

# First Report of Apical Necrosis in Walnut Cultivars from Northern Argentinean Patagonia

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## Abstract

Walnut blight caused by *Xanthomonas arboricola* pv. *juglandis* and apical necrosis are severe diseases that cause premature drop of walnuts all around the world. Evidence from previous studies proposes *Xanthomonas arboricola* pv. *juglandis* together with *Fusarium* spp. and *Alternaria* spp. as causal agents of apical necrosis. While walnut blight is a well-known pathology in Argentina, apical necrosis has not been reported yet in this country. However, during 2013 and 2014 seasons, serious damages were registered in the nut producing region of Río Negro Middle Valley in northern Argentinean Patagonia. Diseased fruits exhibited symptoms that matched those described for walnut blight and apical necrosis. Therefore, the aim of this study was to determine the incidence of these pathologies and the microorganisms involved in their etiology. As a result, it was possible to identify morphologically and molecularly the bacteria isolated from damaged fruits, confirming their identity as *Xanthomonas arboricola* pv. *juglandis*. Moreover, *A. tenuissima* was found in a high prevalence in damaged as well as in healthy fruits, whereas *Fusarium* spp. was isolated with a low frequency in both. When pathogenicity tests were performed, it was demonstrated the ability of the three microorganisms to produce typical lesions in healthy nuts. Thus, we can presume that both diseases occurred during this couple of years in this region. This is the first report of apical necrosis in Argentina

**Keywords:** Apical necrosis; *Xanthomonas arboricola* pv. *Juglandis*; *Alternaria tenuissima*; *Juglans Regia* L

## Introduction

Walnut blight caused by the bacterium *Xanthomonas arboricola* pv. *juglandis* (syn. *Xanthomonas campestris* pv. *juglandis*) is an important pathology affecting up to 50% of a walnut cultivar (*Juglans regia* L.) yield, as reported in France [1]. At a global scale, this disease occurs practically in all the countries where walnut trees are grown. This pathology, also known as “blackwood”, “bad dry” or “black plague” of walnut was first reported in Argentina by Marchionatto in 1944 in Buenos Aires province [2]. It was also found in the provinces of Mendoza, La Rioja, Catamarca, Río Negro and Chubut [3]. When fruits are infected by this pathogen, during the pre-blooming or blooming periods, lesions locate typically at the apical end and consist of small, circular or irregular and moist areas that subsequently collapse and sink into the internal tissue. When the infection occurs after blooming, small circular patches are observed on the sides of the walnut, with well-defined margins and a humid and depressed appearance as the first lesion originated at the end of the fruit [4].

During the last years, it has been reported a new disease that affects walnut trees causing premature fruit to drop and significant yield losses in Mediterranean regions such as Spain, France, Turkey [5,6] and Italy [7]. The typical symptoms of this pathology consist of an apical necrosis that originates at the stigmatic end of the fruits. A characteristic apical necrosis that causes severe fruit fall was defined by Belisario et al. [7] as Brown Apical Necrosis (BAN) and was recently described in some walnut producing regions of Spain and Italy. Brown apical necrosis was first reported and observed in Italy in 1998 [7].

Information collected after several years of studies on apical necrosis in walnut cultivars of Spain and Turkey led to the description of the internal and external symptoms of affected fruits that remain attached to the tree and fruits fallen to the ground. The external symptoms begin to be generated after the fruit set at the apical end and they become more evident as the fruit grows. Lesions are usually small and dark brown or black, not moist, that progress until they are approximately 2 mm to 15

mm in size with relatively regular and usually circular margins, in the form of a patch. Unlike the fruits that remain attached to the tree, the external necrosis in the fallen fruits can reach the equatorial zone of the same and, sometimes, they are usually covered of a superficial white fungal mycelium at its ends. With respect to internal symptoms, the infection progresses through the tissues and can reach the seed, causing a brown or black rot of the seeds [6,8]. This description matches with the symptoms found in fruits with brown apical necrosis from cultivars of Italy, described by Belisario et al. [7]. Although necrosis at the apical end is always present, there is no definite correlation between the extent of external and internal lesions [7].

Because of the similarity of symptoms, external apical necrosis may be confused with walnut blight during the early stages of walnut development, and the differentiation between the two diseases requires a detailed observation of the symptoms presented by the internal and external tissues of the walnut. [6].

