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NOTE

A fluorometric assay to estimate pea leaf weevil (Coleoptera: Curculionidae) larval feeding damage using leghaemoglobin in root nodules of faba bean (Fabaceae)

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Abstract

The pea leaf weevil, *Sitona lineatus* Linnaeus (Coleoptera: Curculionidae), is an invasive pest of field pea, *Pisum sativum* Linnaeus, and faba bean, *Vicia faba* Linnaeus (Fabaceae). Larvae feed on leguminous root nodules and associated *Rhizobium leguminosarum* Frank (Rhizobiacea) nitrogen-fixing bacteria. Larval feeding causes economic damage, but the current method to assess nodule damage is laborious. Leghaemoglobin, the oxygen-carrying molecule in root nodules, is degraded as larvae feed. Measurement of leghaemoglobin could be an alternative method to estimate larval damage. Here, we developed a fluorometric assay to measure the variation in haem fluorescence, which relates to leghaemoglobin content, from nodulated roots. Roots were collected from caged faba bean plants with or without weevil infestation. Faba bean yield and haem fluorescence were inversely correlated with the percent damaged nodules. A plant growth score was positively correlated with haem fluorescence. This method can be used to assess nodule damage in pulse crops from pea leaf weevil and potentially from other biotic stresses, and it may have wider application to studies of nitrogen fixation.

Introduction

Pea leaf weevil, *Sitona lineatus* Linnaeus (Coleoptera: Curculionidae), is native to Europe and North Africa and is an invasive pest of field pea, *Pisum sativum* Linnaeus, and faba bean, *Vicia faba* Linnaeus (both Fabaceae) in several temperate legume-growing regions (Jackson 1920; Vankosky *et al.* 2009). Pea leaf weevil adults are oligophagous on legumes (Fabaceae) and feed on foliage, making characteristic U-shaped notches (Jackson 1920). Plants compensate for adult feeding damage, and only at high weevil densities does adult feeding cause economic damage (Havlíčková 1980; Williams *et al.* 1995). Field pea and faba bean support larval development and are the main reproductive hosts of pea leaf weevil (Landon *et al.* 1995). Egg laying, however, can occur in the soil near other legumes such as alfalfa, *Medicago sativa* Linnaeus (Fabaceae) (Schotzko and O'Keeffe 1986), even though larvae do not develop on alfalfa (Wijerathna 2021). Larvae feed on nitrogen-fixing *Rhizobium leguminosarum* Frank (Rhizobiacea) bacteria associated with root nodules (Fig. 1), along with nodule tissues of reproductive host plants (Johnson and O'Keeffe 1981; Hamon *et al.* 1987), and to a lesser

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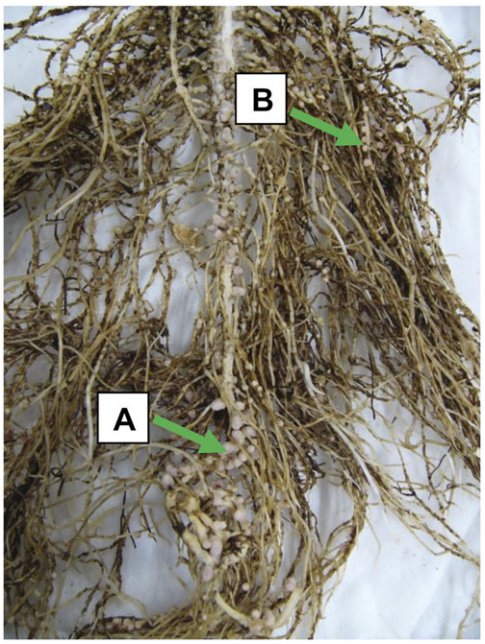

Fig. 1. Root nodules on **A,** main and **B,** lateral roots of faba bean. Light pink colour of nodules indicates presence of leghaemoglobin.

extent, on chickpeas (Williams *et al.* 1991). Pea leaf weevil larval feeding reduces nitrogen availability for field pea plants (Cárcamo *et al.* 2015), seed nitrogen content, soil nitrogen input (Doré and Meynard 1995; Corre-Hellou and Crozat 2005), and yield (Hunter 2001; Corre-Hellou and Crozat 2005). In faba bean, larval feeding reduces yield (Wijerathna *et al.* 2021) and pod production (El-Dessouksi 1971).

