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Role of bacteria isolated from a plant waste-based compost producing bioactive substances in the control of *bacterial spot* syndrome caused by *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

Most of the pathogenic microorganisms that are present in a compost pile are eliminated by various mechanisms of biotic and abiotic origin. Some important biotic factors in this regard are the production of lytic enzymes, antibiotics, siderophores, phenolic acids and other substances of microbial origin that favor the mechanisms of microbial competition in the compost pile. The main objective of this work was to determine the possible biotic factors involved in the suppression of *Xanthomonas campestris* pv. *vesicatoria* throughout a process of composting, and take advantage of this fact to prevent the bacterial spot caused by this pathogenic bacterium in tomato plants artificially infected. So, a collection of bacteria isolated in different stages of a plant waste-based composting was obtained. The strains were *ex situ* tested by *dual cultures* against the growth of *Xanthomonas campestris vesicatoria,* which causes serious damages in *Solanaceae* crop. Once the best strains of the collection were selected in terms of their capacity to inhibit *in vitro* the growth of this phytopathogenic bacterium, a screening was performed to detect the qualitative and quantitative production of siderophores and salicylic acid. A strain identified as *Pseudomonas putida*, producing siderophores and salicylic acid, was selected to test *in vivo* its ability to control bacterial spot disease in tomato seedlings. This strain was able to significantly reduce the effect of the artificially applied phytopathogenic agent by indirect and direct mechanisms, as well as to provoke a stimulating effect on the development of the plant root system.

1. Introduction

Plant diseases are an important threat to many agricultural and horticultural crops. The use of soil fumigants is being restricted in Europe and other areas of the world and, therefore, alternative methods for the plant pathogen control are needed (Termorshuizen et al., 2006). The use of compost to suppress plant diseases has great potential among biological control practices (Noble and Coventry, 2005). Several studies have shown the suppression of numerous plant diseases by the use of organic amendments from heterogenic sources such as agro-industrial waste, fresh plant material, olive mill waste, manure, rubbish and sludge (Ntougias et al., 2008; Suárez-Estrella et al., 2007).

Composting is the biological decomposition of organic matter under controlled and aerobic conditions, into a humus-like stable product called compost that can be used as an organic amendment. Composting is due to the combined activity of a wide variety of microbial populations, and several physical and chemical factors govern the succession of the different communities appearing throughout the process (Moreno and Mormeneo, 2008). The intense microbial activity leads to a rapid increase in temperature during the bioxidative phase of the process, beginning at ambient temperature values and with a microbial community residing in the original organic material. This is the main factor that is considered to eliminate the pathogens inside the compost pile. In fact, the Environmental Protection Agency considers it as a basic criterion to accept an optimum hygienic quality compost (EPA, 2013).

During composting, the bacterial population is mainly responsible for the substrate decomposition and heat generation (López-González et al., 2014). However, other microbial products and activities support the sanitation capacity of the high temperatures inside the composting process. The effect of the antagonistic microbiota involves a variety of actions such as competition for nutrients and ecological niches, microbial production of lytic enzymes and/or antibiotics, fatty acid degradation, parasitism and changes in nutrient availability (Borrero et al., 2006, 2009).

Plant diseases such as bacterial spot disease of tomato caused by *Xanthomonas campestris* pv. *vesicatoria* (Xcv) (Jones et al., 2004), can

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greatly decrease crop production. Bacterial diseases are traditionally managed with copper compounds, although this control is inadequate due to the prevalence of copper-resistant strains and weather conditions that often favour bacterial diseases in the field (Ritchie and Dittapongpitch, 1991). Both in conventional and organic tomato production systems, additional disease management strategies are therefore needed to reduce economic losses caused by bacterial outbreaks (Bardin et al., 2015). Numerous authors have corroborated that compost obtained from heterogeneous vegetable wastes shows important suppressive effects against diseases caused by several bacterial plant pathogens such as *Xanthomonas campestris* pv. *vesicatoria* (Elorrieta et al., 2003; Suárez-Estrella et al., 2014a). These mechanisms can be carried out directly, through the microbial production of antibiotics, lytic enzymes or other antimicrobial compounds, or indirectly through the microbial production of siderophores (molecules that compete for iron), or by activating the plant defence thanks to the production of salicylic acid or other phytohormone-like substances (Adesina et al., 2007; Lucas-García et al., 2004; Rammamoorthy et al., 2001; Ramos-Solano et al., 2008).

Based on the above statements, it would be desirable to find an efficient biological control approach to prevent the destructive effect of this plant pathogen by broad spectrum mechanisms. In this sense, this work focused on the presence and characterization of biopesticide agents against Xcv inside a composting pile based on plant remains. A first series of experiments was carried out based on the detection of the antagonistic effect of the collection of strains against the *in vitro* growth of the phytopathogenic bacterium. As the component of the plant did not intervene in this case (experiments performed in the absence of plants), this was considered a protocol for the detection of control mechanisms directed to the growth of the pathogen (direct control mechanisms). In addition, the most effective biological control agents were evaluated in relation to their ability to produce siderophores and salicylic acid (control indirect mechanisms), as well as stimulate the plant root system and suppress Xcv in artificially infected tomato seedlings.