Current studies indicate that the microorganisms responsible for this disease could involve the bacterium *Xanthomonas arboricola* pv. *Juglandis* as responsible for the initial infection and also to a diverse group of fungi among which *Fusarium* stands out and a complex of different *Alternaria* taxa [9]. The latter can be separated into three morphologically different groups, each of them typified by a representative *Alternaria* species: The *A. alternata* group, the *A. tenuissima* group and the *A. arborescens*

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Received June 23, 2017; Accepted July 17, 2017; Published July 20, 2017

**Citation:** Temperini CV, Pardo AG, Pose GN (2017) First Report of Apical Necrosis in Walnut Cultivars from Northern Argentinean Patagonia. J Plant Pathol Microbiol 8: 414. doi: [10.4172/2157-7471.1000414](https://doi.org/10.4172/2157-7471.1000414)

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group [10,11]. The presence of these fungi would be associated with an opportunistic colonization of apical lesions that were originated in the first place by the bacterium.

It has been noted that nutrient deficiency and soil properties (acidity, light texture, low manganese and magnesium content, and phosphorus and calcium deficiency) in addition to microbial infection could predispose trees to be infected and increase the severity of apical necrosis [1]. This pathology could also be related to climatic factors such as rainfall or temperature. Although the environmental conditions that favor its development are not completely defined, it was observed that percentages of relative humidity higher than 70% and average temperatures higher than 24°C during the initial growth period of the fruits can trigger the development of the disease [6].

In northern Patagonia at the Middle Valley region of the province of Río Negro, Argentina, walnut culture with innovative characteristics began to develop, with varieties of lateral load, use of rootstocks, greater density of plantation and modern systems of conduction. Approximately 400 ha are cultivated by 50 producers in this valley, and the main variety is Chandler, followed in importance by Franquette, Cisco, Tulare, Ivarto and Mayette. The most used rootstocks are *Juglans regia* and *Juglans hindsii*. Producers in the region integrate a public-private initiative called "Río Negro Dry Fruits Cluster", which brings together farmers, businessmen, technicians, and government people from the middle valley, the lower valley and the upper valley region of Río Negro related to the production and commercialization of nuts, hazelnuts and almonds [12].

During the 2013 and 2014 seasons, in several walnut cultivars (*Juglans regia*) of the middle valley region, severe damage was observed due to a disease affecting walnut trees, which caused the early fall of the fruits and the progressive injury of those which were attached to the trees. Special climatological conditions took place such as average maximum temperatures exceeding the threshold of 16°C, and the occurrence of precipitations during the period of susceptibility to the disease (September to December) that generate propitious conditions (free water on the surface of the plant organs) [12]. Walnut producers have registered losses of up to 40% in local production, which means a very important economic loss for this productive sector [13]. The percentages of losses recorded in previous seasons due to walnut blight were lower than those during the 2013 and 2014 seasons [13]. This numbers allow us to suspect the presence of a new disease in the region.

Although there is knowledge about the existence of walnut blight in walnut cultivars from several provinces of Argentina [2,3], cases of apical necrosis were found only in this region [14]. According to information provided by the National Institute of Agricultural Technology staff, this new disease is currently emerging in other places of Argentina such as Mendoza and Tucumán. However, after an exhaustive bibliographical search, we have not found formal reports of apical necrosis in our country. In addition, considering the situation in the middle valley region in relation to a disease that causes significant losses to the producing sector, as well as its fast spread through the region, the aim of this work is focused on studying the microorganisms involved in the pathology, identifying them and determining their role, in order to later establish control strategies, as well as early detection, to avoid large economic losses to the productive sector and maintain cultivars health.

## Materials and Methods

### Sampling and disease incidence

During 2013 and 2014 seasons, 79 symptomatic fruits were

randomly collected from diseased walnut trees (*Juglans regia* L.) of commercial cultivars of Chandler and Tulare varieties located in Choele Choel and Luis Beltrán towns (middle valley region, province of Río Negro, north of Patagonia, Argentina). Based on external symptoms, they were classified as affected by walnut blight, apical necrosis, or coexistence of both pathologies to determine their incidence. Then, 30 of these fruits, 15 with walnut blight and 15 with apical necrosis were cut to check and record the presence and extension of internal lesions. Later on, different pieces from these damaged tissues were used for the microbiological study.

