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Profiling of non-pathogenic bacterial population by MALDI-TOF mass spectrometry in stone fruits

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Abstract
The study was carried out to investigate the status of non-pathogenic bacteria isolated from infected plant tissues in stone fruit orchards including almonds, apricots, cherries, mahaleb, olives and plums in Adıyaman, Diyarbakır and Mardin provinces of Turkey. Surveys were performed in the mentioned provinces between March and August in 2019-2021. Survey studies showed that, 87 samples with typical bacterial disease symptoms were collected from 34 different stone fruit orchards. Hypersensitivity (HR) and host pathogenicity tests were performed following isolation from diseased plant tissues in the samples. A total of 70 isolates, which were found to be non-pathogenic with negative HR and host pathogenicity tests, were definitively diagnosed by MALDI-TOF analysis method. Finally, it was specified that bacteria of *Bacillus* and *Pseudomonas* genera were more densely colonized in different tissues of stone fruits. It was concluded that the most concentrated bacteria in the stone fruits was *Stenotrophomonas rhizophila* with 13 isolates, followed by respectively *Bacillus megaterium* with 9 isolates, *Pantoea agglomerans* with 7 isolates, *Bacillus pumilus* with 6 isolates, *Xanthomonas hortorum* with 5 isolates, *Bacillus mojavensis* and *Rahnella aquatilis* with 3 isolates

Keywords
Drupe, MALDI-TOF, Non-pathogenic bacteria, Bacteria population, Agriculture

Introduction

In many countries of the world, especially in Asian and European countries, peach (*Prunus persica* L.), nectarine (*Prunus persica* var. *nucipersica* Schneid.), cherry (*Prunus avium* L.), sour cherry (*Prunus cerasus* L.), apricot (*Prunus armeniaca* L.), plum (*Prunus domestica* L.), olive (*Olea europaea* L.), almond (*Prunus amygdalus* Batsch, syn. *Prunus dulcis* (Miller) DA Webb), cranberry (*Cornus mas* L.), silverberry (*Elaeagnus*) and Mahlep (*Prunus mahaleb* L.) is an important type of stone fruit, except for a few, the others are produced economically. Turkey is the 14th country in the world stone fruit production with 1 985 394 tons on area 14 384 953 decares (da). Southeastern Anatolia Region has an important share in this position with 154 875 tons of stone fruit production in 1 091 852 da area (FAO 2020), (TÜİK 2020). Adıyaman, Diyarbakır and Mardin provinces located in the Southeastern Anatolia Region are important production centers of some stone fruit species, especially almonds and cherries. In many countries, diseases such as fungi, bacteria, viruses, viroids and plant plasmas, which limit the yield and quality of stone fruit species, have been recorded. Considering the limited control possibilities of bacteria among these, it turns out to be of great importance. Some of the bacteria infecting stone fruits can be summarized as bacterial canker and leaf blight of stone fruits (*Pseudomonas syringae* pv. *syringae* van Hall, *Pseudomonas syringae* pv. *morsprunorum* (Wormald) Young), bacterial dieback of peach (*Pseudomonas syringae* pv. *persicae*), bacterial leaf spot of stone fruits (*Xanthomonas arboricola* pv. *pruni*), crown gall (*Rhizobium radiobacter*), leaf scorch of almond (*Xylella fastidiosa*), bacterial canker of olive (*Pseudomonas savastanoi* pv. *savastanoi*), bacterial canker of almond

(*Pseudomonas amygdali*) and European yellows of peach (*Candidatus* Phytoplasma prunorum). The detection of these in stone fruits all over the world goes back to the beginning of the 20th century, but this rate is increasing numerically (Wilson 1953). Bacteria that make disease on stone fruits in Turkey are *Pseudomonas syringae* pv. *syringae* on apricot and *Pseudomonas syringae* pv. *morsprunorum* on cherry was identified about 70 years ago (Bremer 1954). However, over time, additional records were made about the presence, prevalence and damage levels of both these pathogens and new bacteria on stone fruits in different regions (Türkoğlu et al. 1974), (Karaca 1977), (Kavak and Çıtır 1995), (Ogawa et al. 1995), (Kotan et al. 2006), (Kavak and Üstün 2009), (Gormez and Sahin 2012), (Bülbül and Mirik 2014), (Mirik et al. 2016).

