Journal of Phytopathology

SHORT COMMUNICATION

In vitro Evaluation of Wood Preservatives to Prevent Dispersal of Pitch Canker Pathogen, *Fusarium circinatum*

Yolanda Serrano, Margarita Elvira-Recuenco, María Conde, M. Teresa Troya and Rosa Raposo

Instituto Nacional Investigación y Tecnología Agraria (INIA), Centro de Investigación Forestal (CIFOR), C. Coruña km 7.5, 28040 Madrid, Spain

Keywords
3-iodo-2-propinyl butyl carbamate, EC_{50}, fungal inhibition, propiconazole, tebuconazole, wood block test

Correspondence
R. Raposo, INIA-CIFOR, Madrid, Spain.
E-mail: raposo@inia.es

Received: April 29, 2014; accepted: September 2, 2014.

doi: 10.1111/jph.12321

Abstract

Fusarium circinatum, the causal agent of pitch canker disease on pines, can be disseminated by wood produced in infested areas. The purpose of the study was to evaluate the effect of wood preservatives, commonly used against sapstain and wood-decay fungi, on growth and sporulation of *Fusarium circinatum*. Seven active ingredients of antisapstain and anti-wood-decay preservatives were evaluated by their inhibition of mycelial growth. Propiconazole, tebuconazole, and 3-iodo-2-propinyl butyl carbamate (IPBC) were effective against *F. circinatum*, whereas hydroxycarbonate of cooper was not. An assay was also conducted to evaluate the efficacy of three commercial antisapstain and two anti-wood-decay preservatives on *Pinus radiata* sapwood blocks that were previously inoculated with *Fusarium circinatum*. The product with the best efficacy was an antidecay preservative composed of tebuconazole, propiconazole, and dichlofluanid. None of the antisapstain preservatives tested was effective even though they contained fungicidal ingredients. Effects of dosage, product application, and formulation on the efficacy of these preservatives are discussed.

Introduction

Fusarium circinatum Nirenberg & O'Donnell (teleomorph: *Gibberella circinata* Nirenberg & O'Donnell) is a fungal pathogen that causes pitch canker disease, one of the most serious threats to pines (Correll et al. 1991). The disease has been recently detected in Europe, in localized areas in Spain (Landeras et al. 2005), Portugal (Braganca et al. 2009), France (EPPO 2011), and Italy (Carlucci et al. 2007). Consequently, quarantines and regulations are in force to eradicate the pathogen and restrict its spread, and to prevent disease establishment in pathogen-free zones.

Fusarium circinatum can infect branches and main stem of susceptible pine trees, causing sunken cankers with resin exudation (Dwinell et al.1985). The pathogen initially colonizes the cortex and phloem, and later reaches the pith and invades the xylem (Martin-Rodrigues et al. 2013). Infection is initiated by air-disseminated conidia that survive in the bark (Gordon et al. 2001; EPPO 2009).

Bark and wood from an infested area are potential pathways of spread into new pathogen-free areas. No effective use of chemical fungicides for management of pitch canker disease in forest plantations has been reported (Wingfield et al. 2008). When trees are felled, they are debarked and moved to the sawmill to be processed. Raw wood can be used to make wood packaging material, and then it is heat-treated (56°C for at least 30 min) according to the International Standards for Phytosanitary Measures (ISPM No. 15) to reduce the risk of introduction and spread of quarantine pests associated with the movement in international trade of wood material. When raw wood is to be used as timber in construction, it is usually protected against fungal degradation, using anti-wood-decay preservatives according to the final use class in the market. Logs are also frequently treated with antisapstain fungicides in sawmills, especially when they are stored in spring time or for long time periods.

Potential dissemination of *F. circinatum* is a serious limitation in the international wood trade for timber

produced within an affected area. If wood preservatives commonly used to control sapstain and wood-decay fungi were effective against *F. circinatum*, then the use of these treatments could represent another strategy to prevent the spread of this disease and to overcome trade restrictions. The objective of this work was to determine the *in vitro* sensitivity of *F. circinatum* to the most frequently used antisapstain and anti-decay-wood preservatives.

Materials and Methods

Wood preservatives and fungal isolates

Several commercial products currently used in the market (Table 1) were tested to determine their effect on growth of *F. circinatum* on sapwood wood blocks and on conidial germination. Also, most common active ingredients (a.i.) were used to determine inhibition of fungal growth on culture medium.