### Isolation and identification of microorganisms

Fruits were superficially disinfected with 1% sodium hypochlorite solution (NaOCl) for 2 min and rinsed twice with sterile distilled water for 2 min. Then, walnuts were cut in half and portions of lesions from the stigmatic end and different interior tissues were aseptically taken and cultured on Potato Dextrose Agar (PDA) supplemented with chloramphenicol (0.1 g/L). Petri dishes were incubated at 25°C for 5 days to make differential counting of the genera and species based on their morphological and microscopic characteristics according to Pitt and Hocking [15]. Isolates belonging to the genera *Alternaria* and *Fusarium* were obtained from monosporic cultures on water agar for further species identification. For *Alternaria*, it was followed the criteria established in previous studies [16,17], and for *Fusarium* according to Nelson [18] and Leslie [19].

Bacteriological analysis was carried out with the remaining half of each fruit, submerging it in 100 ml of physiological solution (0.85% NaCl) followed by an incubation in an orbital shaker at 100 rpm, and 25°C for 24 h. Thereafter, 100 µl were scattered with a Drigalsky spatula on Luria Bertani (LB) and Yeast Dextrose Calcium (YDC) media. Plates were incubated at 27°C for 4 days. Those colonies showing the typical morphological characteristics of *Xanthomonas* were cultured on a differential medium for this genus called Xan-D [20], and incubation was carried out at 27°C for at least 4 days to 6 days or until observing a change of color of the medium and/or colonies.

### Morphological, biochemical and physiological assays of bacteria

Colonies showing morphological characteristics of *Xanthomonas* spp. on Xan-D medium were sub-cultured on nutrient agar to perform morphological, biochemical and physiological assays following the standard microbiological protocols proposed by Schaad [21]: Gram staining and presence of fluorescent pigments on F Agar (similar to King B medium), growth at 35°C and NaCl tolerance on 3% nutrient agar, catalase, oxidative and fermentative metabolism of glucose, aerobic fermentation of sugars in broth (glucose, lactose and sucrose), indole and nitrate production, hydrolysis of aesculin and starch, digestion of proteins (casein and gelatin), lipolytic activity (hydrolysis of Tween 80) and urease activity [9].

### Molecular methods

DNA extraction: Fifteen bacterial isolates from nutrient agar cultures showing specific morphological and biochemical characteristics of *Xanthomonas* were resuspended in sterile distilled water reaching an  $OD_{600\text{ nm}}=0.2$  to achieve a concentration of  $1 \times 10^8$  CFU/ml to  $5 \times 10^8$  CFU/ml approximately (isolates: 1XVM, 5XVM, 10XVM, 11XVM, 20XVM, 26XVM, 39XVM, 47XVM, 52XVM, 57XVM, 60XVM, 63XVM, 94XVM, J1XVM, J2XVM). Then, suspensions were centrifuged at 5000 × g for 10 min and the supernatant was discarded. Pellets were stored at -80°C until use. DNA extraction was performed with the DNeasy blood

and tissue mini kit with a pretreatment protocol for gram negative bacteria following manufacturer's instructions (Qiagen, Intl). Genomic DNA was quantified with a fluorimeter Qubit 2.0 (Life Technologies, Intl.).

PCR reactions: two different PCR protocols were carried out, in order to identify bacteria:

At genus level: PCR amplifications were performed using *Xanthomonas* genus-specific primers X1 and X2 for 16S rDNA genes, according to Maes [22] protocol with modifications. Reaction tubes were placed in a multigene thermal cycler (Labnet International Cycler) with the following modified running conditions: initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 37°C for 30 sec and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. Water and DNA from *Pseudomonas* spp. were used as negative controls.

At species level: PCR amplifications were performed using *Xanthomonas* specie-specific primers XarbQF and XarbQR for *qumA* gene, a quinate dehydrogenase [23]. Reaction contained a total volume of 40 µl with 50 ng of DNA template, 1X PCR buffer, 0.2 µM of each primer (GBT Oligos, Buenos Aires), 0.2 µM of each dNTP, 2.5 mM MgCl<sub>2</sub>, 1%, DMSO and 1.2 units Taq DNA polymerase (Thermo Fischer Scientific, Invitrogen Argentina S.A.). Reaction tubes were placed in a multigene thermal cycler (Labnet International Cycler) with the following running conditions: initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min. Water and DNA from *Pseudomonas* spp. were used as negative controls.

