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Article

Identification of bacteria associated with symptoms in foliage of garlic in two localities of Guanajuato

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Abstract

Pathogenic bacteria are responsible for major impacts on major crops, causing from moderate damage to total loss. In garlic plants grown during the autumn-winter cycle 2016-2017 in two localities, Celaya and Irapuato (state of Guanajuato, Mexico), symptoms of yellow striae were detected on the leaf margin from two months after sowing. This study aimed to isolate and identify the causal agents associated with these symptoms. From 62 foliage samples with characteristic manifestations of the disease in three garlic materials, 74 pure bacterial isolates were obtained and the 13 most representative were selected according to their colonial morphology and were molecularly identified by amplification and sequencing of the 16S ribosomal gene. The following species were identified for the locality of Celaya: *Pantoea agglomerans*, *Bacillus amyloliquefaciens*, *Erwinia persicina*, *Bacillus megaterium* and *Bacillus mojavensis* and for Irapuato: *Bacillus aryabhattai*, *B. amyloliquefaciens*, *B. megaterium* and *Erwinia persicina*. These bacteria turned out to be of phytosanitary importance in the vegetative development and bulb formation phases, as they were recovered from damaged tissue obtained from previously inoculated plants. To the best of our knowledge, this is the first report of the presence of these bacteria related to the symptoms described in garlic crops and to the deterioration of bulbs during postharvest storage.

Keywords:

Allium sativum L., bacteriosis in garlic, molecular identification, pathogenicity.

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Introduction

Currently, approximately 90% of the plants grown worldwide are propagated by seeds; seeds are considered the most important source for the spread of pathogens, which favors early primary infections (Navarrete-Maya *et al*., 2014).

The presence of phytopathogenic bacteria in crops and their associated diseases significantly reduce yield, with a great economic impact. Infections caused by viruses, bacteria, fungi, and other pathogens alter the physiological processes of the plant, including the absorption of water and mineral nutrients by the root system. This caused root necrosis caused by oomycetes *Phytophthora* spp., some *Fusarium* species and the nematodes *Pratylenchus* spp., the translocation of water into the xylem and nutrients absorbed by healthy roots; it distorts meristem activity by the development of cankers on trunks and branches, promotes root tumors and nodulations (eg., anthracnose, necrotic spots, mildews, powdery mildew, and rust) that distort radiation absorption by reducing photosynthetic efficiency and the redistribution of assimilates (eg., carbons, phytoplasmosis, mildew, powdery mildew, rusts, and viruses) (Jiménez, 2017).

In most reports, there is no reference to diseases caused by bacteria because, previously, it was not common to find them affecting fruit and vegetable crops. Nonetheless, climate change, represented by alterations in soil moisture and rainfall, has triggered an increase in the populations of insects that are vectors of viruses and bacteria in addition to increasing the pathogenic capacity of microorganisms that had not been reported (Hawkes *et al*., 2017).

Garlic is one of the vegetables with the highest production in the world (FAO, 2018); its reproduction occurs by selection of bulbs with good field health and a vegetative propagation system is used, which has caused the accumulation of viruses, bacteria, nematodes, and fungi in plants (O'Neill *et al*., 2018).

The plant-pathogen interaction is also influenced by environmental components, including abiotic elements of the soil, water, and air, the microbiota of the soil, plant surfaces, and tissues, as well as the insects that contribute to the dispersion and transmission of pathogens (Jiménez, 2017).

Garlic is susceptible to attack by pathogens that cause diseases in both the foliage and bulbs. The most frequent diseases in garlic are purple spot (*Alternaria porri* Ellis), white rot (*Stromatinia cepivora* Berk.), downy mildew (*Peronospora destructor* Berk.), gray mold (*Botrytis* spp.), southern blight (*Sclerotium rolfsii*), rust (*Puccinia allii* P.), virosis, and a soft rot (bacteriosis), very common in other vegetables, distributed in warm and temperate regions where garlic and onions are grown (Navarro-León *et al*., 2019).