The pea leaf weevil was first recorded in southern Alberta, Canada in 1997 (Vankosky *et al.* 2009). Current pea leaf weevil chemical management practices in Canada's Prairie provinces rely on monitoring adult feeding damage on field pea (Philip *et al.* 2018) and faba bean (Wijerathna *et al.* 2021), even though larval feeding causes the economic damage. The nominal threshold is 15% and 30% of plants with adult damage on terminal leaves for faba bean and field pea, respectively (Cárcamo and Vankosky 2011; Wijerathna *et al.* 2021). Foliar damage correlates with nodule damage in faba bean (Wijerathna *et al.* 2021) but not in field pea (Cantot 1989; Cárcamo and Vankosky 2011). The current method for estimating larval damage is labour and time intensive, as each nodule is inspected for larval feeding holes (Cárcamo and Vankosky 2011; Wijerathna *et al.* 2021). Because faba bean has more nodules than field pea does, assessment of nodule damage on faba bean is even more laborious (Wijerathna 2021).

Root nodules with *Rhizobium* bacteria contain the oxygen-carrying molecule leghaemoglobin that potentially drives larval feeding of pea leaf weevil (Danthanarayana 1967). Larval feeding on root nodules degrades leghaemoglobin and reduces nitrogen fixation (Cárcamo *et al.* 2015). Leghaemoglobin levels correlate with nitrogenase activity of soybean nodules (LaRue and Child 1979). Faba bean plant growth is positively correlated with the number of red-pigmented root nodules (Wijerathna *et al.* 2021), which indicate active leghaemoglobin (Singh and Varma 2017). In the present study, we hypothesised that root nodule damage by pea leaf weevil larvae can be assessed by leghaemoglobin levels in roots before plant maturity. To that end, our objective was to develop a practical analytical method using fluorometry (LaRue and Child 1979) to assess pea leaf weevil larval damage on root nodules of faba bean.

A series of field cages was established to obtain different levels of pea leaf weevil larval damage on faba bean root nodules. Cages were set up at a faba bean site (58 m × 53 m) near Lethbridge, Alberta, Canada (49.7004° N, −112.7632° W) in 2018. Faba bean seeds (Zero tannin, cultivar

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"CDC Snow Drop") were treated with *Rhizobium* inoculant (1.222 kg/1000 kg seeds; Nodulator® FB Peat, 2008027A; BASF Canada, Mississauga, Ontario, Canada) and fungicide (Trilex EverGol, 1.2 L/ha; Bayer CropScience Canada, Calgary, Alberta, Canada) before seeding. In April 2018, faba bean was directly seeded at 0.2-m row spacing. Field mesh cages (1 × 1 × 1.5 m) were deployed over plants in a randomised block design soon after seeding. Each of 10 blocks contained five treatments along a transect, with 4.49 m between cages. Plant density was maintained at 30 plants per cage.

Pea leaf weevils were collected by sweeping nearby alfalfa fields from late April to early May 2018, and weevils were separated according to sex (Jackson 1920). Pea leaf weevils – 30 females (treatment 1), 60 males (treatment 2), and 15 males and 15 females (treatment 3) – were introduced to cages when plants were at the second- to third-node stage (Saskatchewan Pulse Growers 2018) to obtain roots with varying levels of larval damage. Cages without weevils (treatment 4) and 30 plants without a cage (treatment 5) were used as control treatments. Foliar damage by female weevils is higher than that by males (Wijerathna 2021). Therefore, 60 males (30 × 2) were introduced to provide similar levels of foliar damage as females. The frill (0.5 m) around the bottom of cages was buried in the soil to prevent weevils from escaping through the soil. Weevils remained in cages for approximately 2.5 months to allow for egg hatch and larval feeding on root nodules.

As larval numbers and feeding damage cannot be measured on the same root nodules that are used for leghaemoglobin measurement by fluorometric analysis, two plants from each cage were harvested at early seed pod development stage for assessment (BBCH-scale: 70–73; Weber and Bleiholder 1990). The entire root mass was collected for each sample, and soil around plant roots was collected using a hand trowel to capture larvae in the soil and root nodules. Soil was stored in plastic bags (4.5 L) and soaked in water after transport to the laboratory root-washing station. Plant roots were washed and then blotted dry with paper towels. Both plants from each cage were assessed for nodule health, which contributed to a growth score, adapted from 20/20 Seed Lab Inc. (2019), that included plant growth and vigour, nodule colour and number, and nodule position (Supplementary material). Root nodules from the first randomly selected plant were separated from the roots, counted, and assessed for larval damage. All nodules on each plant were dissected from the plant, and the number of nodules with red pigment (marker for leghaemoglobin) was recorded (Cárcamo and Vankosky 2011). The percentage of damaged nodules per plant ((number of damaged nodules/total number of nodules) *100) was calculated. For the second plant from each cage, the root was separated after washing and weighed to the nearest 0.01 g (XS3001L, Mettler Toledo, Zurich, Switzerland). Root tissues and nodules were then wrapped in foil, frozen in liquid nitrogen, and stored at −80 °C for fluorometric analysis. Remaining faba bean plants in cages ($n = 28$) were harvested by hand, and bean samples were air-dried, cleaned, and weighed to measure yield. Yield per cage was calculated by pooling the yield of 28 plants per cage.