Amplifications for identification at pathovar level could not be performed due to the absence of published sequences for specific primers.

Amplicons were visualized under UV light after running 10 µl of each reaction on 1% agarose gel with Gel Red. Sequencing of *qumA* fragments was done with the XarbQF primer by Macrogen Inc. (Seoul, Korea) and sequence data obtained in this study were deposited in GenBank database under accession numbers MF186174 to MF186188.

### Storage of microbial isolates

Pure isolates of *X. arboricola* were maintained on solid nutrient agar at 4°C and stored in Yeast Peptone Glucose Broth (YPGB) supplemented with 15% glycerol at -80°C for long-term storage [24]. Fungal isolates of *Alternaria* and *Fusarium* were maintained on solid PDA at 4°C and stored in 18% glycerol at -80°C for long-term storage.

### Pathogenicity tests

Immature fruits (40 days after fruits set) were disinfected superficially with tap water, brushing the surface and the stigmatic end, to avoid opportunistic infections. Then the procedure was repeated with a 20% NaCl solution for 5 min, and thereafter rinsed twice with sterile distilled water. Three isolates of *Fusarium* (*F. chlamydosporium*, *F. equiseti* and *F. sambucinum*), 8 isolates of *Alternaria* (7 *A. tenuissima* and 1 *A. alternata*) and 14 isolates of *Xanthomonas arboricola* were used to test their pathogenicity on healthy walnuts from Chandler variety. To evaluate bacterial pathogenicity, disinfected fruits were punctured two times at the equatorial zone and once at the stigmatic end with a needle impregnated with bacteria taken from a 4-day-old nutrient agar colony [24]. Controls were inoculated with sterile distilled water. To evaluate fungal pathogenicity, 5 mm plugs of 7-day-old PDA cultures

were placed on the stigmatic ends of disinfected fruits. Negative controls were performed by puncturing healthy fruits without adding any microorganism. In each case, the assay was carried out by duplicate. Inoculated fruits were put in glass jars containing a piece of sterilized cotton soaked in water, in order to assure 90% RH. They were incubated at 25°C and 16-h photoperiod for 15 days. After the incubation period, fruits were visually examined to detect external lesions. They were cut transversally at the inoculations points to observe the progression of the disease. In order to determine severity, we used indexes to qualify the infection: 0 (no infection); 1 (necrosis located at the inoculation point); 2 (necrosis extending from the inoculation point through the green tissue); 3 (necrosis extending through the mesocarp and reaching the endocarp) and 4: necrosis affecting the seed. This method is described by Aletà et al. [5]. In order to fulfill Koch's postulates pieces of necrotic lesions were processed as described previously and cultured on LB and PDA, for re-isolation of bacteria and fungi, respectively. Moreover, combined tests following the above-mentioned protocols were assayed mixing bacteria with each fungal genus and a final one with the three microorganisms altogether. Lesions were then recorded and classified as previously mentioned.

### Natural occurrence of *Alternaria* in healthy walnuts

When conducting pathogenicity assays with bacteria, we noticed the issue of contamination from inoculation points at the stigmatic end with *Alternaria*. This forced us to test several disinfection protocols to avoid it and also pushed us to determine natural occurrence of the genus *Alternaria* in healthy walnuts. Twenty-five healthy walnut fruits from Chandler variety were superficially disinfected with 1% sodium hypochlorite solution (NaOCl) for 2 min and rinsed twice with sterile distilled water for 2 min. Then pieces from the stigmatic end were placed on PDA agar medium supplemented with Chloramphenicol (0.1 g/L). Cultures were incubated at 25°C for 7 days and identification of *Alternaria* genus was made by means of morphological characterization [16,17].