To date, there is no certified commercial garlic seed in Mexico; producers select it from their harvests each production cycle, with a high risk of recycling non-visible pathogenic microorganisms that are transmitted by the bulbil-seed. This research aimed to identify pathogenic bacteria associated with striae formed on one or both leaf edges and tip necrosis in garlic plants during the vegetative development phase, applying morphological and molecular methods.

Materials and methods

Garlic genetic material

Three garlic materials of the Taiwan variety were evaluated: LPM, Tacátzcuaro Sta. Anita, and Incrementos ICA.

Location of experiments and cultivation cycles

The planting of the biological material was carried out in two locations: the experimental field of the National Technological Institute of Mexico, Roque *Campus* in Celaya, Guanajuato, Mexico (ITR, for its initialism in Spanish), on September 29, 2016, and in the Experimental Agricultural Field of the Life Sciences Division of the Irapuato-Salamanca Campus of the University of Guanajuato in Irapuato, Guanajuato, Mexico (DICIVA-CIS-UG, for its initialism in Spanish), on September 26, 2016.

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The ITR is located at 20° 31' 44" north latitude and 100° 48' 54" west longitude, altitude of 1 767 m and has silty clayey and sandy clayey soils with high permeability; its climate varies between semi-dry and semi-warm, with an average rainfall of 575.3 mm per year (Inafed, 2017a). The DICIVA-CIS-UG is located at 101° 34' 09" west longitude, 20° 51' 18" north latitude, altitude of 1 730 m with clayey soil; its climate is semi-warm subhumid, with a rainfall of 800 mm per year (Inafed, 2017b).

Evaluation and collection dates

Samples for the detection of bacteria were taken by sampling garlic foliage at 135 days after planting (dap) in the localities of Irapuato (31 samples) and Celaya (31 samples), for a total of 62. A one-leaf sample was obtained from 10 representative plants for each presumptive symptom of bacteriosis: 1) necrosis at the tip of foliage; 2) striping on one side, St1 and 3) striping on both sides of the leaf, St2, and the sample of an apparently healthy plant (Control). Once registered, they were placed in polyethylene bags and kept refrigerated (4 °C) until processing.

Sample processing

The processing of samples and the diagnosis and identification of the etiological agents of the disease were carried out at the Molecular Biology Laboratory of the ITR, Celaya, Guanajuato, Mexico.

Each foliage sample was washed and placed on sterile filter paper. For the samples that presented visible damage, samples were obtained with 50% healthy tissue and 50% diseased tissue of 0.5 to 1 cm; for the controls, samples were taken from different parts of the leaf. All samples were then treated with 1% sodium hypochlorite for 30 s, 1 min in sterile distilled water, 1 min in 70% ethanol, and 1 min in sterile distilled water, placed in a Petri dish with potato dextrose agar (PDA, difco), and incubated at 28 ± 2 °C for 24-48 h. Subsequently, isolation was carried out in culture medium to obtain each of the pure strains, from which the colonial morphology of the bacteria identified in Celaya and Irapuato, respectively, was described. In samples, the controls did not present pathogen development at 72 h of incubation.

Molecular identification of bacteria

From the pure strains of the isolated bacteria, the DNA was extracted directly (Wizard Genomic DNA Purification Kit, Promega^{MR}, Cat. A1120) according to the manufacturer's protocol. DNA concentration and integrity were verified by UV spectrophotometry at 260 nm on a nanodrop 2000c spectrophotometer (Thermo Scientific^{MR}) and 0.8% agarose gel electrophoresis.

The primers of the PCR reaction were 16S (5' AGAGTTTGATCMTGGC 3') and A20 (5' CCGTCAATTCMTTTGAGTTT 3') for the amplification of a fragment of the 16S rDNA gene (Klindworth *et al*., 2013). The reaction was carried out in a thermal cycler (Life Technologies^{MR} SimpliAmp Thermal Cycler)with an alignment temperature of 56 °C. PCR amplification products were analyzed by 0.8% agarose gel electrophoresis and were photodocumented on a BIO-RAD Gel Doc^{MR} EZ Imager.

