Regional variation in UK populations of *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker) on oilseed rape

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Abstract

Phoma stem canker, causal agents *Leptosphaeria maculans* and *L. biglobosa*, is a major disease of oilseed rape worldwide. Cankers decrease yield by disrupting water and nutrient transport in the stem, resulting in premature pod ripening. Infection by *L. maculans* results in basal stem cankers that are more severe than the upper stem lesions caused by *L. biglobosa*. Surveys of populations of *L. maculans* and *L. biglobosa* in England and Wales were done in 2001, 2002, 2003 and 2006. Oilseed rape stems with basal cankers or upper stem lesions were obtained from different UK regions in June/July before harvest. Identification of *L. maculans* and/or *L. biglobosa* was by PCR using species-specific primers. Of the lesions tested, approximately two thirds were identified as colonised by *L. maculans* and one third as colonised by *L. biglobosa.* There was regional variation in the occurrence of *L. biglobosa*; the greatest proportion of *L. biglobosa* detected from lesions was in north-east England and the least in southern regions of the UK. The results of ongoing experiments into sensitivity of *L. maculans* and *L. biglobosa* to fungicides will be combined with the results of this study to improve targeting of fungicide applications for control of stem canker in different regions of the UK.

Key words: *Brassica napus,* geographical distribution, PCR

Introduction

Phoma stem canker is the most economically important disease of oilseed rape in Europe, Australia and North America (Fitt *et al.*, 2006a). Phoma stem canker is caused by a complex of species that comprises several distinct species that are morphologically similar (*Leptosphaeria*)(Rouxel & Balesdent, 2005). Two of these closely related species, *L. maculans* and *L. biglobosa*, can be separated on the basis of differences in pseudothecial morphology (Rouxel & Balesdent, 2005) as well as germination, growth, biochemical traits, molecular patterns, pigment diffusion and pathogenicity to *B. napus* (Fitt *et al.*, 2006a). In Europe the majority of *Leptosphaeria* isolates occurring on Brassica crops are related to *L. maculans* 'brassicae' that causes damaging basal stem cankers or *L. biglobosa* 'brassicae' that causes upper phoma stem lesions (Rouxel & Balesdent, 2005). *Leptosphaeria maculans* and *L. biglobosa* are able to co-exist on oilseed rape and have similar life cycles (Fitt *et al.*, 2006b).

Ascospores, released from pseudothecia that develop on the debris of the previous season's crop, act as the main inoculum source to initiate phoma stem canker epidemics each season (Aubertot *et al.*, 2006). Both *L. maculans* and *L. biglobosa* are able to survive as saprophytes and form pseudothecia on crop debris after harvest (Salam *et al.*, 2003). Pseudothecial maturation is dependent on temperature and moisture (Toscano-Underwood *et al.*, 2003). Huang *et al.* (2003) showed that maturation of *L. biglobosa* pseudothecia is slower than that of *L. maculans* pseudothecia at temperatures of less than 10°C (average UK winter temperature), whilst pseudothecial maturation of both species is similar at 15 – 20°C. This may explain why there are differences in the occurrence of *L. maculans* and *L. biglobosa* phoma leaf spots. *Leptosphaeria maculans* ascospores are released earlier than those of *L. biglobosa.* Therefore, numbers of *Leptosphaeria maculans* leaf spots are greatest in autumn/winter whilst numbers of *L. biglobosa* leaf spots are greatest in spring when temperatures are higher (Huang *et al.*, 2003; Fitt *et al.*, 2006b). From the leaf, both *L. maculans* and *L. biglobosa* then grow asymptomatically down through the petiole to the plant stem where they are able to invade and kill host cell tissue resulting in the formation of cankers or stem lesions in the spring/summer (West *et al.*, 2001). Autumn leaf infections are considered to result in the most severe stem cankers as temperatures are higher and *B. napus* plants are smaller in autumn than in winter, allowing the pathogen to quickly reach the stem (Thurwachter *et al.*, 1999; Fitt *et al.*, 2006b). Therefore, *L. maculans* is associated with damaging basal stem cankers whilst *L. biglobosa* is associated with less damaging upper stem lesions (Fitt *et al.*, 2006b). It has been suggested that climatic differences between regions of the UK may contribute to differences in the proportions of *L. maculans* and *L. biglobosa* in populations. This study aims to investigate UK regional variation in the occurrence of *L. maculans* and *L. biglobosa.*

