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e-Xtra*

Phylogeny and Identification of *Pantoea* Species Associated with Bulb Rot and Bacterial Leaf Blight of Onion Crops in Uruguay

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Abstract

Onion is among the most consumed vegetables in Uruguay, grown in the northwestern and southern regions of the country. The onion supply presents interannual variations associated with significant postharvest losses, mainly caused by bacterial rots. Besides bulb rotting, onion leaf lesions as well as infections on seed-stalks during seed production may be devastating for some varieties under conducive conditions. This research aimed to identify the causal agents of bulb rots and leaf blight of onion crops in Uruguay. Symptomatic bulbs, seeds-stalks, and leaves were collected from commercial fields from 2015 to 2020. Bacterial colonies were isolated and identified at genera level using physiological tests and 16S rRNA gene sequence analysis. A collection of 59 *Pantoea* spp. isolates was obtained (11 from bulbs and 48 from leaves and seeds-stalks). Multilocus sequence analysis using four housekeeping genes (*rpoB*, *gyrB*, *leuS*, and *fusA*) allowed the assignment of the isolates to five *Pantoea* species: *P. ananatis*, *P. agglomerans*, *P. allii*, *P. eucalypti*, and *P. vagans*. The last two species were not previously reported as onion pathogens elsewhere. The ability to cause disease symptoms was tested by leaf inoculation and red onion scale assays. *P. ananatis* isolates showed the highest aggressiveness in both assays. Specific isolates from *P. allii* (MAI 6022), *P. eucalypti* (MAI 6036), *P. vagans* (MAI 6050), and *Pantoea* sp. (MAI 6049) ranked second in aggressiveness on onion leaves, whereas only three isolates belonging to *P. eucalypti* (MAI 6036 and MAI 6058) and *P. agglomerans* (MAI 6045) exhibited the same scale-clearing phenotype as *P. ananatis*. Leaf inoculation assays were also performed on a set of eight onion cultivars and breeding lines. Overall, *P. ananatis* MAI 6032 showed the highest aggressiveness in all tested cultivars, followed by *P. eucalypti* MAI 6036. The presence of new reported bacterial species leads to complex disease management and highlights the need for further studies on virulence factors and the epidemiology of these pathogens.

Keywords: *Allium cepa*, center rot, *Pantoea* spp., pathogen diversity

The cultivation of onion (*Allium cepa* L.) is among the four main vegetable crops in Uruguay, whether for the acreage, the number of producers, or its gross production value (Rodríguez et al. 2017). This relevance is also observed on a worldwide scale (FAOSTAT 2018). Onion is grown in the southern farming region of Uruguay, mainly for postharvest conservation, and 500 km away in the northwestern region, mainly for early harvest and fresh consumption (Ackermann et al. 2014).

Onion supply presents significant interannual variations in Uruguay, mainly associated with losses during postharvest conservation as a result of bacterial bulb rotting under the humid climate of the rainy "pampas." Besides bulb rotting, onion leaf lesions and infections on seed-stalks during seed production may be devastating for some varieties under conducive conditions. The problem is roughly called "bacteriosis," with scant background information on the pathogens involved (Arboleya et al. 2013). Using classical physiological techniques, Alonso and Riva (1999) identified the genera *Pseudomonas* spp. and *Erwinia* spp. as the main causes of postharvest rots, although many taxa belonging to these genera have been renamed respectively as *Burkholderia* (Yabuuchi et al. 1992) and *Pantoea* (Gavini et al. 1989). In addition, *Pseudomonas viridiflava* was identified as a pathogen causing leaf spots in Uruguay (Pérez et al. 2004). These determinations were based on classical techniques and did not involve currently available molecular tools.

Worldwide, many organisms causing onion bacteriosis have been reported as belonging to *Pantoea*, *Burkholderia*, *Enterobacter*, and *Pseudomonas* (du Toit et al. 2016; Schwartz and Mohan 2008). The genus *Pantoea* covers a diverse group of yellow-pigmented, rod-shaped, gram-negative bacteria in the Erwiniaceae family (Adeolu et al. 2016). Several strains within this group were recognized as plant pathogens causing galls, wilting, soft rot, and necrosis in a variety of agriculturally relevant plants (Walterson and Stavrinides 2015). Onion leaf blight and bulb rot were reported as caused by five known *Pantoea* species; namely, *P. agglomerans* (*Erwinia herbicola*) (Edens et al. 2006; Hattingh and Walters 1981); *P. ananatis* (Gitaitis et al. 2002; Gitaitis and Gay 1997); *P. allii* (Brady et al. 2011); *P. dispersa* (Chang et al. 2018), and *P. stewartii* subsp. *indologenes* (Stumpf et al. 2018). Disease symptoms caused by these five species cannot be easily distinguished. Initially, lesions appear as small water-soaked spots that rapidly develop into bleached-white streaks that often extend along the affected leaf. These streaks turn necrotic and blighted as the disease progresses. Leaf infections may progress down the leaves and the neck, resulting in invasion of the bulb. As bulb tissues become diseased and discolored, they can be colonized by secondary soft rot pathogens that liquefy the interior and produce fetid odors (Gitaitis et al. 2002; Schwartz and Mohan 2008).

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Center rot of onion, caused by *P. ananatis*, was first reported in Georgia in 1997 from symptomatic onion foliage (Gitaitis and Gay 1997). Thereafter, it was reported in onion-growing areas of Colorado, Michigan, and New York (Carr et al. 2010; Gitaitis et al. 2002; Schwartz and Otto 2000). Walcott et al. (2002) suggested that the pathogen was possibly introduced on infested seed lots from South Africa (Goszczynska et al. 2006). A similar disease caused by *P. agglomerans* (*E. herbicola*) was reported in South Africa in 1981 (Hattingh and Walters 1981) and many years later in the United States (Edens et al. 2006).

Another species causing center rot of onion is *P. allii*. Initially, the bacterial strains pathogenic on onion were tentatively identified as members of the genus *Pantoea* based on phenotypic tests. However, after genotypic characterization, including sequencing of 16S rRNA and housekeeping genes (*gyrB*, *rpoB*, *infB*, and *atpD*) among other assays, the strains were identified as a novel species named *P. allii* (Brady et al. 2011). Using the same phylogenetic analyses, Stumpf et al. (2018) reported the inclusion of *P. stewartii* subsp. *indologenes* as the fourth member in the center rot complex of onion. Finally, the fifth member in this complex was *P. dispersa*, assigned after identification of isolates causing bulb decay in Taiwan (Chang et al. 2018).

As crop technology improves the control on onion yield determinants, the focus of research points to new constraints. This is the case for leaf and bulb bacteriosis of uncertain origin affecting onion crops in Uruguay. There is a need to generate knowledge about which bacterial species cause bacteriosis in Uruguay. A better understanding of the biology, epidemiology, and distribution of these species will facilitate better management practices for center rot of onion. The goals of this research were to isolate, characterize, and phylogenetically identify *Pantoea* species causing bulb rot and leaf stripes in Uruguay and to evaluate their virulence on a set of locally spread onion cultivars.