Sequencing of PCR-amplified fragments

The sequencing of the amplified fragments was carried out at the National Laboratory of Genomics for Biodiversity (Langebio-CINVESTAV-IPN Irapuato) with the Applied Biosystems^{MR} 3730 equipment using the Taq-FS Dye-terminator cycle-sequencing fluorescence-based method, with the Sanger technique and capillary technology.

The electropherograms and sequences obtained were analyzed with the FinchTV v 1.4.0 program (Geospiza Inc.); the editing was carried out by eliminating the extreme regions with ambiguities in sequence and the sequences obtained in both directions of the complementary

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chains were compared; subsequently, the Blast tool (the basic local alignment search tool: http://blast.ncbi.nlm.nih.gov/) was used for the alignment with the GenBank database of the National Center for Biotechnology Information (NCBI). Once confirmed, the sequences were published to be recorded as isolates obtained in the present research (Table 1) in the NCBI database.

Table 1. Molecular identification of bacteria isolated in foliage of garlic plants (*Allium sativum* L.) in Celaya and Irapuato, autumn-winter 2016-2017.

Sample	Locality	Description	No. accession	Code of the strain
S1	Celaya	*Pantoea agglomerans*	MN699684	ITR01
S3	Celaya	*Bacillus amyloliquefaciens*	MN699685	ITR03
S4	Celaya	*Erwinia persicina*	MN699686	ITR04
S5	Celaya	*Erwinia persicina*	MN699687	ITR05
S6	Celaya	*Erwinia persicina*	MN699688	ITR06
S7	Celaya	*Bacillus megaterium*	MN699689	ITR07
S8	Irapuato	*Bacillus aryabhattai*	MN699690	ITR08
S9	Irapuato	*Bacillus amyloliquefaciens*	MN699691	ITR09
S10	Irapuato	*Bacillus megaterium*	MN699692	ITR10
S11	Irapuato	*Bacillus aryabhattai*	MN699693	ITR11
S12	Irapuato	*Erwinia persicina*	MN699694	ITR12
S13	Irapuato	*Bacillus megaterium*	MN699695	ITR13
S14	Celaya	*Bacillus mojavensis*	MN699696	ITR14

Pathogenicity testing: experimental design

The LPM material was used to carry out the pathogenicity tests, selecting bulbs with a healthy appearance, which were established in greenhouse conditions (temperature of 12 to 14 °C) (Khade *et al*., 2017); they were left to germinate and develop to the four leaf stage; it was verified that they maintained a healthy state until before inoculation with pure strains of the isolates.

Suspensions of 10 000 CFU ml^{-1} corresponding to these isolates were used as inoculum. Of each bacterial suspension, 100 µl, corresponding to 1 000 CFUs, was inoculated with a disposable syringe 2 cm above the neck. The inoculated strains were identified as 1, 2, 3, 6, 8, 9, 10, 11, 12, 13 and 14; a control was also included, to which sterile distilled water was inoculated. The experiment was set up in a completely randomized design with three replications.

Statistical analysis

The pathogenicity data were analyzed by the chi-squared test with 11 degrees of freedom using the following formula:

$$x^2=\sum\frac{(fo-fe)^2}{fe}$$

Where: fo= the observed frequency and fe corresponds to the expected frequency for each inoculated strain.

Results and discussion

Colony morphology in bacterial isolates in Celaya and Irapuato

From the different symptoms (tip, St1 and St2), we obtained a total of 45 pure strains with variable morphology from the locality of Celaya, and from these, seven representative strains were selected for molecular identification. Similarly, we isolated 29 pure strains with different morphology from the Irapuato locality and six representative strains were selected for molecular identification (Table 2 y 3).

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Table 2. Results of colony morphology and molecular identification of bacteria isolated in foliage of garlic (*Allium sativum* L.) plants with symptoms, grown in the open field in Celaya, autumn-winter 2016-2017.

