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Identification of *Pseudomonas viridiflava*, causal agent of onion (*Allium cepa* L.) bulb rot

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ABSTRACT

Objective: To phenotypically and molecularly characterize and identify the causal agent of onion (*Allium cepa* L.) bulb rot in Morelos, Mexico.

Methodology: Fluorescent bacteria from onion bulb tissue with symptoms of rot were isolated; the LOPAT test was used to describe them and subsequently they were identified by partial 16S rRNA gene amplification. Pathogenicity in vegetables and plants was evaluated injecting a suspension with 108 CFU mL^{-1} of the pathogen.

Results: Phenotypic characterization and 16S rRNA nucleotide sequencing showed 100% identity with *Pseudomonas viridiflava* as the causal agent of onion bulb rot. The pathogen caused infection in broccoli (*Brassica oleracea* L.), spring onion (*Allium fistulosum* L.), purple onion (*Allium cepa* L.), cauliflower (*Brassica oleracea* var. *botrytis* L.), leek (*Allium porrum* L.), and carrot (*Daucus carota* L.), as well as in plant species such as jalapeño pepper (*Capsicum annuum* var. *annuum* L.), bean (*Phaseolus vulgaris* L.), and tomato (*Solanum lycopersicum* L.).

Implications: This information is important for agriculture in Mexico. *Pseudomonas viridiflava* is a bacterial pathogen with high potential to infect new hosts. This is the first report of *P. viridiflava* causing onion rot in Mexico.

Conclusions: *Pseudomonas viridiflava* is the causal agent of onion bulb rot in Morelos, Mexico. Other vegetables (such as spring onions and leek) can be potential new hosts in Mexico.

Keywords: pectinolytic bacteria, 16S rRNA, pathogenicity, vegetables.

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INTRODUCTION

Onion (*Allium cepa* L.) is the second most important vegetable crop in Mexico. Its export —mainly to the United States— is valued at 3.5 million dollars per year (TecnoAgro, 2017). *Pseudomonas viridiflava* is a bacterial pathogen with a wide range of hosts; it is genetically included within the *Pseudomonas syringae* species complex which is the most important phytopathogenic bacteria worldwide (Mansfield *et al.*, 2010; Bartoli *et al.*, 2015). *P. viridiflava* populations are characterized by their pectinolytic activity and the lack of oxidase and arginine dihydrolase. This sets it apart from the *P. syringae* species complex, according to the LOPAT determinative test (levana - oxidase - potato rot - arginine dihydrolase - hypersensitivity in tobacco [*Nicotiana tabacum* L.]) (Lelliot and Stead, 1966).

In other countries, *P. viridiflava* can cause rot in bulbs, stems, and fruits in a wide range of hosts. Currently, many of these hosts are crops of economic

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importance in Mexico, for example, vegetables and fruits such as: tomato (*Solanum lycopersicum* L.), pepper (*Capsicum annum* L.), melon (*Cucumis melo* L.) (Al-Karablieh *et al.*, 2017), watermelon (*Citrullus lanatus* L.), carrot (*Daucus carota* L.), lettuce (*Lactuca sativa* L.) (Nuebling *et al.*, 2016), bean (*Phaseolus vulgaris* L.) (González *et al.*, 2003), grapes (*Vitis vinifera* L.) (Goumans and Chatzaki, 1998), and citrus (Beiki *et al.*, 2016).

It also affects ornamental flowering plants, such as chrysanthemums (*Chrysanthemum* sp.) (Goumans and Chatzaki, 1998) and the family Rosaceae (Choi *et al.*, 2020). Although *P. viridiflava* is considered an opportunistic pathogen that can survive as a saprophyte and epiphyte, records indicate that severe epidemics have caused significant economic losses in crops such as melon (*Cucumis melo* L.), tomato (*Solanum lycopersicum* L.), chrysanthemum (*Chrysanthemum* sp.) (Goumans and Chatzaki, 1998), onion (*Allium* sp.) (Gitaitis *et al.*, 1998), celery (*Apium graveolens* L.) (Hunter and Cigna, 1981), carrot (*Daucus carota* L.) (Godfrey and Marshall, 2002), and potato (*Solanum tuberosum* L.) (Macagnan *et al.*, 2007).

There are not many studies about onion bulb rot in Mexico. Bulbs with symptoms of rot —probably caused by a bacterial infection— were observed in field onion crops of the State of Morelos, Mexico. Therefore, the objective was to phenotypically and molecularly characterize and identify the causal agent of onion bulb rot, evaluating the *in vitro* pathogenicity in different vegetables and plants species under greenhouse conditions, as well as the *in vitro* sensitivity of the agent to the bactericide.