## Results

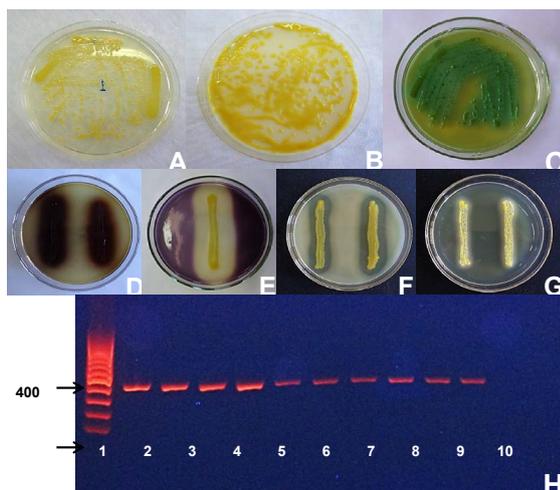
### Incidence and symptoms of diseases from detached fruits

37.7% of the analyzed fruits showed symptoms of walnut blight. Chandler nuts exhibited an external mottled of dark brown to black wet patches that sank into the tissue and spread over the surface of the fruit and sometimes they covered it entirely. A wide range of severity grade and extension of symptoms was observed (Figure 1a). In some cases the presence of white filaments at the stigmatic end corresponding to fungal development could be visualized. Regarding to internal symptoms, in most fruits the exocarp tissue got necrotic in the same places where the external mottling was observed. Sometimes the lesions extended and affected deeper internal tissues such as mesocarp, endocarp and the seed (Figure 1b). In nuts of Tulare variety, the external mottled was larger almost becoming a single spot, which was usually much darker and wetter than in Chandler variety (Figure 1c). Respect to internal lesions, these appeared to be limited only to the tissue underlying the shell (Figure 1d), 46.84% of the analyzed fruits showed symptoms of apical necrosis. They all corresponded to Chandler variety. Externally, there was a single brownish spot, which varied in color intensity, covering only the stigmatic end of the fruit. Sometimes, it appeared surrounded by a defined line at the boundary of it (Figure 1e). Internally, the tissue was affected only at the stigmatic end and from there it could, in some cases, propagate to the seed (Figure 1f).

In fruits affected with walnut blight and in fruits with apical necrosis they showed no correlation between the severity of the



**Figure 1:** Symptoms of walnut blight and apical necrosis: A) external symptoms of walnut blight on Chandler variety; B) Internal symptoms of walnut blight on Chandler variety; C) External symptoms of walnut blight on Tulare variety; D) Internal symptoms of walnut blight on Tulare variety; E) External symptoms of apical necrosis on Chandler variety; F) Internal symptoms of apical necrosis on Chandler variety.



**Figure 2:** Morphological and molecular characterization of *Xanthomonas arboricola* pv. *juglandis*: A) Growth on LB medium; B) on YDC medium and C) on Xan-D medium. D) hydrolysis of aesculin; E) hydrolysis of starch; F) digestion of casein and G) hydrolysis of Tween 80. H) Amplification of *qumA* gene (402 bp fragment): lane 1: DNA molecular size marker 100 bp ladder; lanes 2 to 11: 1XVM, 10XVM, 11XVM, 20XVM, 26XVM, 47XVM, 52XVM, 63XVM, 94XVM, J1XVM; lane 12: water negative control; lane 13: *Pseudomonas* sp. negative control.

external symptoms and the internal ones of the same fruit, as reported by Belisario et al. [7]. Finally, 15.19% of analysed fruits showed a combined symptomatology of both diseases spreading through the surface. They all belonged to Chandler's variety.

### Isolation and identification of microorganisms

In 66.7% of fruits with walnut blight and in 53.3% of fruits with apical necrosis, the isolated bacteria showed the typical morphological characteristics of *Xanthomonas* genera on the three-media used to its culture. On LB, colonies were yellow, mucoid and convex with well-defined edges (Figure 2a). On YDC, colonies were similar but larger and the yellow color was more intense turning to slightly orange in time

(Figure 2b). Finally, on Xan-D medium, and after at least six days of incubation, colonies started to show a change of color on themselves and the media to green or blue, and they were mucoid, convex and circular surrounded by a small clear zone of skim milk hydrolysis and a bigger zone of Tween 80 lipolysis (Figure 2c). This confirmed that these bacteria belong to the *Xanthomonas* genera. Bacteria were isolated most frequently from internal than external tissues, even reaching the seed.