(%) of plants tip* with isolated strains	(%) of plants St1 with isolated strains	(%) of plants St2 with isolated strains	(%) of plants with symptoms of isolated strains, total	Colony morphology color, shape, diameter (mm), elevation, appearance, edge.	Species identified by PCR
15.55	13.33	13.33	42.22	Whitish, circular, 4.5-5, central, mucoid, entire	*Bacillus amyloliquefaciens*
8.88	8.88	13.33	31.11	White, circular, 1-1.5, central, creamy, entire	*Erwinia persicina*
6.66	11.11	2.22	20	White, circular, 3, central, mucoid, smooth	*Bacillus megaterium*
-	2.22	2.22	4.44	Strong yellow, circular, 1.5, slightly central, bright creamy, smooth	*Pantoea agglomerans*
-	2.22	-	2.22	Straw yellow, irregular, 3, convex, aqueous-waxy, undulated	*Bacillus mojavensis*

* = symptom in the tip; St1= striping on one side; St2= striping on two sides in garlic foliage.

Table 3. Results of the colony morphology and molecular identification of the bacteria isolated in three different symptoms of garlic (*Allium sativum* L.) plants in Irapuato, autumn-winter 2016-2017.

(%) of plants tip* with isolated strains	(%) of plants St1 with isolated strains	(%) of plants St2 with isolated strains	(%) of plants symptoms with isolated strains, total	Colony morphology color, shape, diameter (mm), elevation, appearance, edge	Species identified by PCR
6.9	13.79	13.79	34.48	Whitish, circular, 4.5-5, central, mucoid, entire	*Bacillus amyloliquefaciens*
3.45	6.9	13.79	24.14	Straw yellow, circular, 3-5, central, bright creamy, entire	*Bacillus aryabhattai*
10.34	6.9	6.9	24.14	White, circular, 1-1.5, central, creamy, entire	*Erwinia persicina*
10.34	6.9	-	17.24	White, circular, 2-3, central, creamy, entire	*Bacillus megaterium*

*= symptom in the tip; St1= striping on one side; St2= striping on two sides in garlic foliage.

In apparently healthy foliage (asymptomatic), there was no growth of bacterial strains after 72 h of incubation. As a result of the molecular identification, each verified ribosomal sequence (16S) obtained was assigned to an accession number in the NCBI database and registered for open access (Table 1).

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From the pathogenicity tests carried out, it is concluded that the isolated strains correlate with the symptoms identified in the symptomatic plants since the reisolation of the bacteria from the affected tissue allowed the recovery of the same inoculated microorganism, with a chi-square value of 1.33 (critical value= 3.05, $p \leq 0.01$). The sampled plants reached maturity and the bulbs recovered from inoculated plants showed evident damage and lower weight compared to the control.

In the autumn-winter (A-W) cycle 2015-2016, the first symptoms of bacterial disease were observed in the locality of Irapuato. As there is no national record of the damage caused to garlic by the pathogens identified, the results obtained in this research evidence the causality of these bacteria in the damage of garlic crops in Mexico for the first time.

To promote the sprouting of the garlic seedling without negative effects on commercial yields, a temperature of 14 to 17 °C combined with relative humidity of 60% has been established (Burba, 2009). At higher temperatures, the cataphylls that cover the garlic bulbils change their color from mottled to transparent pink when fresh and there is necrosis, which darkens the tissue, and they disintegrate almost completely when the invasion is very severe, mainly of the genus *Bacillus* and some phytopathogenic bacteria (Philip, 2017).

Due to their high water content and availability, most fruits and vegetables display water activity (Aw) values above 0.85 (Badui, 2012), which favor the rapid growth of various microorganisms and the activation of different metabolic functions. When there is not good control of the causative agent during the development of the garlic crop, there is a generalized soft rot in the garlic bulb, called 'bacterial putrefaction'. This process has been previously documented and is related to the attack of bacteria as a causative agent of the disease (Navarrete-Maya *et al*., 2014).

Regarding the pathogenic bacteria identified during this research, in the two study localities, the most frequent bacterial species were *Bacillus amyloliquefaciens* (76.7%), *B. megaterium* (37.24%), *B. aryabhattai* (24.14%), *Erwinia persicina* (55.25%), and *Pantoea agglomerans* (4.44%) (Tables 2 and 3). It has been documented that bacteria are microorganisms that are especially frequent in vegetables and greens that grow in direct contact with the soil, mainly of the genus *Bacillus* and some phytopathogenic bacteria (Philip, 2017).