Plants interact with bacteria in various ways. This connection is not limited to pathogen and host interaction. There are species of endophyte bacteria that can colonize the inner plant tissue and reproduce without harming the plant, or that can reveal strong plant defense mechanisms (Reinhold-Hurek and Hurek 2011). On the other hand Epiphytic bacteria are in contact with plants in various ways such as increasing frost damage in plants, changing plant growth through exogenous phytohormone production, and being a plant disease agent. In general, non-pathogenic epiphytic bacteria that can multiply on the plant surface may not harm the plant they are on but in some cases, they can be beneficial or harmful in various ways (Kinkel et al. 2000), (Gnanamanickam and Immanuel 2007). It can be found in bacteria that colonize plant wounds and become pathogenic when the plant becomes weak, that can compete with pathogenic bacteria and suppress them or increase their activity. Bacteria colonized in plant tissues where there are symptoms of bacterial disease in stone fruits can be effective in many ways such as competition in the pathogenic bacteria-plant relationship, promoting systemic resistance. In this context it is important to diagnose and reveal their status in terms of control strategies.

There are different methods based on many basics such as protein, fatty acid and biochemical properties for the diagnosis of bacteria. MALDI-TOF MS (Matrix-assisted laser desorption ionization time of flight mass spectrometry) is a protein-based technique widely used in the diagnosis and classification of microorganisms (Ernst et al. 2015). MALDI-TOF MS allows rapid identification of bacteria by comparing mass spectra from bacteria with data from the reference library (Ahmad et al. 2012).

Contrary to the common understanding today, apart from the "pathogen x plant" interaction, the effects of microorganisms that are included in the pathosystem but are not pathogenic are evaluated. In this context, the understanding of the effect of the biotic environment is changing our perspective on struggle. In this study, it was aimed to diagnose by MALDI-TOF MS and reveal the status of non-pathogenic bacteria isolated from bacterial disease symptoms in the phyllosphere of plants in stone fruit orchards in Adıyaman, Diyarbakır and Mardin provinces.

Materials and Methods

Bacterial isolates constituting the material of the study were obtained from trees with symptoms of bacterial disease in stone fruit (almond, apricot, cherry, mahaleb, olive and plum) orchards established in Adıyaman, Diyarbakır and Mardin provinces. Samples were taken from the parts where typical symptoms of bacterial diseases such as bacterial ooze, cancer and galls were observed in different organs of the trees such as the main stem, shoot, bud and flower. Survey studies were carried out in the aforementioned provinces between in 2019-2021, during the period between flowering and harvesting of stone fruits.

Isolation method

Isolation study was applied as soon as possible to the samples which were kept in the cooling unit with ice molds and brought to the laboratory. Firstly, the samples were washed in tap water to remove dust etc. factors have been removed. Surface disinfection was done by applying ethanol (70%) to the dried samples and they were placed in a sterile laminar cabinet to dry. After drying, small pieces including healthy and diseased tissue were taken, crushed with a sterile scalpel on a sterile slide, transferred to Eppendorf tubes containing 1 mililitre (ml) of phosphate buffer solution and waited for 1 hour. Then both the plant parts in the eppendorf tube and the buffer solution containing the plant extract were inoculated into King B medium. The planted petri dishes were incubated at 25 ±2 ˚C with daily observation. Purification of bacterial cultures was performed by inoculating on the same medium from the colonies that developed during incubation (Lelliot and Stead 1987), (Popović et al. 2021).

Hypersensitivity (HR) in Tobacco and Pathogenicity Test

Hypersensitivity (HR) test was applied in order to determine whether the bacterial isolates, which were purified, were plant pathogens. The bacterial cultures to be tested were prepared as a suspension at a density of 10^8-10^9 colony forming units (cfu)/ml or at a concentration of 0.3-0.4 absorbance on spectrophotometer (Ultraviolet (UV) visible, %Transmittance: 60% at 600 nanometers (nm) wavelength). Prepared suspension was injected with a hypodermic syringe needle into the mesophyll of the leaf lamina extending along the edge of the lateral veins of the tobacco, in two replications for each isolate. For this procedure, fine needles with an outer diameter of approximately 0.6 millimeter (mm) and a volume of 2 ml were used. Sterile water was used as negative control, and *Pseudomonas syringae* pv *syringae* and *Pseudomonas syringae* pv *morsprunorum* isolates obtained from Van Yüzüncü Yıl University, which caused typical hypersensitive reaction and were molecularly diagnosed, were used as positive control. The injected leaf laminae were labeled by writing the isolate codes on the adhesive labels. The plants were incubated at 25-28 ˚C and 60-80% relative humidity in climate room conditions. In tobacco plants, a collapse and the appearance of water absorption, which was limited to the place where the inoculum was given within 24 hours, followed by dry and light brown necrosis of the tissue within 72 hours was evaluated as a positive reaction. Yellowing or browning without precipitation was not considered a positive reaction

