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Disease Prevalence and Symptoms Caused by *Alternaria tenuissima* and *Pestalotiopsis guepinii* on Blueberry in Entre Ríos and Buenos Aires, Argentina

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Abstract

About 60% of blueberry *(Vaccinium corymbosum)* plantations in Argentina are located in the provinces of Entre Ríos and Buenos Aires. *Alternaria tenuissima* and *Pestalotiopsis guepinii* have been reported as pathogens of blueberry, causing leaf spots and branch cankers. The aims of this research were to estimate the prevalence of these microorganisms on leaves and fruits taken from crops located in Entre Ríos and Buenos Aires, as well as to differentiate leaf symptoms after target inoculations with each pathogen individually and in mixtures. Both fungi were present in blueberry fields from 2010 to 2013. *A. tenuissima* was the most prevalent pathogen, as most of the symptoms detected in the fields had been caused by this species. As a result of inoculations on cv. O'Neal, injured tissues showed symptoms before undamaged ones. Leaf symptoms caused by *A. tenuissima* differed from those caused by *P. guepinii* because of their predominant reddish color and the absence of drop-off of the central part of the lesions. When inoculated in a mixture, incubation period on leaves was intermediate between the registered for individual inoculations. The leaves showed reddish-brown spots typical of *A. tenuissima* and dark brown spots typical of *P. guepinii*, both with red margins. Blight, defoliation and canker symptoms caused in each case were undistinguishable.

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Keywords

Blueberry, Diseases, *Alternaria tenuissima*, *Pestalotiopsis guepinii*, Argentina

1. Introduction

Blueberries (*Vaccinium* spp.) are perennial shrubs belonging to the Ericaceae, which is a large and diverse family with a nearly cosmopolitan distribution [1]. Some species were introduced in South America in the 1980's to be evaluated as fruit crops. While only a small area had been planted in Argentina by 1993 [2], approximately 3100 hectares are dedicated to growing blueberries (*V. corymbosum* L.) at present. Total country production has increased steadily in recent years and it exceeds 16,500 ton nowadays. Half of Argentinean production is harvested in the province of Entre Ríos, mainly in the department of Concordia (37% of the cropping area); and 20% is harvested in the province of Buenos Aires [3]. Crop areas are characterized by either low chilling or no-chilling during winter, warm springs and hot summers. Fruits are harvested from September to April [4]. Blueberry industry has developed mainly due to good prices in the off-season fresh market in North America and Europe, and also to worldwide increasing demand for blueberries. Most of the production is exported as fresh fruits [2]; 60% to The United States, 16% to Continental Europe and 16% to the United Kingdom [3]. Argentina ranks second as exporter in the Southern Hemisphere, and contributes with 2% of world production [5]. Highbush blueberry (*V. corymbosum* L.) is the most commonly planted type in Argentina [6] [7]. At the beginning of the century, the most frequent cultivars were O'Neal and Misty; while nowadays they are Abundance, Blue Crisp, Emerald, Jewel, Millennia, Misty, O'Neal, Primadonna, Snow Chaser, Spring High and Star [3].

A good understanding of the physiological processes that regulate dormancy, flower bud induction and differentiation, as well as fruit development in blueberry plants is required for successful commercial production [4]. Additionally, there is a need of increasing knowledge of major diseases, in order to plan crop health management.

Alternaria Nees is a dictyosporic genus of the family Dematiaceae, order Hyphomycetes, Fungi Imperfecti, with a widespread distribution in Nature [8]. Many species are common saprophytes in soil, air and a variety of other habitats; some are ubiquitous agents of decay and plant pathogens [9] [10]. The genus *Pestalotiopsis* Steyaert belongs to the family Melanconiaceae (Coelomycetes) [11]. Its species have a worldwide distribution, particularly in tropical and temperate ecosystems [12] [13]. Most species are plant pathogens [14] and some are saprobes [15] or grow on decaying wild fruits [16]. *Alternaria tenuissima* (Kunze) Wiltshire and *Pestalotiopsis guepinii* (Desm.) Steyaert have been reported causing disease on 329 and 57 hosts, respectively [17] and both are important aerial pathogens of blueberry in Argentina. Injuries facilitate their penetration in plant tissues [18]-[20]. They are able to infect leaves, branches and fruits. Simultaneous isolation of the two species from symptomatic blueberry leaves is usual, but the existence of co-infection has not been demonstrated. The aim of this research was to estimate the relative importance of *A. tenuissima* and *P. guepinii* in crops located in Buenos Aires and Entre Ríos, as well as to differentiate their symptoms after target inoculations. To our knowledge, there are not previous studies on the subject.

