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PREVALENCIA Y ETIOLOGÍA DE MICROORGANISMOS ASOCIADOS A NECROSIS APICAL CAFÉ EN FLORES Y FRUTOS DE NOGAL (*Juglans regia* L.) BAJO CONDICIONES DE CAMPO DE LA ZONA CENTRO SUR Y SUR DE CHILE

Tesis para optar al grado de Magister en Ciencias Agronómicas con mención en Producción y Protección Vegetal.

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RESUMEN

La necrosis apical café (BAN) es una enfermedad del nogal que se caracteriza por la necrosis apical y una caída inusual y prematura de los frutos. Esta enfermedad esta asociada a especies de *Fusarium* y *Alternaria*, aunque esta sintomatología puede estar asociada a *Xanthomonas arboricola* pv. *juglandis* agente causal de la peste negra del nogal. Recientemente en Chile se ha descrito una notoria caída prematura de frutos presentando necrosis apical que ha sido asociada a BAN. Los objetivos del presente trabajo fueron determinar la prevalencia de los principales microorganismos asociados al síntoma de necrosis apical y caída de frutos, evaluar el nivel de patogenicidad de las especies de microorganismos involucrados en el complejo BAN cuando actúen de forma separada o interactuando entre ellos y determinar él o los estados fenológicos más susceptibles a la infección bajo condiciones experimentales de campo durante dos temporadas. Los resultados muestran que *Alternaria* sp., *Fusarium* sp. y *Xanthomonas arboricola* pv. *juglandis* prevalecieron sobre frutos con síntomas de BAN, y que su prevalencia fue diferente entre huertos y entre temporadas. Las pruebas de patogenicidad demostraron la capacidad de los tres microorganismos, ya sea de forma individual o como infección mixta, para producir síntomas y caída prematura de frutos inoculados. Los estados fenológicos de mayor susceptibilidad fueron los estados Ff2 y Gf, mientras que las aplicaciones de cobre disminuyeron la severidad de la enfermedad. Estos resultados sugieren que BAN es una enfermedad causada por un complejo de microorganismos y que su severidad depende del estado fenológico en donde ocurre la infección.

ABSTRACT

Brown apical necrosis (BAN) is a walnut disease that causes apical necrosis and premature fruit drop of walnut, resulting in important yield losses. The disease is associated with *Fusarium* and *Alternaria* species, although symptomatology can also be caused by *Xanthomonas arboricola* pv. *juglandis* (Xaj), which is the causal agent of walnut blight. Increased premature fruit drop has been described in Chile in recent years, with presence of apical necrosis associated with BAN. The objectives of this study were to: determine the prevalence of the main microorganisms responsible for apical necrosis in walnut orchards of south-central and southern Chile; evaluate the level of pathogenicity of the species of microorganisms involved in the BAN complex when they act separately or interact with each other; and determine the development stage(s) when walnut flower or fruit is more susceptible to be infected by microorganisms associated with BAN under field conditions during two seasons. Results showed that *Alternaria* sp., *Fusarium* sp. and Xaj prevailed on fruits with symptoms of BAN, and that their prevalence varied between orchards and seasons. Pathogenicity trials demonstrated that the three microorganisms, acting either individually or as a mixed infection, can cause symptoms and premature drop of inoculated fruits. Ff2 and Gf were the development stages with the greatest susceptibility to BAN, while copper sprays decreased disease incidence. These results suggest that BAN is not caused by a single agent but by a combination of microorganisms and that disease incidence depends on the phenological stage in which the infection occurs.

INTRODUCCIÓN GENERAL

El nogal (*Juglans regia* L.) en Chile ocupa una superficie de aproximadamente 40.800 hectáreas lo cual representa un 11,9% de la superficie dedicada a la producción de distintos frutales en el país. La superficie establecida se concentra entre las regiones de Coquimbo y Biobío, siendo la Región Metropolitana la que concentra la mayor superficie plantada [34,6% del total], aunque el mayor crecimiento en superficie se ha localizado en la zona centro sur y sur de Chile entre las regiones del Maule y la Araucanía, que representan alrededor de un 25% de la superficie del país (Larrañaga y Osores, 2019).

El cultivo del nogal, al igual que la mayoría de los cultivos, se ve afectado por diversas enfermedades, causadas principalmente por pseudo-hongos, hongos y bacterias. Entre las enfermedades que afectan al nogal está la peste negra del nogal causada por *Xanthomonas arboricola* pv. *juglandis* (Pierce) (Sharma *et al*., 2012; Hassan y Ahmad, 2017).

Xanthomonas arboricola pv. *juglandis* puede afectar todos los tejidos nuevos de la planta, tales como: amentos, flores femeninas, hojas, frutos y brotes (Frutos, 2010). Los síntomas generales de la enfermedad se presentan en forma de manchas marrón oscura a negras sobre las hojas, tallos jóvenes y la cáscara del fruto (Moragrega *et al*., 2011; Ninot *et al*., 2002). Los síntomas en las hojas comienzan como pequeñas manchas de color marrón oscuro rodeadas por un halo amarillento, que conduce a una necrosis negra que es más extensa en etapas avanzadas (Frutos, 2010). Las infecciones leves en los frutos aparecen como lesiones pequeñas, circulares o irregulares y húmedas, que posteriormente se deprimen hacia el tejido interno. Estas lesiones pueden ocurrir tanto al costado como en la zona apical del fruto (Miller y Bollen, 1946), donde las infecciones graves en los frutos producen la caída de estos, mientras que aquellos que logran permanecer en el árbol tienden a ennegrecerse y secarse quedando, muchas veces, en el árbol (Frutos, 2010). De acuerdo a la sintomatología producida por la bacteria, se cree que esta no produciría la muerte de la planta huésped (Frutos, 2010), aunque se ha demostrado que algunas poblaciones de esta bacteria desarrollan cancros en la madera del árbol, causando una enfermedad denominada VOC (del inglés: *vertical oozing canker*) (Hajri *et al*., 2010). Las pérdidas de producción asociadas a peste negra pueden variar de un 50% al 70% cuando las condiciones ambientales son favorables para el desarrollo de la enfermedad (Hajri *et al*., 2010; Lang y

Evans, 2010). Por ello, en la actualidad esta bacteria fitopatógena es la más importante a nivel de las especies del género *Juglans* (Arquero *et al*., 2006).

No obstante lo anterior, durante la temporada de 1998 en Italia se presentó una caída inusual y prematura de los frutos en un huerto productivo. En las cáscaras de los frutos recolectados, a menudo se presentaron manchas de color marrón a marrón oscuro, que se originaban en su región estigmática, tendiendo a agrandarse, llegando al interior de la nuez, la cual presentó una superficie suave y acuosa. A este síndrome se le denominó necrosis apical café o BAN (del inglés: *brown apical necrosis*; Belissario *et al*., 2001). La sintomatología externa asociada a esta nueva enfermedad comienza en la zona apical del fruto una vez que este ha cuajado. Las lesiones suelen ser pequeñas, normalmente circulares, de color marrón oscuro, medianamente regulares alcanzando tamaños de 2 a 15 mm. Las lesiones internas ocurren una vez que el patógeno avanza por el tejido hasta alcanzar las partes internas produciendo podredumbre de color marrón oscura a negra. En la fruta caída, los síntomas externos se agravan, cubriendo la mayor parte del fruto, y en ocasiones, presentando desarrollo de signos de los posibles patógenos (Belisario *et al*., 2001; Belisario *et al*., 2002; Belisario *et al*., 2004; Moragrega y Ozaktan, 2010; Moragrega *et al*., 2011; Akat *et al*., 2016; Temperini *et al*., 2017). De acuerdo a los síntomas antes mencionados, BAN se podría confundir con la sintomatología que presentan los frutos con *X. arboricola* pv. *juglandis*, pero a diferencia de los frutos con peste negra, los frutos con BAN no presentan lesiones acuosas, que se deprimen hacia el tejido interno y la infección inicial está limitada a la zona apical del fruto (Moragrega y Ozaktan, 2010) . Los estudios posteriores al primer reporte de esta enfermedad fueron enfocados en asociar la sintomatología, antes descrita, con un agente causal, ya que ha existido una controversia asociada a cuál es la real importancia de los hongos que han sido aislados desde frutos inmaduros enfermos. Estudios han demostrado que frutos inoculados con diferentes especies de *Fusarium* producen una mayor caída de éstos en las estados más juveniles de la flor o del fruto (Scotton *et al*., 2015), aunque otros autores plantean que esta caída de frutos ocurre por alteraciones fisiológicas (Garcin y Duchesne, 2001). Los pocos antecedentes que existen sobre este nuevo síndrome sugieren que especies de *Fusarium*, serían los agentes causales de BAN (Belissario *et al*., 2001; Belissario *et al*., 2002) ya que a pesar de aislar distintas especies de *Alternaria* a partir de frutos sintomáticos, al realizar inoculaciones artificiales de estos hongos sobre frutos sanos arrojaron que solo *Fusarium* sp. fue capaz de reproducir la sintomatología asociada a BAN. Por otra parte otras investigaciones concluyen que solo *X. arboricola* pv. *juglandis* sería el agente causal de esta caída de frutos y que *Fusarium* sp. actuaría aumentando la patogenicidad cuando fueron inoculadas en conjunto (Akat *et al*., 2016). Del mismo modo se ha sugerido que BAN se origina debido a un complejo de microorganismos patógenos, los cuales puede coexistir en la misma planta y/o fruto, estos microorganismos incluirían a *X. arboricola* pv. *juglandis, Fusarium* sp. y *Alternaria* sp. (Ustun *et al*., 2016; Belissario *et al*., 2002; Scotton *et al*., 2015; Temperini *et al*., 2017).

