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RESEARCH NOTE

Effect water activity on *in vitro* mycelial growth of *Neofusicoccum* spp. infecting blueberry

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Abstract

B.A. Latorre, G.A. Díaz, and M.P. Reed. 2012. Effect of water activity on mycelial growth of *Neofusicoccum* spp. infecting blueberry. Cien. Inv. Agr. 39(1): 221-228. Stem canker in blueberries (*Vaccinium corymbosum* L.) is a disease which is widely distributed around the world and of growing importance in Chile, associated with *Neofusicoccum* species. The purpose of the present work was to study the effects of water activity (a_w) and temperature on mycelial growth in vitro of *N. arbuti, N. australe* and *N. parvum*, obtained from blueberries with symptoms of stem canker. According to the results obtained, 25 ºC was the optimum temperature for mycelial growth of *N. arbuti*, *N. australe* and *N. parvum.* These species presented minimal growth at 35 ºC and none at 0 and 5 ºC. The effect of a_w on mycelial growth was significant ($P \leq 0.001$) and varied with the species of *Neofusicoccum* and the temperature. At 25 ºC, the optimum temperature, the three species of *Neofusicoccum* presented minimum growth with a_w of 0.900 and 0.920, while optimum mycelial growth was found with a_w equal to 0.990. Independent of the *Neofusicoccum* species, the effect of temperature (T) and a_w on mycelial growth was best explained by $y = -721.62 + 0.24T + 788.92a_w$. ($R^2_{adj} = 0.77$, $P \leq 0.001$), suggesting that T and a_w jointly explained 77% of the total variation on mycelial growth of *Neofusicoccum* spp. The results obtained provide important information for a better understanding of the biology of these plant pathogenic fungi, and suggest that temperature and a_w could be important parameters for understanding factors that affect the inoculum production, colonization and infection by *Neofusicoccum* spp. in blueberry plantations.

Key words: Ecological determinants, *Neofusicoccum arbuti*, *Neofusicoccum australe*, *Neofusicoccum parvum*, stem canker, dieback, *Vaccinium*, water potential.

Introduction

The blueberries (*Vaccinium corymbosum* L.) are highly important in Chile. There are at present 10,763 ha planted with blueberries, distributed between Coquimbo (29°57' S) and Osorno (40°34' S), along a north-south axis of approximately 1,400 km, leading to great variations in climate and soil conditions (INE, 2010). Under these conditions, stem canker caused by species of genus *Neofusicoccum* has acquired great economic importance. *N. arbuti*, *N. australe*, and *N. parvum*, have been associated with blueberry stem canker in Chile (Espinoza *et al*., 2009).

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Most species in the family Botryosphaeriaceae have a wide host range and geographical distribution and they are favored by drought-stress (Desprez-Loustae *et al*., 2006; Ma *et al*., 2001). However, free moisture and high relative humidity appear to be necessary for conidial germination and infection of species of Botryosphaeriaceae (Arauz and Sutton, 1989; Sutton and Arauz, 1991). Optimum temperatures for germination of conidia and mycelial growth vary among species in the family Botryosphaeriaceae. For instance, the optimum temperature for mycelial growth of *N. arbuti, N. australe* and *N. parvum* was 25 ºC and 30 °C for *Botryosphaeria dothidea* (Espinoza *et al*., 2009; Kohn and Hendrix, 1982). The optimum temperature for conidial germination of most Botryosphaeriaceae species has been found between 25 and 30 ºC, but 40 °C for pigmented conidia of *Lasiodiplodia theobromeae* (Sutton and Arauz, 1991, Úrbez-Torres *et al*., 2010). Similar to other hosts, it has been postulated that pycnidia, formed on disease wood and pruning debris in blueberry plantations, release conidia during rainy periods and /or under high relative humidity conditions (van Niekerk *et al*., 2010).

Temperature and water activity (a_w) are the most important abiotic parameters determining the potential for mycelial growth of fungal pathogen on the phyllosphere (Magan and Lacey, 1984), being a_w the same value of relative humidity under equilibrium conditions. Knowledge of the thermal and humidity requirements necessary for spore germination and mycelial growth has been employed in the past in formulating predictive models. These models make it possible to predict favourable conditions for infection, and thus, to orientate chemical control of numerous diseases (Arauz and Sutton, 1989; Bendek *et al*., 2007; Broome *et al*., 1995; Latorre *et al*., 2002; Sutton and Arauz, 1991). The effect of temperature and a_w on mycelial growth in species of *Neofusicoccum* which cause canker in blueberries has not been investigated. Nevertheless, it is considered that this information is necessary for a better understanding of the epidemiology of stem canker infection. The purpose of this work was therefore to study the effect of a_w and temperature on the mycelial growth of three species of *Neofusicoccum* previously described in blueberries in Chile (Espinoza *et al*., 2009).

