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The potential for pesticide trunk injections for control of thousand cankers disease of walnut

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Abstract. Thousand cankers disease, caused by the pathogen *Geosmithia morbida* vectored by the bark beetle *Pityophthorus juglandis*, has emerged as an important disease of walnut trees in Europe. The present study was performed to evaluate the efficacy of trunk injections of four commercial fungicides and one insecticide for control of the fungus and its vector. Laboratory tests indicated that fungicides containing prochloraz + tetraconazole were the most effective. Field trials on non-infected trees allowed for the selection of a mixture containing prochloraz and tetraconazole (Binal Pro), the insecticide abamectin (Vertimec EC) and the adjuvant 2-(2-ethoxyethoxy) ethanol (Carbitol™) as having rapid host uptake. Injections of this formulation in naturally infected black walnut trees reduced the presence of *G. morbida*, supporting trunk injection as an efficient and low impact technique to manage fungal damage on infected trees.

Keywords. Prochloraz, tetraconazole, abamectin, *Geosmithia morbida*, *Pityophthorus juglandis*.

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INTRODUCTION

Widespread dieback and mortality of black walnut (*Juglans nigra* L.) has occurred in the United States of America since the mid-1990s (Kolařík *et al.*, 2011; Utley *et al.*, 2013). The causal agents were determined in 2008 to be a combination of infestation by the bark beetle vector *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae, Scolytinae) and infection by the fungus *Geosmithia morbida* M. Kolarik, E. Freeland, C. Utley & N. Tisserat (Kolařík *et al.*, 2011).

Adults of *P. juglandis* carry spores of *G. morbida* on their bodies and infect host trees via the galleries they create in the bark of host tree branches and trunks. *Geosmithia morbida* then grows within and around the insect

Table 1. Commercial products, active ingredients, and concentrations tested for their fungicidal effects on colony growth of *Geosmithia morbida*.

Active ingredient	Commercial product	Active ingredient concentration (μg mL^{-1})	Manufacturer	Range of active ingredient tested (μg mL^{-1})
Thiabendazole	TECTO 20S	2.2×10^5	Syngenta Crop Protection s.p.a.	$7.44 \times 10^3 - 7.44 \times 10^{-1}$
Procloraz	SPORTAK 45 EW	4.5×10^5	BASF Italia s.p.a	$1.52 \times 10^4 - 1.52$
Allicin	CONQUER	5×10^3	Neem Biotech Ltd.	$1.69 \times 10^2 - 1.69 \times 10^{-2}$
Procloraz + tetraconazole	BINAL PRO	$2.3 \times 10^5 + 4.1 \times 10^4$	GOWAN Italia s.p.a.	$7.78 \times 10^3 - 7.78 \times 10^{-1}$ and $1.39 \times 10^3 - 1.39 \times 10^{-1}$

feeding sites and galleries. This combined damage/infection process was named "thousand cankers disease" (TCD) by Tisserat *et al.* (2009). Cankers developing from the numerous fungus introduction points gradually coalesce, compromising phloem transport efficiency (Tisserat *et al.* 2011). The disease symptoms include flagging leaves, and thinning and dieback of the host canopy. Over a period of some years, larger branches are progressively killed, and the disease often leads to tree death (Utley *et al.* 2013; Montecchio *et al.*, 2016; Hefty *et al.*, 2018).

In 2013, *P. juglandis* and *G. morbida* were detected for the first time in Italy and Europe, on black (*J. nigra* L.) and European (*J. regia* L.) walnuts (Montecchio *et al.*, 2014; Montecchio and Faccoli, 2014). The pest and pathogen were included in the EPPO A2 List (EPPO, 2018).

Effective TCD management options have been limited to sanitation efforts, based on cutting, chipping and burning infected trees (Haun *et al.*, 2010; Mayfield *et al.*, 2014). No information is available on effects of pesticide treatments to save infected trees or to protect healthy ones.

