1.2	-
Pseudomonas gessardii	Lt 13	4.6	3.9
	Lt 14	2.2	1.4
	Lt 15	2.2	1.8
	Lt 16	3.1	2.8
	Lt 17	2.8	2.1
	Lt 18	3.1	2.0
	Lt 19	2.9	1.5
	Lt 20	2.4	2.6
Pseudomonas putida	Lt 22	3.1	2.8
Pseudomonas jessenii	Lt 21	1.1	-
Pseudomonas caricapapayae	Lt 23	-	-
Citrobacter braakii	Lt 28	-	-
Rheinheimera soli	Lt 30	-	-
Bacillus mojavensis	Lt 24	4.0	3.4
Not reliable identification	Lt 25	1.1	-
	Lt 26	1.1	-
	Lt 27	1.4	-
	Lt 29	-	-
	Lt 31	-	-
	Lt 32	-	-

Identification of inhibitory effect of endophytic bacteria in vitro

In vitro inhibitory effect of the strains on pathogenic *Pc* and *Pv* strains were evaluated according to the antagonistic index value which was calculated by proportioning the

diameter of the transparent inhibition zone around the EB colony to the diameter of the EB colony (Table 1). The most inhibitory effects were obtained from fluorescence *Pseudomonas* strain Lt 13 and *Bacillus* strain Lt 24 and were identified by MALDI-TOF MS analysis as *Pseudomonas gessardii* (*Pg*) and *Bacillus mojavensis* (*Bv*), respectively (Figure 1a, b). *Pg* strain LT 13 and *Bm* strain Lt 24 were selected for *in vivo* tests and were applied as the antagonistic EB to confirm the inhibitory effect on lettuce plantlets against pathogenic *Pc* and *Pv* strains.

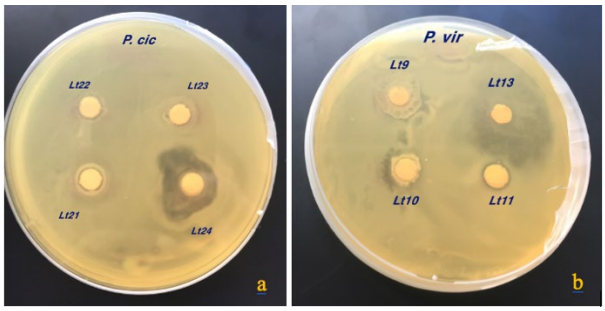

Fig. 1. *The inhibitory zone of different endophytic bacteria obtained from healthy lettuce leaves against Pc (a) and Pv (b) on NA after 24h incubation at 28℃.*

Molecular identification by 16S rRNA sequence analysis

The universal 16S rRNA primer pair 63f/1396r was used for PCR analysis and sequencing of the strains. When the obtained sequences were blasted, strain Lt 13 was found as *P. gessardii* with 99,79% and Lt 24 was found as *B. mojavensis* with 99,39% similarity to the respective reference strains. The sequences were submitted to GenBank under the accession number MT_856830 for Lt 13 and MT_856906 for Lt 24.

Application of Bs strain QST713 and endophytic bacteria to lettuce

The first symptoms on inoculated lettuce plantlets were observed at the 5th dai for both pathogenic bacteria. The effect of treatments and application time were assessed at 14th dai. The statistical analysis showed that there were differences between application time and treatments on *Pc* and *Pv* infections on lettuce (Table 2, Table 3). When the results were evaluated for *Pv* infection (Table 3), *Bs* strain QST 713 was found effective at 0, 24, 48, and 72 h prior applications. All-time applications of Lt 13 showed a preventive effect against *Pv* statistically ($p<0.05$). *Bm* strain Lt 24 was also found effective statistically at all application times ($p<0.05$). The most effective application times of the treatments were 0h and 24h prior applications of Lt13 and Lt 24, and 24h prior application of *Bs* strain QST 713 (Fig. 2).