Materials and Methods

Sample collection and bacterial isolation. Symptomatic bulbs, leaves, and seeds-stalks were collected from commercial crops and experimental stations fields from 2015 to 2020 (Fig. 1). The processing, isolation, and identification of the bacterial cultures were carried out following the procedure suggested by Schaad et al. (2001). Pieces of tissue from the disease progression zone were removed with a sterile scalpel, surface sterilized with hypochlorite at 1% for 1 min, rinsed in sterile water, and macerated in Eppendorf tubes with 1 ml of sterile water. Then, serial dilutions of the extract were plated on nutrient broth yeast (NBY) agar medium and incubated at 28°C for 2 days. Reisolation of representative colonies was carried out until pure cultures were obtained from the analyzed samples. Isolates were grown in nutrient broth for 24 h at 28°C and stored in 10% glycerol at –70°C.

Preliminary identification of isolates. All isolates were characterized by colony morphology, pigment production, cell morphology, Gram stain, oxidase reaction, catalase reaction, and Hugh-Leifson oxidation/fermentation test, according to methods described by Fahy and Hayward (1983).

DNA extraction. Genomic DNA of each isolate was extracted from 2 ml of overnight cultures in NBY incubated at 28°C and 150 rpm (Ausubel et al. 2003). DNA extracts were visualized by electrophoresis with 1% (wt/vol) agarose gel in 0.5× Tris-Borate-EDTA (TBE) stained with GoodView nucleic acid stain (SBS Genetech Co., Beijing, China). After quantification of DNA samples with Nanodrop ND-100 (Nanodrop Technologies), final DNA concentrations were adjusted to 50 ng μl^{-1} and stored at –20°C before use.

16S rRNA gene amplification and sequencing. Preliminary identification of isolates to genus level was performed by PCR using the universal primer pair 27F/1492R (Lane 1991) targeting the 16S rRNA gene. Primer sequences and PCR cycles used for amplification are shown in Table 1. DNA amplifications were performed in 25-µl reactions containing 1× standard Taq reaction buffer, 0.2 mM of each dNTP, 0.2 µM of each primer, 0.625 units of Taq DNA polymerase (New England BioLabs, Rowley, MA), and 20 ng of DNA template. PCR products were visualized in 1% (wt/vol) agarose gel in 0.5× TBE stained with GoodView nucleic acid stain. They were purified by desalting and sequenced with both forward and reverse primers (Macrogen Inc., Seoul, South Korea). Sequences were assembled and edited using Geneious 8.0.5 software (Biomatters, Auckland, New Zealand) and compared with existing sequences in the GenBank database using the Nucleotide BLAST tool.

Multilocus sequence analysis. Isolates were subjected to multilocus sequence analysis (MLSA) using partial sequences of four housekeeping genes (*fusA*, *gyrB*, *leuS*, and *rpoB*) based on previous MLSA schemes for *Pantoea* and *Enterobacteriales* (Delétoile et al.

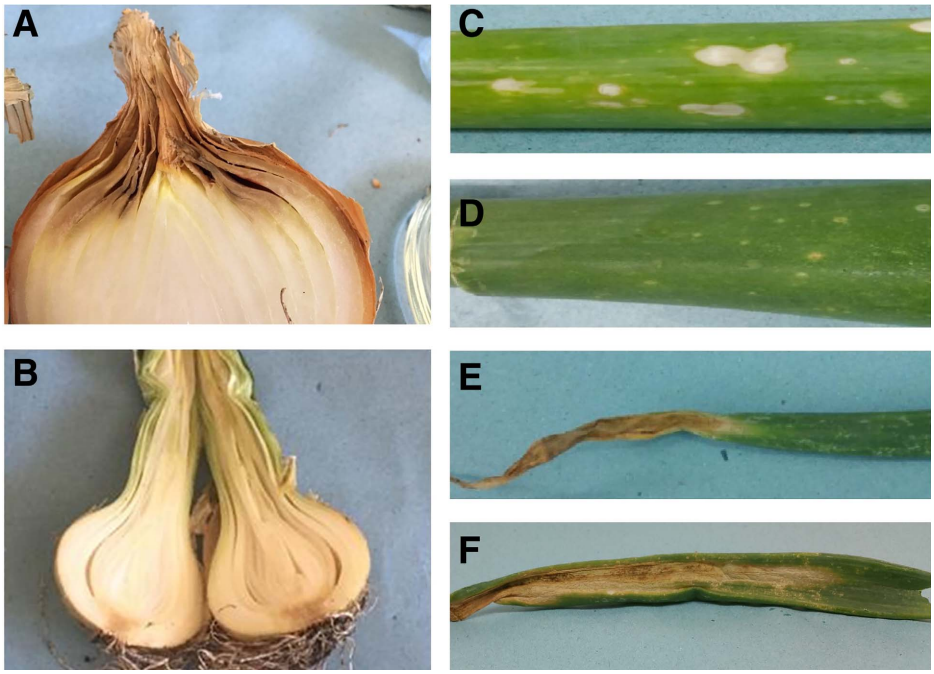

Fig. 1. Symptoms observed on foliage and bulbs of onion plants. **A,** Rotten scales progressing downward on onion bulb. **B,** Discoloration and internal water-soaking of an onion plant. **C,** Chlorotic spots on seed-stalk. **D,** Small white pustules on seed-stalk. **E and F,** Typical foliar center rot symptoms (white streak and tissue necrosis).

2009; Salerno et al. 2007; Stice et al. 2018). Primer sequences and PCR cycles are outlined in Table 1. Each PCR reaction was performed in a 50-µl reaction mix consisting of 1× standard Taq reaction buffer, 0.2 mM of dNTPs, 0.2 µM of each primer, 1.25 units of Taq DNA polymerase, and 50 ng of template DNA. Amplification products were visualized by 1% (wt/vol) agarose gel in 0.5× TBE stained with GoodView nucleic acid stain. Two molecular size markers, 1-kb and 100-bp DNA ladders (Thermo Fisher Scientific, Waltham, MA), were used. PCR products were purified by desalting and sequenced with reverse and forward primers by Macrogen Inc. Editing and analysis of chromatogram traces were performed using Geneious 8.0.5. Each base of the selected template region was confirmed by at least two chromatograms (forward and reverse). Sequences were then aligned, and the alignments were trimmed so that sequences of each given gene were of the same length. The concatenation of the four housekeeping genes represented a total of 2,005 bp. Finally, consensus sequences were used to generate a maximum-likelihood tree based on the Tamura-Nei Model (Tamura and Nei 1993) of MEGA-X 10.1.0 software with 1,000 bootstrapping repetitions. Reference and type strains of *Pantoea* spp. were selected from Tambong (2019) (Table 2), and the correspondent partial gene sequences were retrieved from the NCBI database and included in the MLSA analysis. *Pectobacterium carotovorum* DSM 30168 (accession no. NZ_FQWI00000000.1) was used as an outgroup.