The aims of the present study were: 1) to evaluate and compare the antifungal activity of four commercial fungicides against *G. morbida*; 2) to formulate an injectable pesticide blend with sufficient uptake and activity against the pathogen and vector; and 3) to assay the efficacy of the pesticide blend against *G. morbida* and its vector in infected black walnut trees.

MATERIALS AND METHODS

Pathogen culture

The strain of *G. morbida* (designated LM13GMN) used in this study, selected for its pathogenicity, was originally isolated from a symptomatic *J. nigra* branch collected in May 2014 from an infected black walnut plantation (Santorso, Vicenza, 45°72′ N, 11°40′ E; Montecchio and Faccoli, 2014; Montecchio *et al.*, 2015). Pure cultures of the fungus were maintained on potato dextrose agar (PDA, Difco Laboratories) and stored at 8(±1)°C in the culture collection of the Department TeSAF, University of Padova, Italy. The ITS sequence of this isolate is available in GenBank (accession number MH503927).

In vitro experiment

Four commercial fungicide products - Tecto 20S (active ingredient (a.i.) thiabendazole; Syngenta Crop Protection), Sportak 45EW (a.i. prochloraz; Basf Italia), Conquer (neem, a.i. allicin) and Binal Pro (a.i. prochloraz + tetraconazole; Gowan Italia), (Table 1), were tested *in vitro* at a range of concentrations to determine the LC_{50} values (lethal concentrations for 50% of the colonies) for *G. morbida*.

Each product was diluted with sterile de-mineralized water (at 100%, 75%, 50%, 25%, 10%, 1%, 0.1% or 0.01%), and 0.35 mL of the unbuffered suspensions were evenly spread on the surfaces of 10 mL of PDA in 94 mm diam. Petri dishes (Dal Maso *et al.*, 2014), with 27 replicates per treatment and concentration. The range of the a.i. concentrations was 1.52×10^4 to 1.69×10^{-2} μg mL^{-1} (Table 1). In total, 972 plates were processed. Each PDA plate was inoculated centrally with a 5 mm diam. agar/mycelium plug taken from the margin of an actively growing *G. morbida* colony on PDA, with the aerial mycelium facing the inoculated agar surface (Aloj *et al.*, 1993; Secor and Rivera, 2012). After an incubation at 28±1°C in the dark for 3 days, plugs were transferred to untreated PDA and kept in the same conditions (Aharoni *et al.*, 1997; Allen *et al.*, 2004; Suleiman, 2010; Dal Maso *et al.*, 2014). The effects of the fungicides on subsequent fungal growth were checked weekly using a microscope (up to ×200 magnification) for 4 consecutive weeks. Growing colonies were classified as "viable", and those that failed to grow were classified as "in-active".

The fungus growth data were statistically analyzed using R cran with extension package *drc* (Ritz *et al.*, 2015; R Core Team, 2018). For each fungicide product, a regression curve was fitted using dose-response analyses for binomial outcomes. The best model function was chosen based on Akaike's information criterion (AIC), standard errors and residual analyses of the fitted models (Secor and Rivera, 2012). The LC_{50} values were then calculated and compared among the commercial fungicide products by means of one-way analysis of variance (ANOVA, $P < 0.05$), with the R extension package *rpsychi* (Cohen, 2002). Multiple comparisons were evaluated, and 95 % confidence intervals were computed for each active ingredient.

The products Sportak 45 EW and Binal Pro produced the least LC_{50}s, and were therefore selected for *in planta* experiments.

In planta experiments

Sportak 45 EW and Binal Pro are not formulated for trunk injection, so their solubility and uptake rates at different concentrations were evaluated in triplicate on asymptomatic 12-year-old *J. nigra* trees (N45°39', E11°32', Montecchio Precalcino, VI). The fungicide products were applied using a Bite® injection tool (Montecchio, 2013; Dal Maso *et al.*, 2014). A total of 28 formulations were tested, differing in concentrations of two adjuvant chemicals –[(2-(2-ethoxyethoxy) ethanol (Carbitol™) or acetic acid (1.2 %)], and one commercial insecticide [abamectin 1.84 % w/w, effective against bark beetles and registered for trunk injection (Vertimec EC, Syngenta Crop Protection)] (Table 2).

