Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker) in oilseed rape

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Abstract

Controlled environment (CE) and field experiments were done to investigate effects of fungicide on growth of *L. maculans* and *L. biglobosa* in relation to development of phoma leaf spots and phoma stem canker on oilseed rape. In CE experiments, for plants inoculated with *L. maculans*, fungicide treatment decreased lesion size and amount of *L. maculans* DNA in leaves; for plants inoculated with *L. biglobosa*, fungicide did not affect lesion size or amount of pathogen DNA. In field experiments in 2006/07 and 2007/08, fungicide treatment decreased phoma leaf spot incidence in autumn and stem canker severity at harvest, and increased yield. Fungicide treatment decreased stem canker severity more on cv. Courage, with a good yield response, than on cv. Canberra. In 2006/07, fungicide decreased the amount of *L. maculans* DNA more than that of *L. biglobosa* in stem tissues (measured by quantitative PCR). There was a linear relationship between the amount of *L. maculans* DNA in stems and the stem canker severity score at harvest, but there was no clear relationship between the amount of *L. biglobosa* DNA in stems and the stem canker severity score. These results suggest that effects of fungicides on interactions between *L. maculans* and *L. biglobosa* might affect severity of phoma stem canker and yield response.

Keywords*:* blackleg, *Brassica napus*, disease control, fungicide, phoma stem canker

Introduction

Phoma stem canker, a disease of worldwide economic importance on oilseed rape and brassica vegetables, responsible for worldwide losses worth more than £1000M each growing season (at a price of £300 t^{-1}) (Fitt et al., 2011), is caused by the two closely related species *Leptosphaeria maculans* and *L. biglobosa* (Shoemaker & Brun, 2001; Fitt et al., 2006). *L. maculans* is more damaging, causing stem base canker; *L. biglobosa* is generally less damaging, causing upper stem lesions (West et al., 2002; Huang et al., 2005). The proportion of the two species in local populations has been shown to affect the severity of stem canker epidemics (Stonard et al., 2010). Previous *in vitro* studies indicate that *L. maculans* and *L. biglobosa* differ in their sensitivity to triazole fungicides. In terms of *in vitro* mycelial growth, isolates of *L. maculans* were more sensitive to the triazole fungicides flusilazole and tebuconazole than isolates of *L. biglobosa* (Eckert et al., 2010). Whether triazole fungicides have the same effects on growth of the two species *in planta* as *in vitro* is not clear. To optimise the use of fungicide, there is a need to investigate whether application of fungicides to winter oilseed rape crops affects the proportions of the two *Leptosphaeria* species, and thus the severity of phoma stem canker epidemics.

Materials and methods

Controlled-environment experiments

Plants of cvs Courage and Canberra were grown in pots with one plant per pot in controlled conditions at 20°C with a 12h photoperiod. There were three inoculation treatments: (1) *L. maculans* only; (2) *L. biglobosa* only; (3) mixture of *L. maculans* and *L. biglobosa* (suspensions of *L. maculans* and *L. biglobosa* ascospores were mixed in a 1:1 ratio). Suspensions of *L. maculans* or *L. biglobosa* were prepared as previously described (Huang et al., 2003). There were three fungicide treatments: (1) untreated (control, sprayed with distilled water); (2) sprayed with the commercial fungicide product Punch C at 6 days post inoculation (dpi) (early spray); (3) sprayed at 11 dpi (late spray). Phoma leaf lesions were assessed by measuring lesion diameters at 20 dpi; symptomless growth of *L. maculans* or *L. biglobosa* in the leaf tissues was measured by quantitative PCR (qPCR) (Huang et al., 2009).

Field experiments

In field experiments in 2006/07 and 2007/08, plots were arranged in a randomised block design with three replicates. The fungicide Punch C was applied as an early spray or late spray during each growing season. Phoma leaf spotting (% plants affected) was assessed between September and February. Phoma stem canker severity was assessed before harvest by randomly sampling 20 plants from each plot and cutting the stem base of each plant, then scoring the area of necrotic tissue in the cross-section using a 0−6 scale. To assess the effects of fungicide application on growth of *L. maculans* or *L. biglobosa* in stems, ten plants were randomly sampled from each plot in 2007 before harvest. A 6 cm long piece of stem base was sampled from each plant and freeze-dried for DNA extraction and qPCR.