2. Material and methods

2.1. Composting process

The composting process was carried out using horticultural waste, specifically composed of tomato plants (lacking fruits) which were directly collected after cropping in different greenhouses located at El Ejido (Almería, Spain). Three composting piles were built. Pile dimensions were 3.0 m length × 1.5 m witdh × 1.0 m height. Piles were prepared by mixing plant wastes with pine chips in order to get an appropriate C:N ratio (around 25). The piles were subjected to forced aeration at a rate of 7.5–9.0 L Kg^{-1} every 4 h in order to prevent the oxygen concentration inside the piles decrease below 10%. Piles were turned when the temperature inside them dropped for three consecutive days. The moisture content was initially set between 50 and 55% and it was maintained within this range by watering during turning operations. These management operations were applied during the bioxidative phase that lasted 63 days. After this period, the piles were statically maintened in maturation for an additional period of four months (126 days), so the process lasted for a total of 189 days. Temperature values inside the piles were continuously measured using a Pt 100 temperature probe connected to a data logger. pH, bulk density and electrical conductivity were monitored at sampling times throughtout the whole process. Compost samples were obtained by properly mixing and homogenizing subsamples extracted from nine different locations inside each pile. Samplings were carried out at six different stages of the composting process, according to the thermal values prevailing inside the piles (RM: Raw Material, MES: Mesophile Phase, THER: Thermophile Phase, COOL: Cooling Phase, MAT: Maturation Phase, FP: Final Product).

2.2. Isolation of potential antagonistic microorganisms

Ten grams of compost were suspended in 90 mL of sterile saline solution (0.9% w/v NaCl) and shaken for 30 min at room temperature. Ten-fold serial dilutions were made in sterile saline solution (0.9% w/v Na Cl) and 100 μL of appropriate diluted suspension was spread out in plate count with Nutrient Agar (NA; CM0003,Oxoid Ltd. UK). Bacteria were isolated on NA plates and incubated at 30 °C during 24 h.

After incubation, plates were photographed (Canon EOS 450D camera, lens Zoom EF-S18-55 mmf/3.5–5.6 IS). Each different colonial type (according to size, morphology, pigmentation and texture) in each plate transferred to a new plate with fresh medium. Plates were incubated (same time and temperature as before indicated), checked for purity and stored at 4 °C (working pure cultures) or preserved in cryoballs Cryoinstant (Deltalab, Spain) for long-term conservation. Pure cultures were also photographed and all morphotypes were compared. In addition, microscopic observations, Gram and spore stains, catalase and oxidase tests were performed in order to eliminate repeated isolates.

2.3. Bacterial phytopathogenic agents

A phytopathogenic strain was supplied by Spanish Type Culture Collection (CECT). Bacterial culture of *Xanthomonas campestris* pv. *vesicatoria* CECT 792 (Xcv) was kept in slant on NA at 4 °C.

2.4. Characterization of biopesticides by dual cultures

This first set of experiments was based in the detection of antagonist effect by *in vitro* dual cultures (direct mechanisms). Each potentially antagonistic strain was cultured on Nutrient Broth (NB; CM0001, Oxoid Ltd. UK) for 24 h prior to use. Suppressive effect of the strains towards Xcv was demonstrated using a modified version of the technique described by de Boer et al. (1999). First, 2% water agar (WA) plates were prepared and, after solidification, two 8-mm-diameter steel hollow cylinders were placed equidistantly from the edge of the plate. A second layer of NA was added on the WA plates. Once NA had solidified, the cylinders were removed and two empty wells were obtained. A liquid culture of Xcv was streaked on the plate surface with a sterile swab.

Wells were then filled with 50 μL of the potentially antagonistic bacterial culture to be assayed. After 2 h at room temperature plates were incubated at 30 °C for 48 h and then the plates were observed for clear inhibition zones around the wells. Two replicated plates were used for each antagonist-Xcv combination. Inhibition index (I) was expressed as percentage of Xcv growth inhibition in the presence of the antagonistic strain (Suárez- Estrella et al., 2014b; c). To calculate the Inhibition Index (I), the diameter of the clear zones was taken into account in the presence of potentially antagonistic bacterial cultures, as well as the maximum growth diameter that the phytopathogenic bacterium could present in the absence of antagonism (in this case it would be 90 mm, since the bioassays were carried out in 90 mm Petri dishes).

2.5. Siderophore production: qualitative and quantitative assay

Siderophore production was evaluated by the modified method of Schwyn and Neilands (1987). All glassware was previously acid-washed for 48 h, and then rinsed with distilled water. Bacteria were inoculated in 3 mL of King B broth consisting of g L^{-1}; peptone, 20.0; $MgSO_4$, 1.5; K_2HPO_4, 1.5; and glycerol, 10 mL (pH 7.0). Cultures were incubated at 30 °C during 24 h. Then, cells was removed from the medium by centrifugation (500 g, 5min) and 0.5 mL of supernatant was blended with 0.5 mL of Chrome Azurol S (CAS reagent). The mixture was incubated at room temperature and colour change since blue to orange-brown in 10 min indicated the presence of siderophores.