The relative frequency of leghaemoglobin in root nodules was estimated using the fluorometric assay (Fig. 2). The washed root samples (whole root mass and nodules) reserved for fluorometric analyses were removed from the freezer and transferred to a solution of 0.2% (w/v) potassium ferricyanide and 0.1% (w/v) sodium bicarbonate at 6 mL per gram of tissue. Roots were incubated in the solution at room temperature for 5–10 minutes. Samples were ground in stainless steel mini-containers for 1-L blenders (Waring, McConnellsburg, Pennsylvania, United States of America) using the two-speed laboratory blender at 22 000 rpm for three 30-second bursts. Debris was pelleted by centrifugation at 6750 × *g* for 30 minutes. Five 100-µL aliquots of supernatant were transferred to each of five 2-mL microcentrifuge tubes containing 1 mL saturated oxalic acid (approximately 2 M) and then mixed by vortexing. Three of the five tubes were heated by autoclaving at 120 °C, 15 psi, for 30 minutes. The two remaining unheated tubes were kept as negative controls. In the presence of heat, oxalic acid removes the iron group from leghaemoglobin, releasing a stable fluorescent product, protoporphyrins

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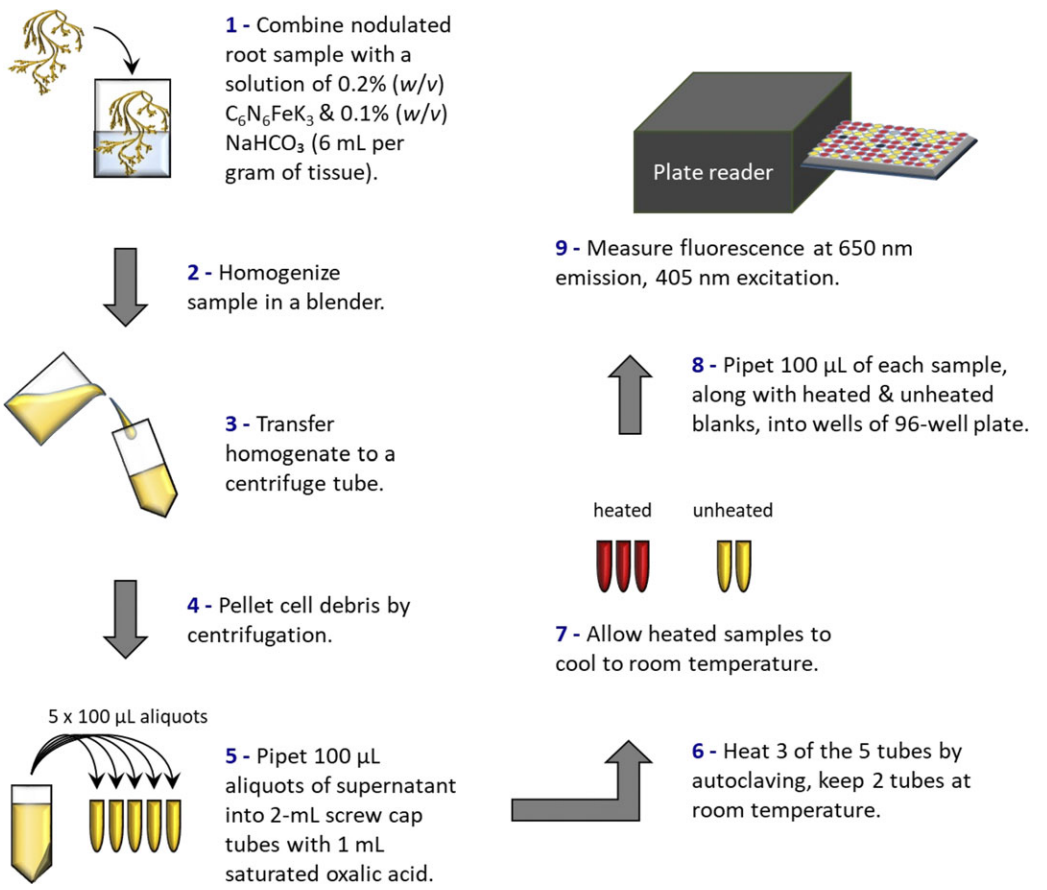

Fig. 2. Workflow for fluorescence detection of leghaemoblobin in faba bean roots.