DNA extraction and quantitative PCR (qPCR)

Affected leaves or stems were freeze-dried and ground into powder. DNA was extracted from a 20 mg sub-sample using a DNA extraction kit (DNAMITE Plant Kit, Microzone Ltd, UK) and quantified on a Nanodrop ND-1000 spectrophotometer (Labtech International, UK). The amounts of *L. maculans* or *L. biglobosa* DNA in each leaf or stem sample were quantified using a Sigma SYBR Green qPCR kit (Sigma, Gillingham, UK) with specific primers LmacF/LmacR (for *L. maculans*) and LbigF/LmacR (for *L. biglobosa*) (Huang et al., 2009). Standard curves were generated by using known amounts of *L. maculans* or *L. biglobosa* DNA (from 1 to 10^4 pg μL^{-1}) from pure cultures. Results were expressed as amount (pg) of *L. maculans* or *L. biglobosa* DNA in 50 ng total DNA from affected plant tissue.

Results

Effects of fungicide treatment on growth of *L. maculans* and *L. biglobosa* in leaf tissues in controlled environment experiments

There was a significant difference in size of leaf lesions between fungicide treatments (P<0·001, SED 0 ·12, 68 df); spraying with the fungicide Punch C significantly decreased size of leaf lesions of *L. maculans* that developed on cvs Courage and Canberra (Table 1). There was also a significant difference in size of leaf lesions between inoculum treatments (P<0·001, SED 0·13, 68 df) but no difference between cultivars. There was an interaction between spray timing and inoculum treatment (P<0·001, SED 0·21, 68 df). For plants inoculated with *L. biglobosa* alone or with a mixture of *L. maculans* and *L. biglobosa*, there was no significant difference in lesion development between cultivars or between fungicide treatments. There were significant differences in amount of pathogen DNA between fungicide treatments (P<0·001, SED 0·10, 68 df), inoculum (P<0·001, SED 0·12, 68 df) and cultivars (P<0·001, SED 0·08, 68 df). When the data for *L. maculans* and *L. biglobosa* DNA were analysed separately, sprays of Punch C decreased amounts of DNA of *L. maculans* (P=0·025, SED 0·27, 5 df) and *L. biglobosa* (P=0·047, SED 0·18, 5 df) in the leaf tissues. There was more *L. maculans* DNA in leaves of Courage than leaves of Canberra but there was little difference between the two cultivars in amount of *L. biglobosa* DNA.

Table 1 Effects of the fungicide Punch C on development of lesions of *Leptosphaeria maculans* or/and *L. biglobosa* on leaves of oilseed rape cvs Courage or Canberra in a controlled environment experiment. Plants were inoculated with ascospores of *L. maculans* (Lm), *L. biglobosa* (Lb) or a mixture of *L. maculans* and *L. biglobosa* (LmLb). There were three fungicide treatments; untreated, sprayed at 6 or 11 days post inoculation (dpi)*.

Inoculum	Cultivar	Untreated	Sprayed at 6 dpi	Sprayed at 11 dpi
Lm	Courage	0.70	0.06	0.22
	Canberra	1.09	0.05	0.31
Lb	Courage	0.50	0.46	0.38
	Canberra	0.46	0.51	0.39
LmLb	Courage	0.38	0.34	0.36
	Canberra	0.39	0.31	0.36

*The size of lesions was measured at 20 dpi.

Effects of fungicide on phoma leaf spots and stem canker in field experiments

In both growing seasons, early fungicide treatment decreased the incidence of phoma leaf spot in autumn. In 2006/07, incidence of phoma leaf spotting reached 40% in late October with a maximum of 78% plants affected in late November in untreated plots. In 2007/08, although leaf lesions were observed in early October, the incidence of leaf spot did not reach 60% until mid-December with a maximum of 70% plants affected in early January in untreated plots. Fungicide treatment decreased

the severity of phoma stem canker in 2006/07 and 2007/08. In 2006/07, fungicide treatment significantly decreased the final stem canker severity (P <0·001, SED 0·21, 10 df). However, there was no difference in stem canker severity between early sprayed and late sprayed plots (Fig. 1). There was a significant difference in stem canker severity between cultivars (P <0·001, SED 0·17, 10 df). In 2007/08, there was a significant difference in stem canker severity at harvest between cultivars (P<0·01, SED 0·22, 10 df) but there was no difference between fungicide treatments (Fig. 1). In both seasons, there were significant differences in yield between treated and untreated plots. Overall, treated plots yielded more than untreated plots.