Siderophore quantification was carried out by the universal assay of Schwyn and Neilands (1987) and expressed as EDTA equivalents. The reaction was started by mixing CAS reagent with the supernatant previously obtained (1:1). Dilutions of EDTA were processed in the same way. The absorbance of the mixture was measured at 630 nm.

2.6. Salicylic acid production: qualitative and quantitative assay

The methodology used for the study of salicylic acid-producing microorganisms was based on the modified Trinder universal test (Trinder, 1954). Bacterial cultures were prepared in King B broth and incubated as mentioned above. After the incubation period, the cultures were centrifuged at 5000 rpm for 15 min and the supernatants were acidified to pH 2 with 2N HCl. Thereafter, 1 mL of the microbial extract was mixed with 1 mL of distilled water and 2 mL of a 0.1% ferric chloride solution. The formation of a purple complex was observed for producers of salicylic acid.

To perform the quantitative test, cultures of salicylic producing-strains were prepared in 20 mL of King B broth at pH 7, incubated for 48 h at 30 °C, and shaked at 150 rpm. After incubation time, the cultures were centrifuged at 5000 rpm for 15 min 5 mL of the supernatant was acidified to pH 2 and then extracted whit ethyl acetate by the protocol described by Visca et al. (1993). Absorbance was measured at 527 nm.

2.7. In vivo suppression of bacterial spot of tomato

On the basis of a restrictive selection, a culture of *Pseudomonas putida* 2082 was prepared in NA medium and incubated at 30 °C for 24 h. After incubation, bacterial biomass was collected in 10 mL of $MgSO_4$, removed from the plate and keep under sterile conditions. The density of this pre-inoculum was checked, obtaining a bacterial count of 10^8 cfu/mL. To obtain the final inoculum, 10 mL of the concentrated biomass was added to 990 mL of tap water just before applying in plant.

Tomato seeds (*Solanum lycopersicum* var. Raf) were germinated at 26 °C in the dark in 77-well trays filled with a steam-sterilized substrate mixture from organic substrate and verrmiculite at a ratio of 3: 1 (v/v).

Greenhouse experiments were performed in duplicate in a randomized experimental design consisting of 3 different treatments, each using 30 plants. Three weeks after seeding, seedlings were transplanted into 10 cm diameter pots with steam sterilized mix. One week later, tomato seedlings were drenched with 25 mL of the potential antagonistic suspension.

After the treatment with the antagonistic strain seedlings allowed to grow for 10 days and then infected with the phytopathogenic agent. Xcv inoculum was obtained from a 48 h old NB culture. Prior to inoculation, leaves of tomato plants were softly injured with a sterile needle and then sprayed over with the Xcv inoculum (10^8 cfu per plant) following the method described by Al-Dahmani et al. (2003). Control plants were sprayed with water.

The control culture consisted of tomato seedling without treatment. Inoculated or non inoculated plants treated with 10 mM MgSO4 buffer served as positive or negative controls, respectively. After inoculations, plants were continuously watered to prevent drying of the substrate. Disease severity was determined every two days, beginning when foliar necrotic symptoms first appeared on tomato plants, based on a symptom severity scale where: 0 = healthy plant; 1 = local necrosis; 2 = vegetative growth deficit; 3 = systemic leaf necrosis; 4 = systemic leaf necrosis and generalized wilting; and 5 = dead plant.

2.8. Promoting root development on tomato seedlings

The effects of bacterial inoculation on plant growth were evaluated as previously described by Santoro et al. (2015). Greenhouse experiments were performed in duplicate for each treatment using a randomized design of 20 plants each: negative control plants (not inoculated) and plants inoculated with the biological control agent. The temperature and dark/light conditions were similar to those used in the experiments cited above.

After collection of the tomato plants from the containers, the roots were rinsed with water to remove the substrate, and the following standard growth parameters were evaluated for each plant: leaf number, stem and root length (cm), dry plant weight (g) and root/stem length ratio.

2.9. Molecular identification of biocontrol agents

The identities of specific isolates were determined based on partial or nearly full length 16S rRNA gene sequence analysis. For genomic DNA extraction, freshly grown colonies were suspended in 500 mL of sterile milliQ water, heated at 97 °C for 5 min and then chilled in an ice bath for 5 min. The 1/10 diluted supernatant was used as template. The amplification was carried out using MyCycler thermal cycler (Bio-Rad, CA, USA). Universal primers were used: 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT- 3′). Protocols for DNA amplification, cleaning and sequencing have been previously published (Jurado et al., 2014).