Fungal development was observed in 93.3% of walnuts exhibiting symptoms of walnut blight and in 100% of walnuts exhibiting symptoms of apical necrosis. The isolations obtained corresponded to the genera *Alternaria* (83.21%), *Epicoccum* (13.74%) and *Fusarium* (3.05%).

Regarding *Alternaria*, isolates were classified in two morphologically different groups: 97.25% corresponded to the *Alternaria tenuissima* group and 2.75% to the *Alternaria alternata* group. All isolates of *Epicoccum* corresponded to the *E. nigrum* species and the species of the *Fusarium* genera were: *F. equiseti*, *F. chlamydosporium* and *F. sambucinum*.

*Fusarium* and *Alternaria* were also found in all tissues of the fruit but differently from bacteria, they were most frequent at external than internal tissues.

### Morphological, biochemical and physiological assays of bacteria

Results obtained indicate a gram-negative bacteria and catalase positive with no production of fluorescent pigments, growth at 35°C and tolerance to 3% NaCl. They have an oxidative glucose metabolism and they produce acid from glucose, lactose, and sucrose under aerobic conditions. They show the ability to hydrolyze aesculin and starch, digest casein and gelatin and possess lipolytic activity (Figures 2d-2g). There were neither production of indole nor nitrates and no urease activity was observed.

### Molecular methods

All the bacterial isolates tested showed the genus-specific amplicon (data not shown). Moreover, all of them displayed the typical 402 bp fragment corresponding to the sequence of *qumA* gene (Figure 2h). Both negative controls showed no amplification. Sequencing of *qumA* PCR products allowed us to confirm the identity as *Xanthomonas arboricola*.

### Pathogenicity tests

All of the fifteen *X. arboricola* isolates resulted to be pathogenic on healthy Chandler walnuts from detached fruits. External lesions were observed after 7 days of incubation and consisted of 2 mm to 3 mm brown to blackish patches with a wet appearance that progressed to a maximum of 5 mm patches at 14 days after inoculations. Regarding internal lesions: 28.6% showed necrosis located at the inoculation point (index 1), 42.7% showed necrosis extending from the inoculation point through the green tissue (index 2), 14.3% showed necrosis extending through the mesocarp and reaching the endocarp (index 3) and 14.3% showed necrosis affecting the seed (index 4) (Figures 3a-3e).

Among *Fusarium* species only *F. equiseti* caused necrosis on walnuts with a lesion consisting of a 10 mm brownish to black spot with irregular boundaries and dry aspect. The infection remained circumscribed only to the exocarp tissue (index 2) (Figures 3f and 3g). All the *Alternaria* isolates (*A. tenuissima* and *A. alternata*) were pathogenic on healthy walnuts. External lesions progressed at 14 days to 5-10 mm brown to blackish patches, sank into the tissue, with a dry aspect and irregular boundaries. As *Fusarium*, necrosis only progressed to the exocarp (index 2) (Figures 3h and 3i).



**Figure 3:** Pathogenicity tests on Chandler walnuts: A) external lesion produced by *Xanthomonas arboricola* pv. *juglandis*; B) index of infection: 1; C) index of infection: 2; D) index of infection: 3; E) index of infection: 4; F) external lesion produced by *F. equiseti*; G) internal lesion produced by *F. equiseti*; H) external lesion produced by *A. tenuissima*; I) internal lesion produced by *A. tenuissima*; J) external lesions produced by combination of the three microorganisms; K) internal lesions produced by combination of the three microorganisms.

Results from all combined tests with bacteria and each fungus consisted of external lesions with similar characteristics to the necrotic external patches caused by fungi and bigger or identical extension, but infection always progressed to the seed (index 4). When the three microorganisms were present all together, morphological characteristics of external and internal lesions remained the same but their extension was wider in some cases. (Figures 3j and 3k)

All negative controls performed for each assay did not show any kind of symptoms on healthy fruits.

### Natural occurrence of *Alternaria* in healthy walnuts

Fungi were found in all of the stigmatic ends of healthy Chandler walnuts. 92% of the fungal isolates corresponded to *Alternaria* spp. and the remaining 8% to *Epicoecum nigrum*.