Materials and methods

Culture media

The effect of temperature was studied *in vitro* in acidified potato dextrose agar (APDA) containing per liter 20 g agar, 20 g dehydrated potato, 20 g glucose and 0.5 mL 92% lactic acid, pH 4.2. The a_w was determined in APDA amended with 0, 17.4, 35.5, 65.7, 115.7 and 142 g L^{-1} NaCl, equivalent to a_w of 0.996, 0.990, 0.980, 0.960, 0.920 and 0.900, respectively (FDA, 2009).

Isolates

The following isolates, obtained from blueberry stem cankers in Chile and previously identified, were used in this study: *N. arbuti* (isolate B1-09, Osorno, Chile), *N. australe* (isolate B1-05, Rapel, Chile) and *N. parvum* (isolate B1-06, Santiago, Chile) (Espinoza *et al*., 2009). These isolates were activated in APDA at 20 ºC and maintained in the same culture medium at 5 ºC.

Incubation chambers

The incubation chambers used were Velp Scientifica FOC 225E (Velp, Usmate, Italy). The inside temperature of the chambers was verified with HOBO PRO temperature sensors (Onset Computer Corp. Bourne, Massachusetts, USA) that were maintained for 5 days before starting the tests with a ± 0.2 ºC error.

Effect of temperature

The effect of temperature on mycelial growth of *Neofusicoccum* spp. was previously reported (Espinoza *et al*., 2009). However, prior to study the effect of a_w, the optimal range of temperature for mycelia growth in vitro was reassessed in this study. With this purpose, a piece of mycelium (5 mm diameter), obtained from pure cultures of 7 days in APDA, was placed in the centre of a Petri dish with APDA and incubated at 0, 5, 10, 15, 20, 25, 30 and 35 ºC (±0.2ºC). The diameters of the colonies developed were determined at 48 h of incubation.

Effect of water activity (a_w)

The effect of a_w on mycelial growth *in vitro* was studied at 20, 25 and 30 ºC. With this purpose, a plug of agar (5 mm diameter) taken from 7 day old cultures of each isolate in APDA at 20ºC, was placed in the centre of a Petri dish. The APDA medium used was amended with 0, 17.4, 35.5, 65.7, 115.7 and 142 g L^{-1} NaCl, resulting in a_w of 0.996, 0.990, 0.980, 0.960, 0.920 and 0.900, respectively (FDA, 2009). The mycelial growth was determined at 48 and 96 h of incubation.

Design and statistical analysis

In the first test, the effect of temperature on mycelial growth was studied as a completely randomized design with a 3x8 (*Neofusicoccum* species x temperature) factorial structure with three replicates for each species and each temperature treatment. In the second test, the effects of the *Neofusicoccum* species, temperature and a_w, were distributed in a completely randomized design, with a 3x3x6 (species x temperature x a_w) factorial structure, replicated three times for each *Neofusicoccum* species, a_w and temperature.

The results were subjected to analysis of variance and means were separated using Tukey's phairwise comparison test ($P \leq 0.05$) using the SigmaStat statistical programme (Systat Software, Inc., USA). The relationship among mycelial growth, temperature and a_w was studied with a multiple linear regression analysis in which the dependent variable was mycelial growth and the independent variables were temperature and a_w. In addition, the relation between a_w and mycelial growth was determined by polynomial regression analysis between a_w = water activity and y = mycelial growth determined at 96 h at 25 ºC. The models $y = f(a_w)$ and $f(a_w, T)$ were tested to observe the relationship between the effect of water activity (a_w) and/or temperature (T) on the mycelial growth (y), using data pooled from three replicates. Regression analysis was used to estimate these parameters. Data were fitted to the model by coefficient of determination (R^2) using SigmaStat statistical programme.