Sequence accession numbers. Sequences generated in this study were deposited in the GenBank database with accession numbers

Table 1. Primers sequences and conditions used for amplification and sequencing

Gene	Primer name	Primer sequence	PCR cycles	Template size (bp)[z]	Reference
16S rRNA	27F	5′-AGA GTT TGA TCC TGG CTC AG-3′	30 s 95°C; 30 s 95°C, 1 min 58°C, 1 min 68°C (30 cycles); 5 min 68°C		Lane (1991)
	1492R	5′-TAC GGY TAC CTT GTT ACG ACT T -3′			
fusA	fusA3	5′-CAT CGG TAT CAG TGC KCA CAT CGA-3′	30 s 95°C; 30 s 95°C, 1 min 58°C, 1 min 68°C (31 cycles); 5 min 68°C	516	Salerno et al. (2007)
	fusA4	5′-CAG CAT CGC CTG AAC RCC TTT GTT-3′			
gyrB	gyrB3	5′-GCG TAA GCG CCC GGG TAT GTA-3′	30 s 95°C; 30 s 95°C, 1 min 58°C, 1 min 68°C (31 cycles); 5 min 68°C	405	Delétoile et al. (2009)
	gyrB4	5′CCG TCG ACG TCC GCA TCG GTC AT-3′			
	gyrB3i	5′-AAC GCW ATC GAC GAA GC-3′	Primers used only for sequencing		
	gyrB4i	5′-TGG AAC CCR TCR TTC CAC-3′			
leuS	leuS3	5′-CAG ACC GTG CTG GCC AAC GAR CAR GT-3′	30 s 95°C; 30 s 95°C, 1 min 58°C, 1 min 68°C (31 cycles); 5 min 68°C	586	Salerno et al. (2007)
	leuS4	5′-CGG CGC GCC CCA RTA RCG CT-3′			
rpoB	Vic3	5′-GGC GAA ATG GCW GAG AAC CA-3′	30 s 95°C; 30 s 95°C, 30 s 50°C, 1 min 68°C (31 cycles); 5 min 68°C	498	Delétoile et al. (2009)
	Vic2	5′-GAG TCT TCG AAG TTG TAA CC-3′			

[z] Size of the sequence template used for sequence comparison.

Table 2. Reference and type strains of *Pantoea* used for genetic analysis

Species	Strain code[z]	Source	Location	Accession no.
P. agglomerans	DSM 3493^T	Human	Zimbabwe	FYAZ00000000.1
P. agglomerans	BD 1274	Onion seed	S. Africa	QQXI00000000.1
P. ananatis	LMG 2665^T	Pineapple	Brazil	JFZU00000000.1
P. ananatis	LMG 20103	Eucalyptus	S. Africa	CP001875.2
P. allii	LMG 24248^T	Onion	S. Africa	NTMH00000000.1
P. allii	PNA 200-10	Onion	U.S.A.	QGHF00000000.1
P. eucalypti	LMG 24197^T	Eucalyptus	Uruguay	VHJB00000000.1
P. eucalypti	NFPP29	–	–	FUWI00000000.1
P. eucalypti	FBS135	Masson pine	China	CP020820.1
P. eucalypti	MHSD5	*Pellaea calomelanos*	S. Africa	PUEK00000000.1
P. vagans	LMG 24199^T	Eucalyptus	Uganda	CP038853.1
P. vagans	9140	–	–	JQNO00000000.1
P. wallisii	LMG 26277^T	Eucalyptus	S. Africa	MLFS00000000.1
P. septica	LMG 5345^T	Human	U.S.A.	MLJJ00000000.1
P. rwandensis	LMG 26275^T	Eucalyptus	Rwanda	MLFR00000000.1
P. rodasii	LMG 26273^T	Eucalyptus	Colombia	MLFP00000000.1
P. latae	AS1^T	*Zamia integrifolia*	U.S.A.	MWUE00000000.1
P. eucrina	LMG 5346^T	Human	U.S.A.	MIPP00000000.1
P. deleyi	LMG 24200^T	Eucalyptus	Uganda	MIPO00000000.1
P. cypripedii	LMG 2657^T	Orchid	U.S.A.	MLJI00000000.1
P. conspicua	LMG 24534^T	Human	France	MLFN00000000.1
P. brenneri	LMG 5343^T	Human	U.S.A.	MIEI00000000.1
P. anthophila	LMG 2558^T	*Impatiens balsamina*	India	VHIZ00000000.1
P. dispersa	CCUG 25232^T	Soil	Japan	NZ_VXKA00000000.1
P. alhagi	LTYR-11Z^T	*Alhagi sparsifolia* Shap.	China	CP019706.1
Mixta theicola	QC88-366^T	Black tea	Japan	NZ_NWUO00000000.1
M. gaviniae	LMG 25382^T	Powdered infant formula	Switzerland	NZ_MLFQ00000000.1
M. calida	LMG 25383^T	Powdered infant formula	Switzerland	NZ_MLFO00000000.1
P. endophytica	596^T	Maize	China	NZ_PJRT01000033.1
P. stewartii subsp. *stewartii*	DC283^T	Maize	U.S.A.	CP017581.1
P. stewartii subsp. *indologenes*	LMG 2632^T	*Setaria italica*	India	JPKO00000000.1
P. coffeiphila	342^T	*Paullinia cupana*	Brazil	NZ_PDET00000000.1
P. sesami	Si-M154^T	–	–	NZ_FQWJ01000034.1
Pectobacterium carotovorum	DSM 30168^T	Potato	Denmark	NZ_FQWI00000000.1

[z] Strains marked with ^{T} are the type strain of the species.

MZ289963 to MZ290021 for 16S, MZ299161 to MZ299219 for *fusA*, MZ299220 to MZ299278 for *gyr*B, MZ299279 to MZ299337 for *leuS*, and MZ299338 to MZ299396 for *rpoB*.

Pathogenicity assays on onion leaves and aggressiveness evaluation. A subset of 38 *Pantoea* isolates covering the diversity of species assigned by MLSA was selected for pathogenicity evaluation on a susceptible onion cultivar ('Pantanoso del Sauce CRS'). Because of space limitations, the 38 isolates were evaluated in two independent inoculation trials, with five isolates evaluated in both trials.

For inoculum preparation, isolates from freezer stocks were plated onto NBY agar medium. After 48 h of incubation at 28°C, single colonies were subcultured in 7 ml of NBY medium and incubated overnight at 28°C on a rotary shaker (MRC, Holon, Israel) with shaking at 150 rpm. After incubation, 2 ml of culture was centrifuged at 8,000 rpm (Thermo Scientific Sorvall Legend MicroCL 17R) for 5 min. The supernatant was discarded, and the pellet was resuspended in 2 ml of sodium chloride solution (0.9% wt/vol). The bacterial concentration was adjusted to an optical density of 0.1 at 600 nm (equivalent to 10^8 CFU ml^{-1}) using an ultraviolet-visible spectrophotometer (Jenway 6705, Stone, UK).

Three-month-old onion plants were inoculated by cutting the first developed leaf 1 cm from the apex with a sterile pair of scissors (Stice et al. 2018). Using a micropipette, a 10-µl drop of bacterial suspension was placed at the cut end of the leaf. Plants were incubated in a growing chamber at 28°C, ensuring 100% RH up to 48 h after inoculation. Six replicate plants were inoculated per isolate, and sterile water was used as mock-inoculated control. Experiments were arranged in a completely randomized balanced design. Ten days after inoculation, lesion length (in centimeters) for each plant was measured and recorded. Koch's postulates were accomplished by isolating bacteria from symptomatic tissue on NBY medium as described above. Yellow-pigmented colonies were isolated and assigned to the genus *Pantoea* by Gram staining and biochemical tests (oxidase reaction, catalase reaction, and Hugh-Leifson oxidation/fermentation test).