(Klement et al. 1964).

Host pathogenicity test was applied to bacterial isolates with positive HR test and high probability of being plant pathogen. In this context, 3-year-old almond and 2-year-old apricot, cherry, mahalep, olive and plum plants grown under field conditions were used in the pathogenicity test.

For the pathogenicity test, 1-day (24 hours) fresh bacterial cultures were used, which were cultivated in King B medium. Before cutting, the one-year shoot surface was wiped with 70% ethanol and disinfected. Then with the help of a sterile scalpel, a 1 cm long and 0.5 cm deep part of the shoot was cut and the wound was opened. 1 ml of bacterial culture was taken and applied to the opened wound with a sterile toothpick (Klement et al. 1990). The cut bark part was placed on the inoculated shoot part and a sterile cotton piece moistened with sterile water was wrapped on it. The inoculated area was tightly wrapped with parafilm and labeled. At the end of the 5-6 week incubation period of the isolates, parafilm and sterile cotton were opened and the pathogen reaction was evaluated and recorded. The definitive diagnosis of the isolates with negative HR and Pathogenicity test results was made with the MALDI-TOF mass spectrometer device.

Diagnosis of Bacterial Isolates with MALDI-TOF Mass Spectrometer

Fresh colonies (24-48 hour) of pure bacterial cultures inoculated in KB medium were extracted by ethanol-formic acid method. Each bacterial isolate was taken with the aid of a sterile wooden toothpick and placed directly on the corrugated stainless steel plate and covered with 1 microliter (µl) of HCCA Matrix (α-Cyano-4-hydroxycinnamic acid) solution. Following air drying, samples were analyzed using a Bruker Ultraflex II MALDI-TOF-MS (Bruker Daltonics). The mass spectra of the samples were analyzed with Flex Control Software (Bruker Daltonics GmbH, Bremen, Germany) and their definitive diagnosis was made by matching them with the reference spectrum data in the library (BIOTYPERTM 1.1 software) (Pavlovic et al. 2012), (Kara et al. 2017).

Results and Discussion

In the study, 87 samples with typical bacterial disease symptoms were collected from 34 different stone fruit orchards in Adıyaman, Diyarbakır and Mardin. HR and host pathogenicity tests were applied to the bacterial isolates obtained as a result of isolation and purification studies from the collected samples and isolates with negative results were selected. The highest bacterial isolates were obtained from almond (39) plants and followed by apricot (12), mahaleb (9), cherry (6), olive (3) and plum (1) plants, respectively (Table 1). Although bacterial cultures were obtained mostly from shoots and main stems of stone fruit plants, samples from different plant tissues such as gall, bud, flower and leaf were also obtained albeit in small numbers. Colonization of bacteria in stone fruit plants is controlled by many factors of plant, microorganism and environmental origin. The wounds opened by harvesting, pruning etc. in the shoot and main body of stone fruits provide an opportunity for bacteria that have already adapted to the phyllosphere of the plant to enter the plant and colonize it (Manceau and Kasempour 2003). In this respect the presence of bacteria originating from shoots and main stems may be higher in stone fruits.

The definitive diagnosis of bacterial isolates determined to be non-pathogenic by HR and host pathogenicity tests was made by MALDI-TOF analysis method. As a result of the diagnosis it was determined that the majority of bacterial isolates were included in the genus *Bacillus*. A total of 27 isolates were identified from 7 different *Bacillus* species and *Bacillus megaterium* is the main isolated bacteria with 9 isolates in that genus. Aktan and Soylu (2020) obtained similar results in a study they carried out in Diyarbakır province, where the most isolated bacteria on almond trees was *Bacillus* genus.