Results

Effect of temperature

Neofusicoccum spp. grew at temperatures between 10 and 35 ºC. There was no growth at 0 and 5 ºC (Figure 1). The optimum temperature for mycelial growth was 25 °C. At this temperature, *N. parvum* had the greatest radial growth of the mycelium with 72.7 mm, while *N. australe* and *N. arbuti* had an average mycelial growth of 61.3 and 37.5 mm, respectively. The temperature and *Neofusicoccum* spp. had a significant effect ($P \leq 0.001$) on mycelial growth. Likewise the interaction between species and temperature was significant ($P \leq 0.001$). The mean differences in mycelial growth obtained between the *Neofusicoccum* species were statistically significant (P=0.05) (Table 1).

Effect of water activity (a_w)

All the *Neofusicoccum* species grew in media with a_w between 0.900 and 0.996. Independent of temperature, *N. parvum* attained optimum mycelial

Table 1. Analysis of variance of the effect of water activity (a_w) as a function of temperature on the mycelial growth of *Neofusicoccum arbuti, N. australe* and *N. parvum*[1]

Cause of variation[2]	g.l.	CM	F	P
Test 1				
Neofusicoccum spp. (N)	2	939.69	124.182	≤0.001
Temperature, ºC (T)	7	4039.05	533.772	≤0.001
N x T	14	203.50	26.89	≤0.001
Error	48	7.57		
	Diff. of means, mm	P≤0.05		
N. parvum vs *N. arbuti*	12.1	yes		
N. parvum vs *N. australe*	3.3	yes		
N. australe vs *N. arbuti*	8.8	yes		
Test 2				
Neofusicoccum spp. (N)	2	593.03	87.2	≤0.001
Temperature, ºC (T)	2	262.60	38.6	≤0.001
Water activity (a_w)	5	31409.60	4616.5	≤0.001
N x T	4	19.40	2.9	0.027
N x a_w	10	484.39	71.2	≤0.001
T x a_w	10	266.10	39.1	≤0.001
N x T x a_w	20	79.35	11.7	≤0.001
Error	108	6.80		

[1]Mycelial growth in APDA determined after 96 h, except N. arbuti, which was incubated for 48 h.

[2]The following isolates were used: B1-06 (N. parvum), B1-05 (N. australe) and B1-09 (N. arbuti).

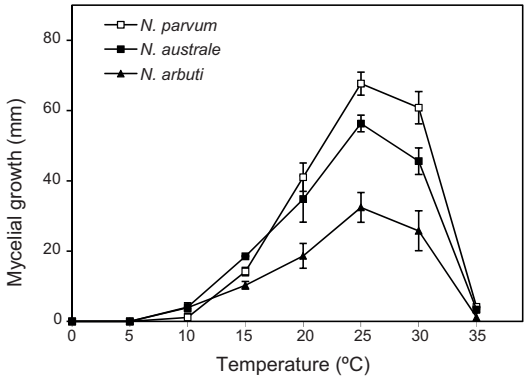

Figure 1. Effect of temperature on the mycelial growth of species of *Neofusicoccum* causing stem canker in blueberries, incubated in acidified potato dextrose agar for 48 h. Bars = standard deviation.

growth at a_w of 0.996, while *N. australe* and *N. arbuti* attained optimum mycelial growth at a_w of 0.990 (Figure 2)**.** The species, temperature and a_w had a significant (P≤0.05) effect on mycelial growth, with significant (P<0.027) interactions between *Neofusicoccum* species (N) and temperature (T), between N and a_w, T and a_w, and between N, T and a_w (Table 1).

The relation between the a_w at 25 ºC and mycelial growth was explained by polynomial models where $y = 14917\ a_w^2 - 27480\ a_w + 12657$ (R^2 = 0.95) for *N. arbuti* $y = 13036\ a_w^2 - 23889\ a_w + 10950$ ($R^2 = 0.93$) for *N. australe*, and $y = 15763\ a_w^2 - 29087\ a_w + 13417$ ($R^2 = 0.97$) for *N. parvum* (Figure 3). Independently of *Neofusicoccum* species, the mycelial growth (y) was modelled by the following quadratic function $y = -721.62 + 0.24T + 788.92a_w$, ($R^2_{adj} = 0.77$, P≤0.001), where T = temperature and a_w = water activity. Standard errors were: 0.52, 59.08 and 15.62 for T, a_w and for the estimates, respectively.