Recientemente en Chile, se ha descrito en la región del Biobío una notoria caída de frutos de nogal en huertos productivos presentando sintomatología asociada a BAN (Moya, 2017). Debido al poco conocimiento epidemiológico que existe de la necrosis apical café y a la alta ocurrencia que ha tenido esta enfermedad en las últimas temporadas en la Región del Biobío, se hace necesario determinar cuáles son las causas que generan este síndrome, así como él o los estados fenológicos en que ocurre la infección de la flor y/o fruto y las interacciones que ocurren entre los microorganismos que participan en este síndrome.

HIPÓTESIS

La necrosis apical café que afecta a los frutos de nogal es generada por la interacción de la bacteria *Xanthomonas arboricola* pv. *juglandis,* y hongos fitopatógenos de los géneros *Fusarium* y *Alternaria,* y el daño causado depende del estado fenológico de la flor o fruto en que ocurre la infección.

OBJETIVO GENERAL

Determinar la interacción entre microorganismos asociados a necrosis apical café y los estados fenológicos de la flor y fruto en que estos agentes fitopatógenos causan este síndrome en huertos comerciales de la Región del Biobío, Chile.

OBJETIVOS ESPECÍFICOS

- Determinar la prevalencia de los principales microorganismos causantes de BAN en nocedales comerciales entre la región Metropolitana y la Araucanía.

- Evaluar el nivel de patogenicidad que alcanzan las especies de microorganismos involucrados en el complejo BAN cuando actúan de forma separada o interactuando entre ellos.
- Determinar él o los estados fenológicos en que la flor o fruto son susceptibles a la infección por microorganismos asociados a BAN.
- Evaluar el impacto de las aplicaciones de cobre en la expresión de la enfermedad en frutos inoculados con los microorganismos asociados a BAN.

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Prevalence and etiology of microorganisms associated with brown apical necrosis in flowers and fruits of walnut (*Juglans regia* L.) under field conditions in south-central and southern Chile.

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Abstract

Brown apical necrosis (BAN) is a walnut disease that causes apical necrosis and premature fruit drop of walnut, resulting in important yield losses. The disease is associated with *Fusarium* and *Alternaria* species, although symptomatology can also be caused by *Xanthomonas arboricola* pv. *juglandis* (Xaj), which is the causal agent of walnut blight. Increased premature fruit drop has been described in Chile in recent years, with presence of apical necrosis associated with BAN. The objectives of this study were to: determine the prevalence of the main microorganisms responsible for apical necrosis in walnut orchards of south-central and southern Chile; evaluate the level of pathogenicity of the species of microorganisms involved in the BAN complex when they act separately or interact with each other; and determine the development stage(s) when walnut flower or fruit is more susceptible to be infected by microorganisms associated with BAN under field conditions during two seasons. Results showed that *Alternaria* sp., *Fusarium* sp. and Xaj prevailed on fruits with symptoms of BAN, and that their prevalence varied between orchards and seasons. Pathogenicity trials demonstrated that the three microorganisms, acting either individually or as a mixed infection, can cause symptoms and premature drop of inoculated fruits. Ff2 and Gf were the development stages with the greatest susceptibility to BAN, while copper sprays decreased disease incidence. These results suggest that BAN is not caused by a single agent but by a combination of microorganisms and that disease incidence depends on the phenological stage in which the infection occurs.

Keywords: Brown apical necrosis; Walnut blight; Complex disease; Etiology.

Introduction

Walnut (*Juglans regia* L.) orchards occupy an area of approximately 40,800 hectares in Chile, which represents 11.9% of the total cultivated area of different fruit trees in the country. The main production area is concentrated between the Coquimbo and Biobío Regions, but the largest planted area (34.6% of the total) is found in the Metropolitan Region. During the last decades, walnut orchards have steadily increased between the Maule and Araucanía Regions (south-central and southern Chile), representing around 25% of the country's total planted surface of this nut crop (Larrañaga and Osores, 2019).

Walnut production in Chile is affected by different diseases caused by pseudo-fungi, fungi and bacteria. The main pathogens are the pseudo-fungi *Phytophthora cactorum* (Lebert and Cohn) and *Phytophthora cinnamoni* (Rands), which cause root and stem rot (Guajardo *et al*., 2019). In addition, fungi *Diplodia mutila* (Díaz *et al*. 2018) and different species of the *Botryosphaeriaceae* family have been described causing wood cankers (Chen *et al*. 2014), whereas the fungus *Microstroma juglandis* (Berenger) Sacc., causes downy leaf spot on leaves. Bacterial diseases such as crown gall caused by *Agrobacterium tumefaciens* (Smith and Townsend), and walnut blight caused by *Xanthomonas arboricola* pv. *juglandis* (Pierce) (Xaj) (Ninot *et al*. 2002; Yakabe *et al*. 2012; Sharma *et al*. 2012; Hassan and Ahmad, 2017) have been reported, with Xaj being the most important pathogen affecting this nut crop in Chile (Esterio *et al*. 2006; Moya-Elizondo *et al*. 2018), particularly in the south-central and south areas of the country because frequent spring rains and moderate temperatures favor the development of the disease (Moya-Elizondo *et al*. 2018; Moya-Elizondo, 2020). *Xanthomonas arboricola* pv. *juglandis* (Xaj) can affect all the new tissues of the plant, such as catkins, female flowers, leaves, fruits, and shoots (Frutos, 2010). The general symptoms of the disease appear in the form of dark-brown to black spots on the leaves, young stems, and fruits (Moragrega *et al*. 2011; Ninot *et al*. 2002). Mild infections in walnut fruit appear as small, circular, or irregular, and moist lesions that subsequently collapse and sink into the internal tissue. These lesions can occur both on the side of the husk and in the apical zone of the walnut fruit (Miller and Bollen, 1946). Serious infections cause early fruit drop, while fruits that manage to remain on the tree tend to blacken and dry out (Frutos, 2010).

Yield losses associated with walnut blight can vary from 50% to 70% when environmental conditions are favorable for the development of the disease (Hajri *et al*. 2010; Lang and Evans, 2010). For this reason, Xaj is the most important pathogen involved at the level of the *Juglans* genus species (Arquero *et al*. 2006). In Chile, Moya-Elizondo (2020) has estimated yield losses of 20% between the Maule and Araucanía Regions, with a cost of US$ 2,200 per hectare, and annual expenses of US$ 10,342,700 derived from walnut blight control in this area.

Since the first report of premature fruit drop in a productive walnut orchard in Italy, which was associated with the syndrome called brown apical necrosis or BAN (Belissario *et al*. 2001), several studies have described the importance of this disease (Belisario *et al*. 2001; Belisario *et al*. 2002; Belisario *et al*. 2004; Moragrega and Ozaktan, 2010; Moragrega *et al*. 2011; Akat *et al*. 2016; Temperini *et al*. 2017). BAN causes brown to dark-brown spots of 2 to 15 mm, which originate in the stigmatic region and tend to enlarge, reaching the inside of the walnut. The disease is mainly associated with species of fungi of the genera *Fusarium* and *Alternaria* (Belissario *et al*. 2001) whose external symptoms begin in the apical zone of the fruit (Moragrega and Ozaktan, 2010). Internal lesions occur once infection progresses through the tissue, reaching the internal parts of the fruit and producing dark brown to black rot. In fallen fruit, the external symptoms are aggravated, covering most of the fruit, and, in some cases, showing signs (mycelium) of possible fungal pathogens (Belisario *et al*. 2001; Belisario *et al*. 2002; Belisario *et al*. 2004; Moragrega and Ozaktan, 2010; Moragrega *et al*. 2011; Akat *et al*. 2016; Temperini *et al*. 2017). The aforementioned symptoms can be confused with those presented by fruits infected with Xaj, the causal agent of walnut blight (Moragrega *et al*., 2011). However, fruits infected with BAN do not present moist areas that then collapse and sink towards the internal tissue, eventually affecting the entire fruit (Moragrega and Ozaktan, 2010). In general, the importance of BAN is controversial because there is no agreement on the real pathogenicity that isolated fungi have on diseased immature fruits. Even though fruit drop can occur due to physiological alterations (Garcin and Duchesne, 2001), studies have shown that fruits inoculated with different species of *Fusarium* produce greater premature drop at early stages of flower or fruit development (Scotton *et al*. 2015). The limited information existing on this disease suggests that *Fusarium* species would be the causal agents of BAN because, even though different species of *Alternaria* were isolated from symptomatic fruits, only *Fusarium* sp. was able to reproduce the symptoms associated with BAN when performing artificial inoculations with these fungi on detached healthy fruits (Belissario *et al*. 2001; Belissario *et al*. 2002). On the other hand, Moragrega *et al*. (2011) and Akat *et al*. (2016) concluded that only Xaj would cause apical necrosis and fruit drop and that *Fusarium* sp. would only increase pathogenicity in case of a combined infection. In this sense, other researchers have suggested that BAN is caused by a combination of pathogenic microorganisms, which can coexist in the same plant and/or fruit, such as Xaj and different species of *Fusarium* and/or *Alternaria* (Ustun *et al*. 2016; Belissario *et al*. 2002; Scotton *et al*. 2015; Temperini *et al*. 2017).

In recent years, premature fruit drop has been observed in walnut orchards of the Ñuble and Biobío Regions (37° 35'S 72° 28'W), with symptoms associated with BAN (Moya, 2017; Moya-Elizondo *et al*. 2021). However, there is a lack of quantitative information on BAN disease prevalence and disease severity in the walnut production of south-central and southern Chile. Yield losses caused by the disease, which local growers have associated with walnut blight, have varied from year to year. Additionally, recent reports on resistance to copper by Xaj strains obtained from productive walnut orchards (Moya-Elizondo *et al*, 2018) have influenced this perception. Therefore, it is important to determine the causal agents that generate BAN, the development stage (s) in which the infection of the flower and/or fruit occurs, and the interactions that occur between microorganisms that cause this disease.

The objectives of this study were to: (I) determine the prevalence of the main genera of microorganisms that cause BAN in commercial orchards of south-central and southern Chile; (II) evaluate the level of pathogenicity by the species of microorganisms involved in

BAN complex disease when they act separately or interact with each other; and (III) determine the development stage(s) in which the flower or fruit is more susceptible to be infected by microorganisms associated with BAN under field conditions.