Pseudomonas spp. with 6 different species and 9 isolates in total is following that. Although it is not a prominent species in the genus *Pseudomonas*, it is generally isolated from 3 different plants such as almond, apricot and cherry. Many bacterial species in the genus *Pseudomonas* are common in stone fruits, especially in almonds (McGarvey et al. 2014).

Following these, many different types of bacteria such as *Stenotrophomonas*, *Pantoea*, *Xanthomonas*, *Acinetobacter*, *Agromyces*, *Erwinia*, *Ochrobactrum* have been isolated. Among the 21 different bacterial species isolated in the study, *Stenotrophomonas rhizophila* was the most isolated non-pathogenic bacterium from stone fruits with a total of 13 isolates (Figure 1). Many studies have documented that bacteria of the genus *Stenotrophomonas* promote plant growth and are antagonistic to soil-borne pathogens (Berg et al. 1994), (Dunne et al. 1998), (Ryan et al. 2009). It also has plant protective properties against abiotic stress conditions (Alavi et al. 2013). At the same time, the aforementioned bacterium was detected in all of the almond, mahaleb, cherry, olive and plum plants in the study, except for apricot. Abiotic and biotic stress occurring in many tissues with typical bacterial disease symptoms such as shoots, leaves and galls in 5 different stone fruit plants creates a suitable environment for the colonization of *Stenotrophomonas rhizophila*.

Pantoea agglomerans with a total of 7 isolates, on the other hand, is the 3rd bacteria isolated from the stone fruits in the study after *Bacillus megaterium*. Six of 39 bacterial isolates isolated from almonds were diagnosed as *Pantoea agglomerans* and it comes first in the presence of non-pathogenic bacteria in the plant in question. *Pantoea agglomerans* which was isolated from almonds in the study, is present in many plants as epiphytic and endophyte. In addition to stimulating plant growth, there are species that can stimulate gall formation in plants such as gypsum and beets (Barash and Manulis-Sasson 2007). Furthermore, in a study conducted by Marchi et al. (2006); they determined that *Pantoea agglomerans* actively helped to increase the population of *Pseudomonas savastanoi*, which is the factor of bacterial canker of olive, which causes tumor formation in the olive plant in the inoculation region. But in the same study; they concluded that when the population of *Pantoea agglomerans* is high, it suppresses the presence of *Pseudomonas savastanoi* in competition for nutrients and space, possibly through antibiotic production.

Bacillus pumilus with a total of 6 isolates obtained from mahlep, olive and almond, is another bacterial that comes to the fore in stone fruits. Five *Xanthomonas hortorum* isolates were identified from the samples taken from shoot parts of almond and apricot plants. There are strains of *Xanthomonas hortorum* bacteria that cause bacterial blight and spot disease on plants such as carrots, lettuce and tomatoes. In addition, non-primary asymptomatic strains of *Xanthomonas hortorum* were isolated from plants such as peony, lavender, pot marigold and avocado (Costa et al. 2021). In this context, some bacteria such as *Xanthomonas hortorum* can be found in different hosts without showing symptoms or as a weakness parasite.

These bacterial species which are more intensively isolated are respectively followed by *Bacillus mojavensis* and *Rahnella aquatilis* with 3 isolates each, *Bacillus niacini*, *Pseudomonas graminis*, *Bacillus altitudinis*, *Bacillus vallismortis*, *Bacillus subtilis*, *Pseudomonas libanensis*, *Pseudomonas orientalis* with 2 isolates each, *Pseudomonas lutea*, *Acinetobacter lwoffii*, *Erwinia herbicola*, *Agromyces mediolanus*, *Pseudomonas aeruginosa*, *Ochrobactrum intermedium* ve *Pseudomonas cedrina* with one isolates each. The presence of bacterial in different tissues in stone fruits may differ in relation to their interactions with each other and with the plant.