As uptake rate is known to be a limiting factor for tree trunk injection treatments (Dal Maso *et al.*, 2014), weekly tests (from the first week of May to the second week of September), were carried out to select the best formulation that, when injected at 25 cm from the ground, allowed for an uptake rate of 1 mL cm^{-1} of trunk circumference (at breast height) within 24 h.

According to the results obtained in the preliminary test, the formulation no. 21, containing Binal Pro, Vertimec EC and Carbitol™ (Table 2), was selected and used in a subsequent fungicide efficacy experiment. Twelve trees showing symptoms of thousand canker diseases, in a naturally infected 17-year-old black walnut plantation (N45°38', E11°39', Bressanvido, VI; Montecchio and Faccoli, 2014), were treated during the first week of September 2016. Six trees were injected with 1 mL cm^{-1} circumference of the no. 21 formulation, six trees were injected with the same volume of water, as in experimental controls (average of 100 mL per tree). Injections were each made through a single port 20 cm above-ground.

After 310 d from treatment (July 2017), two black walnut trees per treatment were randomly selected. Three twigs for each cardinal direction (N, E, S or W) were collected from each tree at 11-13 m above-ground. For each twig, four cankers were carefully debarked to detect *P. juglandis* insects or galleries. Each entire sample was then incubated under humid conditions for 2 weeks, at 24±1°C in the dark and observed each day. The proportions (percent) were recorded of necrosis from which hyaline mycelium developed, with conidiophores and conidia typical of *G. morbida* (Kolařík *et al.*, 2011). Fungus identity was confirmed by analysis of the internal transcribed spacer region (ITS1-5.8S-ITS2) of rDNA.

Percentages of samples positive for *G. morbida* were arranged by treatment in contingency tables, then Fisher's Exact Tests for count data were processed in R cran (R Core Team, 2018).

RESULTS

In vitro experiments

All the tested fungicides inhibited mycelium growth of *G. morbida*, with LC_{50} values ranging from 5.48 to 4.4×10^2 µg mL^{-1}. Analysis of variance showed significant differences among the four commercial products for efficacy to limit growth of *G. morbida* colonies ($F(968, 3) = 110.77$; $P < 0.01$). Tecto 20S (thiabendazole) was the least effective compound, with an LC_{50} of 4.4×10^2 µg mL^{-1}, followed by Conquer (allicin) with an LC_{50} of 1.5×10^2 µg mL^{-1}. Sportak 45 EW (prochloraz) and Binal Pro (prochloraz + tetraconazole) gave the greatest inhibition of *G. morbida*, with LC_{50} values of, respectively, 5.48 and 5.84 µg mL^{-1}.

In planta experiments

The field tests showed that, on asymptomatic trees, the formulation with the most rapid uptake rate was the no. 21 (containing 1.90×10^4 µg mL^{-1} prochloraz, 3.4×10^3 µg mL^{-1} tetraconazole, 0.9×10^3 µg mL^{-1} abamectin, and 8.38×10^5 µg mL^{-1} Carbitol™: Table 2), with the greatest uptake rate detected the first week of September 2016 (16 weeks after injection).

The percentage of samples showing cankers 310 d after treatment, from which *G. morbida* developed, were evenly distributed among replicates in the equivalent treatment classes ($P > 0.05$), and among for the water treated plants ($P > 0.05$).

Table 2. Average volumes of solutions injected into *Juglans nigra* trees during 60 min, for 28 different formulations tested on at atmospheric pressure or manually applied external pressure, as obtained in the pesticide uptake rate test. a = Sportak 45 EW commercial product; b = Binal Pro commercial product.

