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# The dominant modes of action of macrocidins, bioherbicidal metabolites of *Phoma macrostoma*, differ between susceptible plant species

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ABSTRACT

The bioherbicidal fungus *Phoma macrostoma* produces macrocidins, which induce chlorosis in susceptible plant species by interfering with the carotenoid biosynthesis. Macrocidins inhibit the enzyme phytoene desaturase (PDS) and likely act on other components of the carotenoid biosynthetic pathway. It was hypothesized that macrocidins' mode(s) of action differ between susceptible plant species that respond differently to these phytotoxins. This idea was tested by exploring the impact of macrocidins on the carotenoid and carotenoid precursor profiles over time, as well as chlorophyll fluorescence parameters, symptom severity and biomass, in dandelions, groundsel and chickpea. While PDS was inhibited in all three plants, this impact was strongest in dandelion and weakest in groundsel. However, groundsel showed the most severe macrocidin-induced symptoms and biomass reduction. In solution, macrocidin A bound iron and magnesium cations. Macrocidin-induced changes in OJIP chlorophyll fluorescence parameters are consistent with inhibited electron transfer from $Q_A$ to $Q_B$ (potentially due to iron-binding), and uncoupling the light-harvesting complex (LHC) of photosystem II (PSII) from the reaction centre, leading to an increase in photoprotective xanthophylls. This latter impact was stronger in groundsel than chickpea or dandelion. The decrease in total carotenoid and carotenoid precursor content in macrocidin-treated plants could be explained by macrocidins' magnesium-binding activity reducing the efficiency of 1-deoxy-D-xylulose (DXP) reductoisomerase (DXR) and/or phytoene synthase (PSY). The putatively metal-binding-related modes of action of macrocidins occurred more rapidly than inhibition of PDS. These inter-specific variations in, and diversity of, modes of action suggest that the risk of resistance developing to macrocidins is very low.



## 1. Introduction

*Phoma macrostoma* isolate 94–44B is registered as a bioherbicide in Canada and United States for the suppression of broadleaf weeds in turfgrass (Bailey and Derby, 2001). Macrocidins, polyketide secondary metabolites of *P. macrostoma* (Graupner et al., 2003), are able to induce photobleaching in susceptible plants, even in the absence of the living fungus (Hubbard et al., 2014) by inhibiting carotenoid biosynthesis. Hubbard et al. (2015) determined that macrocidins inhibit the enzyme phytoene desaturase (PDS) in the carotenoid biosynthesis pathway in the susceptible weeds (dandelion and thistle) but not in the resistant plants (pumpkin and wheat). However, Hubbard et al. (2015) also observed that macrocidin-treatment led to a drop in the β-carotene to lutein ratio and total carotenoid and carotenoid precursor content not seen in plants treated with diflufenican (DF), a PDS inhibitor (Wightman and Hayes, 1985). These findings suggest that macrocidins have additional modes of action. By examining the changes in carotenoid profiles over time, rather than at a single post-treatment time point, in plants treated with

*Abbreviations:* ABA, abscisic acid; a.i., active ingredient; ANOVA, analysis of variance; DF, diflufenican; DXP, 1-deoxy-D-xylulose; DXR, 1-deoxy-D-xylulose reductoisomerase; ESI–MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LCY-b, lycopene β-cyclase; LHCII, light harvesting complex of photosystem II; LSD, least significant difference; MU, macrocidin units; NIC, nicotine; NIC+DF, nicotine and diflufenican; PDS, phytoene desaturase; PSII, photosystem II; PSY, phytoene synthase.

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macrocidins or synthetic herbicides with known modes of action, it was hoped these additional modes of action could be elucidated.

Nicotine (NIC), a known inhibitor of lycopene β-cyclase (LCY-b) (Howes, 1974), was used as a control to determine if macrocidins inhibit the activity of this enzyme, testing the hypothesis that LCY-b inhibition contributes to the change in β-carotene to lutein ratio observed by Hubbard et al. (2015). NIC-treatment was also combined with the application of DF (the combined treatment will be referred to as NIC+DF), which is known to inhibit PDS (Wightman and Hayes, 1985), to assess if the combination of these two modes of action could mimic that of macrocidins.

Macrocidins are structurally similar to other natural products with metal-chelating capacity (Schobert and Schlenk, 2008). Hence, it is possible that metal binding could play a role in the biological activity of macrocidins. Iron plays critical roles in the photosynthetic apparatus (see review by Krohling et al. (2016)), particularly in the passing of electrons from plastoquinone A ($Q_A$) to plastoquinone B ($Q_B$) and in the Rieske FeS protein of the cytochrome $b_6$/f complex (see Fig. 2 in Stirbet et al. (2014)). Plants grown with insufficient iron display chlorosis in new growth, decreased total chlorophyll and carotenoid content, as well as changes in carotenoid composition (Abadia et al., 1999; Larbi et al., 2004; Morales et al., 1990), reminiscent of the symptoms induced by macrocidin treatment. While magnesium deficiency can also lead to chlorosis, these symptoms tend to appear in older growth first, unlike macrocidin-induced yellowing. Magnesium forms the centre of chlorophyll, meaning that a lack of availability of this element can lead to breakdown of chlorophyll in older leaves in order to make it available for new growth (see Verbruggen and Hermans (2013) for a review).