Table 1. MALDI-TOF analysis results of non-pathogenic bacteria obtained from different tissues of stone fruits

No	IP	Diagnosis results	MALDI-TOF similarity index	PTI	No	IP	Diagnosis results	MALDI-TOF similarity index	PTI
1	Almond	*Pseudomonas lutea*	1.950	Shoot	36	Almond	*Bacillus megaterium*	1.922	Shoot
2	Almond	*Bacillus niacini*	1.918	Shoot	37	Almond	*Pseudomonas libanensis*	1.989	Shoot
3	Almond	*Pseudomonas graminis*	2.210	Shoot	38	Almond	*Rahnella aquatilis*	1.857	Shoot
4	Almond	*Pseudomonas graminis*	1.673	Shoot	39	Almond	*Stenotrophomonas rhizophila*	1.893	Shoot
5	Almond	*Bacillus pumilus*	2.059	Shoot	40	Apricot	*Bacillus subtilis*	1.643	Flower
6	Almond	*Bacillus niacini*	1.982	Bud	41	Apricot	*Pseudomonas aeruginosa*	1.400	Shoot
7	Almond	*Bacillus pumilus*	1.966	Bud	42	Apricot	*Ochrobactrum intermedium*	1.368	Shoot
8	Almond	*Bacillus altitudinis*	1.912	Bud	43	Apricot	*Bacillus megaterium*	1.934	Shoot
9	Almond	*Bacillus niacini*	1.485	Shoot	44	Apricot	*Xanthomonas hortorum*	2.299	Shoot
10	Almond	*Xanthomonas hortorum*	2.052	Shoot	45	Apricot	*Bacillus megaterium*	2.219	Shoot
11	Almond	*Acinetobacter lwoffii*	2.097	Shoot	46	Apricot	*Bacillus megaterium*	1.897	Shoot
12	Almond	*Erwinia herbicola*	2.190	Shoot	47	Apricot	*Bacillus megaterium*	2.001	Shoot
13	Almond	*Bacillus pumilus*	2.055	Shoot	48	Apricot	*Pantoea agglomerans*	2.169	Shoot
14	Almond	*Bacillus altitudinis*	1.970	Shoot	49	Apricot	*Bacillus vallismortis*	1.580	Shoot
15	Almond	*Xanthomonas hortorum*	1.88	Shoot	50	Apricot	*Bacillus mojavensis*	1.827	Shoot
16	Almond	*Pantoea agglomerans*	2.083	Shoot	51	Apricot	*Xanthomonas hortorum*	2.246	Shoot
17	Almond	*Pantoea agglomerans*	1.912	Shoot	52	Mahaleb	*Stenotrophomonas rhizophila*	2.063	Shoot
18	Almond	*Pantoea agglomerans*	1.930	Shoot	53	Mahaleb	*Stenotrophomonas rhizophila*	2.093	Shoot
19	Almond	*Pantoea agglomerans*	1.834	Shoot	54	Mahaleb	*Bacillus pumilus*	2.019	Shoot
20	Almond	*Xanthomonas hortorum*	2.121	Shoot	55	Mahaleb	*Stenotrophomonas rhizophila*	2.151	Shoot
21	Almond	*Agromyces mediolanus*	1.445	Shoot	56	Mahaleb	*Rahnella aquatilis*	1.906	Shoot
22	Almond	*Bacillus vallismortis*	1.566	Shoot	57	Mahaleb	*Stenotrophomonas rhizophila*	2.150	Leaf
23	Almond	*Bacillus mojavensis*	1.481	Shoot	58	Mahaleb	*Stenotrophomonas rhizophila*	2.037	Shoot
24	Almond	*Xanthomonas hortorum*	2.122	Shoot	59	Mahaleb	*Stenotrophomonas rhizophila*	2.116	Shoot
25	Almond	*Bacillus megaterium*	1.642	Shoot	60	Mahaleb	*Bacillus pumilus*	2.144	Shoot
26	Almond	*Pantoea agglomerans*	2.140	Shoot	61	Cherry	*Pseudomonas orientalis*	2.130	Shoot
27	Almond	*Pantoea agglomerans*	1.737	Shoot	62	Cherry	*Pseudomonas cedrina*	2.197	Shoot
28	Almond	*Bacillus megaterium*	1.523	Shoot	63	Cherry	*Pseudomonas orientalis*	2.053	Shoot
29	Almond	*Rahnella aquatilis*	2.000	Main stem	64	Cherry	*Stenotrophomonas rhizophila*	2.086	Shoot
30	Almond	*Bacillus megaterium*	1.729	Shoot	65	Cherry	*Stenotrophomonas rhizophila*	2.099	Shoot
31	Almond	*Bacillus mojavensis*	1.838	Shoot	66	Cherry	*Stenotrophomonas rhizophila*	2.225	Shoot
32	Almond	*Bacillus pumilus*	2.224	Shoot	67	Olive	*Stenotrophomonas rhizophila*	2.365	Gall
33	Almond	*Bacillus subtilis*	1.629	Shoot	68	Olive	*Stenotrophomonas rhizophila*	1.835	Gall
34	Almond	*Pseudomonas libanensis*	2.038	Shoot	69	Olive	*Bacillus pumilus*	1.964	Gall
35	Almond	*Bacillus megaterium*	1.925	Main stem	70	Plum	*Stenotrophomonas rhizophila*	2.017	Shoot