The data of both experiments were combined, and a linear mixed model was carried out, with isolates as fixed effect and trial as random effect in the model. The linear mixed model analysis was followed by a post hoc multiple comparison with Tukey's honestly significant difference test at $P < 0.05$ level. The analysis was performed using the "lmer" package (Bates et al. 2015) in R statistical software version 3.6.1 (R Core Team 2016).

Aggressiveness of *Pantoea* isolates on a set of onion cultivars. Additional evaluations were performed with four *Pantoea* isolates selected based on the results obtained in the previous assay: *P. ananatis* MAI 6032, *P. eucalypti* MAI 6036, *P. allii* MAI 6022, and *P. eucalypti* MAI 6051. The aggressiveness of these isolates was evaluated on a set of eight onion cultivars and breeding lines from Northwest and South Uruguay: 'Albana', 'Naqué', 'Canarita', 'Regia', '9719', 'INIA Fagro Dulce', and '22E' and 'Pantanoso × Regia (PxR)'. Five replications (individual pot plants) per isolate and cultivar combination were used in a unique experiment. Sterile water was used as mock-inoculated control. The experiment was arranged in a completely randomized balanced design. Seedlings of each cultivar were inoculated as described above. Ten days after inoculation, lesion length (in centimeters) for each inoculated leaf was measured and recorded.

A linear model (two-way analysis of variance) was carried out to analyze the data, estimating the effects of isolate, cultivar, and interaction between isolate and cultivar. The linear model analysis was followed by a post hoc multiple comparison with Tukey's honestly significant difference test at $P < 0.05$ level. The analysis was run using "lm" function (package agricolae) (Chambers 1992) in R statistical software version 3.6.1 (R Core Team 2016).

To confirm that symptoms observed were caused by *Pantoea* isolates, bacteria from symptomatic tissue were isolated from a region adjoining the necrotic and healthy tissue as described above. The identification of the resulting colonies was made using classic tools (Gram staining, preliminary biochemical tests). Yellow-pigmented colonies were isolated and assigned to the genus *Pantoea*.

Red onion scale assay. Asymptomatic red onion bulbs 'Naqué' were used to evaluate the ability of all *Pantoea* isolates to cause scale necrosis through the red onion scale assay described by Stice et al. (2018). Prior to inoculation, bulbs were surface sterilized with sodium hypochlorite 1% for 1 min and rinsed in sterile water. Thereafter, red onions were sliced to remove any diseased tissue and cut to approximately 3-cm-wide scales. Individual scales were placed in plastic cupcake containers on two layers of paper towels premoistened with 1 ml of distilled water. Individual onion scales were wounded in the center of the inner surface with a sterile pipette tip, and 10 µl of a prepared bacterial suspension (10^8 CFU/ml) was inoculated into the wound, as described earlier (Stice et al. 2018). Four replicate onion scales were inoculated with each isolate. Sterile water was used as negative control. After inoculation, onion scales were incubated at 28°C for 72 h in the darkness. Pathogenicity was recorded as the ability to clear the red anthocyanin pigment and cause tissue maceration.

Results

***Pantoea* is the main bacterial genera affecting onion crops in Uruguay.** Symptomatic bulbs, leaves, and seeds-stalks were collected from commercial crops and experimental stations fields from 2015 to 2020. Bacterial colonies were isolated and identified at genera level using classical tools and 16S rRNA gene sequence analysis. Isolates from bulb rots and superficial bulb wounds were assigned to several genera, including *Burkholderia*, *Enterobacter*, *Pantoea*, and *Pseudomonas*, which have been previously reported as phytopathogens in onion postharvest (du Toit et al. 2016; Schwartz and Mohan 2008). On the other hand, isolates obtained from symptomatic onion leaves and seed-stalks were assigned mainly to *Pantoea*. Overall, the genus *Pantoea* was the most predominant (i.e., 43%) and widely distributed in our collection; thus, we focused the study on this genus.

The survey of onion-growing regions in Uruguay during 2015 to 2020 allowed us to generate the first local collection of *Pantoea* spp. isolates affecting onion crops in Uruguay. Fifty-nine *Pantoea* isolates were obtained from symptomatic onion leaves, seed-stalks, and bulbs (11 from bulbs and 48 from leaves and seed-stalks) (Table 3). Isolates from the southern region (Canelones) were obtained from bulb rots and symptomatic leaves, whereas isolates from the northwest region (Salto) were obtained from superficial bulb wounds, leaves, and seed-stalks. All isolates produced yellow-pigmented colonies in NBY-agar plates and corresponded to gram-negative, rod-shaped, facultatively anaerobic bacteria, oxidase negative and catalase positive. 16S rRNA gene sequences were determined for all 59 isolates. Using the BLASTn tool, a high degree of sequence identity (≥98%) was found with previously determined sequences of bacteria belonging to the genus *Pantoea*.

Diversity of *Pantoea* species identified by MLSA. The sequences of internal portions of four protein-coding genes (*rpoB*, *gyrB*, *leuS*, and *fusA*) were obtained for the 59 *Pantoea* isolates. Phylogenetic analysis of the concatenated and aligned sequences allowed the assignment of isolates to five *Pantoea* species: *P. ananatis*, *P. agglomerans*, *P. allii*, *P. eucalypti*, and *P. vagans* (Fig. 2). The most prevalent species was *P. eucalypti*, with 36 of the 59 obtained isolates. The presence of several subclusters in the phylogenetic tree suggested a great diversity within this species. A second group was made up of five isolates that clustered separately from all reference and type *Pantoea* strains used in this phylogenetic analysis. These five *Pantoea* sp. isolates formed a distinct, well-supported cluster, with *P. eucalypti* being the closest known species. A third cluster consisted of 11 isolates that were grouped with reference and type strains of *P. agglomerans*. This group was phylogenetically close to a fourth cluster, with only one isolate (MAI 6050) identified as *P. vagans.* A fifth cluster corresponded to *P. ananatis* and was made up of only two isolates (MAI 6032 and MAI 6039) obtained from symptomatic seed-stalks collected in Salto in 2018. Finally, the sixth cluster grouped reference and type strains of *P. allii* and included four isolates obtained from symptomatic leaves in Canelones in 2018.

Isolates identified as *P. eucalypti*, *P. agglomerans*, and *Pantoea* sp. showed a wide distribution and were obtained from symptomatic bulbs, leaves, and seeds-stalks collected in different years and crop regions along the country. In contrast, *P. ananatis* and *P. allii* showed a limited distribution, being isolated from the northwestern (Salto) and southern (Canelones) regions, respectively.