Effects of fungicide treatment on growth of *L. maculans* and *L. biglobosa* in stem tissues

The growth of the pathogens in stem tissues was measured by quantification of the pathogen DNA using qPCR. Fungicide treatment significantly decreased the amount of *L. maculans* DNA in the stem base tissue (P<0·001, SED 0·24, 72 df).There was no significant difference between cultivars in amount of *L. maculans* DNA and no interaction between cultivar and spray timing. Fungicide treatment significantly decreased the amount of *L. biglobosa* DNA in cv. Canberra (P<0·001, SED 0·16, 74 df) but not in cv. Courage. When *L. maculans* and *L. biglobosa* DNA were analysed together, there was no difference between cultivars but there was a difference between spray treatments (P = 0·017, SED 2·86, 5 df). The amount of *L. maculans* DNA in the stem base was significantly greater than the amount of *L. biglobosa* DNA (P<0·001, SED 2·03, 6 df). Fungicide treatments decreased the amount of *L. maculans* DNA more than amount of *L. biglobosa* DNA. There was a linear relationship between the amount of *L. maculans* DNA in stems and the stem canker severity score.

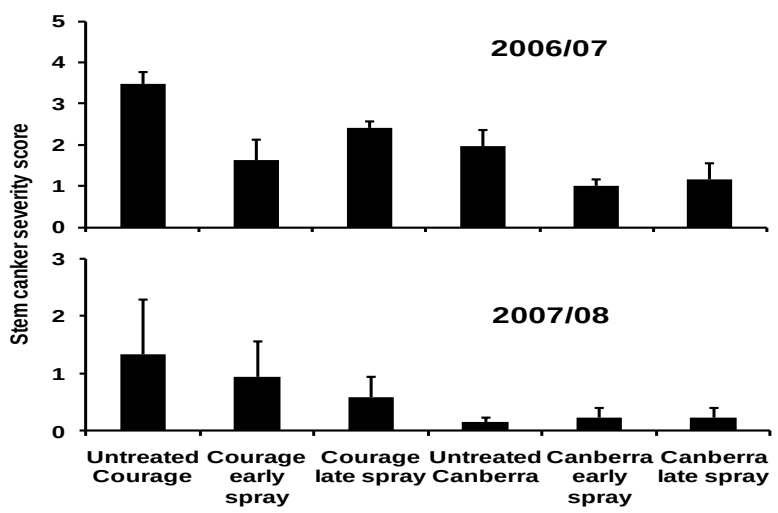

Fig. 1 Effects of fungicide Punch C treatments on severity of basal phoma stem canker on winter oilseed rape cvs Courage or Canberra in the 2006/07 and 2007/08 growing seasons at Rothamsted. Severity of basal stem cankers was assessed 2 weeks before harvest. Vertical bars are standard deviations.

Discussion

The results of both controlled environment and field experiments indicate that the fungicide Punch C (flusilazole plus carbendazim) decreased *in planta* growth of *L. maculans* in oilseed rape more than that of *L. biglobosa*. This suggests that Punch C is more effective in controlling the growth of *L. maculans* than that of *L. biglobosa* in oilseed rape leaf tissues. Thus these *in planta* results confirm the conclusions from previous *in vitro* experiments which showed that the triazole fungicides flusilazole and tebuconazole were more effective against *L. maculans* than against *L. biglobosa* (Eckert et al., 2010). This conclusion is further supported by results from the field experiments. Although fungicide treatment decreased the amount of DNA of both *L. maculans* and *L. biglobosa* in stem tissues by harvest in 2007/08, the amount of *L. maculans* DNA was decreased more than that of *L. biglobosa* DNA. These results suggest that use of fungicides influences the proportions of these two co-existing *Leptosphaeria* species on oilseed rape. These results suggest that effects of fungicide on interactions between *L. maculans* and *L. biglobosa* in autumn play an important role in determining the relationship between autumn fungicide timing and severity of phoma stem canker the following summer.

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References

Eckert MR et al., 2010. *Pest Management Science* **66,** 396-405; Fitt BDL et al., 2006. *Annual Review of phytopathology* **44**, 162-182; Fitt BDL et al., 2011. *Plant Pathology* **60**, 44-53; Huang YJ et al., 2003. *Plant Pathology* **52**, 245-55; Huang YJ et al., 2005. *European Journal of Plant Pathology* **111**, 263-77; Huang YJ et al., *Plant Pathology* **58,** 314-23; Huang YJ et al., 2011. *Plant Pathology* **60**, in press; Shoemaker & Brun, 2001. *Canadian Journal of Botany* **79**, 412-419; Stonard JF et al., 2010. *European Journal of Plant Pathology* **126,** 97-109; West JS et al., 2002. *Plant Pathology* **51,** 311-21.