The forward and reverse sequences were edited, assembled and aligned using the programs Sequence Scanner v1.0 (Applied Biosystem), Reverse Complement (www.bioinformatics.org/sms/rev_comp.html), Clustal X v2.0.11, and MEGA 5 v5.2. The partial or nearly full-length sequences were compared for similar nucleotide sequences with the BLAST search of the National Center of Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Identify utility at the EzTaxon server (www.ezbiocloud.net/eztaxon/identify).

2.10. Statistical analyses

Data obtained were subjected to statistical analysis using Statgraphics Centurion XVII. A multifactorial analysis of variance (ANOVA) and a multiple comparison test (Fisher's Least Significant Difference) were performed to compare mean values for different levels of sampling ($P < 0.05$). To select the most effective strains from a suppressive point of view, scatter plots were performed. To identify groups of interrelated variables, a principal component analysis (PCA) was accomplished.

3. Results and discussion

3.1. Identification and characterization of biopesticides by dual cultures

Antibiosis is an antagonistic process mediated by microbes through specific or non-specific metabolites, lytic enzymes, volatile compounds, antibiotics or other toxic substances (Fravel, 1988). The production of this kind of bioactive substances is very common among compost microbes, and the effect can be detected by inhibition of pathogenic growth in a dual culture test.

More than 600 microbial morphotypes were isolated from different samples, collected from a horticultural waste based-composting process. The methodology used for isolating the collection of bacterial strains tested in this work has been widely explained in section 2.2.

Results derived from *in vitro* antagonism tests using dual culture techniques are next described. Data were subjected to statistical analysis using Statgraphics Centurion XVII (StatPoint, Inc., Virginia) and expressed according to the temporary origin of the strains, that is, days since the beginning of the composting process. Fig. 1 shows the proportion of antagonist bacteria against Xcv in relation with the different thermal phases of the composting process (see section 2.1.). Results evinced a higher significant proportion of antagonist bacteria during the Maturation period, more specifically after the cooling phase of the composting process. On the other hand, the proportion of strains that acted as biological control agents was lower during the composting phase in which higher temperatures were reached (Thermophilic Phase).

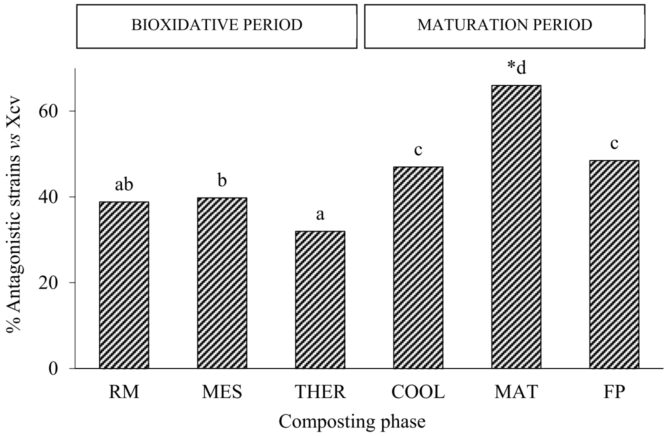

Fig. 1. Proportion of bacterial strains isolated throughout a composting process that were effective against *in vitro* growth of *Xanthomonas campestris pv. vesicatoria* (Xcv). Least Significance Means are indicated in lowercase letter for each phase of the composting process according to Fisher's LSD test at P = 0.05.

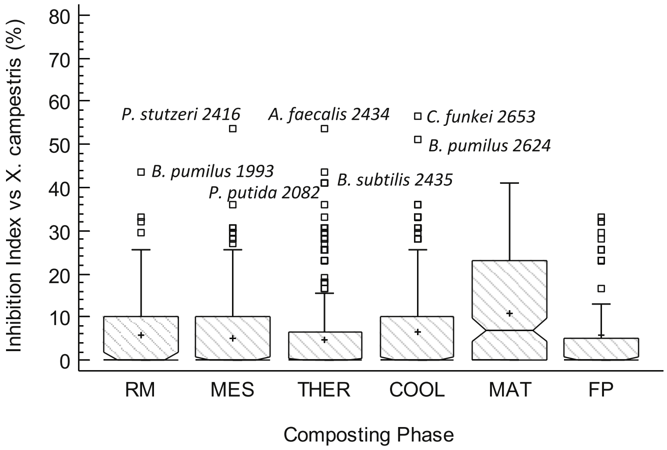

Fig. 2. Box and Whisker plot showing the indices of inhibition from the isolated microbial collection of compost against the growth of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) *in vitro*. Values are indicated as the percentage inhibition of Xcv growth relative to a control plate in the absence of antagonist strains. The most relevant strains are identified at the species level in the graph. All results are averages of 4 repetitions.

In order to select those microbial strains which showed greater potential as control agents, results were arranged in a box and whisker plot (Fig. 2). While Fig. 1 reveal qualitative results derived from de dual cultures (that is, a strain was or not antagonist against Xcv), Fig. 2 shows the quantitative results relative to the strength of the inhibition *versus* Xcv (Inhibition Index, see section 2.4.). The representation of the data using box and whisker plot complemented what has already been described in Fig. 1. That is, the highest average inhibition rates were detected during the cooling and maturation phases of the composting process. However, sporadically, some strains isolated during the bio-oxidative period also showed very high inhibition rates (Fig. 2).