### Discussion

This study provides information about the different microorganisms that appear to be involved in diseases causing early drop in walnut fruits of the Río Negro Middle Valley region. We give a detailed description of the characteristic symptoms of each disease, matching in every case to those described for walnut blight [4] and for apical necrosis [9]. As regards their incidence, and particularly referring to these couple of years with special and differential climatological features, both pathologies were present with similar prevalence in cultivars of the region, but apical necrosis showed a higher incidence. It is also important to mention the appearance, in a low frequency, of fruits with both symptomatology reflecting a mixture of both diseases in a single walnut. In respect to varieties response to walnut blight, Tulare seems slightly more sensitive to the disease than Chandler. However, in a study conducted by Buchner et al. [25] evaluating blight response in different walnut varieties, Tulare was not statistically different from Chandler both showing "low blight sensitivity".

Typical morphological characteristics on isolation media and differential medium Xan-D, together with biochemical and physiological assays led to presume the identity of the bacteria as *Xanthomonas* spp. [9,20,21]. Molecular tests provided a confirmation of the species assuring its identity as *Xanthomonas arboricola*. Although

we could not identify it at a pathovar level due to the absence of published sequences for specific primers, we could presume that it is *Xanthomonas arboricola* pv. *Juglandis*. This is supported by the fact that it was isolated from damaged walnuts of *Juglans regia* trees and the absence of reported information of the ability of pathovars to cause disease in different hosts.

Among the genera isolated, the genus *Alternaria* stands out for its frequency and this result could be related to the existence of *Alternaria* spp. as a natural agent at the stigmatic end of healthy nuts. In fact, Belisario et al. [7] reported its presence in healthy fruits and also in diseased fruits with a high frequency of isolation. Within this genus, the group-species *A. tenuissima* predominates. Different from other parts of the world such as Mediterranean places like Italy [7] or Spain [9], *Fusarium* is less meaningful due to its low frequency of isolation from detached fruits at this latitude, though it has the ability to cause lesions on healthy fruits.

### Conclusion

In conclusion, morphological and biochemical tests along with molecular analysis contributed to identify the causal agents of both pathologies. Moreover, PCR tests could be performed directly from infected tissues, with a previous DNA extraction step, to only detect the bacterium [26] or the bacterium along with the fungus when running a PCR test with universal primers for identifying fungal genera, such as internal transcribed spacer (ITS) of nuclear ribosomal DNA [27]. This could be useful to rapidly discriminate whether the disease is walnut blight or apical necrosis.

Even though, *Xanthomonas arboricola* pv. *Juglandis* has been proven to cause lesions under pathogenicity trials [9], *Alternaria* has not been considered as a pathogen [7,9]. However, taking into account results from test pathogenicity, both *Xanthomonas arboricola* pv. *Juglandis* and *A. tenuissima* can produce external typical lesions of walnut blight and apical necrosis on healthy immature fruits. Actually, *A. tenuissima* generates external bigger necrotic patches, but it does not progress to internal tissues, as *Xanthomonas arboricola* pv. *Juglandis*. When both microorganisms are present, the extension and progression of diseases is bigger and faster.

All these data contribute to support the idea that during 2013-2014 seasons, walnut blight and apical necrosis diseases took place under special climatological conditions of the Río Negro Middle Valley producing region. Moreover, apical necrosis showed a higher incidence. The finding of fungi in all of the analyzed fruits with apical necrosis and bacteria in almost half of them, along with their ability to cause disease in healthy walnuts led us to propose *A. tenuissima* together with *Xanthomonas arboricola* pv. *Juglandis* as the microorganisms involved in the development of apical necrosis. On the other hand, the presence of *A. tenuissima* in a high percentage of fruits with walnut blight suggests this fungus as responsible for progression and deterioration of infected fruits to walnuts exhibiting both symptomatology. Further studies on each phenological stage of walnuts should and will be conducted to unravel the role of each microorganism in the development of both diseases.

### Acknowledgments

The authors wish to thank the members and collaborators of the Río Negro Dry Fruits Cluster, Javier Alonso and Dr. Mirta Rossini (INTA). To CONICET, UNRN, UNQ and ANPCYT for financial support.

### Conflict of Interest

The authors declare no conflict of interest.

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