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Efficacy tests on commercial fungicides against ash dieback *in vitro* and by trunk injection

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ABSTRACT

Ash dieback, caused by *Hymenoscyphus pseudoalbidus* Queloz et al. (anamorph *Chalara fraxinea* Kowalski), has emerged as a critical disease in urban areas and in the forests of many European countries. This study was conducted to evaluate six fungicides for their potential to control the disease. *In vitro* assays with different concentrations of the products against five different strains of the pathogen, illustrated that thiabendazole, propiconazole and allicin exhibited lower median lethal doses, procloraz completely killed half of the samples at higher concentrations, whereas copper sulphate and potassium phosphite were totally ineffective. Subsequently, the antifungal activities of the best three compounds were investigated *in planta* against *H. pseudoalbidus* by trunk injection. The rate of necroses development following artificial inoculation of 24 *F. excelsior* was significantly slowed down in the growing season by the treatment with thiabendazole and allicin. In the phenological phase and climatic conditions tested, and with the chosen formulation and injection method, propiconazole injections were impracticable. The results of this study, along with some technical suggestions for application in the field, support the idea of using organic and chemical endotherapic products to combat ash dieback symptoms in *Fraxinus* spp., with the safe and very low impact method of trunk injection.

Introduction

Over the last 14 years, an increasing decline in ash trees (*Fraxinus excelsior* L. and *F. angustifolia* Vahl) has been noted in Northern and Central Europe. According to Kowalski (2006) and Queloz et al. (2011), this has been caused by the Ascomycete *Hymenoscyphus pseudoalbidus* Queloz et al. (anamorph *Chalara fraxinea* Kowalski). Also pathogenicity was noted against the European *Fraxinus ornus* L., the North American *F. nigra* Marsh., *F. pennsylvanica* Marsh., *F. americana* L. and the Asian *F. mandschurica* Rupr. (Drenkhan and Hanso, 2010; Kirisits et al., 2010).

All age classes are affected, resulting in terminal decline. Infection takes place on leaves or at the leaf rachises, after wind dispersal of ascospores in summer from apothecia developed from pseudosclerotial plates in infected leaf remnants in the litter (Cleary et al., 2013; Gross et al., 2014). Infected leaves desiccate and the pathogen develops inside the stem, spreading into the phloem below the bark, into the parenchymatic rays and into the xylem, causing a brown discoloration in the wood followed closely by crown dieback (Schumacher et al., 2010; Dal Maso et al., 2012; Gross et al., 2014). Due to the ease of its spread and pathogenicity, the fungus was included in EPPO Alert List (EPPO, 2007).

During recent years, research has focused on the study of the *in vitro* mycological characteristics (Brasier and Webber, 2013; Kirisits et al., 2013), apothecia and ascospores role in the disease (Gross and Holdenrieder, 2013; Hietala et al., 2013; Kowalski et al., 2013), different host susceptibility, genetic variability of the pathogen (Kraj and Kowalski, 2013; Stener, 2013; MacLean, 2014; McKinney et al., 2014; Thomasset et al., 2014), pathogen detection techniques (EPPO, 2013; Gherghel et al., 2013; Pham et al., 2013) and, finally, the ecological consequences of the disease (Pautasso et al., 2013; Lõhmus and Runnel, 2014; Lygis et al., 2014).

Attention is now being centered on phytosanitary protection of ash trees from the pathogen. At the present time there are no effective measures to control the disease, but biosecurity protocols on disinfection to prevent the spread of *H. pseudoalbidus* have been recommended. In particular, Cooke et al. (2013) proposed various physical and chemical methods to restrict the production and spread of ascospores, including the removal of plant debris from infected sites, preventing movement of infected plant material to new sites, the use of disinfectants to treat contaminated footwear, clothing and equipment and the use of fungicides and biocides for

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Table 1
Commercial products and respective active ingredients tested for their fungicidal effect against *H. pseudoalbidus*.

Commercial product	Active ingredient	Strength	Manufacturer
TECTO 20S	Thiabendazole	220 g/L	Syngenta Crop Protection S.p.a.
SPORTAK 45 EW	Procloraz	450 g/L	BASF Italia S.p.a
ALAMO	Propiconazole	14.3%	Syngenta Crop Protection S.p.a.
CONQUER	Allicin	5000 ppm	JCA Limited
FOSFISAN	$P_4O_{10} + K_2O$	30%, 20%	Agrofill by Adriatica S.p.a.
BIOYETHI CU	Copper sulphate	2%	Summerfruit S.r.l.

the treatment of infected debris. In addition, hot water treatments were suggested by Hauptman et al. (2013) for the disinfection of plant propagation material or growing plants, considering the sensitivity of *H. pseudoalbidus* to relative high temperature.

Until now there are no complete reports on fungicides effective against the disease (Cooke et al., 2013), but the potential for a cure was considered to be high with procloraz and carbendazim, being able to stop the production of apothecia after fungicidal treatments (Hauptman et al., 2012).

Considering the lack of information on phytosanitary measures against ash dieback, the first aim of this study was to ascertain the *in vitro* lethal dose of six commercial fungicides against the pathogen. The best performing ones were then used for trunk injections (Tattar et al., 1998; Young, 2002; Takai et al., 2003) in artificially infected trees, in order to determine their potential to control the disease *in planta*.