Materials and methods

Study sites

The prevalence study of the microorganisms that cause BAN in commercial walnut orchards, and the experiments to evaluate pathogenicity and the development stage with greater susceptibility to infestation were carried out in three 8-year-old ‘Chandler’ walnut orchards. The orchards are located in Chillán, Paso Alejo Sector (36°37'S; 71°57'W), Ñuble Region; Los Angeles, Santa Elcira sector (37°21'S; 72°26'W); and Negrete (37°35'S; 72°28'W) in the Biobío Region. Walnut trees were sampled during two seasons (2018 and 2019) for the prevalence study. In addition, four productive ‘Chandler’ orchards were sampled during the 2019 season in Paine (33°43'S; 70°45'W), Metropolitan Region; Talca (35°34'S; 71°19'W), Maule Region; Coihueco (36°37'S; 71°51'W), Ñuble Region; and Perquenco (38°21'S; 72°24'W, Araucanía Region. These four surveyed orchards were sampled to obtain a broader view of the ocurrence of BAN disease along a more extensive area of walnut production in Chile.

Prevalence study of the main microorganisms that cause BAN in commercial orchards

One hundred immature fruits presenting symptoms associated with BAN (presence of necrosis in the distal area of the drupe) during the phenological stage J: fruit thickening (Muncharaz, 2000) were collected from different trees in the orchards under study between November and December of 2018 and 2019. The fruits were collected in a transept that was carried out in a “zigzag” pattern within an area of approximately 2 ha in each orchard. The fruits were taken to the laboratory, disinfected for 2 min in 75% ethanol and rinsed twice with sterile distilled water (SDW). Subsequently, the fruits were cut in half longitudinally; one of the halves was placed in a humid chamber, while two pieces of affected tissue were obtained from the remaining half. One of the sub-pieces was mantained on potato dextrose agar (PDA) culture medium supplemented with streptomycin (200 mg L^{-1}) to favor fungal isolation; whereas the other sub-piece was placed on yeast extract dextrose calcium carbonate agar (YDCA) culture medium, which allows differentiating *Xanthomonas* species by the yellow color of the colonies (Lelliott and Stead, 1987). As there was mycelium growth from the pieces, this was reisolated by taking hyphal tips that were placed on PDA medium. All the material used for the isolation of the microorganisms was previously sterilized in an autoclave at 120ºC at a pressure of 1.2 kg cm^{-2} for 15 min. The isolated fungi and bacteria were identified by observing morphological structures under an optical microscope and comparing them with taxonomic keys.

Inoculation of BAN-associated microorganisms in walnut flowers and fruit.

Production of inoculum of microorganisms.

The microorganisms used corresponded to an isolate of *Alternaria alternata* (F122) [(ITS GenBank accession numbers: MT482505; SSU 33 rRNA: MT489690; btub1: MT495609)], an isolate of *Fusarium oxysporum* (H02) [ITS: MT482501; SSU rRNA: MT489686; tef1-α: MT511739] (Moya *et al*., 2021) and *Xanthomonas arboricola* pv. *juglandis* Negrete strain, which belong to the collection of microorganisms of the Laboratory of Phytopathology of the Faculty of Agronomy, Universidad de Concepción, Chillán campus. The microorganisms were isolated from walnut fruits presenting symptoms of BAN in orchards from the Biobío Region during the 2017 season.

Bacteria were grown in test tubes containing Luria Bertani (LB) broth, incubated for 48 h at 25° C and 150 rpm. Subsequently, the bacteria were centrifuged at 5000 rpm for 6 min to remove the supernatant and resuspended in 8.9% saline solution prior to inoculation. For fungi, 5 mm mycelium discs of each fungal isolate were added to 90 mm diameter Petri dishes with 20 mL of PDA medium. The plates were incubated in a culture chamber at a temperature of 25 ºC ± 0.5 ºC in the dark for 7-10 days for the growth and sporulation of the fungus. The conidia suspension was obtained by applying 15 mL of sterile distilled water (SDW) on the surface of the sporulating fungus inside the Petri dish, and the conidia were detached by using a glass rod. The resulting suspension was filtered using a sterile cheesecloth to remove mycelium debris and obtain conidia only. Once filtered, the conidial concentration of the suspension was determined using a Neubauer chamber.

Pathogenicity trials: microorganisms associated with BAN at different flower and fruit development stages.

For the pathogenicity trials in the 2017 season, *A. alternata* and *F. oxysporum* were used in suspension at a concentration of 10^6 cfu mL^{-1}, while inoculation with suspension of Xaj was at 10^7 cfu mL^{-1}. These microorganisms and a control, which used SDW, were applied individually on the stigmas of the flower and on the apical zone of the fruit, using a suspension volume of 10 µL (Scotton *et al*. 2015). The inoculated fruits were from trees previously treated with copper hydroxide (134.5 g kg^{-1} of $Cu(OH)_2$ [250 g Kocide® 2000 WP, ANASAC Chile S.A.]) and untreated trees. The microorganisms were inoculated in two different development stages: Gf or stigma wilting (stigmas are no longer receptive and begin to dry out) and stage I (fruit set) (Muncharaz, 2000). Finally, the inoculated flowers and fruits were covered with a transparent plastic clamshell to prevent external contamination by other pathogens. Ten fruits per tree were inoculated for each microorganism used, considering the fruits of a tree as an experimental unit and three replicates. This procedure was performed for each development stage. Samples were collected when the fruits reached the phenological stage J: thickening of the fruit (Muncharaz, 2000).

Pathogenicity of the inoculated microorganisms was evaluated based on their ability to reproduce symptoms associated with BAN and cause fruit drop, and the development stage in which the symptoms are more severe. The reisolation procedure of the inoculated microorganisms was similar to that described for the prevalence study. This experiment was conducted in the orchard located in Paso Alejo Sector, Chillán.

In the 2018 season, the different microorganisms were evaluated individually [*Alternaria alternata* (Alt), *Fusarium oxysporum* (Fus) and *Xanthomonas arboricola* pv. *juglandis* (Xaj)], and combined (Alt + Xaj, Fus + Xaj, Alt + Fus, and Alt + Fus + Xaj). The microorganisms were used in suspension at a concentration of 10^6 cfu mL^{-1} and inoculated at three different stages of flower and/or fruit development: Ff2: Full female flowering (stigma opening and end of the period of stigma receptivity); Gf: stigma wilting (stigmas are no longer receptive and begin to dry out) and stage I: fruit set (Muncharaz, 2000). Ten fruits per tree were inoculated with individual or combined microorganisms, considering one tree as an experimental unit and three replicates. The inoculation process was carried out using the methodology described above. However, for combined inoculations, the final volume of 10 µL was obtained by adding equal parts of each microorganism. Collection of inoculated fruits and their evaluation, as well as the reisolation of pathogens for each treatment, were conducted as described for the experiment of the 2017 season. The experiment was replicated in the three 'Chandler' orchards under study: Chillán, Los Ángeles, and Negrete, using trees that were treated with copper hydroxide (134.5 g kg^{-1} of $Cu(OH)_2$ [250 g Kocide® 2000 WP, ANASAC Chile SA]) during the season.

The prevailing environmental conditions during the field trials, which was approximately 80 days in each season and location, are shown in Table 1.

Experimental design and statistical analysis

The data on microorganism prevalence obtained from the nine 'Chandler' walnut orchards sampled (three during season 2018 and six during season 2019) were evaluated based on the groups of microorganisms observed, while incidence of each group was compared using a χ squared goodness-of-fit test to determine differences between the study sites, considering the average prevalence for the number of orchard assessed each season as the expected value (EV) and the prevalence obtained for each orchard in each study season as the observed value (OV). In the pathogenicity trials, effects of the phytopathogenic microorganisms were analyzed based on the symptoms associated with BAN and fruit drop resulting from the inoculation carried out at two or three different development stages. Both experiments were carried out using a completely randomized design with a factorial arrangement, the factors being the inoculated microorganism(s) (A), development stage (B) and orchard management (C) for the experiment carried out in the 2017 season, while only factors A and B were considered in the three experimental sites in the 2018 season. The experimental unit was the walnut tree where the inoculations on fruits were carried out. Pathogenicity data were subjected to an analysis of variance and comparison of means using Fisher's least significant difference (LSD) test ($\alpha = 0.05$). Prior to ANOVA, the data were analyzed to determine normality of distribution and homogeneity of variance using the Shapiro-Wilks and Levene tests, respectively. Percentages of premature fruit drop were transformed by using the formula $y = \sqrt{(x + 0.5)}$, where x = percentage value (Little and

Hills, 1978) to normalize them. All statistical analyzes were conducted using Infostat software version 2008 (Balzarini *et al*., 2008).

Results

Prevalence study

The results of the prevalence study conducted in the 2018 and 2019 seasons are shown in Table 2. During the 2018 season, Xaj recorded the highest prevalence, affecting the apical portion of walnut fruit, and showing yellow-colored colonies on LB medium. When grown on YDC culture medium, those colonies maintained their color, whereas fungal colonies of *Fusarium* showed a pinkish-reddish colored mycelium and *Alternaria* showed a dark brown mycelium on PDA medium. According to the results of the χ^2 test, the prevalence of microorganisms on fruits with symptoms of BAN (2018 season) showed statistical differences between the three sampled orchards for almost all the groups of microorganisms determined ($p < 0.05$; Table 2), with the exception of the combined infection by *Alternaria* and *Fusarium* species (Alt+Fus), and the combination of these two fungal genera with Xaj (Alt+Fus+Xaj). The prevalence of Xaj in symptomatic fruits was higher in Los Angeles orchard (58%), followed by Chillán (26%) and Negrete (3%) (Table 2). Higher prevalence of fungi associated with BAN was observed in Negrete where individual species of *Alternaria* (28% of symptomatic fruits) and *Fusarium* genera (18% of symptomatic fruits) or the combination of both taxonomic fungal groups (Alt+Fus, 11% of symptomatic fruits) had the highest prevalence. BAN pathogens were less recurrent in Los Ángeles compared with Chillán, where *Fusarium* species were observed in 11% of the symptomatic fruits, while *Alternaria* species behaved similarly in both orchards. Fruit infectation by *Alternaria* sp. alone and combined with Xaj showed no differences between the orchards under study. However, the infection caused by *Fusarium* sp. alone and combined with Xaj recorded higher prevalence in Los Angeles (17% incidence), reaching only 4% and 3% in Chillán and Negrete, respectively. Furthermore, the infection caused by Alt+Fus+Xaj was not recurrent among the assessed walnut orchards. Other microorganisms were observed colonizing the apical necrosis of the fruit, such as *Cladosporium* sp., *Penicillium* sp. and bacteria of the genus *Bacillus*, which reached an average of 19.3% of incidence, with higher values in the walnut orchards of Negrete and Chillán (Table 2).