2. Materials and Methods

2.1. Pathogens' Isolation and Prevalence

O'Neal crops located in the provinces of Buenos Aires and Entre Ríos were surveyed to collect symptomatic samples during the growing seasons 2010-1011, 2011-2012 and 2012-2013. A hundred of spotted leaves were taken during November and a hundred of rotten fruits were taken during December. **Table 1** summarizes sampling locations. Disease prevalence was estimated as percentage of samples with infection of each pathogen.

Sampled leaves and fruits were cut into small pieces that were surface disinfected by immersion in ethanol:water (70:30 v/v) during one minute, 2% (v/v) of Cl as NaOCl during 1 minute, and washed with sterilized deionized water. The materials were plated on Merck potato dextrose agar (PDA) at 22˚C, incubated during 7 - 10 days, and examined for pathogen development. The emerging colonies were successively transferred to PDA plates for purification, and finally to PDA slants that were maintained at 7˚C. Those that showed similar charac-

Table 1. Blueberry sampling locations.

Department	Province	Latitude	Longitude
Concordia	Entre Ríos	31˚23'32"S	58˚01'01"W
Gualeguaychú	Entre Ríos	33˚00'28"S	58˚30'40"W
San Pedro	Buenos Aires	33˚40'33"S	59˚39'47"W
Zárate	Buenos Aires	34˚05'45"S	59˚01'27"W
Mercedes	Buenos Aires	34˚39'02"S	59˚25'53"W
La Plata	Buenos Aires	34˚55'17"S	57˚57'16"W

teristics were grouped after preliminary observations under the lens. One isolate was chosen from each group as representative for further taxonomical identification and coded as A-BL-30, A-BL-31, A-BL-32, A-BL-33, A-BL-34, A-BL-35 and A-BL-36. The cultural and morphological characteristics of the isolate were recorded for identification.

2.2. Inoculations of *A. tenuissima* and *P. guepinii*

Isolates A-BL-1 (*A. tenuissima*) and A-BL-15 (*P. guepinii*) were grown on PDA slants so as to increase inocula. The isolates had been obtained from diseased organs, and had been already confirmed as pathogenic on blueberry [19] [20].

Healthy blueberry plants were inoculated with *A. tenuissima*, *P. guepinii* or *A. tenuissima* + *P. guepinii*. Pathogen inocula consisted of spore suspensions (1×10^5 conidia/mL) that were sprayed over the plants. Ten leaves and five branches per plant were injured with flamed needles previous to inoculation. Controls were sprayed with sterilized water. Each plant was individually enclosed in a polyethylene bag and placed at random in a climatic chamber at 22˚C. The bags were removed after 48 hr. The plants remained in the chamber and were regularly monitored so as to describe and measure leaf and branch lesions. Spots were measured until they became coalescent, so that only well-defined leaf and branch lesions were taken into account. The diameter of leaf spots and the length of branch injuries were registered along time. Data of the last day of observations were statistically analyzed by analysis of variance using Infostat software (www.infostat.com.ar). The inoculated pathogens were recovered after immersion of portions of diseased organs in diluted bleach (2% Cl) during 1 min and plating on PDA.

3. Results

3.1. Pathogens' Isolation and Prevalence

As a result of morphological and biometrical studies, the isolates were identified to genus or species, as follows:

Isolate A-BL-30. Colonies that developed on potato carrot agar (PCA) reached a diameter of 5 cm at seven days. They showed a dark olive pigmentation on both plate sides and produced abundant light brown, obclavate, muriformly septate conidia born in chains. Spores measured 15 - 42 × 5 - 17 µm (27 × 13 µm) and had a beak of 2 × 10 (6 µm). Some of them showed a typical constriction at the medium transverse septum. Cultures grown on malt agar (MA) during seven days showed unbranched conidial chains when observed under the dissecting microscope. These characteristics were coincident with the descriptions provided for *A. tenuissima* [21] [22].