Some of the strains tested were able to inhibit the *in vitro* growth of Xcv at a rating of over 30%. One of the most interesting strains capable affecting the growth of *X. campestris* pv. vesicatoria were identified as *Bacillus pumilus* 1993, *Pseudomonas putida* 2082*, P. stutzeri* 2416, *Alcaligenes faecalis* 2434, *B. subtilis* 2435, *Cellulosimicrobium funkei* 2653 and *B. pumilus* 2624 (Fig. 2). In the last five years some of the benefits of these microbial species have been previously described by other authors (Bernal et al., 2017; Chandrasekaran et al., 2017; Georgieva et al., 2018; Suárez-Estrella et al., 2014b,c).

During the bioxidative stage of the composting process, it is evident that the dynamics of the dominant microbial communities depend on the drastic changes that the temperature undergoes inside the pile. Traditionally it has been considered that the high temperatures generated during the biooxidative phase of the composting process are the main factor involved in the disinfecting capacity of the process. However, as can be seen in terms of the results described so far, a multitude of microorganisms present in the partially or fully composted material compete for available nutrients, produce enzymes, antibiotics, phytohormones, cyanide or other substances that destroy or inhibit other phytopathogenic microorganisms (Wu et al., 2015).

This first set of data highlights the fact that the control of plant pathogens during a composting process could take place not only during the thermophilic stages, but throughout the whole process, occurring continuously even in those phases that govern the mesophilic temperatures.

3.2. Production of agronomic interest substances: siderophores and salycilic acid

It is well known that benefits in a greenhouse due to the application of biocontrol agents would be achieved at three levels: i) niche colonization (Lucas-García et al., 2004), ii) preventing pathogen growth by iron chelation (siderophores), antibiosis and/or enzymatic (Adesina et al., 2007), and iii) inducing systemic resistance, activating plant's defensive mechanisms (Ramos-Solano et al., 2008).

Microbial siderophores are small molecules with high affinity for ferric ions enhancing iron uptake by plants, thus triggering plant defensive responses to biotic and abiotic stresses (Beneduzi et al., 2012). Additionally, iron is one of the limiting factors during competition of nutrients between pathogenic and antagonistic microorganisms causing a decrease in the severity and onset of disease symptoms in plants. In this sense, siderophore producing microorganisms protect plants at two levels, first, limiting growth of pathogenic microorganism and secondly, triggering plant's defensive metabolism (Bevivino et al., 1998; Bernal et al., 2017; Georgieva et al., 2018). Therefore, siderophores produced by this microbiota could play a vital role in nutrient competition among plant pathogens and beneficial microorganisms.

Concerning salicylic acid, it is usually involved in signal transduction pathways directed to the activation of plant acquired systemic resistance; this compound usually is produced in response to a local lesion caused by abiotic stress or by the entry of some phytopathogenic agents (Raskin, 1992). Other authors also argue that, similar to other phenolic-like molecules, salicylic acid has an important antimicrobial effect against numerous plant pathogens (Surendran et al., 2017).

Thanks to *ex situ* experiments performed in this work, it has been checked that an important number of native bacteria isolated from a plant based-composting process are able to produce bioactive substances lead to biological control of plant pathogens (Fig. 3). Results derived from this second set of experiments highlighted the presence of siderophore and salicylic acid producing microorganisms throughout the entire process of composting. In general, the proportion of siderophore producing strains was higher than that of salicylic acid producers. It should be noted that a greater proportion of siderophore producing strains was detected during the bio-oxidative period of the composting process, when higher temperatures were detected (Fig. 3). Several authors support the idea that some microorganisms present in composts excrete siderophores at the medium and could collaborate in inhibiting the growth of phytopathogenic agents present in the raw materials starting (Diánez et al., 2006). Although the proportion of bacteria producing salicylic acid was lower, this microbial group could be detected continuously throughout the entire composting process.

The effectiveness of a biological control agent will not only depend on its potential to produce antimicrobial substances against a specific pathogen (direct mechanisms of action) but also on its ability to compete at the rhizospheric level with other pathogenic or non-pathogenic microbial groups, such as its role as inducer of plant resistance (indirect

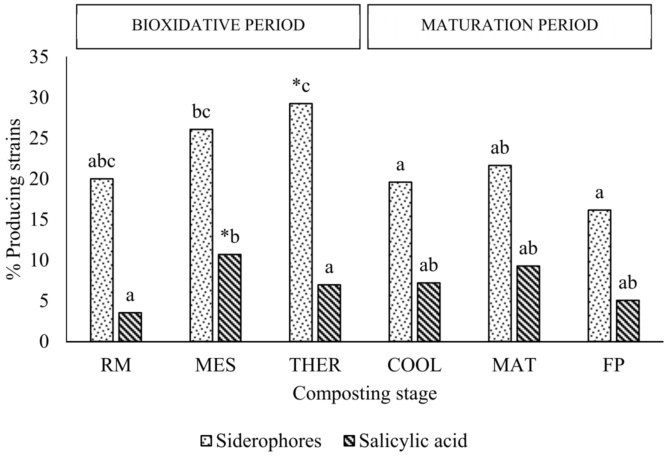

Fig. 3. Proportion of siderophores and salicylic acid producing strains during a composting process of horticultural waste. Least Significance Means are indicated in lowercase letter for each phase of the composting process according to Fisher's LSD test at P = 0.05.