During the 2019 season, the prevalence of microorganisms on symptomatic fruits showed differences in the prevalence of Xaj ($p < 0.05$), reaching an average of 40% among the seven surveyed walnut orchards, being higher in Los Angeles (71%), followed by Negrete (60%), Perquenco (50%), Chillán (40%), Coihueco (33%), Paine (26%) and Talca (10%) (Table 2). Differences in prevalence between the orchards were also observed in the combined infections. The interaction between *Alternaria* sp. and Xaj (Alt + Xaj) resulted in an average of 9.0%, reaching the highest value in Talca (20%), followed by Paine (14%), while Negrete, Coihueco, Chillán, and Los Ángeles recorded values below a 6%. Combined infection of *Fusarium* sp. and Xaj (Fus + Xaj) reached in average 8.0%, with higher values in Talca (26%), followed by Paine (14%); whereas the rest of the orchards recorded prevalence below 5%. Significant differences were also observed with *Alternaria* sp. and BAN fungi combined (Alt + Fus), with higher incidence in Paine (7%), followed by Talca (4%) and Chillán (2%), while this combined infection was not observed on the same fruit in the rest of the orchards. The southermost walnut orchard, Perquenco, did not show walnut fruits infested by the combined infection by Xaj and the two genera of BAN fungi. There were differences among the walnut orchards due to the combined natural infection of the three pathogens of the BAN complex, with values below 2% only in Negrete and Coihueco. The infection caused by the microorganisms associated with BAN (Alt or Fus, separately) did not show differences in prevalence among the assessed orchards (values below 8%), except for Talca since no prevalence of these two fungi was observed (Table 2). Presence of other genera of fungi, such as *Cladosporium, Penicillium* or *Bacillus* bacteria, reached an average of 23% (Range: 43 to 9%), with statistical differences among the orchards (Table 2), with Chillán and Coihueco (Ñuble Region) showing the highest values.

In general, Xaj was the most prevalent pathogen causing necrosis in the apical portion of walnut fruit in the nine orchards sampled along the two seasons, while the combined inoculation with bacteria and BAN fungi resulted in more frequent infections than those caused by both fungal pathogens separately. Infection by fungi was orchard and year dependent. Fungi were higher in Chillán and Negrete orchards in the 2018 season, whereas both walnut orchards showed a high infection by Xaj during the 2019 season. During this latter period, a higher prevalence of Xaj was observed in all the ‘Chandler’ walnut orchards under study, while infection by *Alternaria* isolates showed a higher prevalence than *Fusarium*, but without exceeding 8% of prevalence.

Pathogenicity trial with microorganisms associated with BAN at different development stages of the flower and fruit, 2017 season. In the experiment conducted the season before the prevalence study, it was observed that the inoculated microorganisms generated external and internal symptoms that were similar to those reported for BAN. Initial symptoms consisted of small dark-brown circular lesions located in the apical part of the fruit, which enlarged to more irregular brown spots reaching the interior of the fruit. The quantity of fruits that presented symptoms of BAN and the quantity of dropped fruits showed significant differences ($P < 0.05$; Table 3). The percentage of symptomatic fruit was only statistically significant in the case of orchard management, but no differences were observed for the rest of the assessed factors. In terms of orchard management, the percentage of symptomatic fruits from the trees not treated with copper hydroxide was significantly higher (22.8%) than in the treated trees (Fig. 1). The percentage of fruit drop showed significant differences for factors such as inoculated microorganism, development stage, and orchard management, with factor interaction between inoculated microorganism and orchard management, and development stage and orchard management (Table 3). The simple effect of fruits inoculated with the different microorganisms in copper-treated trees (Fig. 2) revealed that *A. alternata* resulted in a significant increase in premature fruit drop with respect to the non-inoculated control, while *F. oxysporum* and Xaj caused fruit drop of 43% and 35%, respectively, being statistically similar to *A. alternata* and the non-inoculated control. The simple effect analysis of the fruits inoculated with the different microorganisms in untreated trees showed that *F. oxysporum* and Xaj resulted in 26% and 16% more fruit drop than the non-inoculated control (Fig. 2), whereas *A. alternata* caused 37% fruit drop and was statistically similar to the untreated control. Regarding orchard management for each inoculated microorganism, *F. oxysporum* and Xaj caused higher rates of dropped fruits in untreated trees, while *A. alternata* tended to greater fruit drop in copper hydroxide-treated trees (Fig. 2).

Regarding factor interaction between development stage and orchard management, fruit drop was 21% higher in fruits inoculated at Gf stage in untreated trees (no copper hydroxide). At stage I, fruit drop did not show differences between copper-treated and untreated trees. When analyzing inoculated fruit at different development stages, fruit drop was greater at Gf stage compared to stage I, with no differences between trees treated or not treated with copper hydroxide (Fig. 3).

Reisolation of the three inoculated microorganisms from symptomatic and asymptomatic fruits varied between 50 and 97% (Table 4). Regardless of the development stage, reisolation of *A. alternata* was slightly higher and *A. alternata* inoculation was not affected in fruits of trees treated with copper hydroxide. *F. oxysporum* was reisolated in a higher percentage with respect to *A. alternata* and its reisolation frequency increased when fruits were inoculated with this fungus at stage Gf in untreated trees, while at stage I, it was the opposite. Reisolation of Xaj was higher at stage I, with slight differences between copper-treated or untreated trees; at Gf stage, its reisolation was higher when the bacteria were inoculated in fruits of untreated trees (Table 6).

The experiment allowed demonstrating that the inoculated microorganisms were able to reproduce the symptoms associated with BAN, such as brown necrosis in the apical portion of the fruit and premature fruit drop, *F. oxysporum* and Xaj being more pathogenic than *A. alternata*. Walnut fruit was more susceptible to infection by BAN pathogens at Gf stage rather than at stage I. Disease incidence was lower in walnut trees treated with copper hydroxide, while reisolation varied between inoculated microorganisms and management treatment with copper.

Experiments on the interaction between microorganisms associated with BAN in different flower and fruit development stages, 2018 season. In the experiments conducted during the 2018 season, it was possible to reproduce the symptoms of BAN on walnut fruits. Premature fruit drop of flowers/fruits inoculated with the different pathogens under study (applied separately and combined) was evaluated in three different orchards (Table 5). Statistical differences were found in terms of symptomatic fruits and fruit drop ($p < 0.05$) in Chillán and Negrete, while Los Angeles showed differences only in fruit drop rate (Table 5).

Symptomatic fruits. The interaction between pathogen inoculation and development stage had a significant effect on the walnut orchards in Chillán and Negrete (Table 5). In Chillán, the simple effect analysis on the symptomatic fruits inoculated with microorganisms at different development stages showed that only the combined inoculation with *A. alternata* and Xaj (Alt+Xaj) presented symptomatic fruits at Ff2 stage (Table 6). At Gf stage, fruits of the non-inoculated control and those inoculated with *A. alternata* recorded 58.3% and 41.4% of fruits with symptoms, respectively; values were higher than and statistically different from the rest of the inoculated microorganisms (Table 6). In general, the percentage of symptomatic fruits associated with BAN was higher at Gf stage in Chillán (Table 6). In Negrete, Alt+Fus resulted in the highest rate of symptomatic fruits (23%) at Ff2 stage, being significantly different from the rest of the inoculated microorganisms and non-inoculated control (Table 6). The combined inoculations of Alt+Xaj and Alt+Fus+Xaj did not result in fruits with symptoms at this development stage. At Gf stage, *F. oxysporum*, Xaj and Alt+Fus developed symptoms in inoculated fruits, but these treatments were not different from the non-inoculated control. Inoculation during stage I showed that *A. alternata* and Alt+Fus developed BAN symptoms. The inoculation of microorganisms at late development stages decreased the symptoms generated in the inoculated fruits, with the exception of *A. alternata* and Alt+Fus since symptoms were observed in fruits inoculated at stage 1 (Table 6).

Premature fruit drop. In Chillán, there was no significant interaction between inoculated microorganisms and development stage (Table 5). However, the three inoculated microorganisms and their respective combinations caused premature fruit drop. The combinations of fungi and Xaj (Fus + Xaj, Alt + Xaj, and Alt + Fus + Xaj) caused the highest fruit drop, ranging from 83.3 and 70.8%. The microorganisms applied individually were not different from the control, while Alt+Fus was statistically similar to the inoculations that presented higher fruit drop, but also similar to the non-inoculated control (Table 7). Ff2 stage presented on average a higher fruit drop rate (81.3%) compared to Gf, which presented 41.7% fruit drop (Table 7).

In Los Ángeles, significant interaction was found between the inoculated microorganism and development stage (Table 5), showing that the microorganisms *F. oxysporum*, *A.*

alternata and Xaj inoculated at Ff2 stage generated the highest fruit drop rate, ranging from 83.3% to 73.3% (Table 7). At Gf stage, the highest premature fruit drop were caused by Xaj and the combinations Fus + Xaj, and Alt + Fus, reaching 50%, 50%, and 40%, respectively. Fruit drop associated with the different inoculated microorganisms did not present significant differences at stage I, while Xaj did not infect fruits. Individual or combined inoculation with BAN microorganisms were less infective, and walnut fruit drop was significantly reduced when inoculatations were conducted in late stages of fruit development (Table 7).