Isolate A-BL-31. White cottony colonies developed on PDA, reaching a diameter of 3 cm in five days. Numerous black, acervular conidiomata exuding conidial masses were produced. Fusiform, straight, 5-celled conidia measured 20.0 - 28.7 µm (22.8 µm) × 6.0 - 8.6 µm (7.4 µm). Their three olivaceous median cells were 16.1 - 19.2 µm (17.3) µm long, while apical and basal cells were hyaline, with three apical appendages 17.4 - 32.5 µm (20.9) µm long and one basal appendage 4.1 - 7.8 µm (6.1 µm) long. Based on [23] and [24], the isolate was identified as *Pestalotiopsis guepinii* (Desm.) Steyaert.

Isolate A-BL-32. Colonies on PDA were initially whitish and rapidly turned grayish brown. They sporulated profusely producing conidia arranged in botryose clusters. Conidia were hyaline, smooth-walled, oval, with a distinctive slightly protuberant hilum, and measured 8.2 - 11.9 µm (10.1 µm) × 5.8 × 7.9 µm (6.7 µm). Numerous, 2.2 - 3.9 mm (3.0 mm) × 1.9 - 3.0 mm (2.6 mm), black, round to elliptical sclerotia developed in circles near the margin of 10-days colonies. According to the observed characteristics, the isolate was identified as *Botrytis cinerea* Pers. [25].

Isolate A-BL-33. Colonies grown on PDA were initially white, becoming light to dark gray with the onset of sporulation. Black, spherical to sub-spherical, one-celled, free conidia measured 14 - 19 µm (17.2 µm) × 12 - 17 µm (16.0 µm). Conidia were borne on a hyaline vesicle at the tip of the conidiophore. These characteristics agree with published descriptions of *Nigrospora sphaerica* (Sacc.) E.W. Mason [26] [27].

Isolate A-BL-34. The isolate grew rapidly on malt extract agar (MEA) and produced round unicellular conidia borne in columns from ampulliform phialides. On the basis of these features, it was identified as *Penicillium* sp. [28].

Isolate A-BL-35. On PDA, colonies developed profuse white cottony mycelium and produced sclerotia which were large, black, irregularly shaped and measured 2.1 - 8.9 mm (6.2 mm) in length. They were distributed at random on the culture mediums, especially at the periphery of the colonies. No reproductive structures were observed. Based on the descriptions provided by [29] and [30] the isolate was identified as *Sclerotinia sclerotiorum* (Lib.) de Bary.

Isolate A-BL-36. The colonies grew fast on PDA. Conidiophores enlarged at its apex to form a rounded vesicle. The fertile area of the vesicle gave rise to a layer of phialides that produced long chains of black conidia. These features led to the identification of the isolate as *Aspergillus* sp. [31].

A. tenuissima was the most prevalent pathogen isolated from the samples of spotted leaves (**Figure 1**). Minor leaf pathogens as *Nigrospora sphaerica* and *Botrytis cinerea*-in decreasing importance—were also obtained, at percentages that ranged from 1% to 5%. Fruit rot pathogens were mainly *A. tenuissima* and *P. guepinii* (**Figure 2**). Other pathogens, recovered from 34% to 57% of the symptomatic fruits were *Botrytis cinerea*, *Nigrospora sphaerica*, *Penicillium* sp., *Sclerotinia sclerotiorum* and *Aspergillus* sp. Among them, *B. cinerea* and *N. sphaerica* were the most frequent.

3.2. Inoculations of *A. tenuissima* and *P. guepinii*

3.2.1. Alternaria Disease

Initial symptoms consisted of reddish pinpoint spots which appeared two days after inoculation on punctured leaves and four days after inoculation on undamaged ones. Symptom progress curves were similar and the highest leaf spot diameter was statistically equal between them. Developed leaf spots were light brown or reddish-brown, with or without red margins. Blight developed from leaf apexes and margins in 10% of punctured leaves from day nine on, and caused defoliation. Branch lesions appeared as tiny reddish-brown spots 24 and 32 days from inoculation of punctured and undamaged branches respectively, and developed into cankers at 40 days.