MATERIALS AND METHODS

Pathogen isolation

In 2019, Blanca Morelos variety onion bulbs with symptoms of rot were collected in field in the town of Cuautla, Morelos (18° 48' 45" N; 98° 57' 17" W). The tissue samples were disinfected with 1% sodium hypochlorite for 1.0 min and washed three times with sterile distilled water. Tissue pieces (0.5 g) were stirred in 20 mL of saline solution (0.85% NaCl) for 1 h; 20 μL of this suspension were placed in plates with King's B (KB) culture medium and were incubated at 28 °C for 72 h. Five fluorescent colonies from that suspension were isolated in ultraviolet light (25W Transilluminator TFL-40, California, USA); an isolate was selected for the subsequent study from one these colonies which had the same morphological characteristics.

Physiological and biochemical characterization

The fluorescent isolate was characterized for the LOPAT determinative test (Lelliot and Stead, 1966) and as per the protocols described by Schaad *et al.* (2001).

In vitro sensitivity to bactericides

Fifteen commercial bactericides (including four biological products) were evaluated using the dose recommended on the label and a modification of the procedure described by Klančnik *et al.* (2010): 100 μL of a bacterial suspension were inoculated with 10^8 CFU. mL^{-1} in KB medium and evenly distributed on the culture medium surface; then, 0.5-cm wide filter paper discs, previously embedded in the bactericide solution, were placed.

Plates were incubated for 72 h at 28 °C. Sensitivity was determined by the formation of a bacterial growth inhibition halo around the filter paper embedded with the bactericide.

Molecular identification

DNA was obtained from pure colonies using the CTAB method (William and Copeland, 2012). The partial 16S rRNA gene amplification was performed with the 8F:5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R:5'-GGTTACCTTGTTACGACTT-3 primers and under the PCR conditions described by Weisburg (1991). Amplified fragments were sequenced in Macrogene Inc. (Korea); sequences were assembled and edited using the BioEdit Sequence Aligment Editor Software v.7.2.6 (Hall, 2005); the consensus sequence generated was compared with those contained in the National Center for Biotecnology Information (NCBI), with the BLAST nucleotide (dvV5) 2.10.0 option.

Pseudomonas viridiflava pathogenicity

Pathogenicity was evaluated *in vitro* in 15 vegetables and in 8 plants species, under greenhouse conditions. Vegetables were disinfected with soapy water, alcohol (70%), and three washings with sterile distilled water. Inoculation was carried out by 100-μL injection of suspension with 10^8 CFU.mL^{-1}. The treatments were kept in a humidity chamber and incubated at 28 °C for 72 h. Each vegetable was inoculated with three repetitions. The control was inoculated with sterile distilled water. A completely randomized design was used. The plants species were sown in pots with a sterile substrate of agrolite, peat moss, and soil (2:2:1). They were kept in a greenhouse with >60% relative humidity and a 25-30 °C temperature. A BD Plastipak hypodermic syringe was used to inoculate 0.5-mL infiltration of a suspension with 10^8 CFU.mL^{-1} in the abaxial surface of three leaves, which were then kept in a greenhouse for 35 d. Each plant species was inoculated with five repetitions. The control plants were inoculated with sterile distilled water.

RESULTS AND DISCUSSION

Biochemical characterization

The physiological and biochemical characterization of the onion bulb isolated strain showed high similarity to the metabolic profile described in other studies about *P. viridiflava* (Heydari *et al.*, 2012; Sarris *et al.*, 2012). Such characterization is 91% identical to the characteristics of the *P. viridiflava* ATCC 13223 (American Type Culture Collection) reference strain and nine isolates identified as *P. viridiflava* that cause rot in melon (Al-Karabieth *et al.*, 2017) (Table 1).

Using the LOPAT test, the isolated strain from onion produced a fluorescent pigment in KB medium and induced a hypersensitivity reaction in tobacco leaves; it also had negative results for oxidase and arginine dihydrolase; it did not produce levana and caused rot on potato slices.

According to Lelliot and Stead (1966), it was identified as *P. viridiflava* group II of Pseudomonas and had 100% similarity with *P. viridiflava* strain identified in tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.), celery (*Apium graveolens* L.), and amaranth (*Amaranthus blitum* L.) (Al-Karablieh *et al.*, 2017).