The nucleotides sequence trees for each gene (Supplementary Figs. S1 to S4) showed similar groupings to those of the concatenated nucleotide sequence tree, with some exceptions. In the *fusA* nucleotide sequence tree (Supplementary Fig. S1), *P. eucalypti* and *P. agglomerans* reference and type strains were not differentiated and were grouped in a unique cluster together with 52 isolates from Uruguay. The topology of the tree based on *gyrB* sequences (Supplementary Fig. S2) was similar to that of the concatenated nucleotide sequence tree and allowed the assignment of the isolates to the same five *Pantoea* species: *P. ananatis*, *P. agglomerans*,

Table 3. *Pantoea* isolates collected in Uruguay from symptomatic onion

Isolate	MLSA identification	Source	Geographic location	Isolation year	Red onion scale assay[x]	PCR assays[y]	
						HiVir	*alt*
MAI 6000	*P. agglomerans*	Onion (bulb rot)	Uruguay (Canelones)	2015	–	–	+
MAI 6001	*P. agglomerans*	Onion (bulb rot)	Uruguay (Canelones)	2015	–	–	+
MAI 6002	*P. agglomerans*	Onion (bulb rot)	Uruguay (Canelones)	2015	–	–	+
MAI 6003	*P. eucalypti*	Onion (bulb rot)	Uruguay (Canelones)	2015	–	–	–
MAI 6004	*P. allii*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6005	*P. agglomerans*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6006	*P. allii*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6007	*P. allii*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	–
MAI 6008	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	–
MAI 6009	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6010	*P. agglomerans*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6011	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6012	*P. agglomerans*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6013	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6014	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6015	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6016	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6017	*P. agglomerans*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6018	*P. agglomerans*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6019	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6020	*Pantoea* sp.	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6021	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	–
MAI 6022	*P. allii*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6023	*P. agglomerans*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6024	*P. eucalypti*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6025	*P. agglomerans*	Onion (leaf)	Uruguay (Canelones)	2018	–	–	+
MAI 6026	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2018	–	–	+
MAI 6027	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2018	–	–	–
MAI 6028	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2018	–	–	+
MAI 6029	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2018	–	–	+
MAI 6030	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2018	–	–	+
MAI 6031	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2018	–	–	+
MAI 6032	*P. ananatis*	Onion (seed-stalk)	Uruguay (Salto)	2018	+	+	+
MAI 6033	*P. eucalypti*	Onion (seed-stalk)	Uruguay (Salto)	2018	–	–	+
MAI 6034	*P. eucalypti*	Onion (seed-stalk)	Uruguay (Salto)	2018	–	–	+
MAI 6035	*P. eucalypti*	Onion (seed-stalk)	Uruguay (Salto)	2018	–	–	+
MAI 6036	*P. eucalypti*	Onion (seed-stalk)	Uruguay (Salto)	2018	+	–	+
MAI 6037	*P. eucalypti*	Onion (seed-stalk)	Uruguay (Salto)	2018	–	–	–
MAI 6038	*P. eucalypti*	Onion (seed-stalk)	Uruguay (Salto)	2018	–	–	+
MAI 6039	*P. ananatis*	Onion (seed-stalk)	Uruguay (Salto)	2018	+	+	+
MAI 6040	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2018	–	–	+
MAI 6041	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2018	–	–	+
MAI 6042	*P. eucalypti*	Onion (bulb surface)	Uruguay (Salto)	2018	–	–	+
MAI 6043	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2019	–	–	–
MAI 6044	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2019	–	–	+
MAI 6045	*P. agglomerans*	Onion (leaf)	Uruguay (Salto)	2019	+	–	+
MAI 6046	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2019	–	–	+
MAI 6047	*P. eucalypti*	Onion (leaf)	Uruguay (Salto)	2019	–	–	+
MAI 6048	*P. eucalypti*	Onion (seed-stalk)	Uruguay (Salto)	2019	–	–	–
MAI 6049	*Pantoea* sp.	Onion (bulb surface)	Uruguay (Salto)	2019	–	–	+
MAI 6050	*P. vagans*	Onion (seed-stalk)	Uruguay (Salto)	2019	–	–	–
MAI 6051	*P. eucalypti*	Onion (seed-stalk)	Uruguay (Salto)	2019	–	–	+
MAI 6052	*P. eucalypti*	Onion (seed-stalk)	Uruguay (Salto)	2019	–	–	+
MAI 6053	*Pantoea* sp.	Onion (seed-stalk)	Uruguay (Salto)	2019	–	–	+
MAI 6054	*P. eucalypti*	Onion (bulb rot)	Uruguay (Canelones)	2020	–	nd[z]	nd
MAI 6055	*P. eucalypti*	Onion (bulb rot)	Uruguay (Canelones)	2020	–	nd	nd
MAI 6056	*Pantoea* sp.	Onion (bulb rot)	Uruguay (Canelones)	2020	–	nd	nd
MAI 6057	*Pantoea* sp.	Onion (bulb rot)	Uruguay (Canelones)	2020	–	nd	nd
MAI 6058	*P. eucalypti*	Onion (bulb rot)	Uruguay (Canelones)	2020	+	nd	nd

[x] Isolate ability to cause softening and pigment clearing on red onion scale, 3 days after inoculation (see Fig. 5 and Materials and Methods for description). Isolates that cleared the red anthocyanin pigment and caused scale maceration were depicted as "+" and those that did not were depicted as "–."
[y] HiVir and *alt* clusters PCR assays results from Stice et al. (2021).
[z] nd, not determined.

P. allii, *P. eucalypti*, and *P. vagans*. Finally, the *leuS* and *rpoB* nucleotide sequence trees (Supplementary Figs. S3 and S4) showed *Pantoea* sp. MAI 6057 isolate grouped with reference and type strains of the *P. eucalypti* and *P. agglomerans* species, respectively.

Pathogenicity and aggressiveness of *Pantoea* isolates on onion leaves. Based on the phylogenetic analysis, 38 isolates were selected to assess the pathogenicity and virulence on plants of the susceptible 'Pantanoso del Sauce CRS'. All isolates displayed typical foliar necrosis symptoms on inoculated plants 10 days after inoculation under controlled conditions (Fig. 3). In addition, significant differences among isolates were found considering the lesion length as a measure of strain aggressiveness ($P < 2.2e\text{-}16$; Fig. 4). The highest level of aggressiveness was displayed by *P. ananatis* isolates (MAI 6032 and MAI 6039), followed by *P. allii* MAI 6022, *P. eucalypti* MAI 6036, *Pantoea* sp. MAI 6049, *P. allii* MAI 6006, *P. vagans* MAI 6050, and *P. eucalypti* MAI 6009. On the other hand, 30 isolates assigned to the species *P. eucalypti*, *P. allii*, *Pantoea* sp., and *P. agglomerans* showed mild aggressiveness, causing leaf lesions length ranging from 1.62 to 3.93 cm, although they were not significantly different from the control (0.08 cm).