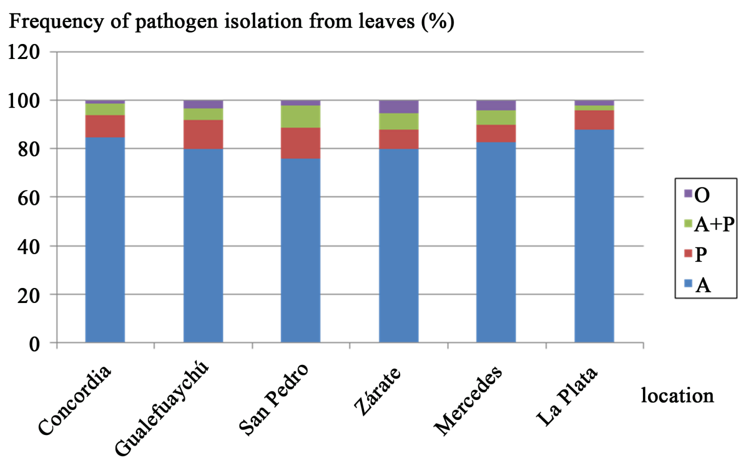

Figure 1. Prevalence of *Alternaria tenuissima* (A), *Pestalotiopsis guepinii* (B), *A. tenuissima* + *P. guepinii* (A + P) and other pathogens (O) in blueberry leaves at different locations. Mean of three observations (100 leaves each).

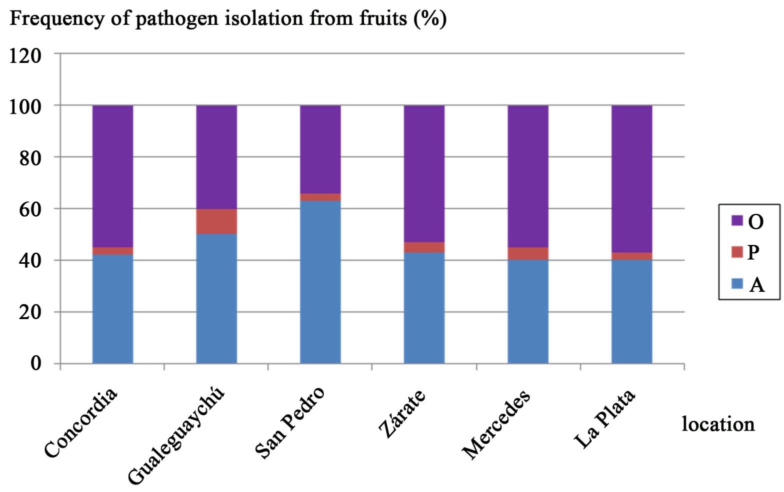

Figure 2. Prevalence of *Alternaria tenuissima* (A), *Pestalotiopsis guepinii* (P), and other pathogens (O) in blueberry fruits at different locations. Mean of three observations (100 fruits each).

3.2.2. Pestalotiopsis Disease

Initial symptoms were evident six and nine days from inoculation, on damaged and undamaged leaves, respectively as light brown pinpoint spots. Coalescent spots were evident on 8% of the leaves at day 16 and defoliation was observed. Leaf spots were light to dark brown after 16 days, and had turned dark brown at day 32. Drop-off of the center of the lesions eventually occurred. Inoculated branches showed light brown discoloration around needle injuries, after 9 days, and cankers were evident at day 24. Spots appeared on undamaged branches 24 days from inoculation and turned into cankers at day 40.

3.2.3. Alternaria + Pestalotiopsis Disease

Initial symptoms began to develop four and six days after inoculation, on damaged and undamaged leaves, respectively. The leaves showed reddish-brown spots typical of *A. tenuissima* and dark brown spots typical of *P. guepinii*, both with red margins. Leaf apex necrosis was prevalent after 16 days, causing defoliation. Incubation period on leaves was intermediate between the registered for individual inoculations. Injured branches showed brown discoloration around needle punctures after 9 days.

Control plants did not develop disease symptoms. The inoculated fungi were recovered from diseased organs. Both pathogens, individually and in a mixture, are able to cause leaf spots as well as branch cankers. Significant interaction was detected between treatments and registration dates ($p < 0.0001$). **Table 2** summarizes symptom characteristics observed in each case and **Figures 3-5** illustrate symptom development on leaves and branches.