A significant interaction between inoculated microorganism and development stage was also found in the 'Chandler' orchard of Negrete (Table 5). *Fusarium oxysporum* and Fus + Xaj, inoculated at Ff2 stage, caused the highest percentages of fruit drop, reaching 50% and 43.3%, respectively, and differing from the non-inoculated control (Table 7). On the other hand, flowers inoculated with the three pathogens combined (Alt+Fus+Xaj) had the lowest fruit drop rate (26.7%). At Gf stage, the combination of microorganisms caused the highest premature fruit drop rate, rangining from 20 to 33.3%, and differed from the non-inoculated control. *F. oxysporum* resulted in 26.7% of dropped fruits and was significantly different from the non-inoculated control. For *F. oxysporum* and the combinations of Fus + Xaj and Alt + Fus, and Alt + Xaj, fruit drop was observed only at stage I. Same as in Los Ángeles, Negrete recorded a decrease in fruit drop when the inoculation was conducted at a late development stage, while the combined inoculation of Alt + Xaj resulted in a significant decrease at Gf stage and an increase at stage I. From Ff2 to Gf stages, the combined inoculation of Alt + Fus and Alt + Fus + Xaj resulted in similar levels of fruit drop, but this symptom decreased significantly at stage I (Table 7).

Reisolation of inoculated microorganism. Table 8 shows the results of the reisolation of the inoculated BAN pathogens from the three experiments conducted during the 2018 season. In Chillán, only fungi were successfully resiolated from the inoculated fruits and, when inoculations included bacteria combined with fungi, there was reisolation only in the combined inoculation with *Alternaria* at Ff2 stage (Table 8). Reisolation of *F. oxysporum* and *A. alternata* individually or as a combined infection was obtained from symptomatic and asymptomatic fruits, whereas reisolation of both fungi on the inoculated fruits was reduced when the inoculation was conducted at late development stages (Table 8). At Gf stage, Xaj was not reisolated from fruits inoculated with this pathogen only or when it was combined with the fungi (Table 8).

In Los Ángeles, all inoculated microorganisms were reisolated from symptomatic or asymptomatic walnut fruit collected at J stage, ranging from 4 to 93%, at the three development stages where they were inoculated. The same was observed in Negrete but Xaj was not isolated from fruits inoculated at Gf and I stages (Table 8). Reisolation of *A. alternata* was lower at late development stages; *F. oxysporum* was successfully reisolated, with no major variations between the different development stages in which this fungus was inoculated and it was obtained in a high proportion at late development stages. When both fungi were combined, neither prevailed over the other. *Xanthomonas arboricola* pv. *juglandis* (Xaj) presented a low percentage of reisolation and was only recovered from fruits inoculated at Ff stage (Table 8).

The results obtained in the three experiments of the 2018 season confirmed those obtained in the 2017 season. However, the ability to reproduce BAN symptoms by the pathogens was lower than in the first season. Regarding premature fruit drop, there were differences between the use of pathogen inoculations and the control, but the infection behaved differently in each experimental site. *F. oxysporum* caused greater fruit drop in Negrete, whereas Xaj was more frequent in Los Angeles walnut orchard. *Alternaria alternata* caused similar rates of premature drop in the inoculated fruits in both seasons, but infection was slightly lower in Negrete. The combined inoculation of BAN microorganisms increased fruit drop incidence in Chillán, whereas no differences were observed between individual or combined inoculations in the other two orchards. Regarding fruit development, early stages of development were more susceptible to infection by pathogenic microorganisms.

Discussion

The prevalence study conducted on fruits with symptoms of apical necrosis in ten ‘Chandler’ walnut orchards located in south-central and southern Chile allowed isolating the complex of pathogens associated with BAN disease. Pathogen frequencies were orchard dependent and varied between years. During both seasons, Xaj prevailed in almost all the orchards, reaching the highest level in Los Angeles during the 2018 season. In Negrete, fungi of the genera *Fusarium* and *Alternaria* prevailed, while frequency of fungi and bacteria was relatively similar in Chillán. However, the prevalence study conducted in 'Chandler' walnut orchards during the 2019 season showed that Xaj was more prevalent than the fungi in the three study sites evaluated in 2018, while the same behavior was also observed in the other four additional surveyed orchards. These results suggest that environmental conditions and orchard management influence the population of microorganisms that occur in each orchard and season in south-central and southern Chile. Different prevalence studies aimed at determining BAN pathogens have shown that fungi of the genera *Fusarium* and *Alternaria*, and Xaj were the most prevalent isolates from apical necrosis in walnut fruits. For example, a study conducted in Turkey by Ustun *et al*. (2016) isolated Xaj, *Fusarium* spp. and *Alternaria* spp. Another study conducted by Temperini *et al*. (2017) in walnut in Rio Negro (Argentinian Patagonia) showed that fungi from the genera *Alternaria*, *Epicoccum*, and *Fusarium* were found in 100% of the sampled fruits, while Xaj reached 53.3%. Apart from the similarity between the recurrency of *Fusarium* and *Alternaria* species and Xaj involved in the symptoms of apical necrosis, our results showed variability in the prevalence of these microorganisms between the different study sites. In this sense, Belissario *et al*. (2002) conducted a study on three walnut orchards (two located in Italy and one in France) and observed that the prevalence of the same microorganism varied between the orchards. According to the mentioned authors, the climatic conditions and phytosanitary management of each orchard can explain this variability.

Of all the pathogen species, associated with BAN symptoms, colonizing walnut fruits in south-central and southern zone of Chile, Xaj was the most prevalent. In previous studies, species of the genus *Fusarium*, identified as *F. oxysporum, F. avenaceum, F. culmorum, F. graminearum*, and *F. equiseti*, tended to prevail in some orchards; species of the genus *Alternaria* (*A. alternata* and *A. tenuisima*) were relatively similar to *Fusarium* in terms of prevalence. In addition, other species of microorganisms belonging to other genera of fungi, such as *Cladosporium*, *Penicillium* and *Chaetomium*, were also observed, varying between orchards and seasons. In this sense, pathogen frequency in some orchards and seasons suggests that further studies are required to evaluate the pathogenic implication of these other genera of fungi. In the present study, identification at the level of individual species was not considered because of the the high number of samples collected, so the data and identification grouped the fungal isolates obtained only at the genera level.

The experimental work conducted showed that BAN is a disease complex that varies in incidence according to the development stage in which infection occurs, pathogen(s) involved, and orchard management (copper spraying). Similarly, the interaction between these factors directly affects the development and incidence of the disease. In the experiment conducted in Chillán during 2017 season, *F. oxysporum* and Xaj caused premature fruit drop in the inoculated fruits, whereas *A. alternata* was less frequent. Belissario *et al*. (2002) and Belissario *et al*. (2010) demonstrated that *Fusarium* species, such as *F. semitectum* and *F. graminearum*, were the causal agents of BAN, while Moragrega *et al*. 2011 showed that *X. arboricola* pv. *juglandis* was the main causal agent of apical necrosis in walnut fruits, concluding that this symptomatology would be a new manifestation of walnut blight disease. Although frequently isolated from symptomatic fruits collected from the nine walnut orchards under study, the inoculation with the isolate of *A. alternata* did not cause major symptoms in the inoculated fruits. This agrees with Belissario *et al*. (2002) and Moragrega *et al*. (2011), who indicated that *Alternaria* species should be more a saprophyte than a pathogen on walnut fruits showing apical necrosis. The treatments not inoculated and treated with a 10 µL drop of SDW also presented premature fruit drop, particularly at Ff2 stage. Therefore, distilled water might generate an osmotic imbalance that leads to floral abortion, or this SDW drop could be related to increased pistillate flower abortion (PFA), resulting in early fruit drop caused by an excess of pollen on the stigmas, which occurs naturally in walnut orchards (Gonzáles *et al*. 2008). On the other hand, *Bacillus* bacterial colonies or fungi of the genera *Cladosporium*, *Penicillium* and sometimes *Alternaria*, which were observed in the reisolation from non-inoculated fruits, could play a role where these environmental saprophytes microorganisms colonize flower and affect pollination, causing abortion and drop of walnut flowers and fruits.

During the 2018 season, the pathogenic response of the microorganisms associated with the disease were different in the experiments conducted in the three ‘Chandler’ walnut orchards under study. Inoculation with *A. alternata* caused premature fruit drop in the trial conducted in Los Angeles when this fungus was inoculated individually, which is in agreement with a study conducted by Temperini *et al.* (2017) in Argentina. However, this fungus did not present pathogenicity by itself in Chillán or Negrete, but it increased premature fruit drop when combined with *F. oxysporum* and/or Xaj, acting as a secondary pathogen that increases disease incidence. *F. oxysporum* caused more premature fruit drop inoculated alone in Negrete, and when combined with *A. alternata* and/or Xaj at late phenological stages in Chillán and Los Angeles. *Xanthomonas arboricola* pv. *juglandis* caused stable premature fruit drop in the three orchards. Although pathogenicity of each microorganism inoculated individually in walnut flowers or fruits remaining in the tree was confirmed during the second season, their ability to increase premature fruit drop was also observed in combined infections, particularly in Chillán. The inoculation trials conducted by Moragrega *et al*. (2011) with detached immature fruits revealed that the causal agent of apical necrosis in walnut fruits was Xaj, and that *Fusarium* sp. could be involved by interacting with the bacterial infection. In addition, Akat *et al*. (2016) also demonstrated, using walnut detached immature fruits, that the co-inoculation of fungi and Xaj caused an increase in disease incidence. In the present study, we inoculated flowers and fruits that were attached to the walnut plant, which implies that the defense mechanisms of the plant also influence the response to infection by fungi and bacteria. Considering this, our results showed that this combination of pathogens could increase disease incidence in combined infections under certain field conditions.