Formulation No.	Procloraz μg mL^{-1}	Tetraconazole μg mL^{-1}	Carbitol™ μg mL^{1}	Abamectin Mg mL^{-1}	Acetic acid mg mL^{-1}	Injection method	Injection speed mL/60 min
1	2.25×10^{5} [a]	0	0	0	0	Atmospheric pressure, 101325 Pa	0
1	2.25×10^{5} [a]	0	0	0	0	External pressure, 111377 Pa	0
2	2.25×10^{5} [a]	0	0	0	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
2	2.25×10^{5} [a]	0	0	0	1.26×10^{4}	External pressure, 111377 Pa	0
3	2.25×10^{5} [a]	0	2.42×10^{5}	0	0	Atmospheric pressure, 101325 Pa	0
3	2.25×10^{5} [a]	0	2.42×10^{5}	0	0	External pressure, 111377 Pa	0
4	2.25×10^{5} [a]	0	2.42×10^{5}	0	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
4	2.25×10^{5} [a]	0	2.42×10^{5}	0	1.26×10^{4}	External pressure, 111377 Pa	0
5	2.25×10^{5} [a]	0	4.83×10^{5}	0	0	Atmospheric pressure, 101325 Pa	0
5	2.25×10^{5} [a]	0	4.83×10^{5}	0	0	External pressure, 111377 Pa	0
6	2.25×10^{5} [a]	0	4.83×10^{5}	0	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
6	2.25×10^{5} [a]	0	4.83×10^{5}	0	1.26×10^{4}	External pressure, 111377 Pa	0
7	4.5×10^{4} [a]	0	5.80×10^{5}	1.8×10^{3}	0	Atmospheric pressure, 101325 Pa	0
7	4.5×10^{4} [a]	0	5.80×10^{5}	1.8×10^{3}	0	External pressure, 111377 Pa	0
8	4.5×10^{4} [a]	0	5.80×10^{5}	1.8×10^{3}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
8	4.5×10^{4} [a]	0	5.80×10^{5}	1.8×10^{3}	1.26×10^{4}	External pressure, 111377 Pa	0
9	4.5×10^{4} [a]	0	7.74×10^{5}	1.8×10^{3}	0	Atmospheric pressure, 101325 Pa	0
9	4.5×10^{4} [a]	0	7.74×10^{5}	1.8×10^{3}	0	External pressure, 111377 Pa	0
10	4.5×10^{4} [a]	0	7.74×10^{5}	1.8×10^{3}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
10	4.5×10^{4} [a]	0	7.74×10^{5}	1.8×10^{3}	1.26×10^{4}	External pressure, 111377 Pa	0
11	3.82×10^{4} [b]	6.8×10^{3} [b]	5.16×10^{5}	1.8×10^{3}	0	Atmospheric pressure, 101325 Pa	0
11	3.82×10^{4} [b]	6.8×10^{3} [b]	5.16×10^{5}	1.8×10^{3}	0	External pressure, 111377 Pa	0
12	3.82×10^{4} [b]	6.8×10^{3} [b]	5.16×10^{5}	1.8×10^{3}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
12	3.82×10^{4} [b]	6.8×10^{3} [b]	5.16×10^{5}	1.8×10^{3}	1.26×10^{4}	External pressure, 111377 Pa	0
13	3.82×10^{4} [b]	6.8×10^{3} [b]	7.1×10^{5}	1.8×10^{3}	0	Atmospheric pressure, 101325 Pa	0
13	3.82×10^{4} [b]	6.8×10^{3} [b]	7.1×10^{5}	1.8×10^{3}	0	External pressure, 111377 Pa	0
14	3.82×10^{4} [b]	6.8×10^{3} [b]	7.1×10^{5}	1.8×10^{3}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
14	3.82×10^{4} [b]	6.8×10^{3} [b]	7.1×10^{5}	1.8×10^{3}	1.26×10^{4}	External pressure, 111377 Pa	0
15	2.25×10^{4} [a]	4.01×10^{3} [b]	7.74×10^{5}	9×10^{2}	0	Atmospheric pressure, 101325 Pa	0
15	2.25×10^{4} [a]	4.01×10^{3} [b]	7.74×10^{5}	9×10^{2}	0	External pressure, 111377 Pa	0
16	2.25×10^{4} [a]	4.01×10^{3} [b]	7.74×10^{5}	9×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
16	2.25×10^{4} [a]	4.01×10^{3} [b]	7.74×10^{5}	9×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
17	2.25×10^{4} [a]	4.01×10^{3} [b]	8.7×10^{5}	9×10^{2}	0	Atmospheric pressure, 101325 Pa	0
17	2.25×10^{4} [a]	4.01×10^{3} [b]	8.7×10^{5}	9×10^{2}	0	External pressure, 111377 Pa	0
18	2.25×10^{4} [a]	4.01×10^{3} [b]	8.7×10^{5}	9×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
18	2.25×10^{4} [a]	4.01×10^{3} [b]	8.7×10^{5}	9×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
19	1.91×10^{4} [b]	3.4×10^{3} [b]	7.42×10^{5}	9×10^{2}	0	Atmospheric pressure, 101325 Pa	0
19	1.91×10^{4} [b]	3.4×10^{3} [b]	7.42×10^{5}	9×10^{2}	0	External pressure, 111377 Pa	0
20	1.91×10^{4} [b]	3.4×10^{3} [b]	7.42×10^{5}	9×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
20	1.91×10^{4} [b]	3.4×10^{3} [b]	7.42×10^{5}	9×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
21	1.91×10^{4} [b]	3.4×10^{3} [b]	8.38×10^{5}	9×10^{2}	0	Atmospheric pressure, 101325 Pa	0
21	1.91×10^{4} [b]	3.4×10^{3} [b]	8.38×10^{5}	9×10^{2}	0	External pressure, 111377 Pa	2.1 (1.4 - 4.2)
22	1.91×10^{4} [b]	3.4×10^{3} [b]	8.38×10^{5}	9×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
22	1.91×10^{4} [b]	3.4×10^{3} [b]	8.38×10^{5}	9×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
23	9.54×10^{3} [b]	1.7×10^{3} [b]	1.26×10^{5}	4.5×10^{2}	0	Atmospheric pressure, 101325 Pa	0
23	9.54×10^{3} [b]	1.7×10^{3} [b]	1.26×10^{5}	4.5×10^{2}	0	External pressure, 111377 Pa	0
24	9.54×10^{3} [b]	1.7×10^{3} [b]	1.26×10^{5}	4.5×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0