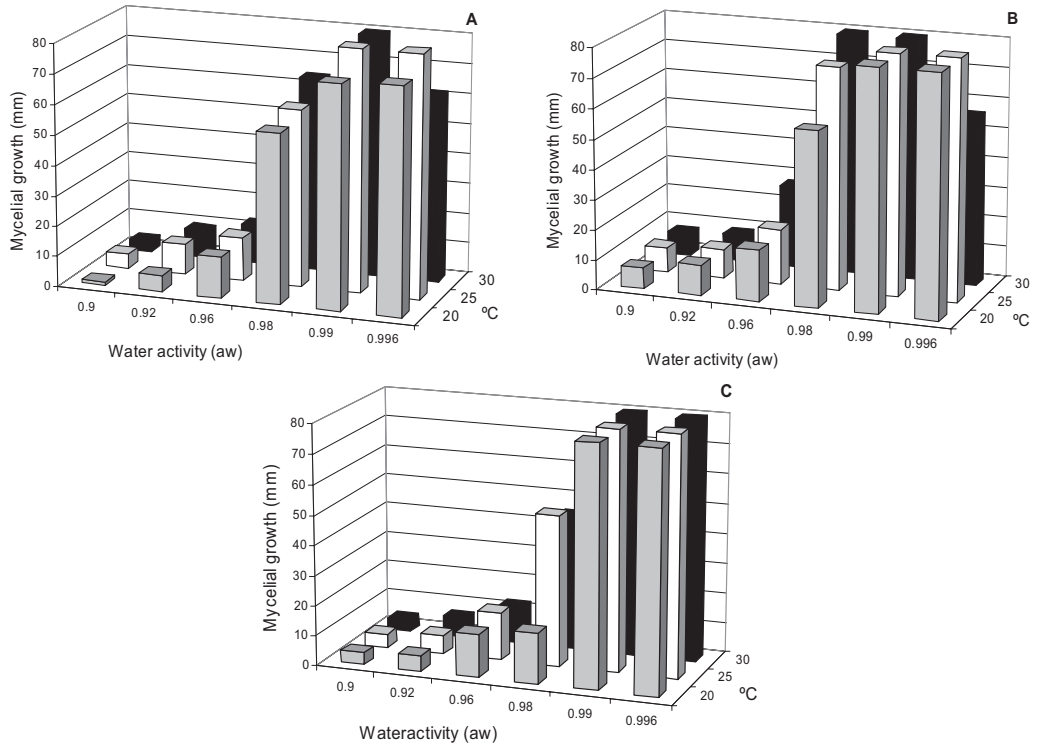

Figure 2. Effect of water activity (a_w) on mycelial growth of *Neofusicoccum* species as a function of temperature in acidified potato dextrose agar. A. *N. arbuti*, B. *N. australe* and C. *N. parvum*. The a_w was adjusted with sodium chloride between 0.900 and 0.996.

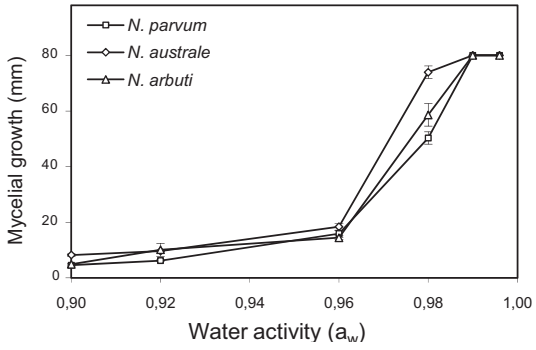

Figure 3. Effect of water activity (a_w) on the average mycelial growth of *Neofusicoccum* species, determined after 96 h incubation at optimum temperature (25 ºC) on acidified potato dextrose agar, pH 4.2. Bars = standard deviation.

Discussion

Several species in the family Botryosphaeriaceae have been associated with stem cankers of blueberries. In Chile, *N. arbuti, N. australe* and *N. parvum* were reported (Espinoza *et al*., 2009), but it is possible that they may coexist with other species of the Botryosphaeriaceae family and other plant pathogenic fungi. For example, *Pestalotiopsis clavispora*, *P. neglecta* and *Truncatella angustata* were frequently found associated with stem canker in blueberries (Espinoza *et al*., 2008).