Two single-conidial isolates of *F. circinatum* were used, one of each mating type (named MAT-1 and MAT-2 types). Isolates were isolated from *Pinus radiata* in a nursery in Asturias, Spain (ID no. 8), and from a plantation in País Vasco, Spain (ID no. 7).

Mycelial growth assay with active ingredients

Active ingredients dissolved in acetone (99% v/v) were added to potato dextrose agar (PDA) medium to have final concentrations of 100, 10, 1, and 0.1 mg a.i./l. The maximum concentration in the growth medium was 3 ml of acetone/l. Non-amended growth medium was used as a control, because in a previous trial, our results showed that acetone had no effect on mycelial growth. Mycelial plugs (5 mm diameter) were removed from 10-day-old PDA cultures, transferred to fungicide-amended PDA plates, incubated for 10–15 days at 20°C, and then two perpendicular colony diameters were measured. For each isolate and active ingredient, four plates were used. The assay was performed at three independent times.

Conidial germination assay with commercial products

A conidial suspension was prepared from PDA cultures of each *F. circinatum* isolate grown for 8–12 days at 22°C, adding sterile water, scrapping plate surface and filtering the suspension through two layers of sterile cheese cloth. Number of conidia was estimated with a hemocytometer. Commercial preservatives were dissolved following manufacturer recommendations and then diluted at 100, 10, 1, 0.1, and 0 (used as control) ml product/l of water in a final volume of 50 ml with 10^5 spores/mL. Four 40 µl droplets were placed on glass slides and incubated in a moist chamber at 25°C in darkness for 24 h. The assay was repeated once. Five to ten pictures were taken with a camera (ColorView *I*) mounted on a microscope (Olympus CX41) at randomly distributed spots for every droplet, and the number of germinated and non-germinated spores was determined from a total of 100–250 spores.

Dose–response curves and EC_{50} value comparison

For each isolate and product, inhibition of mycelial growth and conidial germination was calculated as percentage referred to the control treatment. A probit response model was fitted to the data using logarithm base 10 of the product concentration as the independent variable. The two regression lines estimated for each product, one for each isolate, were tested for equality of their intercept and slope by analysis of covariance (Neter et al. 1989), and if they were not different significantly ($\alpha = 0.05$), data from both isolates were pooled to re-estimate a single regression line for that product. Effective concentration values that inhibited mycelial growth or conidial

Table 1 Composition and names of commercial products used to evaluate inhibition of conidial germination and growth on wood blocks

Composition	Product name	Recommended dosage	Final use
DDAC[a] 8.2%	FKRAC	3%	Anti-sapstain
IPBC[a] 3.1%, propiconazole[a] 3.1%	FKR Ecoplus	2%	Anti-sapstain
Copper carbonate[a] 20.5%, 2-aminoethanol carbonate 20%, tebuconazole[a] 0.5%, propiconazole[a] 0.5%, boric acid 5%, polyethylenimine 20%, organic acid 5%	Tanalith	2.7–4%	Anti-decay Use class[b] 4
Cypermethrin 0.22%, propiconazole[a] 0.45%, dichlofluanid[a] 0.45%, tebuconazole[a] 0.45%	Corpol PF3	Ready to use	Anti-decay Use class[b] 3
Propiconazole[a] 3%, IPBC 5%, Bardap-26[a] 3%	Corpol Madera verde	2%	Anti-sapstain

[a]Active ingredients used to evaluate mycelial growth inhibition.
[b]As defined in European Standard EN-335-1 (2012).

germination by 50% (EC_{50}) were obtained from the estimated probit regression line.

Additionally, slopes of the dose–response lines were compared when EC_{50} values for two or more products were close. Slopes represent fungus response per unit change in dose, so a steeper slope means the product is more toxic even though EC_{50} values are similar. Data analysis was performed with STATGRAPHIC *Plus* 5.0 software.

Wood assay

P. radiata blocks of 30 × 15 × 5 mm were sawn from defect-free either green or dry sapwood boards to test antisapstain or anti-wood-decay commercial products, respectively (antisapstain products are usually applied on green wood). Test blocks were autoclaved at 121°C for 20 min, placed on Petri dishes with *F. circinatum* actively growing on PDA, and incubated for 2 weeks at 22°C and 65% RH. Blocks were chemically treated at the recommended dosage (Table 1) by dipping in antisapstain products for 30 min. (surface treatment), or in antidecay products for 24 h (total impregnation). Control blocks were either surface treated or impregnated with water. Then, they were incubated at 22°C and 65% RH for 1 month. Six blocks were treated for each product and fungal isolate, and the assay was repeated once in independent tests.