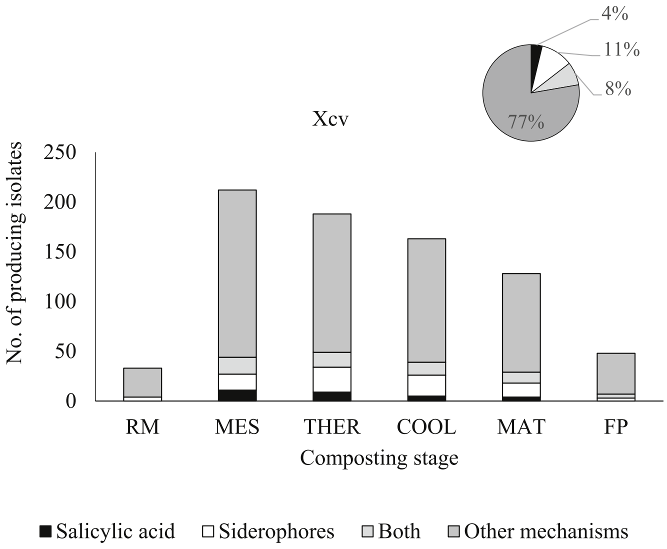

Fig. 4. Number of antagonistic strains against *in vitro* growth of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) that produced siderophores and/or salicylic acid. In the upper right corner, the percentages of antagonist strains producing 1, 2 or none of the indicated bioactive substances are represented.

mechanisms). Therefore, a biological control agent will be more robust, insofar as it is capable of exercising control in both directions. Taking into account this point of view, Fig. 4 includes information on both types of mechanisms. The data shown in Fig. 4 revealed that only 8% of all strains antagonistic to Xcv were capable of producing both siderophores and salicylic acid. Surprisingly, 77% of the antagonistic strains produced neither siderophores nor salicylic acid, but exerted a variable degree of inhibition against Xcv (Fig. 4).

Therefore, it is evident that during the composting process two large groups of mechanisms are produced, both direct and indirect, capable of controlling the growth of Xcv. This fact is reflected in the analysis of principal components shown in Fig. 5. This graph reveals the clear division that exists between the microorganisms isolated from the composting process that can produce siderophores and/or salicylic acid, and those that cause the inhibition of the pathogen directly through the dual culture tests. The complementarity of both types of mechanisms, together with the thermal effect of the process, supports what was indicated above in relation to the sanitizing nature of the composting process.

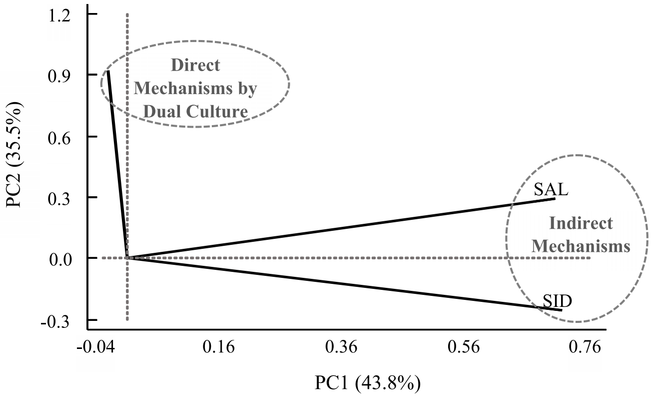

Fig. 5. Results of principal component analysis (PCA) based on the characterization of the microbial collection in relation with the availability to produce siderophores (SID), salicylic acid (SAL) (indirect mechanisms) or inhibition of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) growth by dual cultures test (direct mechanisms).

Table 1
Characterization of biopesticides strains against *Xanthomonas campestris* pv. *vesicatoria* and qualitative production of siderophores and salicylic acid (* indicates an inhibition index greater than 35%; ** indicates an inhibition index greater than 50%).

Code	Identity	%Id Blast	Accession Blast no.	SID[a]	SAL[b]	I *vs* Xcv[c]
1993	*Bacillus pumilus*	98	KC692204.1	–	–	*
2082	*Pseudomonas putida*	100	AB680123.1	+	+	*
2416	*Pseudomonas stutzeri*	99	KC253270.1	–	+	**
2434	*Alcaligenes faecalis*	99	KC199967.1	–	–	**
2435	*Bacillus subtilis*	99	KC990823.1	–	–	*
2449	*Staphylococcus succinus*	99	HQ423378.1	+	–	*
2624	*Bacillus pumilus*	99	KC842270.1	+	–	**
2653	*Cellulosimicrobium funkei*	99	JQ659848.1	+	–	**
2708	*Bacillus stratosphericus*	99	JX680066.1	–	–	*
2788	*Bacillus aerophilus*	98	JX049585.1	+	–	*

[a] SID: Siderophores.
[b] SAL: Salicylic acid.
[c] I *vs* Xcv: Inhibition Index *versus X. campestris* pv. *vesicatoria*.