In the 2017 season, pathogen reisolation from inoculated fruits with BAN pathogens was higher in the experiment conducted in Chillán for all microorganisms, whereas *A. alternata* and *F. oxysporum* were reisolated with greater success than Xaj in the second season, especially when they were inoculated together. This could be related to the applications of copper hydroxide during the development of the experiment in the 2018 season. Despite being covered with a plastic container, bacteria caused symptoms of the disease in fruits, but repeated copper sprays could enter through small openings present in the container causing bacterial death and/or facilitating the entry of opportunistic fungi. However, it is important to note that the reduction in bacterial concentration and amount of suspension volume of inoculated Xaj bacterium could have also affected reisolation; bacterial concentrations in a suspension of 10^7 cfu mL^{-1} and 10^6 cfu mL^{-1} were used for the pathogenicity tests in 2017 and 2018 seasons, repectively. On the other hand, three pathogens were inoculated with 10 µL on flowers the first season; during the second season, when combined microorganisms were inoculated, the final inoculation volume of 10 µL was obtained by adding equal parts of each microorganism. These results suggest that bacterial infection in walnut fruits and flowers could be more dependant on inoculum concentration than on the fungi involved in BAN disease under field conditions.

Environmental conditions (air temperature and relative humidity) did not vary greatly between the orchards. Nevertheless, the number of rainy days and the amount of rainfall varied considerably (Table 1). During the 2017 season, Chillán recorded only 10 rainy days, with a rainfall of 61.3 mm, from pathogen inoculation to fruit removal at stage J in approximately 80 days; during the 2018 season, there were 18 rainy days, with a rainfall of 89.2 mm (45% more than the previous season). Los Ángeles and Negrete recorded 21 and 23 rainy days, with rainfall of 131.4 and 138.3 mm in the 2018 and 2019 seasons, respectively. In terms of rainfall concentration, around 70% of rainy days occurred during the first month of trials, which implied that higher humidity conditions were available for microorganisms inoculated at Ff2 and Gf stages, which would explain the higher incidence of BAN pathogens during those stages under field conditions. High humidity has been suggested as an important environmental variable, significantly affecting walnut fruit drop under BAN infection (Scotton *et al*. 2015). On the other hand, the microorganisms inoculated at stage I were subjected to lower environmental humidity and lack of free water on plant tissues due to a lower rainfall, resulting in less favorable conditions for bacterial and fungal infection. This would explain why the microorganisms were isolated in high percentages at the time of reisolation (stage J), despite the fact that they did not produce major symptoms of the disease when they were inoculated at early development stages. Under field conditions, BAN pathogens are likely to remain latent in the fruit, waiting for favorable conditions to infect.

In south-central and southerner Chile, copper sprays are carried out on weekly basis or even more frequently depending on the magnitude of rainfall events. In order to control the increase in Xaj populations, repeated copper spraying is conducted between September and December, reaching 15-17 spraying events during the season; the period in which the experiments were conducted included 5 to 8 spraying events. Therefore, the inoculation with Xaj caused disease symptoms, but repeated copper spraying reduced bacterial inoculum in walnut fruit, affecting reisolations and favoring fungal isolations as observed in the experiments conducted in treated orchards. Nevertheless, most Xaj isolates from infected fruit could be explained by deficient applications of the bactericide or presence of copper-resistant populations of Xaj as previously reported in Chile (Moya-Elizondo *et al.* 2018)

The inoculation of microorganisms at Ff2 stage caused higher premature fruit drop, decreasing at late fruit development stages (Ff2 > Gf > I). This agrees with Akat *et al.* (2016) and Arquero *et al.* (2006), who suggested the presence of symptomatic fruits in walnut orchards of different varieties at early stages of fruit development. The results of the present study showed that susceptibility to BAN pathogen complex decreases as fruit development progresses, with a marked difference in fruit drop incidence between stages Gf and I. Therefore, considering the etiology of BAN disease, higher susceptibility to infection by this complex of pathogens occurs at Ff2 and Gf stages, period in which fungicides or bactericide sprays need to be conducted to allow for adequate disease control in productive walnut orchards.

Copper hydroxide spraying reduced infection in inoculated walnut fruits. In fact, apical necrosis and fruit drop was more frequent in untreated trees (no copper spraying), showing more effectiveness at Gf stage. However, it was less effective with *A. alternata*, which almost increased its presence under copper spraying. In this sense, the literature has described that copper spraying reduces the populations of Xaj and disease severity caused by the pathogen in walnut (Lee *et al.* 1993; Ninot *et al.* 2002; Lang and Evans, 2010). To our knowledge, there is no information about fungal control by copper in walnut. However, Scotton *et al.* (2015) indicated that a walnut orchard under chemical treatments showed a very low availability of natural inoculum of BAN pathogens. *In vitro* tests have demonstrated the effectiveness of copper nanoparticles in the control of *F. oxysporum, F. culmorum*, and *F. equiseti* (Bramhanwade *et al.* 2016; Viet *et al.* 2016). Nevertheless, Belissario *et al.* (2010) demonstrated the effectiveness of the fungicides Tebuconazole and Mancozeb in the control of BAN disease.

The results of the present study contribute to a better understanding of the etiology of BAN in 'Chandler' walnut trees grown under field conditions in south-central and southern Chile. BAN should be considered a new disease affecting walnut in this area. Xaj, *Fusarium* and *Alternaria* species are involved in disease development, which varies in each orchard. *Fusarium oxysporum* and Xaj and, to a lower extent, *Alternaria alternata*, can cause BAN symptoms as individual pathogens or combined. Flowering and the initial stage of fruit expansion are the most susceptible walnut development stages, where disease control measures should focus on the control of walnut blight and BAN by using bactericides and fungicides. Variability in the prevalence of the three main BAN pathogens between walnut orchards suggests that further research is required to evaluate pathogen frequency in order to develop better management practices to control the disease. Finally, it is also necessary to continue evaluating the impact of climatic conditions on BAN disease as fruit development stage is relevant in the expression of this disease.

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TABLES AND FIGURES

Table 1. Environmental conditions during the experimental period in walnut orchards cv. Chandler in Chillán, Los Ángeles, and Negrete, Chile.

Study site	Air temperature (°C)	Relative Humidity (%)	Rainfall (day)	Rainfall (mm)
Chillán (2017)	18.1	64.2	10	61.3
Chillán (2018)	17.7	64.0	18	89.2
Los Ángeles (2018)	18.8	64.9	21	131.4
Negrete (2018)	17.3	63.2	23	138.3

Meteorological information obtained at www.agrometereologia.cl
Fuente: Elaboración propia.

Table 2. Results of the prevalence of microorganisms on walnut fruits cv. Chandler presenting symptoms associated with brown apical necrosis or BAN observed in different walnut orchards located in south- central and southern Chile and comparison using the Chi square (χ^2) goodness of fit test for each group of microorganisms found. Values obtained from a sample of 100 symptomatic fruits from each orchard during two seasons.

	Microorganism prevalence (%)^z							
Season 2018	Alt	Fus	Xaj	Alt+Xaj	Fus+Xaj	Alt+Fus	Alt+Fus+ Xaj	Other microorganisms
Chillán	6	11	26	5	4	1	3	22
Los Ángeles	2	4	58	10	17	0	1	6
Negrete	28	18	3	2	3	11	1	30
Average	12.0	11.0	29.0	5.7	8.0	4.0	1.7	19,3
χ^2	32.7	8.9	52.6	5.7	15.3	18.5	1.6	15,5
p-value	<0.001	0.012	<0.001	0.056	<0.001	<0.001	0.44	<0.001
Season 2019								
Chillán	3	0	40	3	0	2	0	43
Los Ángeles	6	2	71	3	1	0	0	9
Negrete	6	1	60	6	4	0	2	17
Paine	4	5	26	14	14	7	0	13
Talca	0	0	10	20	26	4	0	20
Coihueco	8	4	33	5	5	0	1	38
Perquenco	5	5	50	0	0	0	0	25
Average	4.0	2.0	39.9	9.0	8.0	2.0	0.5	23,0
χ^2	8.1	12.1	62.9	41.7	77.9	24.2	8.7	40.6
p-value	0,230	0,060	<0,001	<0,001	<0,001	<0,001	0,189	<0,001

[z] Symbol description of microorganisms: Xaj = *Xanthomonas arboricola* pv. *juglandis*; Alt = *Alternaria* sp. Fus = *Fusarium* sp.

Fuente: Elaboración propia.

Table 3. Analysis of factors and their interaction on the percentage of symptomatic fruits and dropped fruit inoculated with three BAN pathogens in a walnut orchard cv. Chandler, in Chillán, Chile, during 2017 season.

Factors	p value Symptomatic fruit	p value Dropped fruit
Inoculated microorganism	0.37	0.003
Development stage	0.13	< 0.001
Orchard management (O.M.)	0.04	0.015
Inoculated microorganism * Development stage	0.15	0.300
Inoculated microorganism * Orchard management	0.35	0.015
Development stage * Orchard management	0.33	0.009
Inoc. microorganism * Development stage * O.M.	0.68	0.740

Fuente: Elaboración propia.

Table 4. Reisolation of microorganisms from walnut fruits inoculated with microorganisms associated with BAN in walnut cv. Chandler at stages Gf and I in Chillán, Chile, during the 2017 season.

Development stage[y]	Orchard Management[z]	Inoculated Microorganism	Isolate (%)
Gf	Treated	*Alternaria alternata*	50
		Fusarium oxysporum	67
		Xanthomonas arboricola pv. *juglandis*	70
	Untreated	*Alternaria alternata*	47
		Fusarium oxysporum	73
		Xanthomonas arboricola pv. *juglandis*	50
I	Treated	*Alternaria alternata*	60
		Fusarium oxysporum	67
		Xanthomonas arboricola pv. *juglandis*	83
	Untreated	*Alternaria alternata*	47
		Fusarium oxysporum	60
		Xanthomonas arboricola pv. *juglandis*	97

[y] Gf development stage: wilting of the stigmas; and stage I: fruit growing (Muncharaz, 2000).