(haem group). After cooling to room temperature, 100-µL aliquots from each of the five tubes per extraction were pipetted into individual wells of a 96-well plate (96 Well Black/Clear Bottom Plate, TC Surface; Thermo Scientific, Waltham, Massachusetts, United States of America). Fluorescence was measured at the 405-nm excitation wavelength, and the emission spectrum was recorded at 650 nm on a BioTek Gen5 plate reader (BioTek, Winooski, Vermont, United States of America). The average fluorescence of the two unheated samples was calculated and subtracted from the fluorescence of each of the three heated samples. This difference in haem fluorescence intensity between heated and unheated samples correlates with the relative frequency of leghaemoglobin (haem protein concentration) within the sample (LaRue and Child 1979). The average haem fluorescence intensity per root was calculated by averaging the haem fluorescence of three heated samples and used in the data analysis. A standard curve was established, using haemoglobin in 0.1 M phosphate buffer, pH 7.4, and 0.05% bovine serum albumin.

Data were analysed using R, version 3.6.3 (R Core Development Team 2020). Model residuals were checked for normality using the Shapiro–Wilk test. Models were checked for homogeneity of variance using Levene's test and for over-dispersion using a one-sample Kolmogorov–Smirnov test (DARMa package; Hartig 2018). Model fit was tested using qq-plots. Mixed-effect models were analysed using the lme4 library (Bates *et al.* 2015).

The effect of treatment on the percentage of damaged nodules by larvae was analysed using a generalised mixed-effect model. Separate general mixed-effect models analysed the relationship

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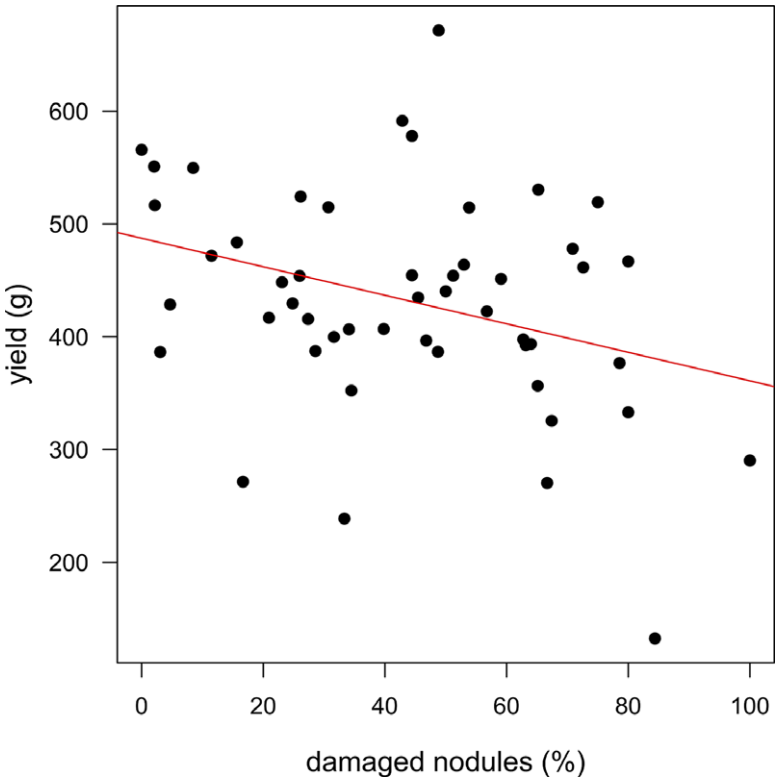

Fig. 3. Relationship between percent damaged root nodules and faba bean yield ($y = -1.26x + 487.36$). Pea leaf weevils were introduced to cages ($n = 30$ plants/cage). Two plants per cage were harvested; one plant was used to assess root nodule damage, and the other plant was used to conduct fluorometric analysis of its root nodules. To measure faba bean yield, 28 plants per cage were harvested, weighed, and pooled for analysis.

between percent damaged nodules and faba bean yield and between percent fed nodules and haem fluorescence. The relationship between plant growth score and haem fluorescence intensity was analysed using a generalised mixed-effect model with a Poisson error distribution. "Block" was the random factor in each model. The percentage of damaged nodules varied with treatment ($\chi^2_4 = 15.27$, $P = 0.004$). Plants that received female weevils had a higher percentage of fed nodules than did plants that received only male weevils (Tukey's *post hoc*, $P < 0.05$). Faba bean yield decreased with increasing percent of damaged nodules ($\chi^2_1 = 5.96$, $P = 0.01$; $r^2 = -0.9$; Fig. 3). Haem fluorescence decreased with increasing percent of damaged nodules ($\chi^2_1 = 8.18$, $P = 0.004$; $r^2 = -0.7$; Fig. 4). The plant growth score increased with haem fluorescence ($\chi^2_1 = 17.76$, $P < 0.0001$).