4. Discussion

A. tenuissima was by far the most frequent species isolated from leaves, and it was highly present on fruits as well; and *P. guepinii* was comparatively less important on both organs. *A. tenuissima* has been identified causing leaf spots on blueberry in Argentina [19], China, the United States and New Zealand [17]. *Pestalotiopsis* sp. and *P. guepinii* were found to infect blueberry leaves in China [15]; Turkey [32] and Argentina [20], respectively. However, most records describe it as pathogen on twigs, e.g. *Pestalotiopsis* spp. were identified as causatives of cankers and dieback in the neighbor country Chile [33]. The other fungal species isolated from symptomatic blueberry organs have been identified as pathogens on *V. corymbosum* already. Blueberry was confirmed as a new host of *N. sphaerica*, on which it causes leaf spot and shoot blight [19]. *B. cinerea* was also reported on blueberry, as causative of flower and branch blights [34] and shoot blight caused by *S. sclerotiorum* was diagnosed [35]. *Penicillium* sp. and *Aspergillus* sp. were previously isolated producing postharvest decay of blueberries in Argentina [36].

The development of epidemics caused by *A. tenuissima* in blueberry is highly dependent on environmental variables and inocula produced from sporulating leaf spots. Disease progress curves obtained in San Pedro, Gualeguaychú and Concordia (Argentina) during crop cycles 2008-2009 and 2009-2010, adjusted to Logistic or

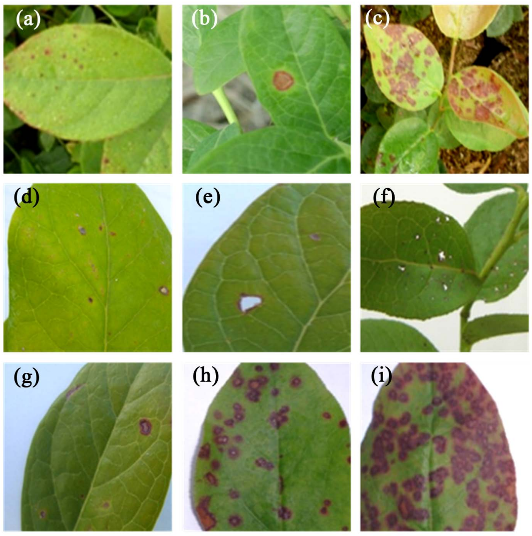

Figure 3. Initial and final symptoms caused by *Alternaria tenuissima* ((a)-(c)) and *Pestalotiopsis guepinii* ((d)-(f)) and *A. tenuissima* + *P. guepinii* ((g)-(i)) on blueberry leaves.

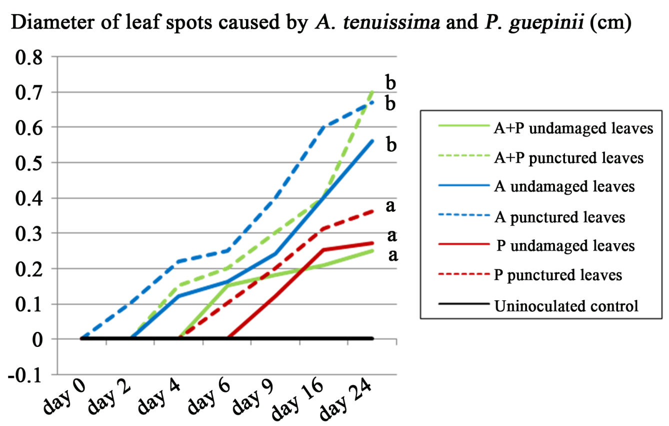

Figure 4. Development of symptoms on blueberry leaves after inoculation with *Alternaria tenuissima* (A), *Pestalotiopsis guepinii* (P), and *A. tenuissima* + *P. guepinii* (A + P). Different letters show significant differences (Tuckey test; $\alpha \leq 0.05$).