Chlorophyll fluorescence parameters, reviewed by Rohacek (2002), are useful for measuring the type and severity of stress experienced by plants. Fv/Fm is a measure of the maximum dark-adapted quantum efficiency of photosystem II (PSII) (Kitajima and Butler, 1975). Fv is a measure of variable fluorescence, which is equal to Fm (maximum fluorescence) minus $F_O$ (initial fluorescence). Lower Fv/Fm values indicate a greater degree of damaged to PSII (Farquhar et al., 1989). F'v/F'm, or effective photosynthetic yield, is the same as Fv/Fm, except that is it measured in light-adapted, or steady-steady, photosynthetic organisms (reviewed by Rohacek (2002)). Decreases in Fv/Fm and/or F'v/F'm have been measured in iron-deficient plants (Abadia et al., 1999; Bertamini et al., 2001; Larbi et al., 2004). The transient fluorescence of chlorophyll, measured by the OJIP transient (Strasser et al., 2000), has been used to evaluate the level of damaged to electron transport chains in plants under adverse conditions, including iron deficiency (Jiang et al., 2007), herbicide, heat, salt (Percival, 2005) and potential bioherbicide (Chen et al., 2015) stress. Because they include points in the fluorescent transient other than the initial fluorescence (O, which is equivalent to $F_O$) and maximum fluorescence (P, or $F_P$, equal to Fm in Fv/Fm) (see Stirbet et al. (2014) for a review), the OJIP parameters can also yield information about the type of inhibition experienced by the electron transport chain (Chen et al., 2005; Chen et al., 2007; Chen et al., 2008; Hirakil et al., 2003).

The symptom severity and percent mortality vary between plant species treated with *P. macrostoma* (Bailey et al., 2011a). Dandelions exhibited both chlorosis and death as a result of treatment with *P. macrostoma*, while groundsel displayed only mortality and chickpea only chlorosis. The hypothesis that macrocidins act by different modes of action on dandelion, groundsel and chickpea, which respond differently to treatment with macrocidins was formulated. The impact and mode(s) of action of macrocidin-treatment were assessed in terms of chlorotic symptoms, biomass, chlorophyll fluorescence parameters and carotenoid profiles over time.

## 2. Materials and methods

### 2.1. Experimental design

The experiments were carried out twice, in 2014 and 2015, with four replicates in each year. Plants were arranged according to a randomized block design. Three plants species, dandelion, groundsel and chickpea, were used. The treatments applied to dandelions were untreated control, 32 macrocidin units (MU) per pot, 128 MU, 256 MU, nicotine alone (NIC), diflufenican (DF) alone and NIC and DF in combination (NIC+DF). Groundsel and chickpeas were subjected to the following four treatments: control (zero), 32, 128 and 256 MU. The macrocidin doses used were equivalent to $^1/_4$, 1 and 2× the label rate of 64 g/m$^2$ (based on a 200 MU/g granular product) for post-emergent weed control. The $^1/_4$ post-emergent rate is also the pre-emergent recommended dose (Bailey et al., 2011a).

### 2.2. Plant material

Chickpeas (*Cicer arietinum*, Sanford kabuli, a gift from Sabine Banniza (Professor, University of Saskatchewan, Saskatoon, SK, Canada)) were planted at a rate of five (first experiment) or seven (second experiment) seeds per pot. Twenty dandelion seeds were planted in each pot (Richters, Goodwood, ON, Canada). Groundsel seeds, collected in Saskatoon, SK, in 2011, were planted 25 seeds to a pot. Prior to treatment, dandelions, groundsel and chickpeas were thinned to five to six, five and three to five plants per pot, respectively. Treatments were applied to four-week-old groundsel and chickpeas and five-week-old dandelions. All plants were grown in soil-less mix in 10 cm × 10 cm pots in a greenhouse at the AAFC Saskatoon Research Centre as described by (Hubbard et al., 2015).

A track sprayer (Halltech Ag GPS, Guelph, ON, Canada) with a TeeJet 8002 XR8002 nozzle (Spraying Systems Co., Wheaton, IL, USA) 40 cm (2014) or 51 cm (2015) above the surface of the soil-less mix was used to apply NIC and DF. NIC was applied at 100 μmol/pot, which is the equivalent of 16.2 kg active ingredient (a.i.)/hectare in a total volume of approximately 0.7 mL/pot in the first experiment. In the second experiment, 90 μmol/pot (14.6 kg a.i./hectare) was applied in 1.1 mL. In the first and second experiments, 50 and 45 μg of DF was applied per pot (the equivalent of 100 and 90 mL/ha or 50 and 45 g a.i./hectare) in a total volume of approximately 0.7 mL and 1.05 mL. Plants treated with both NIC +DF received herbicide in a total volume of roughly 1.4 mL/pot (first experiment) and 2.1 mL/pot (second experiment).

A 1-touch plant mister (Cepia LLC, St. Louis Mo. 63124) was used to spray macrocidins onto leaves and soil surface. Groundsel and chickpea plants were enclosed in plastic sleeves during this process and for several weeks thereafter in order to retain run-off.

### 2.3. Macrocidins and chemicals

Macrocidins were produced by *P. macrostoma* 94-44B grown in a fermenter, prepared and quantified as described by Hubbard et al. (2015).

Dandelions treated with 32, 128 and 256 MU per pot in 2014 received 1.8, 7.2 and 14.4 mL/pot, respectively, of the concentrated solution of batch SCO-11-F12 (17.8 MU/mL), diluted to 15 mL/pot. Batch 2013-01-22 contained 7.6 MU/mL and was applied to groundsel and chickpeas in 2014 at 4.18 mL (32 MU), 16.75 mL (128 MU) and 33.50 mL (256 MU) per pot. Batch 2014-05-12 was applied to dandelion, groundsel and chickpeas in 2015 at 2.1 mL/pot for 32 MU, 8.2 mL/pot for 128 MU and 16.4 mL/pot for 256 MU of the 15.6 MU/mL solution, diluted to 17 mL/pot.