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RESEARCH ARTICLE

Detection of *Neofusicoccum nonquaesitum* causing dieback and canker in highbush blueberry from Southern Chile

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Abstract

Due to increased incidence of wood fungi from genus *Neofusicoccum* in highbush blueberry (*Vaccinium corymbosum* L.) established in various locations in Southern Chile. The objective of this study was the identification of *Neofusicocum* species in two highbush blueberry cultivars from commercial orchards in Southern Chile. During 2011-12 season, stems with basal cankers and twigs with dieback were collected from cultivars Brigitta and Elliott grown in Panguipulli (39º30'S; 72º 19'W) and Teodoro Schmidt (38°58'S; 73°02'W). Tissues were kept in humid conditions and from cirrus conidia were taken and incubated in PDA medium. The mycelia had a cottony consistency, with colour ranging from greyish-white to black; sub-epidermal pycnidia were eruptive, ostiolate, and brown to black in colour. The unicellular conidia were hyaline, smooth-bordered, coenocytic and septate (1-3), with dense granular content, fusiform and elipsoidal with truncated point, measuring 27.2 ─ 29.4 (±3.0) µm × 7.7 ─ 8.4 (±0.9) µm; length/width ratio (L/W) = 3.6 ±0.6 (n=100). The morphometric characteristics corresponded to those of *Neofusicoccum nonquaesitum* and corroborated genetically (100% homologation) by rDNA sequencing ITS and was recorded in CABI under number IMI-500168. Whereas, the sequencing was deposited into Genbank (accession number JX217819.1). The pathogenicity of the fungus was consistent in twigs and stems of highbush blueberry cultivars Brigitta and Elliott.

Keywords: Highbush blueberry, *Neofusicoccum nonquaesitum*, wood fungi

1. Introduction

Highbush blueberry is a fruit-bearing species of economic significance for Chile, with increasing volumes exported both fresh fruit and individually quick frozen (IQF) to North America and some countries in Europe and Asia (Prodorutti *et al*., 2007; Larach *et al.,* 2009; ODEPA, 2014). Commercial orchards are distributed from the Coquimbo to Los Lagos regions. According to The Government Office in Studies and Agricultural Policies (ODEPA), by 2012 the planted area (hectares) with highbush blueberry per region was: Maule (4,365), Bío Bío (4,280), La Araucanía (1,561), Los Ríos (1,519) and Los Lagos (1,141). Total fresh blueberries fruit exported in the 2010-11 season reached 55,012 tons (ODEPA, 2014).

On the other hand, plant health is a crucial factor to obtain a better yield, quality and phytosanitary conditions to blueberry fruit exportation. In recent years, it has been observed an increase of incidence in twigs dieback and stem cankers, associated to wood fungi in highbush blueberry plantations, particularly in the southern Chile; Espinoza *et al*. (2009) reported between 15 and 45% in wood fungi incidence on blueberry orchards. Currently, this situation is critical due to the severe attack may occur on various cultivars, and because wood fungus control strategies are not fully developed for highbush blueberries. These dieback and cankers have been asso-ciated to a complex of fungal species of genera *Botryosphaeria, Neofusicoccum, Pestalotiopsis, Diaporthe* and *Phoma* (Pérez *et al*., 2010; Mc Donald and Eskalen, 2011).

The taxonomy of the genus *Botryosphaeria* and its anamorphs has been unclear in the past; the taxonomic changes and the permanent influence of the literature regarding these fungi have caused the confusion (Shenoy *et al*., 2007; Slippers *et al*., 2007). There are more than 18 anamorph genera associated to *Botryosphaeria*, most of which have been reduced to synonyms of *Diplodia* (pigmented and ovoid conidia, with thick walls) or *Fusicoccum* (hyaline and fusiform conidia, with thin walls) (Perez *et al*., 2008; Phillips, 2010). Nevertheless, conidia are also continuously detected with intermediate morphometric characteristics, which is why the genus *Neofusicoccum* was proposed, by DNA sequencing, distinct from *Fusicoccum sensu stricto* (*F. aesculi*) and which produces two different types of conidia; as a result, many species in *Fusicoccum sensu lato* were situated in the genus *Neofusicoccum*, based on morphometric characteristics but no by genetic analysis (Alves *et al*., 2005; Crous *et al*., 2006).

The genus *Neofusicoccum*, occurs principally in the Southern Hemisphere in angiosperm and occasionally on gymnosperm (De Wet *et al*., 2008), affecting a broad range of native and cultivated trees and shrubs world-wide. In Chile the following species have been reported for genus *Neofusicoccum* i.e. *N. mediterraneum*, *N. australe, N. corticosae, N. arbuti* and *N. parvum* (Espinoza *et al*., 2009; Acuña, 2010; Pérez, 2010). Particularly, *N. nonquaesitum* has been also reported in *Sequoiadendron giganteum* in California, USA (Rooney-Latham *et al*., 2012), *Umbellularia californica* and *Prunus dulcis* in California (USA) (Mycobank, 2010) and, in this same study, its detection in *V.corymbosum* was reported in branches of cvs. Brigitta (Nancagua; Región de O'Higgins, Chile) and Elliott (Río Negro, Región de Los Lagos, Chile) (Inderbitzin *et al*., 2010).