Table 1. Physiological and biochemical characterization, pathogenicity, and in vitro sensitivity to bactericides of *Pseudomonas viridiflava* isolated from onion.

Test	***P. viridiflava* onion**	***P. viridiflava* ATCC 13223[1]**	**Pathogenicity**		***In vitro* sensitivity to bactericides**	
			***In vitro* vegetables**		**Antibiotics and coppers**	
Gram stain	–	ND	Garlic (*Allium sativum* L.)	–	Agricultural Cuprimycin (Oxytetracycline hydrochloride)	–
Fluorescence	+	ND	Broccoli (*Brassica oleracea* var. *Italica* Plenck)	+	Bactrol 2X (Streptomycin and Oxytetracycline	–
Levana	–	–	Cambray onion (*Allium fistulosum* L.)	+	Cuprimycin 17 (Streptomycin sulfate)	–
Oxidase	–	–	Purple onion (*Allium cepa* L.)	+	Agricultural Bactrimicin (Oxytetracycline hydrochloride)	–
Potato rot	+	+	Mushroom (*Agaricus campestris* Fr.)	–	Cuprimycin 500 (Streptomycin sulfate, oxytetracycline, and tribasic copper sulfate monohydrate)	–
Arginine dihydrolase	–	–	Cauliflower (*Brassica oleracea* var. *botrytis* L.)	+	Kasumin (Kasugamycin)	–
Tobacco hypersensitivity	+	+	Ginger (*Zingiber officinale* L.)	–	Final Bacter (Gentamicin sulfate and Oxytetracycline hydrochloride)	+
Catalase	+	ND	Prickly pear cactus (*Opuntia ficus indica* L. Miller)	-	Phyton (Copper sulfate pentahydrate)	–
Gelatin hydrolysis	+	+	Pore (*Allium porrum* L.)	+	Copper oxychloride	–
Nitrate reduction	–	+	Carrot (*Daucus carota* L.)	+	**Biological**	
Starch hydrolysis	–	ND	**Greenhouse plant**		Quatz IV (Quaternary ammonium)	+
Oxidative/fermentative	O	O	Amaranth (*Amaranthus hypochondriacus* L.)	–	Bacter Best (Organic compounds)	–
Use of:			Oats (*Avena sativa* L.)	–	Bioxtermin (*Bacillus thuringiensis* var. *kurstaki*)	–
Glucose	+	+	Jalapeño pepper (*Capsicum annuum* var. *annuum* L.)	+	Serenade powder (*Bacillus amyloliquefaciens*)	+
Lactose	+	ND	Bean (*Phaseolus vulgaris* L.)	+	Fungifree (*Bacillus velezensis*)	–
Maltose	+	+	Tomato (*Solanum lycopersicum* L.)	+		
Cellobiose	+	ND	Purple corn (*Zea mays* L.)	–		
Trehalose	+	ND	Cucumber (*Cucumis sativus* L.)	–		
Dulcitol	–	ND	Weath (*Triticum* sp.)	–		
Inositol	+	+				
Sorbitol	+	+				

[1] ATCC (American Type Culture Collection); Source: Al-Karabieth *et al*., 2017; ND=Test not determined; O=Oxidative metabolism.

Molecular identification

The BLAST analysis of the 16S rRNA nucleotide sequencing of the strain isolated from onion had 100% identity with the RM207.1a strain (16S rRNA gene) of *Pseudomonas viridiflava* (accession AY604845.1 4) which infected Arabidopsis thaliana (Figure 1).