To measure the efficacy of the product, pairs of blocks were placed on semi-selective *Fusarium* agar (SFA) in Petri dishes (Glucose 20 g, KH_2PO_4 0.5 g, $NaNO_3$ 2 g, $MgSO_4*7H_2O$ 0.5 g, yeast extract 1 g, 1% $FeSO_4*7H_2O$ 1 mL, agar 20 g, H_2O to 1 l) (Leslie and Summerell 2006), and incubated for 15 days at 22°C. Blocks were evaluated for the occurrence of *F. circinatum* growing on SFA after 30 days using a scale where 0 means no presence; 1 = *F. circinatum* minimal growth and limited to the wood; and 2 = *F. circinatum* abundant growth and covering the plate. Of each pair of blocks, one was surfaced disinfested by immersion in ethanol (96% v/v) during 5 s and cut in half to facilitate the growth of *F. circinatum* that may have been present inside the block.

Results and Discussion

Propiconazole, tebuconazole, and IPBC were the active ingredients (a.i.) that best inhibited *F. circinatum* mycelial growth as determined by their respective EC_{50} values, which ranged from 0.93 to 2.86 mg a.i./l (Table 2). Furthermore, slopes of the estimated probit regression lines for IPBC (1.3 ± 0.11) and propiconazole (0.45 ± 0.05) were different (P-value<0.000).

Table 2 EC_{50} values (in mg/l) for inhibition of mycelial growth and conidial germination of *Fusarium circinatum*, determined from a dose–response curve in the range of 0–100 mg product/l

	Isolate 7	Isolate 8	Pooled data[c]
Mycelial growth[a]			
DDAC	–	–	26.98
IPBC	–	–	0.93
Hydroxycarbonate of copper	>100	>100	–
Dichlofluanid	–	–	17.60
Propiconazole	–	–	1.94
Tebuconazole	2.86	0.59	–
Bardap-26	19.93	54.84	–
Conidial germination[b]			
FKRAC	–	–	1.40
FKR	–	–	4.23
Tanalith	1–10	0.1–1	–
Corpol PF3	>100	10–100	–
Corpol madera verde	10–100	10–100	–

[a]Mycelial growth inhibition measured using active ingredients.
[b]Conidial germination inhibition measured using commercial products (described in Table 1).
[c]When regression lines between isolates were not statistically different (in intercept and slope) at 0.05 significance level, EC_{50} values were calculated from a regression line estimated from pooled data.

Table 3 Efficacy[a] of commercial products on autoclavated *P. radiata* wood blocks exposed to two isolates of *Fusarium circinatum* for 15 days, following chemical treatment applied as recommended by the manufacturer, and conditioning for 1 month at 22°C and 70% RH

		Isolate 7		Isolate 8	
Product[b]	Wood type	External	Internal	External	Internal
FKRAC	Green	2	2	2	2
FKR	Green	2	2	2	2
Tanalith	Dry	0	1	1	2
Corpol PF3	Dry	0	0	0	0
Corpol madera verde	Green	2	2	2	2
Control[c]	Green/Dry	2	2	2	2

[a]Evaluated after 30 days using the scale: 0 = no fungal presence; 1 = *F. circinatum* scarcely grown and limited to the wood; and 2 = *F. circinatum* abundantly grown and covering the plate. Observations based on 6 blocks.
[b]Described in Table 1.
[c]Comparison with control was performed using its respective wood type.

The compound with the highest EC_{50} value (>100 mg a.i./l) was hydroxycarbonate of copper. Didecyldimethylammonium chloride (DDAC), dichlofluanid, and Bardap-26 EC_{50} values ranged from 20 to 60 mg a.i./l. Approved application rates are 2–3% (Table 1) for

products containing a.i. of medium EC_{50} values, a similar rate to those products with a.i. of low EC_{50}. Comparison of estimated probit regression lines for DDAC and dichlofluanid showed that differences in slope were not significant (P-value=0.743). Thus, we concluded that IPBC is more toxic to *F. circinatum* than propiconazole. According to the European Biocidal Products Directive, IPBC is an ingredient used more frequently in antisapstain products than in antidecay ones, while tebuconazole is mainly included in antidecay products. Propiconazole is used in both types of products.