Materials and methods

Stem samples with cankers or upper stem lesions were supplied by ADAS (2001, 2002 and 2003) and TAG (2006) at harvest (July). Samples were classified by region (Fig. 1). Basal stem cankers (<5 cm above stem base) and upper stem lesions

(>5 cm above stem base) were scored on a 0-5 scale according to the percentage of the stem circumference affected (0% = no symptoms, 1 = 0-25%, 2 = 25-50%, 3 = 50-75%, 4 = 75-100% and 5 = plant dead) (West *et al.*, 2002). Genomic DNA was extracted from pieces of basal stem cankers/upper stem lesions, *c.* 5 mm in diameter (Graham *et al.*, 1994). In 2001, 2002 and 2003, a multiplex PCR approach (Liu *et al*., 2006) was adopted to identify *L. maculans* and/or *L. biglobosa.* In 2006, separate PCR reactions were done to identify either *L. maculans* or *L. biglobosa* in the same genomic DNA sample (Mahuku *et al*., 1995). For the detection of both *L. maculans* and *L. biglobosa* each 15 µl PCR mix comprised 0.3 µl forward primer, 0.3 µl reverse primer, 5.9 µl sterile distilled water, 7.5 µl RED Taq® Ready Mix™ (20mM Tris-HCl, (pH 8.3), 100mM KCl, 3mM $MgCl_2$, 0.002% gelatine, 0.4mM dNTP mix, stabilisers, 0.06 unit/µl Taq polymerase; Sigma, U.K.) and 1 µl target DNA template. Thermo cycling parameters were 95°C for 2 min followed by 40 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 1 min and a final step of 72°C for 10 min, for the detection of *L. maculans*. For the detection of *L. biglobosa* the annealing temperature was 55°C instead. PCR products were separated by electrophoresis on 1% agarose, incorporated with ethidium bromide, in 1x TBE buffer for 1 h at 80 volts and amplicons were positively identified under ultraviolet light.

Figure 1: Division of England and Wales into regions; north-west (NW), north-east (NE), central (C), Wales (WA), south-east and south-west (SW).

Results

Leptosphaeria maculans and/or *L. biglobosa* were identified in 958 stem lesions by PCR over the four years of the study. Unfortunately no samples were received from Wales or north-west England (Table 1).

Table 1: Number of basal stem lesions in which *L. maculans, L. biglobosa* or both *L. maculans* and *L. biglobosa* were detected in 2001, 2002, 2003 and 2006. *Leptosphaeria maculans* and *L. biglobosa* were identified through multiplex PCR, using species-specific primers, in 2001, 2002 and 2003. Separate PCR reactions, using species-specific primers, were done in 2006 to identify *L. maculans* and/or *L. biglobosa*.

Year	Species	Number of lesions				Total
		Region				
		SE	SW	C	NE	
2001	*L. maculans*	22	22	39	6	89
	L. biglobosa	1	2	19	9	31
	L. maculans & *L. biglobosa*	0	2	12	2	16
	Total	23	26	70	17	136
2002	*L. maculans*	39	16	78	5	138
	L. biglobosa	8	5	8	3	24
	L. maculans & *L. biglobosa*	1	0	0	0	1
	Total	48	21	86	8	163
2003	*L. maculans*	31	24	21	18	94
	L. biglobosa	6	1	3	8	18
	L. maculans & *L. biglobosa*	0	0	1	0	1
	Total	37	25	25	26	113
2006	*L. maculans*	36	7	20	8	71
	L. biglobosa	7	1	6	1	15
	L. maculans & *L. biglobosa*	253	27	153	27	460
	Total	296	35	179	36	546