Our findings agree with previous studies (Wijerathna *et al.* 2021) and show that pea leaf weevil larval feeding on root nodules affects faba bean yield. The leghaemoglobin content of root nodules decreased with larval feeding and can be used to estimate pea leaf weevil larval damage on faba bean. Leghaemoglobin is an essential component of nitrogen fixation by *Rhizobium*-containing legume nodules (Appleby *et al.* 1988). *Rhizobium* bacteria require oxygen (O_2) for nitrogen fixation, and leghaemoglobin facilitates oxygen diffusion to the central zone of nodules from the plasma membrane of infected cells of the peribacteroid membrane. Pea leaf weevil larvae feed on root nodules and roots and are suspected to consume *Rhizobium* bacteria (Johnson and O'Keeffe 1981; Hamon *et al.* 1987).

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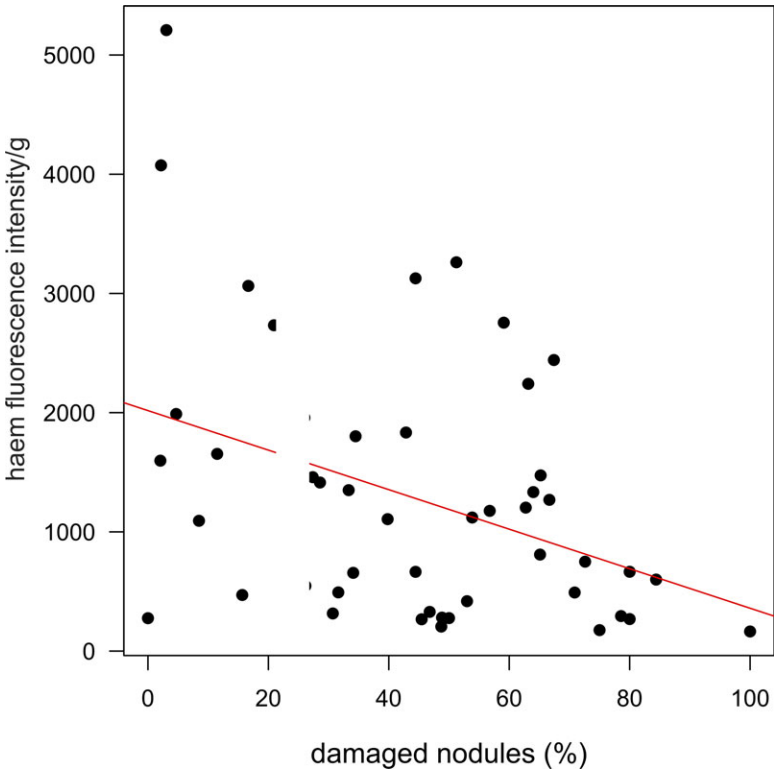

Fig. 4. Relationship between percent damaged root nodules and haem fluorescence intensity of plants ($y = -16.57x + 2017.43$). Two plants per cage were harvested; one plant was used to assess root nodule damage, and the other plant was used to conduct fluorometric analysis of its root nodules. Roots were flash frozen and subjected to fluorometric analysis to estimate the leghaemoglobin content of the root nodules. Fluorescence was measured at 405 nm (excitation) and recorded at 650 nm (emission).

We encountered instances when the haem fluorescent intensity was no more than 500 nm/g in plants with 0–100% damaged nodules by larvae in the current method (Fig. 4). These occurrences may be associated with the nodule age and differences in the efficiency of nitrogen fixation rather than the damage by pea leaf weevil larvae. Based on this, the current method would be accurate to predict pea leaf weevil larval damage when the haem fluorescence intensity is above 500 nm/g.

The fluorometric assay provides an effective alternative method to estimate pea leaf weevil larval damage on faba bean root nodules and would also save time and effort for researchers. The method may also work for field peas and other pulse crops – an application that future assays with samples of those crops could confirm. The technique could be made accessible to producers if they provided samples to researchers with access to fluorometric analysis. This technique can also be used to predict plant health because plant growth scores increase with leghaemoglobin content.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.4039/tce.2022.31.

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Competing interests. The authors declare no competing or financial interests.

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