Fig. 2. *Effect of different treatments on different times against Pv infection on lettuce, a: Lt13 0h, b: Lt 13 24h, c: Lt 24 0h, d: Lt 24 24h, e: Bs QST 713 24h, f: Bs QST 713 96h, g: Disease control, h: Healthy control*

Table 2. Inhibitory effect of Bs strain QST 713, Pg and Bm applications against Pv infection on lettuce plantlets at different times

	0 h	**24 h**	**48 h**	**72 h**	**96 h**
Bs QST 713	0,7±0,0bCD	0,3±0,5bB	0,8±0,0bB	1,2±0,6bB	2,7±0,6aA
Lt 13	0,4±0,6abD	0,0±0,0bB	0,9±0,0aB	0,7±0,6abC	1,0±0,0abB
Lt 24	0,3±0,6aD	0,3±0,5aB	1,0±0,0aB	1,3±0,6aB	0,7±0,6aB
Pv 5.1	3,1±0,0aA	3,1±0,3aA	3,1±0,0aA	3,3±0,6aA	3,0±0,0aA
dH_2O	0.0±0.0aD	0.0±0.0aB	0.0±0.0aC	0.0±0.0aD	0.0±0.0aC

[1] Lowercase letters refer to treatment vs time, [2] Capital letters refer to disease vs time. There is no statistical difference between the same letters following the same column (Tukey's t-test, $P<0.05$)

When the results were evaluated for *Pc* infection (Table 3), *Bs* strain QST 713 was effective at all application times ($p<0.05$). The most effective treatment was found the 24h prior application of *Pg* strain Lt 13 against *Pc* infection. After 24h application, the effect of the strain Lt13 decreased. When *Bm* strain Lt 24 assessed, while it was not effective at the 0h application, it was found effective in the 24 and 48 h prior applications and ineffective in the 72 and 96h to prevent *Pc* infection (Figure 3).

Fig. 3. *Effect of different treatments on different times against Pc infection on lettuce, a: Lt13 24h, b: Lt 24 24h, c: Lt 24 48h, d: Bs QST 713 96h, e: Disease control, f: Healthy control*

Table 3. Inhibitory effect of Bs strain QST 713, Pg, and Bm applications on Pc infection against lettuce plantlets at different times

	0 h	**24 h**	**48 h**	**72 h**	**96 h**
Bs QST 713	0,7±0,5a[1]B[2]	1,0±0,0aB	0,6±0,5aCD	1,3±0,6aB	1,3±0,6aC
Lt 13	0,8±0,4bcB	0,3±0,5cC	2,6±0,5aB	1,8±0,0abB	2,4±0,6aB
Lt 24	2,7±0,5aA	1,0±0,0bB	1,1±0,3bC	2,7±0,6aA	2,9±0,0aAB
***Pc* G5**	3,1±0,3aA	3,0±0,0aA	3,2±0,4aA	3,0±0,0aA	3,0±0,0aA
dH2O	0.0±0.0aC	0.0±0.0aD	0.0±0.0aD	0.0±0.0aC	0.0±0.0aD

[1] Lowercase letters refer to treatment vs time, [2] Capital letters refer to disease vs time. There is no statistical difference between the same letters following the same column (Tukey' t-test, $P<0.05$)

When all treatments and application time data were evaluated together statistically, it can be said that the difference was significant between different treatments and application time (Tukey' t-test, $P<0.05$) to prevent *Pc* and *Pv* infections on lettuce.

Bacterial plant diseases are difficult to be managed with certain pesticides such as fungicides or herbicides in agriculture. Starting with clean propagation material is the most important issue for plant propagation. Since there are plenty of inoculum sources in the environment such as soil, infected plant debris, weeds, vectors, etc. preventive applications are necessary for all crop systems. There are not many studies on bacterial disease management on lettuce crop systems. In this study, the preventive effect of commercial *Bs* strain QST 713 and two different endophytic bacteria against *Pc* and *Pv*, and the effect of application time on the lettuce were investigated.

