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Studies of the interaction between horse chestnut leaf miner (*Cameraria ohridella*) and bacterial bleeding canker (*Pseudomonas syringae* pv. *aesculi*)

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ABSTRACT

The white flowering horse chestnut tree (*Aesculus hippocastanum* L.) was first introduced to the UK ca. 500 years ago. Over the past eight years however, this tree has suffered from severe attack by a mining insect pest known as the horse chestnut leaf miner (HCLM; *Cameraria ohridella*) and, concomitantly a gram negative bacterium (*Pseudomonas syringae* pv. *aesculi*; *Pae*). Although studies have investigated the influence of each problem individually on tree growth and vitality the interaction between HCLM and *Pae* on horse chestnut remains unknown. For this reason four year old horse chestnut seedlings were artificially inoculated with *Pae* in the presence and absence of HCLM. Effects on tree vitality were assessed by monitoring alterations to leaf chlorophyll content, leaf chlorophyll fluorescence (F_v/F_m ratios) as well as key defensive enzymatic activity (β-1,3-glucanase, peroxidase) of woody tissue at the site of *Pae* infection. With respect to mean lesion size, the main proxy of *Pae* success or aggressiveness, lesion size was significantly increased in the presence of HCLM compared to trees inoculated with *Pae* but where HCLM was controlled using insecticide sprays. Results of this study also indicate that suppression of two key defensive enzymes, β-1,3-glucanase and peroxidase, within woody tissue at the site of *Pae* infection, possibly as a result of reduced photosynthetic productivity caused by HCLM defoliation, maybe the underlying reason as to why *Pae* severity is greater in the presence of HCLM.

Introduction

The white flowering horse chestnut tree (*Aesculus hippocastanum* L) was first introduced to the UK ca. 500 years ago (Tryjanowski et al., 2006). Horse chestnuts can now be found in many parks, along avenues, streets, vistas where this single species alone is considered to compose 12% of all UK trees. In spring they produce candle-like blossoms, are also admired for their seeds known as conkers that they produce in the autumn. While recognised of having little economic value for their wood, the horse chestnut tree is one that is of high ornamental value to the British public (Johnson, 2005). Consequently, the demise of this species would be considered a great loss. Since first recorded in 2002, this species has suffered from severe attack by a mining insect pest (Tilbury and Evans, 2003) and, concomitantly a gram negative bacterium (Percival et al., 2011; Steele et al., 2010).

Bacterial bleeding canker of horse chestnut trees caused by *Pseudomonas syringae* pv. *aesculi* (*Pae*) infects the phloem and cambium of the tree on the trunk and aerial woody parts. The bacterium causes necrotic lesions (cankers) which leak exudates that are orange in colour when fresh and turn a dark black colour when dried (Steele et al., 2010). If these cankers encircle the trunk of the tree, then the water supply to the crown will be disrupted and crown death will ensue. Presently it is not known how *Pae* is spread, however, initial research indicates that this could be linked to the water cycle (Morris et al., 2008), being spread by rain or snow. The bacterium carries the *Ina* gene, which enables the bacterium to cause frost damage to plants at temperatures above freezing and thus facilitating a route of entry by damaging epithelial cells which act as portals of entry for *Pae* (Morris et al., 2008). The majority of infections are seen in spring and autumn. Within the UK, thousands of trees now exhibit symptoms of this pathogen. Untreated, trees can decline and die (Green et al., 2009).

A further problem associated with the horse chestnut is defoliation caused by the leaf-mining caterpillar *Cameraria ohridella* commonly known as the horse chestnut leaf miner (HCLM). This

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moth lays its eggs on the leaves. The eggs hatch into larvae that feed on the leaf parenchyma between the two epidermis layers thereby reducing the amount of photosynthetically active leaf tissue (Thalmann et al., 2003). Once the caterpillar has reached maturity it emerges from mined leaf tissue as a moth. In some instances infested leaves die and fall prematurely. If new leaves are formed they are frequently re-infested (Kehrli and Bacher, 2003; Raimondo et al., 2003) as within the UK, normally three HCLM generations occur annually with adult moths from the second generation overlapping with adults of the third generation (Straw and Bellet-Travers, 2004; Tilbury and Evans, 2003). Loss of tree photosynthetic capacity results in detrimental effects on seed production and viability (Percival et al., 2011).

Plant secondary metabolites and some enzymes primarily serve as survival functions for trees, acting as defensive compounds against bacterial, fungal, viral pathogens and insect pests (Nandakumar et al., 2001). Two defensive enzymes that have been shown to play a role in mediating the severity of *Pseudomonas* bacterial pathogens of beans, tomatoes, cherries and tobacco are β-1,3-glucanase and peroxidase (Lippert et al., 2007; Nandakumar et al., 2001; Bashan et al., 1987; Goy et al., 1992; Dalisay and Kuc, 1995; Bulcke et al., 1989). β-1,3-Glucanase is an enzyme that has been shown to influence severity of *Pseudomonas syringae* (Beffa et al., 1996; Bulcke et al., 1989). Peroxidase is an enzyme located in plant cell walls involved in the synthesis of lignin (Taiz and Zeiger, 1991) that has been associated with systemic resistance of plants (Hammerschmidt et al., 1982; Golubenko et al., 2007). The influence of *Pae* and HCLM infestation on β-1,3-glucanase and peroxidase activity in horse-chestnut trees remains unknown although limited studies have associated alterations in β-1,3-glucanase and peroxidase activity with defoliation caused by sawfly and beetle feeding (Karban and Myers, 1989; Barto et al., 2008; Ralph et al., 2006, 2007; Lippert et al., 2007).