Pathogenicity and aggressiveness of *Pantoea* isolates on a set of onion cultivars. Four isolates were selected to evaluate their pathogenicity and aggressiveness on a set of eight onion cultivars and breeding lines. The subset included the most aggressive isolates of *P. ananatis* (MAI 6032), *P. eucalypti* (MAI 6036), and *P. allii* (MAI 6022) as well as a less aggressive *P. eucalypti* isolate (MAI 6051). Results for this assay are outlined in Table 4. The analysis of variance showed significant differences for the main factors of isolate

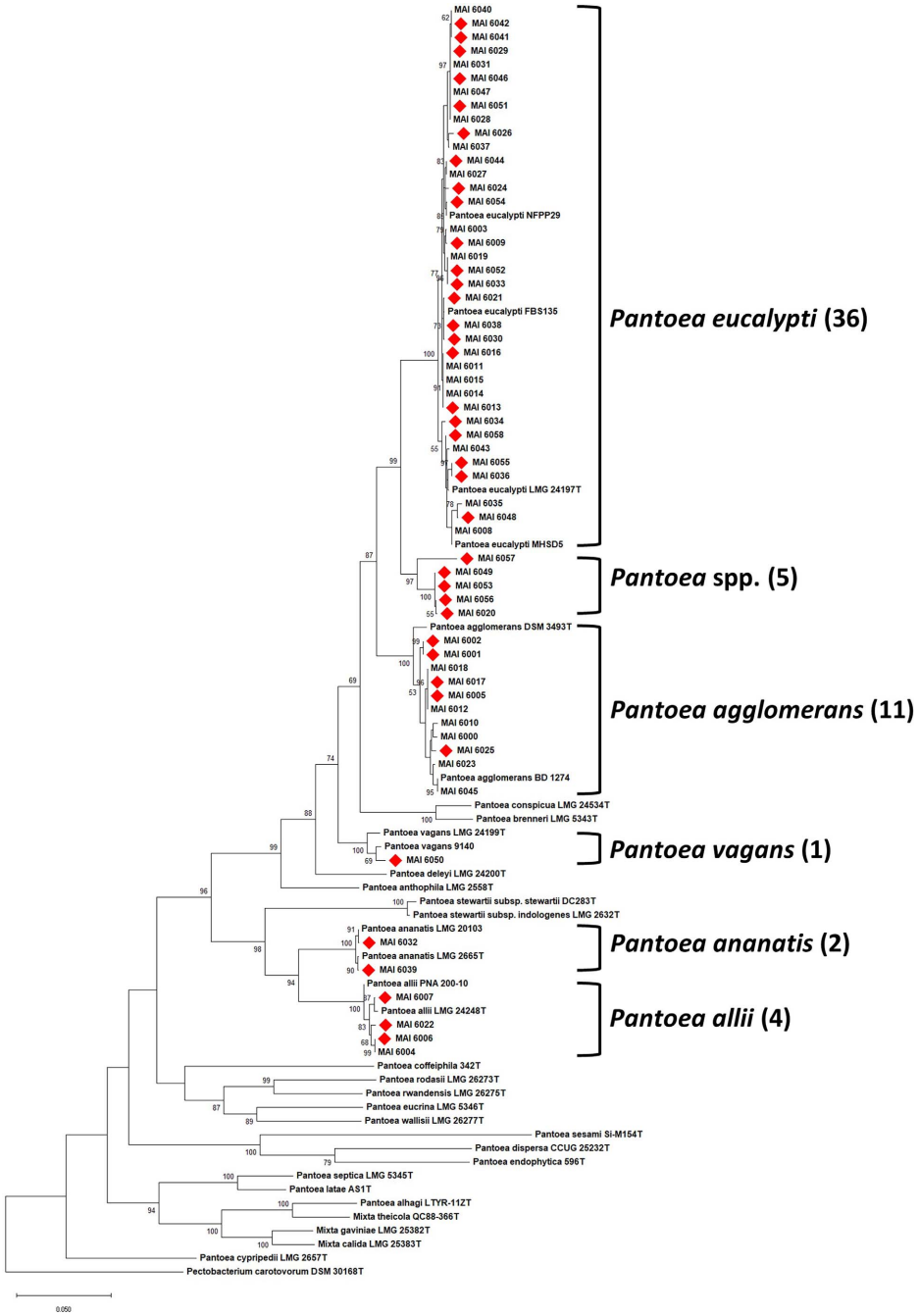

Fig. 2. Maximum-likelihood tree based on the Tamura-Nei Model derived from concatenation of four housekeeping genes (*fusA*, *gyrB*, *rpoB*, *leuS*) of *Pantoea* strains. Bootstrap values are based on 1,000 repetitions, and values >50% are shown at branch nodes. *Pectobacterium carotovorum* DSM 30168 was used as an outgroup. Isolates marked with red rhombus are those selected for pathogenicity and aggressiveness assays on onion leaves. The number of isolates clustered with five reference and type strains of *Pantoea* species is shown in parentheses.

($P < 2.2e-16$) and cultivar ($P = 1.124e-07$) as well as for the interaction between isolate and cultivar ($P = 3.609e-06$). All inoculated plants showed typical foliar necrosis symptoms 10 days after inoculation. However, mean lesion length for each isolate on each cultivar significantly differed according to Tukey's honestly significant difference test. *P. ananatis* MAI 6032 isolate showed the highest aggressiveness in all tested cultivars. *P. eucalypti* MAI 6036 also displayed the highest level of aggressiveness along with *P. ananatis* MAI 6032 in three cultivars (Albana, Canarita, and PxR), whereas an intermediate incidence of foliar necrosis symptoms was observed for the other five. Remarkably, these two isolates were significantly different from the negative control in all cultivars. *P. allii* MAI 6022 was weak to moderately aggressive in all evaluated cultivars. The lowest level of aggressiveness was obtained by *P. eucalypti* MAI 6051 in all

Fig. 3. Foliar necrosis symptoms displayed by onion pathogenic *Pantoea* species. Leaves of onion plants ('Pantanoso del Sauce CRS') were clipped 1 cm from the apex and inoculated by placing 10 µl of bacterial suspension adjusted to 1×10^8 CFU/ml at the cut end. Necrotic lesions on onion leaves infected by *P. ananatis* MAI 6032, *P. alli* MAI 6022, *P. eucalypti* MAI 6036, *P. vagans* MAI 6050, and *Pantoea* sp. MAI 6049. Control plants inoculated with sterile water displayed no foliar symptoms. Inoculated leaves are indicated with an arrow.