To select the most interesting biological control agents, an inhibition index greater than 35% was established as the main criterion. Strains listed in Table 1, showed an antagonistic capacity higher than 35% (*) or 50% (**) against Xcv by dual culture tests. Likewise, six of them also displayed capacity to produce siderophores and/or salicylic acid (*P. putida* 2082, *P. stutzeri* 2416, *Staphylococcus succinus* 2449, *Bacillus pumilus* 2624, *Cellulosimicrobium funkei* 2653 and *B. aerophilus* 2788). Thanks to spectrophotometric quantitative analyses the siderophore production was revealed around 600 μM EDTA in the case *P. putida* 2082 and *S. succinus* 2449 (Fig. 6a). Nevertheless, *Bacillus aerophilus* 2788 evinced the lowest siderophore production (see Fig. 6a).

On the other hand, among the strains listed in Table 1, the main producer of salicylic acid was *P. putida* 2082 with a production close to 10 μg/mL of extract, followed by *P. stutzeri* 2416, with less than 4 μg/mL of extract (Fig. 6b). Supporting these results, it has been suggested that salicylic acid is an essential precursor in the synthesis of bacterial siderophores in some strains of *P. putida* (Bakker et al., 2014; Matthijs et al., 2009).

Although the Inhibition Index derived from the dual culture test between *P. putida* and Xcv was lower than that detected for other strains (Table 1), its capacity to produce significant amounts of siderophores and salicylic acid made it the ideal candidate to perform *in vivo* tests on

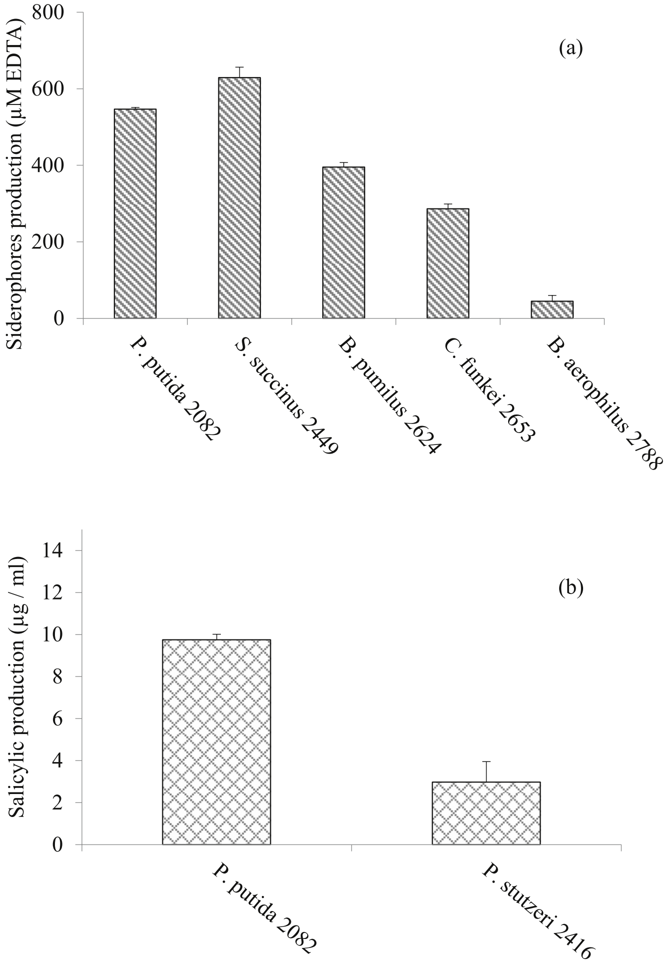

Fig. 6. Quantitative production of (a) siderophores (μM EDTA) and (b) salycilic acid (μg/mL). Black bars indicate error standard values.

artificially infected tomato plants.

3.3. *In vivo suppression of bacterial spot of tomato and promoting root development on tomato seedlings*

Taking into account the above, the potential of the strain *P. putida* 2082 as a biological control agent was evident, both for its direct effect on Xcv growth in dual cultures (inhibition greater than 35%) as well as for its capacity to produce siderophores and salicylic acid. For these reasons, *P. putida* 2082 was selected to perform an *in vivo* test on tomato seedlings artificially infected with Xcv. In view of the results shown in Fig. 7, *P. putida* 2082 could significantly reduce the symptoms of bacterial spot by up to 50% compared to the positive control, in the absence of biological control agent.