[z] Orchard management refers to copper hydroxide spraying (treated trees) applied 8 times in the season at a concentration of 2.5 g * L^{-1} or untreated trees.

Fuente: Elaboración propia.

Table 5. Analysis of factors and their interaction on the percentage of symptomatic fruits and dropped fruit inoculated with three BAN pathogens in three experiments conducted in walnut orchard cv. Chandler in central-south and southern Chile, during 2018 season.

Study sites	Factors	p value Symptomatic fruit	p value Dropped fruit
Chillán	Inoculated microorganism	0.008	0.007
	Development stage	0.0008	< 0.0001
	Inoc. Microor. * Dev. stage	0.01	0.29
Los Ángeles	Inoculated microorganism	0.52	0.015
	Development stage	0.35	< 0.0001
	Inoc. Microor. * Dev. stage	0.10	< 0.0001
Negrete	Inoculated microorganism	0.0001	< 0.0001
	Development stage	0.0001	< 0.0001
	Inoc. Microor. * Dev. stage	0.0001	< 0.0001

Fuente: Elaboración propia.

Table 6. Effect of the inoculation of microorganisms associated with BAN and development stages on the average percentage of symptomatic fruits in two walnut orchards cv. Chandler in Chillán and Negrete, Chile, during the 2018 season.

	Symptomatic Fruit (%)				
	Chillán		**Negrete**		
Inoculated microorganism[x]	**Ff2**[y]	**Gf**	**Ff2**	**Gf**	**I**
Control	0.0 bB[z]	58.3 aA	3.3 cAB	6.7 aA	0.0 bB
Alt	0.0 bB	41.7 abA	13.3 bA	0.0 bB	10.0 aA
Fus	0.0 bA	0.0 cA	10.0 bA	6.7 aA	0.0 bB
Xaj	0.0 bA	16.7 bcA	3.3 cAB	6.7 aA	0.0 bB
Alt+Xaj	41.7 aA	25.0 bcA	0.0 cA	0.0 bA	0.0 bA
Fus+Xaj	0.0 bA	8.3 cA	13.3 bA	0.0 bC	6.7 aB
Alt+Fus	0.0 bA	25.0 bcA	23.3 aA	3.3 abB	0.0 bB
Alt+Fus+Xaj	0.0 bA	8.3 cA	0.0 cA	0.0 bA	0.0 bA

[x] Xaj: *Xanthomonas arboricola* pv. *juglandis*; Alt: *Alternaria alternata* Fus: *Fusarium oxysporum*.

[y] Ff2 stage: Full female flowering; Gf stage: stigma wilting; and stage I: fruit growing (Muncharaz, 2000).

[z] Different letters indicate significant differences according to the Fischer LSD test (α = 0.05), lowercase letters indicate significant differences between inoculated microorganism; and capital letters indicate significant differences between development stages in each study site.

Fuente: Elaboración propia.

Table 7. Effect of the inoculation of microorganisms associated with BAN and development stage on the percentage of dropped fruit in three experiments conducted in walnut orchards cv. Chandler in Chillán, Los Ángeles and Negrete, Chile, during the 2018 season.

	Dropped fruit (%)										
Inoculated	**Chillán**			**Los Ángeles**				**Negrete**			
Microorganism[x]	**Ff2[y]**	**Gf**	**Means**	**Ff2**	**Gf**	**I**	**Means**	**Ff2**	**Gf**	**I**	**Means**
Control	66.7	16.7	**41.7 c[z]**	56.7 cA	26.7 cdB	10.0 abC	**31.1**	40.0 bA	10 eB	0 cC	**16.7**
Alt	75.0	16.7	**45.8 c**	83.3 aA	33.3 bcB	3.3 abC	**40.0**	40.0 bA	16.7 deB	0 cC	**18.9**
Fus	83.3	33.3	**58.3 bc**	83.3 aA	20.0 dB	3.3 abC	**36.6**	50.0 aA	26.7 abcB	10 bC	**28.9**
Xaj	75.0	33.3	**54.2 bc**	73.3 abA	50.0 aB	0.0 bC	**41.1**	36.7 bcA	16.7 deB	0 cC	**17.8**
Alt+Xaj	83.3	58.3	**70.8 ab**	60.0 cA	36.7 bcB	3.3 abC	**33.3**	36.7 bcA	20 cdB	30 aA	**28.9**
Fus+Xaj	91.7	75.0	**83.3 a**	63.3 bcA	50.0 aB	10.0 abC	**41.1**	43.3 abA	23.3 bcdB	10 bC	**25.6**
Alt+Fus	83.3	41.7	**62.5 abc**	60.0 cA	40.0 abB	13.3 aC	**37.8**	30.0 cdA	33.3 aA	10 bB	**24.5**
Alt+Fus+Xaj	91.7	58.3	**75.0 ab**	60.0 cA	36.7 bcB	3.3 abC	**33.3**	26.7 dA	30 abA	0 cB	**18.9**
Means	**81.3 a**	**41.7 b**		**67.5**	**36.7**	**5.8**		**37.9**	**22.1**	**7.5**	

[x] Xaj: Symbol description of microorganisms Xaj*: Xanthomonas arboricola* pv. *juglandis*; Alt: *Alternaria alternata* y Fus: *Fusarium oxysporum.*

[y] Ff2 stage: Full female flowering; Gf stage: stigma wilting; and stage I: fruit growing (Muncharaz, 2000).

[z] Different letters indicate significant differences according to the Fischer LSD test (α = 0.05); lowercase letters indicate significant differences between inoculated microorganisms; and capital letters indicate significant differences between development stages for each study site.

Fuente: Elaboración propia.

Table 8. Reisolation frequency of microorganisms from walnut fruits inoculated with three pathogens associated with BAN on flowers and walnut fruits according to development stages (Ff2 and Gf) in Chillán and (Ff2, Gf and I) in Los Angeles and Negrete, during the 2018 season.

		Development stage[x]					
		Ff2		**Gf**		**I**	
Study site	**Microorganism**	Individual reisolation (%)	**Total reisolation (%)**	Individual reisolation (%)	**Total reisolation (%)**	Individual reisolation (%)	**Total reisolation (%)**
Chillán	*Alternaria alternata*	70	**70**	30	**30**	nc[y]	**nc**
	Fusarium oxysporum	80	**80**	40	**40**		
	X. arboricola pv. *juglandis*	0	**0**	0	**0**		
	Alt+Xaj[z]	65+35	**100**	50+0	**50**		
	Fus+Xaj	90+0	**90**	90+0	**90**		
	Alt+Fus	50+50	**100**	75+25	**100**		
	Alt+Fus+Xaj	15+55+0	**70**	35+35	**70**		
Los Ángeles	*Alternaria alternata*	72	**72**	62	**62**	43	**43**
	Fusarium oxysporum	69	**69**	68	**68**	76	**76**
	X. arboricola pv. *juglandis*	17	**17**	13	**13**	6,9	**6,9**
	Alt+Xaj	35+0	**35**	23+4	**27**	52+8	**60**
	Fus+Xaj	16+0	**16**	45+15	**60**	93+0	**93**
	Alt+Fus	43+45	**88**	43+36	**79**	36+56	**92**
	Alt+Fus+Xaj	52+10+0	**62**	12+38+12	**62**	47+50+0	**97**
Negrete	*Alternaria alternata*	62	**62**	17	**17**	48	**48**
	Fusarium oxysporum	36	**36**	59	**59**	97	**97**
	X. arboricola pv. *juglandis*	12	**12**	0	**0**	0	**0**
	Alt+Xaj	57+0	**57**	34+0	**34**	23+0	**23**
	Fus+Xaj	24+0	**24**	32+0	**32**	71+0	**71**
	Alt+Fus	45+36	**81**	13+52	**65**	27+16	**43**
	Alt+Fus+Xaj	44+20+4	**68**	24+38+0	**62**	10+57+0	**67**

[x] Ff2 stage: Full female flowering; Gf stage: stigma wilting; and stage I: fruit growing (Muncharaz, 2000).

[y] nc: No assessment was conducted

[z] Symbol description of microorganisms: Xaj: *Xanthomonas arboricola* pv. *juglandis*; Alt: *Alternaria alternata* Fus: *Fusarium oxysporum*.