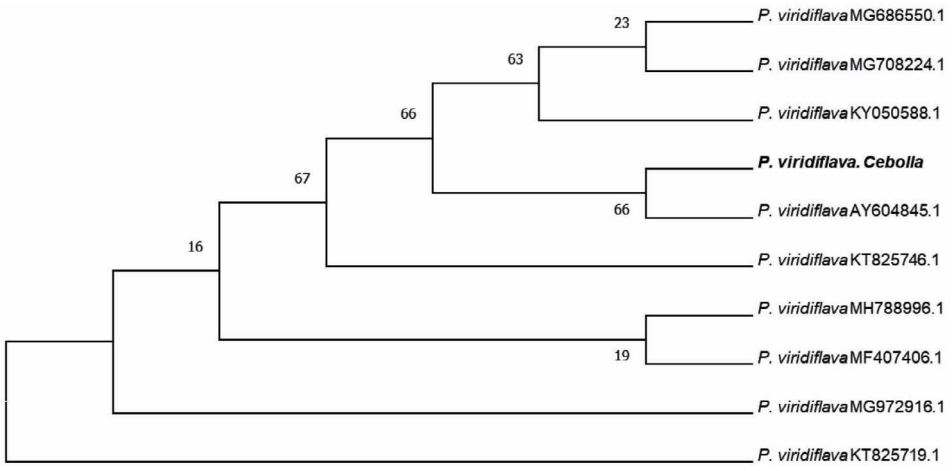

Figure 1. Consensus tree of *Pseudomonas viridiflava* isolated from onion based on partial 16S rRNA gene sequencing, using the Neighbor-Joining (NJ) method. Jukes-Cantor model 500 bootstrap replicates with nine sequences of the gene bank, aligned with the highest similarity index and coverage with Genbank sequences of related bacterial strains.

Other countries have reported significant economic losses as a consequence of the severe onion bulb rot caused by *P. viridiflava* (Gitaitis *et al.*, 1998; Tsuji *et al.*, 2021). In Mexico, *Erwinia chrysanthemi* (de Jesús *et al.*, 2003) and recently *Burkholderia gladioli* (Serret-López *et al.*, 2020) were found to cause rot in onion bulbs. Consequently, this study is the first report of *P. viridiflava* as a causal agent of onion bulb rot in Mexico. P. viridiflava pathogenicity is mainly based on the production of the pectate lyase enzyme —which causes tissue maceration— and the expression of the type III secretion system for the virulence effector production within the host cell (Araki *et al.*, 2007). *P. viridiflava* can survive as an epiphyte on onion leaves; therefore, it is considered as an inoculum source infecting under certain environmental and management conditions. The highest rot severity in onion was associated with epidemics occurring during long rain periods, under excessive fertilization conditions, and with high nitrogen content in leaves (Gitaitis *et al.*, 2003). In the same way, different weed species surrounding onion crops have been identified as the main inoculum source for this pathogen (Gitaitis *et al.*, 1998). *P. viridiflava* infection in citrus leaves was influenced by temperature, humidity, low oxygen concentrations, varietal susceptibility, and pathogen virulence (Beiki *et al.*, 2016).

Pseudomonas viridiflava pathogenicity

The *in vitro* inoculation of *P. viridfilava* isolated from onion in Mexico caused rot in such vegetables as broccoli, spring onions, red onion, cauliflower, leek, and carrot, but not in ginger, mushrooms, garlic, and prickly pear (Table 1). These results are in line with previous reports (Goumans and Chatzaki, 1998); however, based on the bibliography and to our knowledge, this study results provide the first record of experimental rot in spring onions and leek by *P. viridiflava*. *P. viridiflava* caused symptoms of necrosis and foliar chlorosis in jalapeño pepper, tomato, and bean plants, as well as symptoms of plant stunting in beans (Table 1) (Figure 2).