When commercial products were evaluated on wood, the product that inhibited internal and external presence of *F. circinatum* was Corpol PF3 (Table 3), and it worked well for both isolates. This product is used as antidecay of class 3 (UC 3) (European standard EN-335-1), applied to wood or wood-based products that are above ground and exposed to weather conditions, especially rain. Tanalith worked better for isolate 7 than for isolate 8 (Table 3), but its efficacy was limited to the external surface of the wood block, according to the fungal growth observed in blocks half cut. However, conidial germination was best inhibited by this product.

None of the antisapstain products tested was effective when assayed in wood blocks (Table 3). However, IPBC was toxic to *F. circinatum* (Table 2). This suggests that application dosage used here was not appropriate to control *F. circinatum* in wood and it should be determined in subsequent tests. The same applies to product Corpol MV (Table 1) which is composed of propiconazole and IPBC ingredients with high toxicity, and Bardap-26 with medium toxicity (Table 2) against *F. circinatum*; however, fungal growth was not inhibited at all on wood blocks by these materials. Differences in application between antistain and anti-wood-decay products imply that former products were limited to the external wood surface, while latter products are retained in the wood. This could also explain the lack of effectiveness shown by antistain products in this study.

Despite the need for active ingredients to kill *F. circinatum* in naturally infected wood, important restrictions apply for using chemicals in the forest. Here we showed some active ingredients which are commonly included in wood preservatives inhibit pathogen growth, but further work will be needed to establish guidelines for effective use of these materials. Furthermore, our findings indicate that anti-wood-decay products such as Corpol PF3 and Tanalith used routinely in timber for construction could potentially control *F. circinatum*, overcoming trade restrictions for preventing dispersal via log movement.

Acknowledgements

This research was supported by public resources through National Institute for Agricultural and Food Research and Technology (INIA), project numbers RTA2010-00028-00-00 and AT2011-002. Yolanda Serrano was supported by project AT2011-002.

References

Braganca H, Diogo E, Moniz F, Amaro P. (2009) First report of pitch canker on pines caused by *Fusarium circinatum* in Portugal. Plant Dis 93:1079.

Carlucci A, Colatruglio L, Frisullo S. (2007) First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (Southern Italy). Plant Dis 91:1683.

Correll JC, Gordon TR, McCain AH, Fox JW, Wood DL, Schultz ME. (1991) Pitch canker in California: pathogenicity, distribution, and canker development on Monterey pine (Pinus radiata). Plant Dis 75:676–682.

Dwinell LD, Barrowsbroaddus JB, Kuhlman EG. (1985) Pitch canker- A disease complex of Southern pines. Plant Dis 69:270–276.

EPPO European and Mediterranean Plant Protection Organization. (2009) *Gibberella circinata.* Diagnostics. EPPO Bull 39:298–309.

EPPO European and Mediterranean Plant Protection Organization (2011) Plant quarantine data retrieval system (PQR version 5.0). Internet Resource: http://www.eppo.int/DATABASES/pqr/pqr.htm. (verified May 20, 2011)

Gordon TR, Storer AJ, Wood DL. (2001) The pitch canker epidemic in California. Plant Dis 85:1128–1139.

Landeras E, Garcia P, Fernandez Y et al. (2005) Outbreak of pitch canker caused by *Fusarium circinatum* on *Pinus* spp. in Northern Spain. Plant Dis 89:1015.

Leslie JF, Summerell BA. (2006) The Fusarium Laboratory Manual. USA, Blackwell Publishing.

Martin-Rodrigues N, Espinel S, Sanchez-Zabala J, Ortiz A, Gonzalez-Murua C, Dunabeitia MK. (2013) Spatial and temporal dynamics of the colonization of *Pinus radiata* by *Fusarium circinatum*, of conidiophora development in the pith and of traumatic resin duct formation. New Phytol 198:1215–1227.

Neter J, Wasserman W, Kutner MH. (1989) Applied Linear Regression Models. 2nd edtion. Irwin, IL, USA.

Wingfield MJ, Hammerbacher A, Ganley RJ, Steenkamp ET, Gordon TR, Wingfield BD, Coutinho TA. (2008) Pitch canker caused by *Fusarium circinatum* - a growing threat to pine plantations and forests worldwide. Australas Plant Pathol 37:319–334.