(Continued)

Table 2. (Continued).

Formulation No.	Procloraz μg mL^{-1}	Tetraconazole μg mL^{-1}	Carbitol^{TM} μg mL^{1}	Abamectin Mg mL^{-1}	Acetic acid mg mL^{-1}	Injection method	Injection speed mL/60 min
24	9.54×10^{3} [b]	1.7×10^{3} [b]	1.26×10^{5}	4.5×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
25	9.54×10^{3} [b]	1.7×10^{3} [b]	9.03×10^{5}	4.5×10^{2}	0	Atmospheric pressure, 101325 Pa	0
25	9.54×10^{3} [b]	1.7×10^{3} [b]	9.03×10^{5}	4.5×10^{2}	0	External pressure, 111377 Pa	0
26	9.54×10^{3} [b]	1.7×10^{3} [b]	9.03×10^{5}	4.5×10^{2}	1.26×10^{4}	Atmospheric pressure, 101325 Pa	0
26	9.54×10^{3} [b]	1.7×10^{3} [b]	9.03×10^{5}	4.5×10^{2}	1.26×10^{4}	External pressure, 111377 Pa	0
27	0	0	9.67×10^{5}	0	0	Atmospheric pressure, 101325 Pa	0
27	0	0	9.67×10^{5}	0	0	External pressure, 111377 Pa	0
28	0	0	0	0	0	Atmospheric pressure, 101325 Pa	0
28	0	0	0	0	0	External pressure, 111377 Pa	0

Statistically significant differences were found among cardinal directions ($P < 0.05$), with greater numbers of positive necroses (average = 37.5%) detected in the twigs collected from the direction opposite to the injection points, compared with those in the trees treated with formulation no. 21 (average = 9.7 %).

Although all sampled cankers showed scolytid exit holes and galleries, the proportions of necroses positive for the pathogen was significantly less in trees injected with formulation no. 21 (16.7%) than in control trees (42.7 %; Fisher's Exact Tests, $P < 0.01$).