The species of the genera *Erwinia* and *Pantoea* are limited to living on plant surfaces or to playing a secondary role in various infections in addition to the pathogenic species that produce soft rots, where *E. carotovora* and *E. chrysanthemi* (now *Pectobacterium carotovora* subsp., *carotovora* and *P. chrysanthemi*) stand out, whose importance is increased by the wide range of hosts and their worldwide distribution, as well as by their survival and dispersal mechanisms (Navarrete-Maya *et al*., 2014).

Pectobacterium carotovorum, *Burkholderia cepacia*, and *Pantoea ananatis* have been isolated from bulbs (Navarrete-Maya *et al*., 2014), which coincides with the genus *Pantoea* of a bacterium isolated from symptoms of striping on one side and on two sides in garlic foliage grown in the locality of Celaya, Guanajuato

The development of several species of phytopathogenic bacteria of the genus *Erwinia* produces bacterial wet rot by synthesizing enzymes that act on the pectin existing in the middle lamella of the tissues of these products (Palacio-Bielsa *et al*., 2012). Some of the strains isolated during the present research belong to the genus *Erwinia* and are associated with symptoms of tip and striping on one or both sides, as is the case of *E. persicina* as the causal agent of soft rot of the foliage in both Celaya and Irapuato.

In Spain, the record of new bacteria that affect the main woody and horticultural crops has increased and it is due to the introduction of infected plant material; *Erwinia amylovora* has been reported with serious economic losses in numerous Mediterranean countries (Palacio-Bielsa *et al*., 2012). This bacteriosis could also be considered a threat to agriculture in Mexico. The presence of *Erwinia persicina* in garlic crops in the localities of Irapuato and Celaya correlates with this statement. In garlic foliage samples obtained in Celaya, both individual strains and consortia of two strains were isolated in the collected foliage samples, such as *Bacillus amyloliquefaciens* + *B. megaterium* 8.88% of the samples with symptoms in tip and St1; *B. amyloliquefaciens* + *Erwinia persicina* 8.88% in

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symptoms of tip, St1 and St2; *E. persicina* + *B. megaterium* with 6.66% in symptoms of tip and St1; *B. amyloliquefaciens* + *Pantoea agglomerans* 2.22% in striping on one side (St1) and *Pantoea agglomerans* + *E. persicina* with 2.22% in striping on two sides (St2).

The quality of garlic bulbs from Celaya was acceptable because the problem was controlled by applying chemical bactericides during the development of the crop (gentamicin sulfate and oxytetracycline hydrochloride; kasugamycin and tetracycline), whereas in Irapuato, it was considered a total loss of the harvest due to inadequate management and the presence of re-inoculum in the soil due to the allocation of the same crop lot as the previous year. This could be related to the case of a pathogen reported in southeastern Spain; by determining the possible transmission of bacteria through seeds, the presence of *Erwinia aphidicola* in bean seeds was confirmed, causing a decrease of more than 50% in production; in addition, *Bacillus simplex/Bacillus muralis*, *Pseudomonas mendocina*, *Pseudomonas putida* and *Paenibacillus polymyxa* were also identified (Marín *et al*., 2011).

In 2013, some researchers reported the presence of *Erwinia persicina* in Europe, associated with symptoms of soft pink rot in Chinese-type garlic bulbs collected from Tembleque (Toledo, Spain); 50% of the bulbs showed these symptoms in at least one clove and pink coloration along the bulb. Molecular tests identified *E. persicina* as the causative agent, this being the first report in garlic crops in Europe (Gálvez *et al*., 2015).

These results coincide with those found in Celaya and Irapuato since *Erwinia persicina* was isolated in symptoms of tip and striping on one and two sides in garlic foliage; they also coincide with the symptoms in bulbs during storage and at the time of selecting the seed for sowing. Similar cases are the isolates from Celaya and Irapuato that were identified as *E. persicina*, which caused symptoms of tip, St1, and St2 in garlic foliage.

