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 (CarbitolTM) 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.

INTRODUCTION

Widespread dieback and mortality of black walnut (Juglans nigra L.) has occurred in the United States of America since the mid-1990s (Kolafík 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 (Kolafík 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...
feeding sites and galleries. This combined damage/infec-
tion process was named “thousand cankers disease” (TCD) by Tisserat et al. (2009). Cankers developing from
the numerous fungus introduction points gradually coa-
lesce, compromising phloem transport efficiency (Tis-
serat 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 pro-
gressively 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 path-
ogen were included in the EPPO A2 List (EPPO, 2018).
Effective TCD management options have been lim-
ited 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 inject-
able 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; Mon-
tecchio 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 45 EW (a.i. prochloraz; Basf Italia),
Conquer (neem, a.i. allicin) and Binal Pro (a.i. prochlo-
raz + tetraconazole; Gowan Italia), (Table 1), were tested
in vitro at a range of concentrations to determine the
LC50 values (lethal concentrations for 50% of the colo-
nies) for G. morbida.

Each product was diluted with sterile de-mineral-
ized 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 incuba-
tion at 28±1°C in the dark for 3 days, plugs were trans-
ferred 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”.

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

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Commercial product</th>
<th>Active ingredient concentration (μg mL^{-1})</th>
<th>Manufacturer</th>
<th>Range of active ingredient tested (μg mL^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiabendazole</td>
<td>TECTO 20S</td>
<td>2.2 × 10^5</td>
<td>Syngenta Crop Protection s.p.a.</td>
<td>7.44 × 10^{-1} – 7.44 × 10^{-1}</td>
</tr>
<tr>
<td>Prochloraz</td>
<td>SPORTAK 45 EW</td>
<td>4.5 × 10^5</td>
<td>BASF Italia s.p.a</td>
<td>1.52 × 10^{-1} – 1.52</td>
</tr>
<tr>
<td>Allicin</td>
<td>CONQUER</td>
<td>5 × 10^{-1}</td>
<td>Neem Biotech Ltd.</td>
<td>1.69 × 10^{-1} – 1.69 × 10^{-2}</td>
</tr>
<tr>
<td>Prochloraz + tetraconazole</td>
<td>BINAL PRO</td>
<td>2.3 × 10^4 + 4.1 × 10^4</td>
<td>GOWAN Italia s.p.a</td>
<td>7.78 × 10^{-1} – 7.78 × 10^{-1} and 1.39 × 10^{-1} – 1.39 × 10^{-2}</td>
</tr>
</tbody>
</table>
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)].

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 disease, 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 (Kolářík 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^{3} 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^{3} 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.

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

(Continued)
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 LC50 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 Rhychosporium 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 LC50 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 Acimović 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 endo-therapy 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 ofazole 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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LITERATURE CITED


Injections to control a pathogen and its vector