Live *P. juglandis* was recorded in only one experimental control tree.

DISCUSSION

The main goal of the present study was to provide a preliminary evaluation of control of *Geosmithia morbida* through trunk injections of commercial pesticides.

Among the four products tested *in vitro* at eight different concentrations, Sportak 45EW (containing prochloraz) and Binal Pro (prochloraz + tetraconazole) demonstrated the lowest LC_{50} values for colony growth of *G. morbida*. Due to their impacts on ergosterol biosynthesis (Cabras *et al.*, 1998), fungicides belonging to the imidazole and triazole classes are used worldwide for control of many plant pathogens, including as *Fusarium* spp., *Colletotrichum musae*, *Nigrospora* spp., *Hymenoscyphus fraxineus*, *Magnaporthe oryzae*, *Penicillium italicum* and *Rhynchosporium secalis* (El-Goorani *et al.*, 1984; Johanson and Blazquez, 1992; Kendall *et al.*, 1993; Yan *et al.*, 2011; Dal Maso *et al.*, 2014; Fan *et al.*, 2014).

Although demonstrating some fungicidal effect, Conquer (allicin-based) was less effective than prochloraz and prochloraz + tetraconazole mixture products tested in this study. Nevertheless, the LC_{50} obtained (1.5×10^{2} μg mL^{-1}) was in line with those previously recorded for *H. fraxineus* (Dal Maso *et al.*, 2014). Tecto 20S (thiabendazole), known to be fungitoxic at low concentration against a wide range of Ascomycetes (Allen and Gottlieb, 1970; D'Aquino *et al.*, 2013; Zouhair *et al.*, 2014), was active against *G. morbida* only at high concentrations.

Due to their *in vitro* fungicidal efficacy against *G. morbida*, Sportak 45EW and Binal Pro were selected for the formulation of 28 injectable preparations, to determine the formula with the greatest uptake rate for use in the trials on infected trees. The selection of appropriate trunk injection compounds is key to the successful application of trunk injection strategies. Ten months after infected black walnut trees were injected with the formulation no. 21, the percentage of cankers positive for *G. morbida* was significantly less compared to the proportion of positive cankers for the experimental control trees. However, the efficacy changed with injection direction, with reduced effects in the parts of the tree canopies opposite to the injection ports. This was probably due to irregular distribution of the active ingredients within the canopies, as was previously reported by Tanis *et al.* (2012) and Aćimović *et al.* (2014), and this suggests that multi-port injections to individual trees could produce better infections reductions.

The insect vector *P. juglandis* was observed in only one experimental control tree, while insect emergence holes and breeding tunnels were frequently found in the sampled twigs and branches of the symptomatic trees. In Southern Europe, *P. juglandis* usually has two partially overlapping generations each year, from mid-May to late October (Faccoli *et al.*, 2016). Therefore, the emergence holes and the breeding tunnels found in infected walnut trees are probably caused by colonizations that occurred before the chemical treatment carried out in September 2016. This would explain the occurrence of cankers

on trees chosen as symptomatic before the trunk injection with fungicides, as the insect had already infected these hosts. The difficulty finding active insects in the tree branches sampled in July 2017, in the middle of the reproductive season of *P. juglandis*, suggests that the insecticide treatment provided good plant protection against new insect bark colonizations.

Despite the preliminary nature of the research described here, this study has demonstrated that endotherapy of walnut trees can slow the development of *G. morbida* for at least 1 year. However, further investigations are required to fully assess the efficacy of azole fungicides for protection of walnut trees from *G. morbida* infections (Fan *et al*., 2014; Parnell *et al*., 2008).

Because the trial was performed with technical limitations (numbers of trees to inject, injection ports to open), to avoid value losses of timber, more comprehensive trunk injection trials in larger numbers of trees could elucidate important practical details. The could include the number of injection ports necessary to obtain homogeneous distribution of fungicides in the tree crowns, the efficiency of different injection methods, and the potential for applications that prevent thousand canker disease.

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