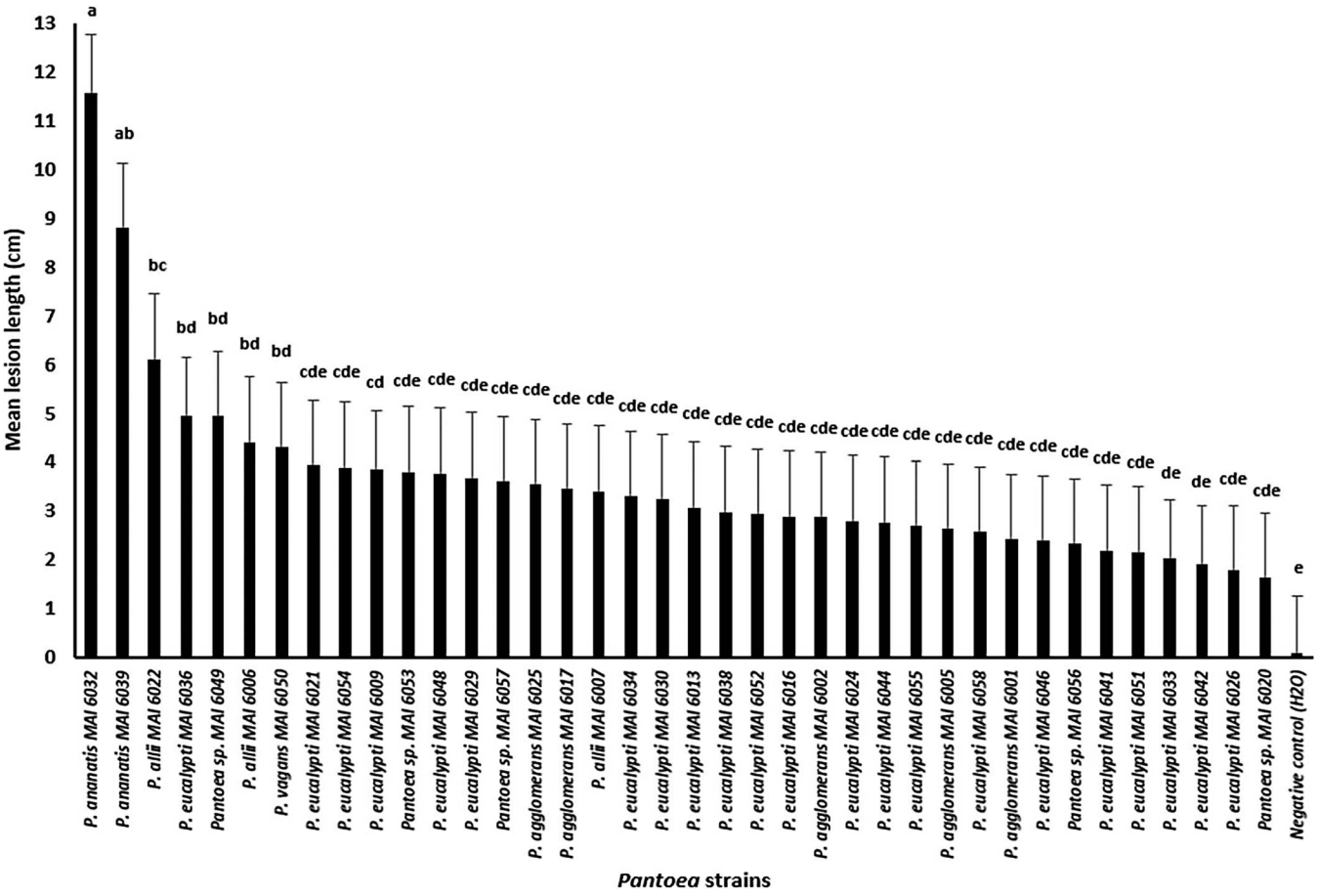

Fig. 4. Pathogenicity and aggressiveness of *Pantoea* isolates on onion leaves. Bar graph illustrating the adjusted mean and standard error (error bars) of lesion length (cm) for 38 *Pantoea* isolates. Different letters (above each bar) indicate significant differences in aggressiveness using Tukey test at 95% confidence interval.

cultivars, confirming the previously observed response in 'Pantanoso del Sauce CRS'.

Different susceptibility levels were observed among cultivars, with Canarita and Albana be averaging the shortest lesion length and 22E and INIA Fagro Dulce displaying the longest mean lesion values.

Red onion scale assay. This assay was performed for the 59 *Pantoea* isolates generated in this study. Isolates that cleared the red anthocyanin pigment and caused scale maceration were depicted as "+" and those that did not were depicted as "–" in Table 3, as defined by Stice et al. (2018). Only five isolates exhibited scale clearing and weakening: *P. agglomerans* MAI 6045, *P. eucalypti* MAI 6036 and MAI 6058, and *P. ananatis* MAI 6032 and MAI 6039. The frequency of isolates for each species that cleared the red onion scales was as follows: 2/2 of *P. ananatis*, 2/36 of *P. eucalypti*, and 1/11 of *P. agglomerans* (Table 3; Fig. 5). The negative control (sterile water) did not exhibit clearing in the onion scale assay.

Discussion

Bacterial diseases are a major constraint for onion production in Uruguay, resulting in significant losses mainly associated with bulb rot during postharvest storage. The survey carried out in this work showed a clear predominance of *Pantoea* genus as the main pathogenic bacteria affecting onion crops in Uruguay. Although 48 of the 59 *Pantoea* isolates from our collection were obtained from symptomatic onion leaves and seed-stalks, it is probable that these strains are also responsible for bulb rot. For instance, it was previously reported for *P. ananatis* that leaf infection in the field can lead to bulb infection and storage losses (Carr et al. 2013). Few isolates identified as members of the genus *Pantoea* were previously obtained from diseased onion plants in the northwest production region in Uruguay (A. Arrauabarrena, personal communication, 2019). However, this finding was limited to a reduced number of isolates, which highlights the need for a more comprehensive survey aiming to identify the most prevalent *Pantoea* species affecting onion crops in the country.

The MLSA approach applied in this work allowed us to identify at least five *Pantoea* species affecting onion crops in Uruguay: *P. ananatis*, *P. agglomerans*, *P. allii*, *P. eucalypti*, and *P. vagans*. Only five isolates could not be identified to species level but formed a separate cluster, with *P. eucalypti* being the most closely related species. Further analyses at both phenotypic and genomic levels are required to determine the phylogenetic position of these isolates and whether they correspond to a new species. *P. ananatis* and *P. allii*, two of the most important onion pathogenic species worldwide, were found in a low number of samples from specific regions. This

Table 4. Adjusted means of lesion length (cm) for each isolate on each cultivar[z]

Pantoea species	Strain	22E	INIA Fagro Dulce	Naqué	P × R	9719	Regia	Albana	Canarita	Average
P. ananatis	MAI 6032	26.0 a	17.7 a	15.0 a	11.8 a	19.0 a	16.3 a	10.7 a	11.5 a	16.0 A
P. eucalypti	MAI 6036	12.0 b	9.9 b	7.6 b	8.8 a	8.0 b	9.2 b	7.4 ab	6.5 ab	8.7 B
P. allii	MAI 6022	7.5 bc	7.4 b	3.9 bc	8.8 a	4.0 bc	5.0 bc	4.5 bc	5.1 bc	5.8 C
P. eucalypti	MAI 6051	5.7 c	5.8 b	4.2 bc	8.0 a	1.8 c	4.5 bc	4.7 bc	3.0 bc	4.7 C
Negative control (water)		0.3 d	0.4 c	0.3 c	0.3 b	0.3 c	0.3 c	0.3 c	0.3 c	0.3 D
Average		10.3 A	8.2 AB	6.2 BC	7.6 BC	6.6 BC	7.1 BC	5.5 C	5.3 C	

[z] Different letters indicate significant differences using Tukey test at 95% confidence interval. Isolate and cultivar main effects are compared by uppercase letters, and isolates for each cultivar are compared by lowercase letters.