Fuente: Elaboración propia

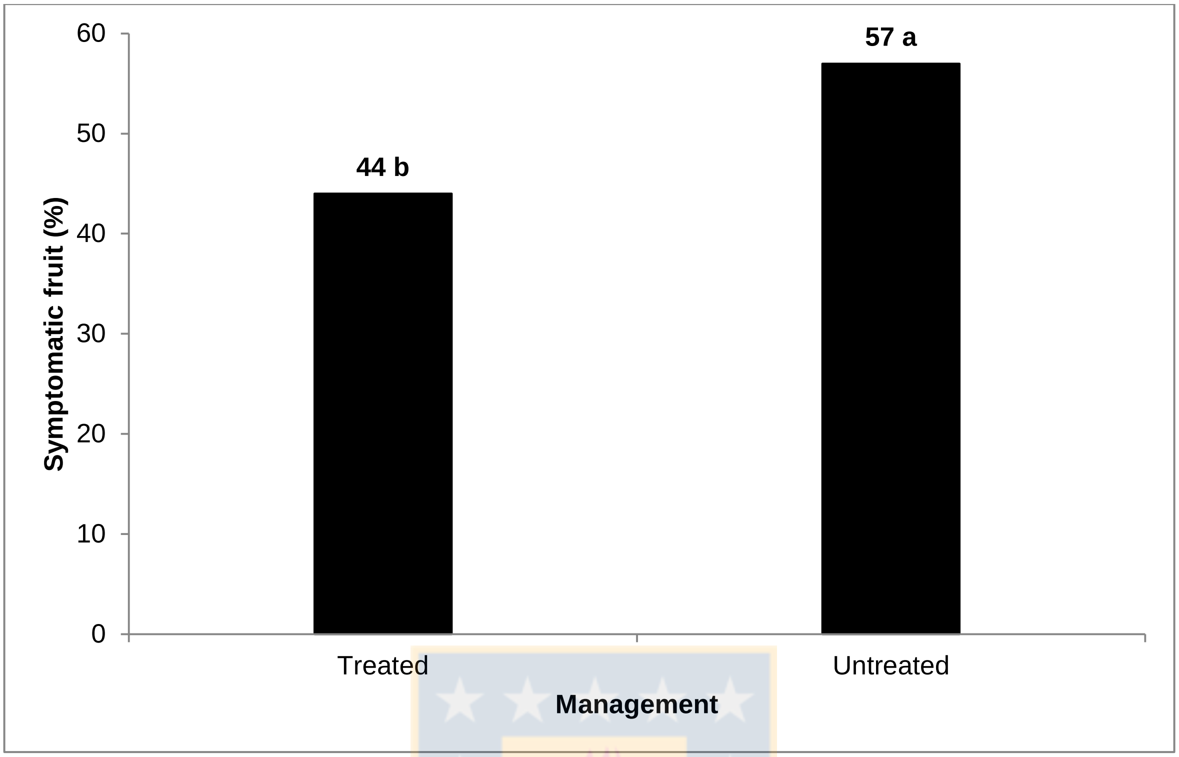

Figure 1. Effect of orchard management (treated and not treated with copper hydroxide applied eight times at a concentration of 2.5 g L^{-1}) on the percentage of BAN symptomatic fruits that were artificially inoculated in a walnut orchard cv. Chandler in Chillán, Chile. Different letters in the columns indicate significant differences according to the Fischer LSD test (α = 0.05).
Fuente: Elaboración propia.

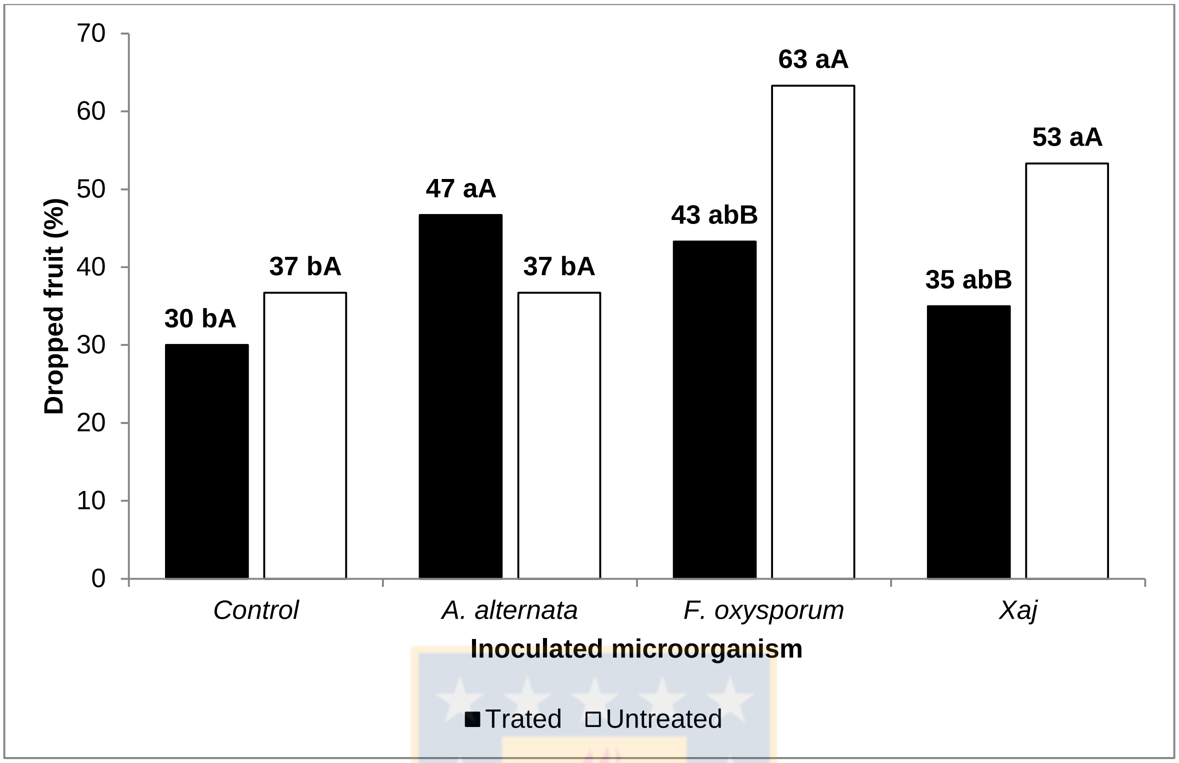

Figure 2. Effect of the interaction between inoculated microorganism and orchard management (with and without treatment with copper hydroxide applied 8 times at a concentration of 2.5 g L^{-1}) on the average percentage of dropped fruits in an orchard of walnut trees cv. Chandler in Chillán, Chile. Different letters indicate significant differences according to the Fischer LSD test ($\alpha = 0.05$); lowercase letters indicate significant differences between inoculated microorganisms; and capital letters indicate significant differences between orchard management practices.
Fuente: Elaboración propia.

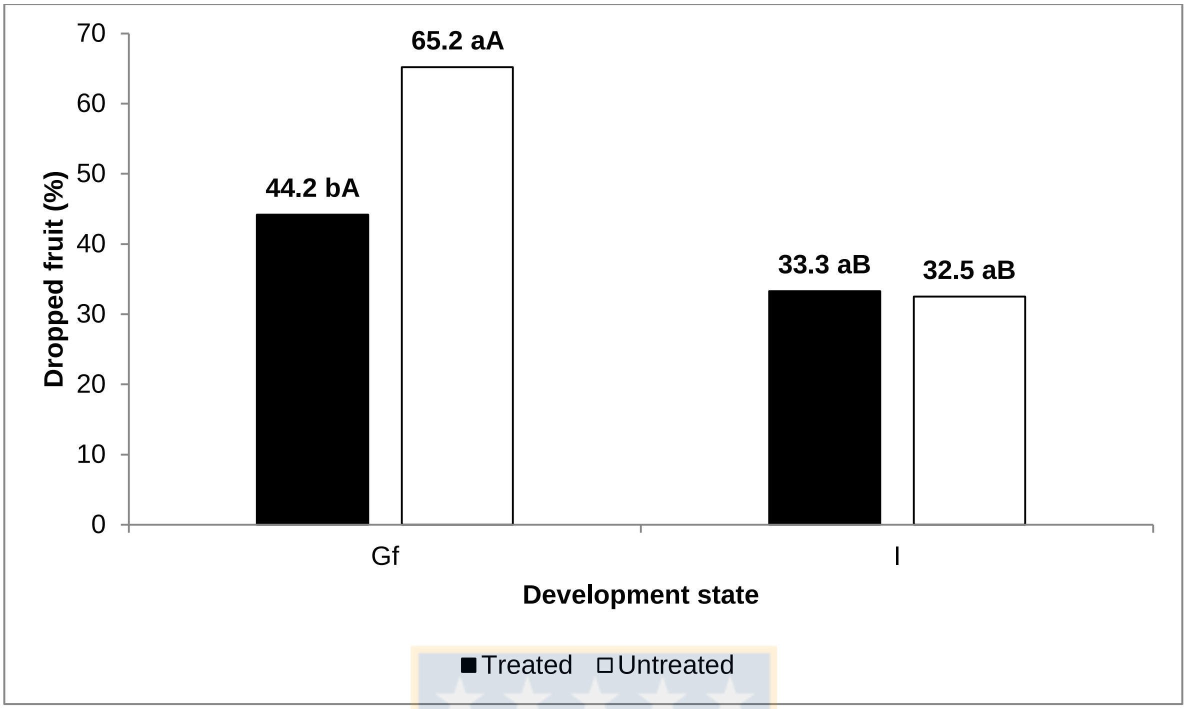

Figure 3. Effect of the interaction between development stage and orchard management (with and without treatment with copper hydroxide applied 8 times at a concentration of 2.5 g L^{-1}) on the average percentage of dropped fruits in an orchard of walnut trees cv. Chandler in Chillán, Chile. Different letters indicate significant differences according to the Fischer LSD test ($\alpha = 0.05$); lowercase letters indicate significant differences between orchard management practices; and capital letters indicate significant differences between development stages.

Fuente: Elaboración propia.

CONCLUSIONES GENERALES

La necrosis apical café o BAN debe ser considerada una nueva enfermedad en el cultivo del nogal en la zona Centro-Sur y Sur de Chile, en donde la expresión de los síntomas de la enfermedad está determinada por un complejo de factores, tales como, el estado fenológico en donde ocurre la infección, las medidas de control fitosanitarias aplicadas en cada huerto y él o los microorganismos que infectan el tejido, siendo la bacteria Xaj y hongos de los géneros *Fusarium* y *Alternaria*, los agentes causales de la expresión de los síntomas de BAN.

Fusarium oxysporum, Xaj y, en menor importancia, *Alternaria alternata*, inducen la aparición de síntomas de la enfermedad BAN ya sea como infección individual o en infecciones combinadas. Del mismo modo, la expresión de la enfermedad es huerto dependiente y varía de una temporada a otra.

Los estados fenológicos de floración y la etapa inicial de expansión del fruto son las etapas más susceptibles, donde las medidas de control de enfermedades deben focalizarse dentro de un programa fitosanitario que controle la peste negra del nogal y BAN mediante el uso de bactericidas y fungicidas.

Las aspersiones de hidróxido de cobre reducen la sintomatología y la caída prematura de frutos inoculados con los microorganismos asociados a BAN.