Also, the species reported include *Fusicoccum putrefaciens* Shear (Guerrero, 1988; Guerrero, 2001), F. aesculi Corda, ananamorph of *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *B. ribis* Grossenbacher & Duggar (Guerrero, 1993; Acuña, 2010) and its anamorph *Fusicoccum* sp.; and the more recently reported *B. australis* Slippers, Crous & M.J. Wingf. (Espinoza *et al*., 2009), *B. parva* Pennycook & Samuels (Espinoza *et al*., 2009); Pérez *et al*., 2010), *Neofusicoccum mediterraneum* Crous & M.J. Wingf. & A.J.L. Phillips (Espinoza *et al*., 2009), *N. corticosae* Crous & Sumerrel (Espinoza *et al*., 2009), *N. arbuti* (D.F. Farr & M. Elliott) Crous, Slippers & A.J.L. Phillips (Espinoza *et al*., 2009), *N. australe* Slippers, Crous & M.J. Wingf. (Espinoza *et al*., 2009), *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (Espinoza, 2008; Pérez *et al*., 2010) and *N. nonquaesitum* Inderb., Trouillas, Bostock & Michalaides (Inderbitzin *et al*., 2010). Thus, the aim of this study was the identification of the *Neofusicoccum* species associated to dieback and cankers in two highbush blueberry cultivars from commercial plantations in La Araucanía and Los Ríos regions.

2. Material and Methods

2.1. Geographical location, collection of samples and isolation.

During 2011-2012 season, 50 samples of twigs (growing season) and stems (one or two years-old) were cut

from the base of bushes (cultivars Brigitta and Elliott) grown in commercial orchards located in Región de La Araucanía (Teodoro Schmidt, 38°58'S; 73°02'W) and Región de Los Ríos (Panguipulli, 39º30'S; 72º19'W) and processed in the Phytopathology Laboratory, Universidad de La Frontera, Temuco, Chile. All samples collected were superficially disinfected with 2% (v/v) sodium hypochlorite, after were washed twice with sterile distilled water and then kept in a humid chamber at 25ºC (±1ºC) and 90% R.H. per seven days. The conidia obtained from the pycnidia cirrus were transferred to Petri plates with potato dextrose agar (PDA, Difco) and streptomycin sulphate (300 ppm) incubated in darkness at 25ºC (± 1ºC). The obtained isolates were purified taking hypha tips in disposed in quadruplicate for each Petri plates.

2.2. Characterization and identification of the isolates

Morphological identification of isolates was made on the basis of the pycnidia and conidia characteristics (size, shape, color, partitioning, wall thickness and conidia texture) by references from the descriptions of Slippers *et al*. (2004a,b) and Oliveira *et al*. (2010); and afterwards corroborated genetically by internal transcribed spacer (ITS) region (ITS1-5.8S rRNA-ITS2-28S rRNA) sequence data compared with sequences Genbank by the Centre for Agricultural Bioscience International (CABI), United Kingdom.

2.3. Pathogenicity test

Pathogenicity test was carried out with the isolated CABI IMI-500168, on healthy plants (two years-old) grown in greenhouse conditions (21ºC ±2ºC, 80% R. H., and 24h of light). Three twigs and stems by plant to each one Brigitta and Elliott, were superficially disinfected with 70% ethanol (v/v) per 30s, and washed once with sterile distilled water. Around to vegetative buds with sterile scalpel was created a wound and inserted by plugs cut from actively growing mycelia on PDA medium. The lesions were covered and sealed with moistened cotton-wool and waterproof tape (Parafilm) during six days.The longitude of the lesion was measured until 21 days and re-isolations of fungus were obtained from the adjacent tissue to lesion. Control treatment with PDA plug was maintained under same conditions. Previous studies from our research group, they were carried out on the development of these fungi in different plant substrates (pine needles, apple and kiwi fruits), verifying information that have noted remarkable variability in the phenotypic and morphometric characteristics of this fungus.