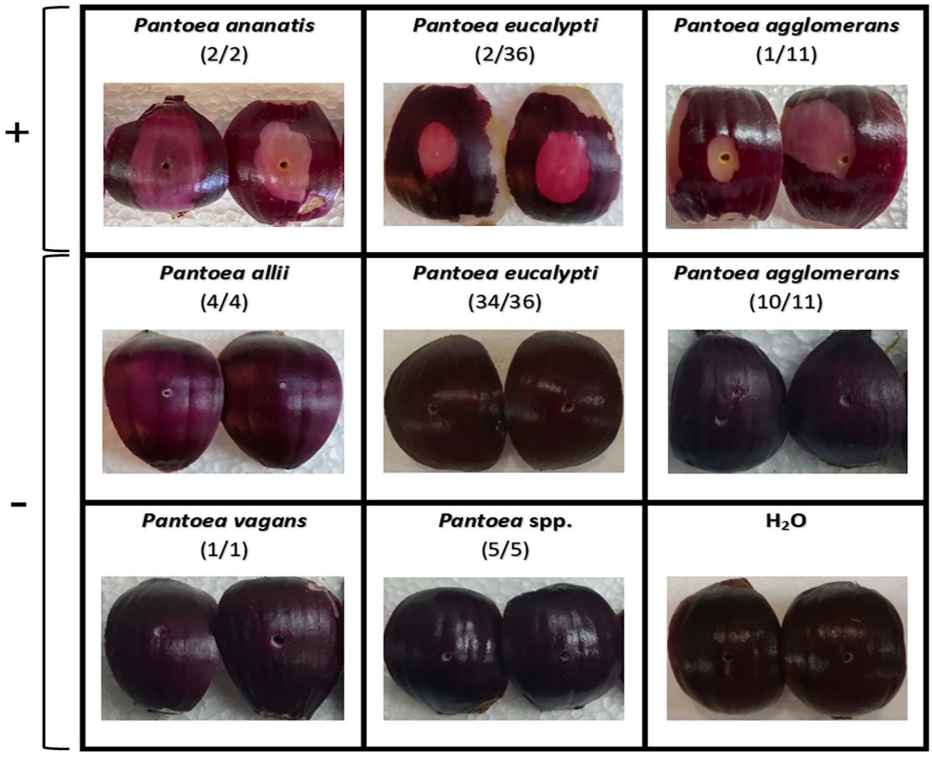

Fig. 5. Red onion scale assay. Individual onion scales were wounded on the center of the inner surface and inoculated with 10 µl of bacterial suspension (1×10^8 CFU/ml) for each isolate. Scales were imaged 3 days after inoculation. *Pantoea* spp. that cleared the red anthocyanin pigment and caused scale maceration were depicted as "+" and those that did not were depicted as "–." The values in parentheses indicate the frequency of isolates for each species that cleared the red onion scales and caused scale maceration and those that did not. Sterile water was used as a negative control.

restricted distribution suggests that *P. ananatis* (northwest region) and *P. allii* (southern region) isolates could have been recently introduced to the country.

The identification of pathogenic isolates such as *P. eucalypti* and *P. vagans* is particularly striking because these species have not been previously reported as onion pathogens elsewhere. *P. eucalypti* isolates stand out for their larger prevalence and wide distribution, having been isolated from symptomatic bulbs, leaves, and seeds-stalks collected in different years and regions. *P. eucalypti* was first nominated as species nova based on polyphasic identification of strains isolated from eucalyptus leaves collected in Uruguay that showed bacterial blight symptoms (Brady et al. 2009). Subsequently, additional *P. eucalypti* strains were reported as endophytes from *Lotus tenuis*, *Solanum lycopersicum*, and *Pinus massoniana* and were studied for their ability to promote plant growth (Castagno et al. 2011, 2014; Romero et al. 2016; Song et al. 2020). It is remarkable that most identified *P. eucalypti* endophytes strains have been isolated in South America, a result consistent with our findings suggesting that this species is highly established in Uruguay and is specifically associated with onion crops.

A red onion scale assay was performed for the collection of 59 *Pantoea* spp. isolates. As expected, the two isolates belonging to *P. ananatis* within the collection (MAI6032 and MAI 6039) cleared the red anthocyanin pigment and caused scale maceration. Interestingly, *P. agglomerans* MAI6045 and two isolates of *P. eucalypti* (MAI6036 and MAI6058) also exhibited scale clearing and weakening, although these isolates did not amplify with the HiVir PCR primers designed based on the cluster sequences retrieved from *P. ananatis* (Stice et al. 2021). These results suggest that these isolates may harbor a divergent HiVir-like cluster distinct from *P. ananatis* or that alternative onion virulence mechanisms may exist among *Pantoea* spp.

A subset of 38 isolates was selected to test pathogenicity and aggressiveness on onion leaves. All inoculated leaves developed white streaking and tissue necrosis, as previously observed in other works (Stice et al. 2018; Stumpf et al. 2018). Although all isolates caused leaf lesions, *P. ananatis* isolates showed the highest aggressiveness, confirming previous reports for this important pathogen of onion (Stice et al. 2018). In addition, specific isolates of *P. allii*, *P. eucalypti*, *Pantoea* sp., and *P. vagans* also displayed moderate levels of aggressiveness, highlighting the need for further studies to elucidate the pathogenic potential of this unexplored onion pathogenic species. Interestingly, *P. eucalypti* MAI6036 displayed the highest level of aggressiveness on onion leaves and was able to clear the red anthocyanin pigment and cause tissue maceration on the red onion assay. Little is known about the pathogenicity mechanisms involved in the virulence of onion pathogenic *P. eucalypti* isolates or other onion pathogenic *Pantoea* spp. isolates. Comparative genome sequencing studies between phylogenetically related highly virulent and mild isolates, particularly for *P. eucalypti*, would add clues on yet unknown pathogenicity mechanisms within this genus. Onion pathogenicity was not experimentally verified for 20 of the 59 isolates of our collection. *Pantoea* spp. have been frequently reported on plants as epi- or endophytic symbionts, establishing beneficial association with its hosts (Dutkiewicz et al. 2016; Walterson and Stavrinides 2015). Therefore, it remains to be determined whether these isolates are truly pathogenic.

The most aggressive isolates of *P. ananatis* (MAI6032), *P. eucalypti* (MAI6036), and *P. allii* (MAI6022), as well as a less aggressive *P. eucalypti* isolate (MAI6051), were selected to evaluate their pathogenicity and aggressiveness on a set of eight onion cultivars and breeding lines. Despite the interaction between isolate and cultivar being significant, *P. ananatis* MAI6032 showed the highest average aggressiveness, followed by *P. eucalypti* MAI6036 in several cultivars, confirming previously observed responses on 'Pantanoso del Sauce CRS'. Although no onion cultivar was resistant to studied *Pantoea* isolates, different responses between cultivars contributed to identifying sources of resistance for onion breeding programs, opening prospects for onion host resistance as a tool in integrated bacteriosis disease management.

The presence of diverse bacterial species leads to complex disease management and highlights the need for further studies on the virulence factors and epidemiology of these species. Furthermore, an improved understanding of variability in resistance to *Pantoea* spp. among onion cultivars would enable onion breeders to select for greater resistance in onion germplasm to reduce losses caused by bacteriosis and assist growers in selecting less susceptible cultivars.

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