Physiological tests of tree vitality in response to pest and pathogen attack are valuable because of their ability to quantify the degree of physical damage induced and so provide a basis for management decisions as well as provide insights into the influence of pest/pathogen attack on plant physiological processes (Berger et al., 2007a, b; Chaerle et al., 2007). Three commonly used tree vitality measurement systems include:

Chlorophyll fluorescence F_v/F_m ratios are an indication of the fate of excitation energy in the leaf photosynthetic apparatus that has been used to provide a rapid and non-destructive diagnostic system of detecting and quantifying injury in tree leaves and needles (Percival et al., 2011).

Leaf chlorophyll content works on the premise that pest and/or pathogen attack can limit the amount of carbohydrates available for growth and reduce nutrient uptake resulting in leaf chlorosis and necrosis (Golawska et al., 2010). Exact knowledge of foliar chlorophyll concentrations consequently provides a robust estimation of tree vitality (Percival et al., 2008). The chlorophyll meter (or SPAD meter) is a commercially available portable piece of equipment that is used to accurately estimate leaf chlorophyll content based on optical responses when a leaf is exposed to light (Loh et al., 2002).

Root carbohydrates are a major storage reserve of woody plants important in the growth and development of stress tolerance (Martínez-Trinidad et al., 2009). Woody plants accumulate carbohydrates during periods of excess production and deplete these reserves when utilization exceeds production (Tromp, 1983). Consequently, the amount of reserve carbohydrates in the root system is crucial for growth and establishment and provides a means of assessing tree vitality, i.e. healthy trees accumulate greater quantities of carbohydrates in their root system (Ritchie and Dunlop, 1980; Struve, 1990).

The influence of the interaction between HCLM and *Pae* remains unknown. For instance does HCLM defoliation increase susceptibility to *Pae* or would the presence of HCLM have little influence on *Pae* severity? Objectives of this study were to (i) investigate the interaction between HCLM and *Pae* bleeding canker on horse chestnut tree vitality. (ii) Gain a greater understanding on how secondary enzymatic activity in response to *Pae* infection may be influenced by this interaction.

Materials and methods

Experimental trees

Two year old, bare rooted stock of horse chestnut (*Aesculus hippocastanum* L.) was obtained from a commercial supplier and planted into 20 L pots containing a general tree compost (loamy texture, with 23% clay, 46% silt, 31% sand, 3.1% organic carbon, pH 6.6) supplemented with the controlled release nitrogen-based N:P:K (29:7:9) fertiliser Bartlett BOOST (The Doggett Corporation, Lebanon, New Jersey, USA) at a rate of 5.0 g/kg soil. Following potting, trees remained outdoors on a free-draining mypex covered surface at the University of Reading Shinfield Experimental Site, Reading, Berkshire (51°43′ N, −1°08′ W) subject to natural climatic conditions and watered as required. Trees were then trained for a further two years to produce a central-leader system to an average height of 1.0 ± 0.15 m with mean trunk diameters of 8.0 ± 1.5 cm at 60 cm above ground level. A fungicide program to prevent *Guignardia* leaf blotch outbreaks were performed based on the synthetic triazole fungicide penconazole (Product name Topas, Headland Agrochemicals Ltd, Saffron Walden, Essex, UK) and applied every four week during the growing season. Sprays were applied using a hand-held sprayer at 3.5 mL penconazole per L^{-1} of water. Trees were sprayed until runoff, generally 0.15 L^{-1} per tree. Consequently no *Guignardia* leaf blotch outbreaks were recorded through-out the trial.

Pae inoculation

Cell densities of *Pae* strain 6619 were adjusted to an OD600 nm of 0.1 (5×10^7 colony forming units [cfu] per mL) and serial dilutions were carried out to reach the inoculation dose (10^4 cfu/mL). Trees were inoculated 30 cm above ground level, through a small incision made in the bark using a sterile scalpel. Following the incision, 1 mL of *Pae* solution was injected into the outer xylem tissue exposed by the open incision (Dorati, 2011). The bark was then replaced on top of the *Pae* inoculated tissue and the inoculation area were sealed with 'Parafilm®' to avoid contamination. Following each injection a new disposable tip was fitted to avoid cross contamination between samples. Trees were inoculated on May 1 2008 when in full leaf (Marsal and Girona, 1997). On this date a threshold of 30 adult moths per trap had been caught by placing HA515-Agralan mini yellow sticky traps at 2.5 m^2 spacings throughout the trial site (Ledieu and Helyer, 1985). A thirty moth threshold is the stage when insecticide treatments against leaf mining pests should occur (Ledieu and Helyer, 1985).

Treatments

Treatments were as follows:

1. 20 non *Pae* inoculated trees were used for control purposes. HCLM were controlled by foliar sprays of the insecticide deltamethrin (Product name Bandu, Headland Agrochemicals Ltd., Saffron Walden, Essex, UK) applied every four week during the growing season (Percival et al., 2011). Sprays were applied using a hand-held sprayer at 0.75 mL deltamethrin per L^{-1} of