3. Results and Discussion

3.1. Detection of Neofusicoccum in commercial plantations

In commercial orchards, diseased plants were characterized by yellowing and fading of foliage, dieback, unilateral stem death, and basal cankers preferably, vascular discoloration and plant death (Figure 1a); the erupting pycnidia were developed on Stem canker (Figure 1b). Mostly cankers with symptoms of fungus were detected at the base of semi and lignified stems. The severity of the necrotic lesions that occurred in young tissues, and a lesser extent in lignified highbush blueberry tissues, may be attributed to a greater translocation of water and nutrients, presence of turgid cells, and less plasmatic membrane resistance to mechanical action exerted by specialized structures of fungi (Ahimera *et al*., 2004; Úrbez-Torres *et al*., 2013). According to Agrios (2005), the necrosis may be attributed to the production of phytotoxins, which are extremely toxic even in low concentrations. Proffer (1989) and Rayachhetry *et al*. (1996) consigned that *Neofusicoccum* affects cell membrane permeability and enzymes can be detected in connection with lesions produced on one side of stem. This might be explained by fungal invasion in vascular tissues: the mycelium moves under epidermis of the stem, in many cases only on one side, since the xylematic vessels are blocked by hyphae and tyloses (Biggs and Britton, 1988).

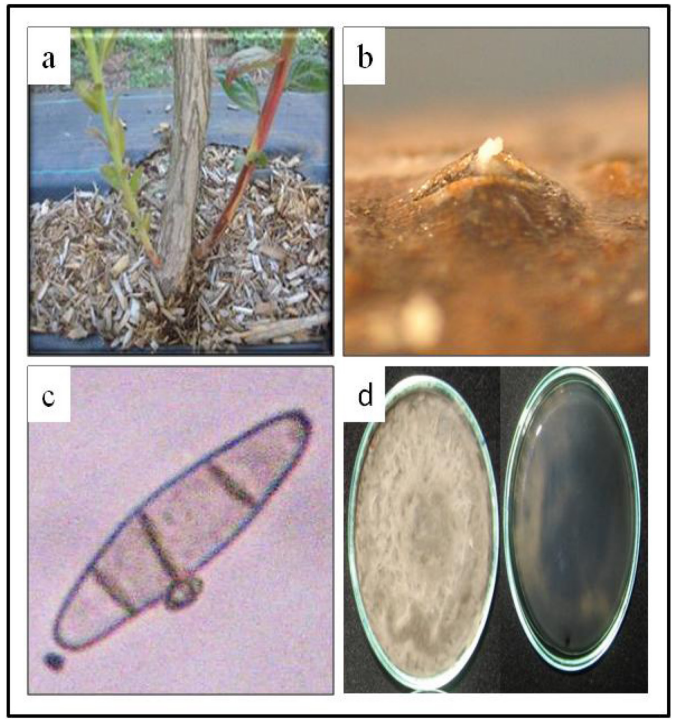

Figure 1. a) Basal cankers in stem (left), b) black erupting pycnidia (10X), c) coenocytic and septate conidia (1-3) (40X), d) olive gray mycelium with cottony growth.

3.2. Morphometric characterization

Pycnidia obtained from pine needle after 21 days, were eruptive, colored brown to black, with diameter ranging from 320 to 480 µm (n=20), agglomerations and hyaline conidiophores, without ramifications. Baskarathevan *et al*. (2009) noted that *N. parvum* and *N. luteum*, subsequent to the inoculation with mycelium, did not produce pycnidia on pine needle substrate, which is consistent with the results of this study. Conidia were unicellular, hyaline to brownish, dense granular content, coenocytic , fusiform and elipsoidal, ovoid base and slightly truncated point (Figure 1c). The reference measurements were in [(22.0-) 27.2-29.4 ±3.0 (-34.0) µm × (6.0) 7.7-8.4 ±0.9 (-10.0)] µm; (L/W) = 3.6 ±0.6 (n=70). In PDA media the mean growth of mycelia by 96h was 25mm diameter (with 24h, darkness), and its appearance greyish-white to olive-gray and chlamydospores were not observed (Figure 1d). Although, those morphometric characteristics were similar to those reported for *N. arbuti* in chilean blueberries by Espinoza *et al.* (2009), Farr *et al*. (2005) indicated that *N.arbuti* has chlamydospores and L/W = [2.3–[3.1 ± 0.4 [–4.2] would be other characteristic to differentiating *N. arbuti* and *N. nonquaesitum* (Inderbitzin *et al*., 2010). Previous study performed on highbush blueberry (cvs. Brigitta and Elliott) reported morphometric characteristics similar to as *Neofusicoccum nonquaesitum* (Inderbitzin *et al.*, 2010).