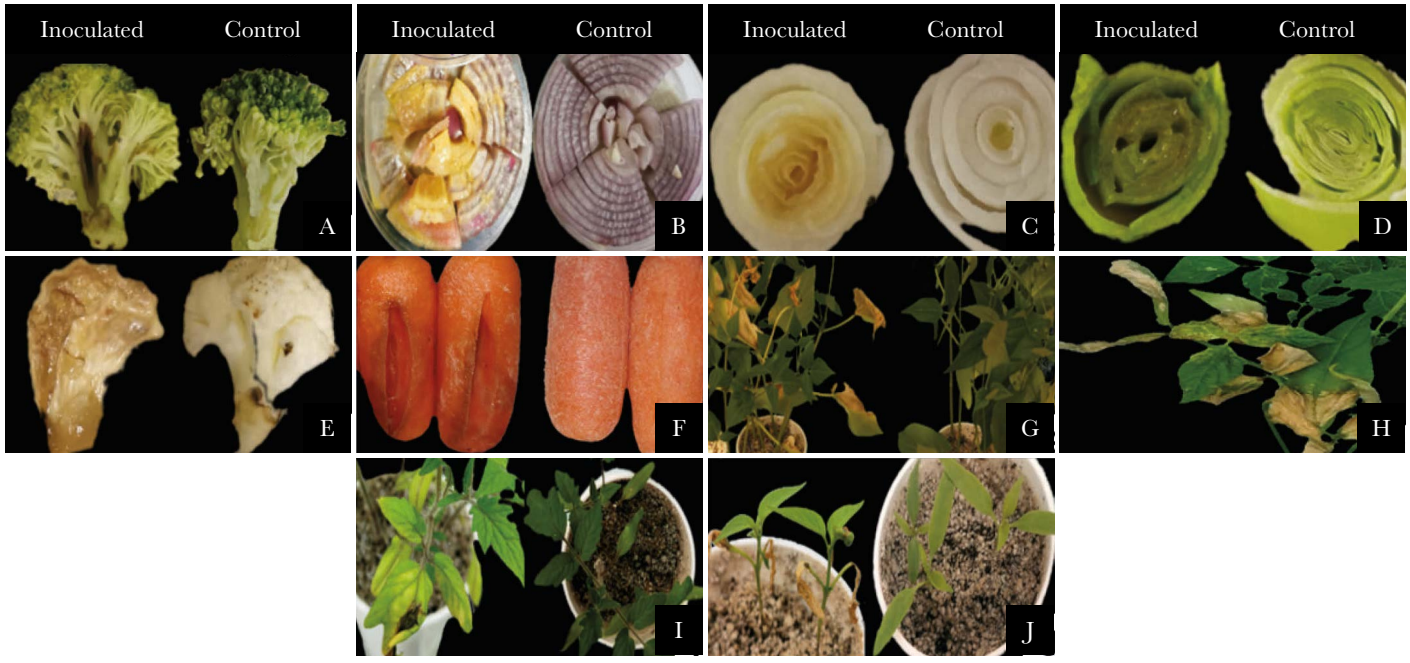

Figure 2. Symptoms caused by *Pseudomonas viridiflava*. *In vitro* rot in vegetables: A) broccoli, B) red onion, C) spring onion, D) leek, E) cauliflower, and F) carrot. Greenhouse plants: G) and H) growth reduction and necrosis in bean leaves, I) necrosis and chlorosis in tomato, and J) necrosis in jalapeño pepper leaves.

The observed symptoms are in line with those reported for these plant species (EPPO, 2019). Growth reduction was observed in infected bean plants; *P. viridiflava* was originally isolated from bean plants with stunting symptoms in Switzerland (Goumans and Chatzaki, 1998). Likewise, stunting symptoms in alfalfa plants (*Medicago sativa* L.) infected with *P. viridiflava* were observed by Heydari *et al.* (2012). *P. viridiflava* pathogenicity and host range varies with the geographic location, plant susceptibility, and pathogen virulence; genetic populations of this pathogen include variants that differ in their proteolytic activity and exopolysaccharide production (Bartoli *et al.*, 2014). No symptoms were developed by vegetables and control plants inoculated with sterile distilled water. From the tissue of infected vegetables and plants, colonies with the same morphological characteristics of *P. viridiflava* were re-isolated in pure culture, fulfilling Koch's postulates. The identity of the inoculated and re-isolated strain was confirmed by PCR, with the amplification and partial 16S rRNA gene sequencing, as well as the above mentioned protocol.

In vitro sensitivity to bactericides

Out of the 14 evaluated bactericides, *P. viridiflava* isolated from onion was sensitive under *in vitro* conditions to Final Bacter (gentamicin + oxytetracycline), Serenade (*Bacillus amyloliquefaciens*), and Quatz IV (quaternary ammonium) (Table 1). The above suggests that this strain has a high resistance capacity to commercial bactericides. Bartoli *et al.* (2014; 2015) identified phenotypes with variable bactericide resistance among *P. viridiflava* strains from different geographic origin and host. However, unlike our results, most strains were susceptible to copper, streptomycin, and oxytetracycline. *B. amyloliquefaciens* has proved

to be effective in the control of *P. viridiflava* in Persian buttercups (*Ranunculus asiaticus*) (Fascella *et al*., 2012); meanwhile, quaternary ammonium is used to disinfect greenhouse facilities and tools.

CONCLUSIONS

Pseudomonas viridiflava is the causal agent of onion rot in Morelos, Mexico. Such vegetables as spring onions and leek may be potential new hosts. *P. viridiflava* isolated from onion in Morelos is resistant under *in vitro* conditions to different antibiotics and coppers. Currently, there is an important legal restriction for the antibiotic application in agriculture; therefore, the use of biological products like Serenade (*B. amyloliquefaciens*) and efficient disinfectants (*e.g.*, quaternary ammonium) should be further evaluated in the field, in order to establish the optimal management of this pathogen.

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