Glick [13] indicated the widely used of EB in agriculture despite the limited understanding of endophytic bacteria-plant interactions. In this study, commercial *Bacillus* strain *Bs* QST 713 was found as the most effective treatment against both *Pc* and *Pv* infection on lettuce based on the treatments. Similarly, *Bs* strain QST 713 and basic copper sulfate mixture were previously used as an effective treatment on lettuce bacterial leaf spot disease caused by *Xanthomonas campestris* pv. *vitians* [14]. *Bacillus subtilis* strain QST 713 is used widely around the world as bio-fungicide [15]. Our results showed that the preventive effect of *Bs* strain QST 713 strain was not only on fungal diseases but also on bacterial plant diseases.

MALDI-TOF is one of the popular instruments used in biological sciences, due to its rapid and precise identification of genus and species of an extensive range of Gram-negative and -positive bacteria [16]. 16S ribosomal RNA sequences have been used extensively in the classification and identification of *Bacteria* and Archaea [17, 18, 19]. The results of MALDI TOF and 16S rRNA sequence results of the promising EB strains, Lt 13 and Lt 24, were found to be compatible with each other.

Pseudomonads have many members of EB which show an antagonistic effect on different plant pathogens. Especially, fluorescent pseudomonads are known as the antagonistic agents through direct antagonistic effect and/or by inducing plant defense system against many diseases [20]. They have important traits in bacterial fitness such as the ability to adhere to soil particles and the rhizosphere, motility, antibiotic synthesis, and hydrolytic enzyme production. Fluorescent pseudomonads were also reported to grow faster than many organisms and to be more competitive in different environments [21]. Except for *P. caripapayae* strain, all fluorescence strains had different levels of the antagonistic effect against both *Pc* and *Pv* strains *in vitro* tests in this study. *In vivo* studies showed that *P. gessardii* strain Lt 13 was more effective on *Pv* infection than *Pc* infection. This result can be explained that *Pg* strain Lt13 may act more competible on *Pv* from the

beginning to the fifth day of application on lettuce than *Pc*. Although both of the plant pathogens belong to the genus *Pseudomonas*, the durability of the pathogenic traits at the same host can show differences. Pseudomonads divided 19 certain groups according to multilocus sequence analysis that *P. gessardii* belong to group II which was named *P. gessardii* group while *Pv* and *Pc* belong to the group XIX was named *P. syringae* group [22]. *Pv* and *Pc* showed difference within the *P. syringe* group genotypically. This difference might be the reason for the difference in tolerance to the same antagonist at the same host.

Bacillus spp. have also important endophytic members which invade plants by entering through stomata on the leaf surface, triggers ISR that protects non-infected plant parts and accelerate closure of the stomata in response to pathogen attack [23, 24, 25]. In this study, *Bm* strain Lt 24 was found effective on 24 and 48h prior applications. As well as the competitive effect of the bacterial strain, another aspect is, some foliar pathogens invade plants by entering through stomata on the leaf surface. This endophyte-induced priming for enhanced stomatal closure represents yet another structural barrier that can delay disease progression in plants. *B. subtilis* FB17-ISR was previously reported to accelerate closure of the stomata in response to pathogen attack [25]. In this case, it can be said that *Bm* strain Lt 24 can play a competitive role with *Pc* at the plant surface at the first 48h after application or it may cause an effect of stoma closure to prevent the infection as reported previously.

Endophytic bacteria can be applied to the plants by seed treatments, dipping the roots to the bacterial suspension or by foliar applications. Vasudevan et al. [26] also reported that some *Bacillus* spp. could decrease rice leaf blight by seed coating, root applications, and foliar spraying. In this study, all the treatments were applied as the foliar application by spraying to the leaf surface and were found effective to prevent pathogenic *Pc* and *Pv* infections on lettuce. The results of this study showed that foliar applications may provide advantageous on leaf pathogens, such as *Pc* and *Pv* because of the direct action on the pathogen on the leaf surface during or after the leaf inoculation.

CONCLUSION

It is difficult to manage bacterial diseases when the host plant was infected. As a part of plant disease management, application time is as important as the selection of the best treatment for control of the diseases. This study revealed the preventive effect of *Bs* QST 713 strain, and two bacterial endophytes isolated from healthy lettuce plants and the effect of the application time against *Pc* and *Pv* on lettuce. Further studies should be conducted to reveal the effect of these effective components in a mixture of different active ingredients and the effect of the strains on the field conditions.

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