B. amyloliquefaciens has been considered a biological control agent and source of antibiotics and other secondary metabolites for the biocontrol of plant pathogens; it can inhibit the growth of pathogenic fungi, such as *Fusarium oxysporum*, *Candida albicans*, and *Penicillium citrinum* (Li *et al*., 2016). This species was isolated as a single strain in the foliage of some garlic plants in Irapuato with symptoms in tip, St1 and St2 and was also isolated in Celaya but in combination with other strains, so it does not seem to be the most recommended as a biocontrol agent in garlic crops; however, further testing is needed to determine its behavior both *in vitro* and in the field.

B. megaterium is a species with phytopathological capacity that has been isolated from seeds, soil, and water. Its presence in garlic foliage was associated with symptoms in tip, St1 and St2 in the locality of Celaya, as well as with tip and St1 symptoms in Irapuato. The A12 (BMA12) strain of *B. megaterium* was recently found to stimulate tomato plant growth under saline conditions (Akram *et al*., 2019).

Although some strains of *Bacillus aryabhattai* contribute to zinc solubilization and can be used as bioinoculants for biofertilization and biofortification (Aketi *et al*., 2014), the results in this research were different since *Bacillus aryabhattai* was isolated individually in the locality of Irapuato and its presence was associated with severe damage to garlic in both foliage and bulbs.

The genus *Pantoea* includes several species generally associated with plants, either as epiphytic or phytopathogenic. The species *Pantoea agglomerans* was only isolated in samples from Celaya. It is characterized by the formation of another type of multicellular structure called symplasmata and maintains the ability to form biofilms (Yang *et al*., 2017). During the development of this research, *P. agglomerans* was isolated from foliage in garlic crops with St1 and St2 symptoms associated with *E. persicina* and *B. amyloliquefaciens*, so it is identified as a pathogen and causative agent of bacteriosis.

B. mojavensis is endophytic with broad-spectrum antibacterial properties (Jasim *et al*., 2016); nevertheless, in Celaya, it was isolated individually, causing damage to garlic foliage and bulbs.

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Conclusions

Emerging diseases were found as six bacteria associated with garlic plants with symptoms of striae in foliage on leaves (one or both edges) and on the tip were identified; 15 bacterial isolates were processed, and six species in the vegetative development phase of the crop were molecularly identified: *Bacillus aryabhattai*, *Pantoea agglomerans*, *E. persicina*, *Bacillus amyloliquefaciens*, *B. megaterium*, and *B. mojavensis*; for the above reasons, they are considered to be phytopathogenic agents capable of affecting both garlic foliage and bulb, so they represent a phytosanitary risk for this crop.

The bacterial species identified in Irapuato were *Bacillus aryabhattai*, *Bacillus amyloliquefaciens*, *Bacillus megaterium*, and *Erwinia persicina*. The strains of the pathogenic bacteria isolated were found alone, capable of reducing yield and causing total crop failure. The bacterial species identified in Celaya were *Pantoea agglomerans*, *E. persicina*, *Bacillus amyloliquefaciens*, *B. megaterium*, and *B. mojavensis*. Seventy-one point one one percent were found alone and 28.88% in a consortium of two strains.

They can act alone or in consortium and can cause soft rot in garlic foliage and bulb. Compared to the isolates in Irapuato, only one colony was found in 100% of the samples. It is possible that *B. amyloliquefaciens* is related to the strain of *E. persicina* or to other species of bacteria; nonetheless, it remains a panorama to be explored. The common pathogens isolated in Irapuato and Celaya were *Bacillus amyloliquefaciens*, *Erwinia persicina*, and *Bacillus megaterium*. They were found in consortia of two strains. It was confirmed that the bacteria recovered in plants inoculated with symptoms corresponded to the inoculated strains.

Recommendations

Remove plant debris at the end of the cultivation, turn the soils with a plow so that weathering reduces the populations of bacteria in the coming year, rotate crops from different botanical families, control insects and weeds that are reservoirs of bacteria, reduce nitrogen fertilization in case of the presence of symptoms of bacteriosis, avoid the prolonged presence of free water on plants and soil, use drip irrigation, disinfect tools used in crop management, and apply chemical control to the bulb-seed during storage, at planting, preventively in the seedling stage, and during the development of the entire crop.

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