Materials and methods

In vitro experiments

Commercial fungicidal formulations of six active ingredients (thiabendazole, procloraz, propiconazole, allicin, potassium phosphite and copper sulphate; Table 1), corresponding to an equal number of chemical classes (Benzimidazole, Imidazole, Triazole, Thiosulfinate, Potassium Phosphonate, Copper compounds), were tested *in vitro* for their effect against five *H. pseudoalbidus* strains (Table 2) previously selected among the ones available in the TeSAF herbarium for their high pathogenicity, according to Ogris et al. (2009).

Each fungicidal agent was diluted with sterile de-mineralized water (100%, 85%, 65%, 50%, 35%, 15%, 5%, 1%, 0.1%, 0.01%, 0.001%, 0.0001%, 0%), and 0.35 mL not buffered suspension was homogeneously spread on the surface of 10 mL PDA (Potato Dextrose Agar, Difco Laboratories, Detroit, MI, USA) in 94 mm diam. Petri dishes (Taiga et al., 2008), accounting 25 replicates per treatment.

After growing the fungal strains on PDA for three weeks at 20 ± 1 °C in the dark, 8 mm diameter. Plugs were removed from the actively growing colony margins, then placed top-down onto five plates per treatment (Aloj et al., 1993; Secor and Rivera, 2012). In this way 1950 plates were produced. After an incubation at 20 ± 1 °C in the dark for 3 days, plugs were transferred to unamended PDA in the same conditions (Aharoni et al., 1997; Allen et al., 2004; Suleiman, 2010), and fungal growth was checked weekly using a microscope (up to 200×) for five consecutive weeks.

Table 2
Isolates of *H. pseudoalbidus* chosen for *in vitro* experiment.

Isolate name	Location	Sample collector
Cf 1005	Cornuda (TV)	Dal Maso E.
Cf 1032	Fusine (UD)	Ogris N.
Cf 1054	Cessalto (TV)	Frigimelica G.
Cf 1056	Falcade (BL)	Frigimelica G.
Cf 1058	C. Agord. (BL)	Frigimelica G.

Growing colonies were classified as "vital", while those which failed to grow as "dead". Growth data were statistically elaborated in R cran (R Core Team, 2013) by means of one-way analysis of variance (ANOVA, $p < 0.05$) to evaluate the growth between strains at different concentrations. Then, for every strain and product, a regression curve fitting was performed by means of Generalized Linear Model considering a binomial outcome, choosing the best between *logit* and *probit* model on the base of Akaike's information criterion (AIC), the coefficient of determination (R^2) and residual analysis (Secor and Rivera, 2012).

LD50s (lethal dose for 50% of the colonies; Aloj et al., 1993) were calculated and then compared among the active agents effective for all the 5 strains by means of Multiple Comparison (Tukey HSD, $p < 0.05$). Shapiro–Wilk Normality Test and Levene test for homogeneity of variance across groups ($p < 0.01$) were performed to check for test assumptions.

The three active ingredients that achieved the smallest LD50s were selected for *in planta* trials.

In planta experiments

The experiment was carried out in a forest of Common ash (*F. excelsior*) (N45°50′26″, E11°58′20″, 180–230 m asl, Cornuda, TV), where *H. pseudoalbidus* has been present since 2010. Specimens ranged from young to mature trees naturally regenerated, to mature trees planted in the 1970s. Due to the need to infect asymptomatic trees with the pathogen, and restrictions made by the forest owner, after careful selection 24 ash trees were chosen for the experiment, ranging from 16.7 to 37.8 cm (ave. 26.45 cm) diam. at breast height (dbh).

Artificial inoculations were performed using the indigenous strain *Cf* 1005, previously grown on PDA added with streptomycin (0.5% w:v) for 60 days at 20 ± 1 °C in the dark. In May 2012, every trunk was wounded 150 cm above the collar with a sterile 7 mm diam. cork borer, penetrating approximately 5 mm, and a plug of the same diameter removed from the colony edge was placed top side inward into the hole, then protected with the bark previously removed.

In June 2013, the edges of the infected wounds were carefully debarked, photographed with a scale bar and their areas were measured by means of ImageJ software (v. 1.46r, Wajne Rasband, National Institutes of Health, USA; Abràmoff et al., 2004). According to both tree diameter and the necrotic areas, the 24 trees were then organized into four comparable groups (Peterson et al., 2009), to be injected with commercial formulations of thiabendazole 24%, propiconazole 24%, allicin 80% (Table 1) and water, as a control.

For the injections, a handheld tool recently developed by the University of Padova (BITE; Montecchio, 2013) was chosen. Preliminary trials to increase the injection speed were made on neighboring trees (*F. excelsior*) in April–May 2013 at different times of the day and with different points of injection (root collar or into the trunk 1.5 m from the soil). Furthermore, as thiabendazole tested formulation is not registered for endotherapy (Table 1), its injection performance at different concentrations of the active ingredient was tested adding a series of chemical adjuvants (acetic acid, acetone, ammonium nitrate, hydrochloric acid, nitric acid,