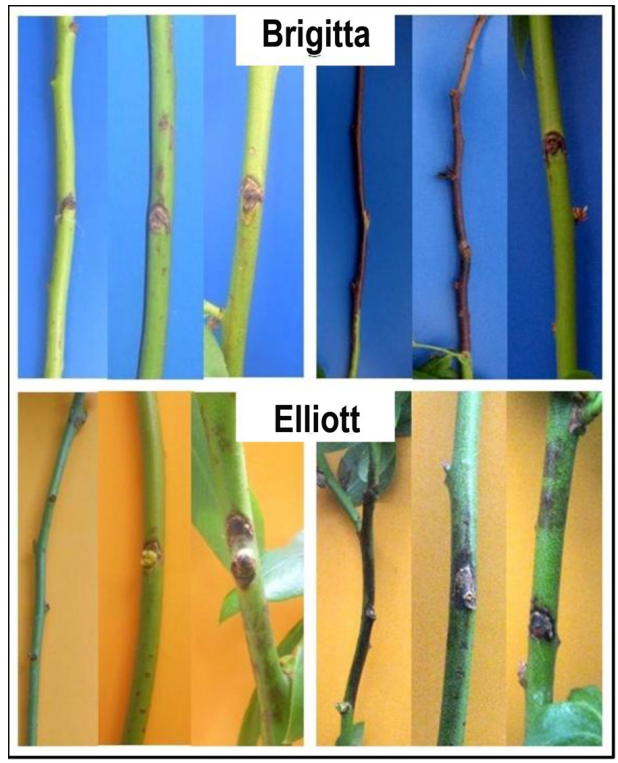

Figure 2. Pathogenicity test on control (left) and cankered stems (right) of cultivars Brigitta and Elliott.

3.3 Identification of the isolates and pathogenicity test

According to reported by CABI, the genetic analysis of obtained isolates was based on rDNA sequencing of ITS region, with total homologation (100%) to fungus *N. nonquaesitum*, available in the CABI collection under the number IMI-500168. Whereas, the sequencing was deposited into Genbank (accession number JX217819.1) based in the products ITS1 (1..177), 5.8S rRNA (178..334), ITS2 (335..488) and 28S rRNA (489..>524) (Table1).

Pathogenicity test were positive in stems of both cultivars, observing a smooth discoloration of adjacent tissue after six days, symptoms were quickly developed, susceptibility varied between cultivars, by 21 day mean length of lesions was for Brigitta 15.5 cm and Elliot 4.9 cm (Figure 2), whereas control plants inoculated with sterile PDA plugs were healthy. The plants under stress conditions due to certain types of handling are frequently affected by *Botryosphaeria* spp., as a result of extensive occurrence and opportunistic nature of these species (Proffer and Jones, 1989; Caruso and Ramsdell, 1995). Spring and winter rains, abundant plant foliage, the size and form of conidia are related to dispersion of inoculum in field conditions (Ahimera *et al*., 2004). The infection produced by *N. nonquaesitum* is inversely proportional to tissue maturity, which is consistent with studies conducted on species of *Botryosphaeria* (Milholland and Meyer, 1984; Creswell and Milholland, 1987; Smith, 2009).

Table 1. *Neofusicoccum nonquaesitum* strain CABI IMI-500168 internal transcribed spacer 1,5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

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1   ccgagttgat tcgagccccg gctcgactct cccaccctat gtgtacctac ctctgttgct
61  ttggcgggcc gcggtcctcc gcaccggctc cctccggggg ctggccagcg cccgccagag
121 gaccacaaaa ctccagtcag cgaacgtctc agtctgaaaa acaagttaat aaactaaaac
181 tttcaacaac ggatctcttg gttctggcat cgatgaagaa cgcagcgaaa tgcgataagt
241 aatgtgaatt gcagaattca gtgaatcatc gaatctttga acgcacattg cgcccttgg
301 tattccgagg ggcatgcctg ttcgagcgtc atttcaaccc tcaagctctg cttggtattg
361 ggctccgtcc tccacggacg cgcctcaaag acctcggcgg tggcgtcttg cctcaagcgt
421 agtagaaaac acctcgcttt ggagcgcacg gcgccgcccg ccggacgaac cttcgaattt
481 ttctcaaggt tgacctcgga tcaggtaggg atacccgctg aact
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After this study, has been frequently the detection of *Neofusicoccum* fungi, especially *N. nonquaesitum* and *N. parvum*, from different location sampling in La Araucanía region. In fact, this study contributes to better understanding and consequently to an effective field control. Also is remarkable that La Araucanía represent a 12% of the total *V. corymbosum* planted area in Chile.

4. Conclusions

The fungus *Neofusicoccum nonquaesitum* associated with twigs dieback and stem cankers in Brigitta and Elliott highbush blueberry cultivars, was identified according to morphological characteristics and genetic sequence, supported by CABI under number IMI-500168; detected in two commercial orchards in La Araucanía and Los Ríos regions, Southern Chile. The pathogenicity test demonstrated that Brigitta, compared with Elliott, was the most susceptibility to infection caused by *N. nonquaesitum*.

We think, in the future is necessary evaluating the incidence of this fungus in different varieties and local conditions. Also, is convenient to study in detail the complex species from *Neofusicoccum* genus reported in Chile.

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