This study has shown mycelial growth of *Neofusicoccum* spp. can be reduced, but not completed inhibited, by lowering the a_w, suggesting that this factor may play an important role in the blueberry infection and colonization of woody tissues under field conditions. High water stress has been reported to enhance canker formation and colonization by species in the family Botryosphaeriaceae (Crist and Schoeneweiss, 1975; Desprez-Loustau *et al*., 2006; Ma *et. al*., 2001; Magar *et al*., 1989). In addition, the influence of a_w and temperature on fungal growth is of

great importance in understanding the ecological relations between species of fungus and to understand the pathogenesis of wood colonizer fungi (Desprez-Loustau *et al*., 2006; Ma *et al*., 2001; Valík and Piecková, 2001). Therefore, the results obtained in this study suggest that a_w could be an important parameter for understand the critical factors affecting the production of inoculum and mycelial colonization of woody tissues in blueberry fields.

Despite the differences that distinguish *N. arbuti*, *N. australe* and *N. parvum*, these species showed a similar growth pattern in response to temperature and showed very similar responses to changes in the a_w of the medium. Corroborating a previous study (Espinosa *et al*., 2009), these *Neofusicoccum* species showed optimal growth at 25 °C and *N. parvum* exhibited the fastest mycelial growth. Similar studies have reported 25 to 35 ºC as the optimum temperature for the germination of the conidia of other species of Botryosphaeriaceae (Pennycook and Samuels, 1985; Sutton and Arauz, 1991; Úrbez-Torres *et al*., 2010; van Niekerk *et al*., 2004; Wright and Harmon, 2010). It is of interest to note that the mycelial growth of *B. dothidea* obtained in Georgia, USA, was optimum at 30 ºC (Kohn and Hendrix, 1983) and that conidia of *N. parvum* from grapevines in California germinated abundantly at 35 ºC, although very little at 40 ºC (Úrbez-Torres *et al*., 2010). The differences in optimum temperature obtained with isolates of *Neofusicoccum* from blueberries in the present work, as compared to *B. dothidea* and other Botryosphaeriaceae, may be attributed to natural variation between species of the Botryosphaeriaceae family and the adaptability of *Neofusicoccum* spp. to environmental conditions.

In conclusion, the results of this study suggest that the a_w, temperature and the interaction of these factors affect the development of the stem canker of blueberry caused by species of *Neofusicoccum*. Nevertheless, before a conclusion is established it will be necessary to validate this information under field conditions.

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Resumen

B.A. Latorre, G.A. Díaz y M.P. Reed. 2012. Efecto de la actividad del agua y temperatura sobre el crecimiento micelial *in vitro* de *Neofusicoccum* spp. de arándano. Cien. Inv. Agr. 39(1): 221-228. La cancrosis de la madera en arándanos (*Vaccinium corymbosum* L.) es una enfermedad de amplia distribución en el mundo y de creciente importancia en Chile, asociada a la acción de especies de *Neofusicoccum*. Este trabajo tuvo como objetivo estudiar el efecto de la actividad de agua (a_w) y de la temperatura sobre el crecimiento micelial i*n vitro* de *N. arbuti, N. australe* y *N. parvum,* obtenidos de arándanos con síntomas de cancrosis de la madera. Según los resultados obtenidos, 25 ºC fue la temperatura óptima para el crecimiento micelial de *N. arbuti*, *N. australe* y *N. parvum.* Estas especies crecieron mínimamente a 35 ºC y no crecieron a 0 y 5 ºC. La a_w afectó significativamente ($P \leq 0.001$) el crecimiento micelial y su efecto varió en función de la especie de *Neofusicoccum* y de la temperatura. A 25 ºC, temperatura óptima, las tres especies de *Neofusicoccum* crecieron mínimamente con a_w de 0,900 y 0,920, siendo óptimo el crecimiento micelial con a_w igual a 0,990. Independientemente de *Neofusicoccum* spp., el crecimiento micelial (y) entre 20 y 30 ºC se explicó a través de $y = -721{,}62 + 0{,}24T + 788{,}92a_w$. ($R^2 = 0{,}77$, $P \leq 0{,}001$). Los resultados obtenidos aporta información para la mejor compresión

de la biología de estos hongos fitopatógenos y sugieren que la a_w es un importante factor que podría afectar la producción de inóculo, colonización y la infección por *Neofusicoccum* spp., en arándanos.

Palabras clave: Actividad del agua, arándano, Botryosphaeriaceae, cancrosis, enfermedades, temperatura, *Vaccinium*.

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