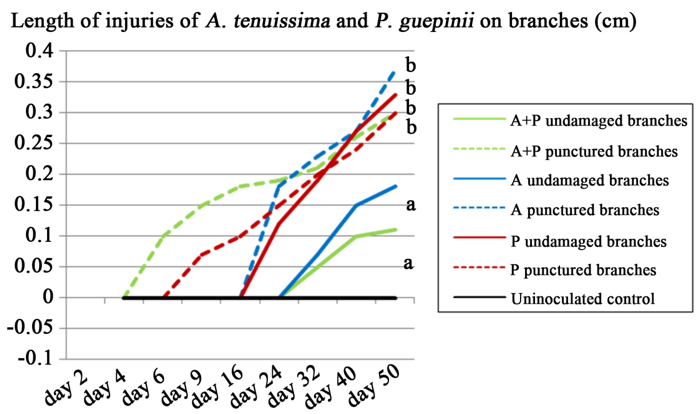

Figure 5. Development of symptoms on blueberry branches after inoculation with *Alternaria tenuissima* (A), *Pestalotiopsis guepinii* (P), and *A. tenuissima* + *P. guepinii* (A + P). Different letters show significant differences (Tuckey test; $\alpha \leq 0.05$).

Table 2. Leaf and branch symptoms registered on blueberry plants inoculated with *Alternaria tenuissima* (A) and *Pestalotiopsis guepinii* (P) separately and mixed.

Disease symptoms	A	P	A + P
Reddish pinpoint initial leaf spots	√		√
Light brown pinpoint initial leaf spots		√	√
Light or reddish-brown leaf spots with or without red margins	√		
Light to dark brown leaf spots		√	
Eventual drop-off of the center of the leaf lesion		√	
Reddish-brown and dark brown spots, both with red margins.			√
Leaf apex or margin necrosis	√	√	√
Branch cankers	√	√	√
Defoliation	√	√	√

Gompertz epidemiological models. A logistic regression model that included leaf senescence rate, rainfall frequency and the interaction between minimum temperature and rainfall frequency, showed a prediction accuracy of 97% [37]. So, a low disease progress rate can be expected during spring (cool weather, young foliage) and a higher probability of high epidemic rates can be expected in summer (increasing air temperature, leaf senescence) [38] [39]. The results added information for the development of a predictive system for leaf spots caused by *A. tenuissima* on cultivars O' Neal and Misty. To our knowledge, no research has been carried out on the epidemiology of symptoms caused by *P. guepinii* on blueberry. Our results add information on the development of symptoms caused by *A. tenuissima* and/or *P. guepinii* on cultivar O' Neal, at cool temperatures.

A. tenuissima species group is one of the most common representatives of the genus *Alternaria* [40]. They may be important as plant pathogens as well as dangerous for human health, as their growth in blueberries is especially problematic since it could result in accumulation of mycotoxins [41]. The genus *Alternaria* was the main component of the blueberry mycobiota (95%). A group of isolates of *A. tenuissima*, as well as *A. alternata* and *A. arborescens* were found to be pathogenic on blueberry fruits in Argentina with different grades of aggressiveness and toxicogenic potential [42]. Further studies are needed to evaluate health risks related to the ingestion of infected fruits. In the same way, toxicogenic potential should be evaluated for the rest of fruit pathogens.

Leaf spots caused by *A. tenuissima* were predominantly reddish, while the spots caused by *P. guepinii* were brownish. Typical *Alternaria* leaf spots were evident after inoculations of isolate A-BL-1 and incubation at 24˚C [20]. Our work was performed with the same isolate at 22˚C, and the different temperature values can explain the comparatively slower development of symptoms registered. Also, *Pestalotiopsis* symptoms developed slowly on leaves when compared with a previous report, maintaining the plants that had been inoculated with the same isolate A-BL-15, at 28˚C [43]. Leaf spot dimensions are similar to those registered by other authors [44] who also observed that spots enlarged and coalesced. When both pathogens were present, they originated leaf spots that were reddish-brown or dark brown, always with red margins. Initial leaf symptoms could be easily differentiated. Symptoms were similar but not identical to those observed after individual inoculations; and both pathogens were recovered from leaf lesions, thus demonstrating the existence of simultaneous infections.

This study was focused on the quantification of prevalence and the differentiation of symptoms caused by main blueberry aerial pathogens on one cultivar in two of the three most important productive region of Argentina. It would be necessary to confirm if other cultivars show the behavior of cultivar O'Neal in these and different locations.

5. Conclusion

A. tenuissima is the major blueberry pathogen that has been detected up till now in Argentina. It is capable of infecting leaves, branches and berries, thus causing important crop losses. Leaf symptoms caused by *A. tenuissima* and *P. guepinii*, which is other important pathogen, can be differentiated at least at initial stages of the diseases on cultivar O'Neal.

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