IP; plant from which is isolated. PTI; Plant tissue from which it is isolated

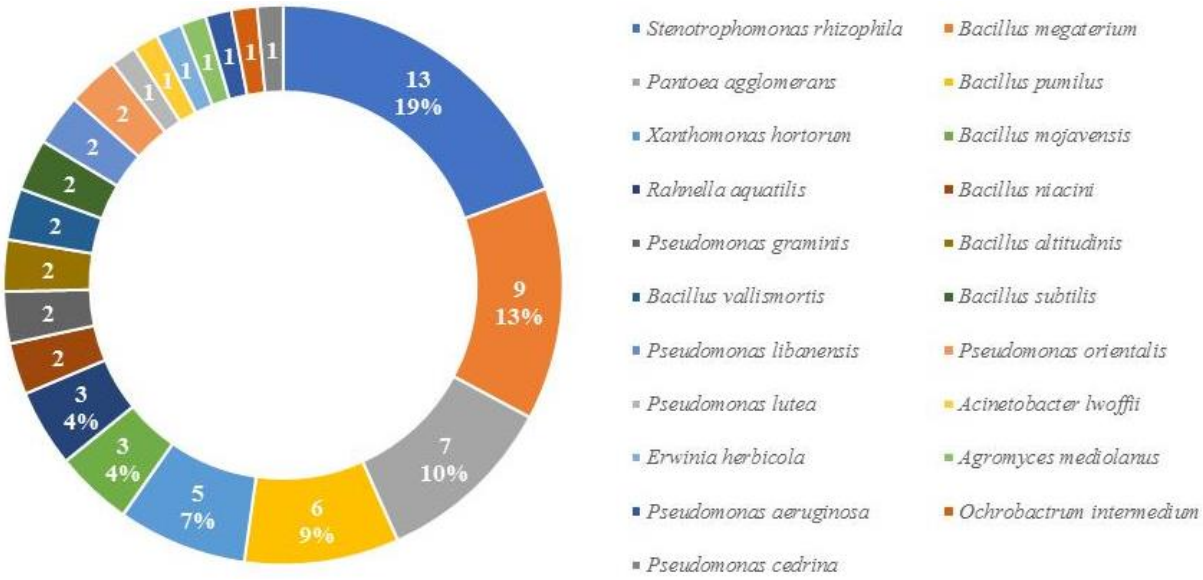

Figure 1. Number of isolates and percent distribution of non-pathogenic bacteria according to MALDI-TOF diagnostic results

Conclusion

Bacillus and *Pseudomonas* genus bacteria came to the fore among the bacteria that were isolated from different tissues of almond, apricot, cherry, mahaleb, olive and plum trees that showed symptoms of bacterial disease and were determined to be non-pathogenic. *Stenotrophomonas rhizophila* bacteria was isolated from all plants of almond, cherry, mahaleb, olive and plum, except apricot. *Stenotrophomonas rhizophila* the most frequently isolated bacteria in the study respectively followed by *Bacillus megaterium*, *Pantoea agglomerans*, *Bacillus pumilus*, *Xanthomonas hortorum*, *Bacillus mojavensis* and *Rahnella aquatilis*. It is important to determine to what extent biotic or abiotic stress conditions affect the presence of *Stenotrophomonas rhizophila* which is intensely isolated from different tissues in stone fruits. How effective the pathogen pressure is in the isolation of bacteria such as *Bacillus* and *Pseudomonas*, which have species that can be used for biological control, can be considered as another research topic. To reveal the effects of differences in plant, bacteria and environment interactions on the presence of bacteria in the plant phyllosphere will be useful to understand the effect mechanisms of bacteria such as *Stenotrophomonas rhizophila*.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal.

All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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