Weller et al. (2002) reported that bacterial species belong to *Pseudomonas* are well known for their antagonistic ability against diseases caused by different phytopathogenic agents. These authors linked up this property mainly with the antibiotic production, although Haas and Défago (2005) also pointed out that this effect could be related with the plant resistance induction and root phytostimulation. In fact, plant promoting effects, which were studied and gathered up in Table 2, showed promising results with *P. putida* 2082, regarding the improvement of the plant root system. Both root length and root/stem ratio displayed just about double value than control plants. No significant differences were observed in leaf number and plant dry weight (Table 2).

Multitude of pseudomonas strains have the ability to stimulate seed germination, as well as the development of shoots and roots of different crops, including chickpea, eggplant, soybeans, tomatoes and other horticultural species. Among these strains, the promoting effect of plant growth by *P. putida* has been recognized in numerous studies, not only to reduce the damage caused by phytopathogenic agents but also to improve the growth of infected plants, and to favor the growth of healthy plants in a disease-free environment. Generally, this effect translates into an increase in the weight of roots and shoots of plants inoculated with this strain. Most likely, the growth promotion capacity of some strains of the *Pseudomonas* genus is derived from a synergistic effect, rather than from a single mode of action (Avis et al., 2008; Georgieva et al., 2018; He et al., 2019; Shameer and Prasad, 2018).

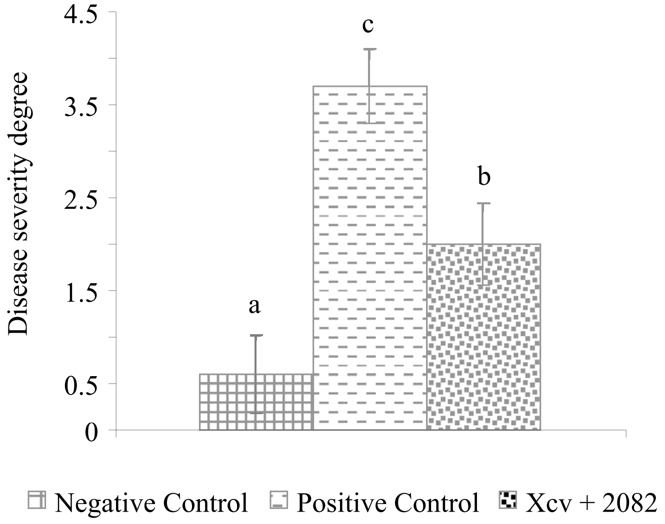

Fig. 7. Suppressive effect of *P. putida* 2082 on bacterial spot severity in tomato seedlings (0: healthy plant; 1: local necrosis; 2: vegetative growth deficit; 3: systemic leaf necrosis; 4: systemic leaf necrosis and generalized wilting; 5: dead plant). Homogenous groups significantly different are indicated with capital letters at a significance level of 95% (Fisher's Least Significant Difference).

4. Conclusions

Bacteria isolated during the composting processes of vegetable waste show a variety of capacities, such as the production of siderophores, salicylic acid, or diverse antimicrobial compounds. Although the high temperatures generated during composting are considered an important factor of sanitation, it seems that the biotic factors mentioned above can significantly influence the elimination or deactivation of harmful agents, such as *Xanthomonas campestris* pv. *vesicatoria,* not only during the composting process, but also after the application of the compost to the crop soils. The results derived from this work show that, among the strains isolated, microorganisms are additionally found to be good promoters of plant growth, which can be considered an additional advantage in the prevention of phytopathogenic infections.

Thanks to this work, we have characterized a group of bacteria isolated from a plant waste-based composting process, in relation to their ability to inhibit the development of phytopathogenic bacteria that cause serious damage in the cultivation of tomato plant, that is the case of *X. campestris* pv. *vesicatoria*. In this work, *bacterial spot* syndrome has shown to be very susceptible to the effect of such biological control agents.

One strain identified as *P. putida* met the necessary criteria to be *in vivo* evaluated as a biocontrol agent in tomato plants. In this case, the biocontrol effect seems to derive from the production of siderophores, salicylic acid and other bioactive substances, which would lead to competition phenomena, induction of plant resistance and antibiosis. In addition, this strain is also an ideal candidate to act as a promoter of plant growth, since it caused a remarkable stimulating effect of root

Table 2

Plant promoting effects of *P. putida* 2082 on tomato seedlings. Homogenous groups significantly different are indicated with capital letters (Fisher's Least Significant Difference).

Treatment	Leaf number	Stem length (cm)	Root length (cm)	Root/Stem Ratio	Plant dry weight (g)
Control	12.80	20.05b	11.79a	0.62a	0.05
P. putida 2082	12.00	15.67a	19.66b	1.35b	0.06
	ns	[a]	[a]	[a]	ns

ns: non-significant differences.

[a] Significant differences at a confidence level of 95%.

growth in tomato seedlings.

Therefore, when establishing biological control phenomena between beneficial agents and phytopathogens, mechanisms of different nature (direct and indirect) must be combined to make the control effect more effective. Therefore, the combination of different control strategies in the same microbial strain makes it a more powerful, competent and robust biological control agent when applied at the rhizospheric level.

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