Of the lesions tested by multiplex PCR in 2001, 2002 and 2003, *L. maculans* alone was detected in 78%, *L. biglobosa* alone in 18% and both *L. maculans* and *L. biglobosa* in 4%. The proportion of *L. biglobosa* was greatest in the north-east and smallest in the south-east (Fig. 2). Of the lesions tested for the presence or absence of *L. maculans* and *L. biglobosa* by separate PCR reactions in the 2006 stem survey, in 80% both *L. maculans* and *L. biglobosa* were identified. Regional weather data (Fig.3) shows lower rainfall in the north-east and central regions than in southern regions.

Discussion

Results suggest that there is regional variation in the proportion of *L. maculans* and *L. biglobosa,* which may be related to regional differences in rainfall and temperature. The occurrence of *L. biglobosa* was greater in the north-east and central regions than in the south-east and south-west, whilst the occurrence of *L. maculans* was greater in the south-east and

south-west regions. Average regional weather data shows that the south-east and south-west experience greater rainfall and higher temperatures than the north-east and central regions in August and September, when pseudothecia are maturing. Basal stem cankers are associated with leaf infections occurring before stem elongation; later infections are associated with upper stem lesions (West *et al.*, 2002). Some ascospore release of *L. maculans* is generally earlier in the season than that of *L. biglobosa,* the majority of basal stem cankers (78% in this study) are caused by *L. maculans*. The lower temperatures in the north-east and central regions will have resulted in slower plant growth (e.g. stem extension) than in the south-east and south-west. Therefore, later infections by *L. biglobosa* may result in basal stem cankers rather than an upper stem lesions in the north-east and central regions.

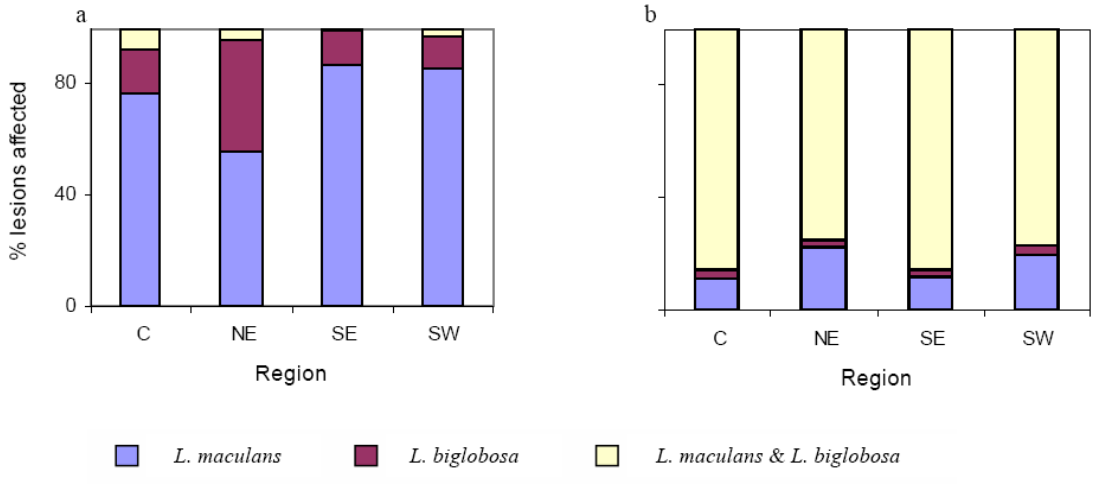

Figure 2: Percentages of phoma stem lesions obtained from different regions of England (central (C), north-east (NE), south-east (SE) and south-west (SW)) in which *L. maculans*, *L. biglobosa* or both *L. maculans* and *L. biglobosa* were identified (a) 2001, 2002 and 2003 stem surveys using a multiplex PCR system and (b) 2006 stem survey using separate PCR reactions to detect *L. maculans* and *L. biglobosa*.

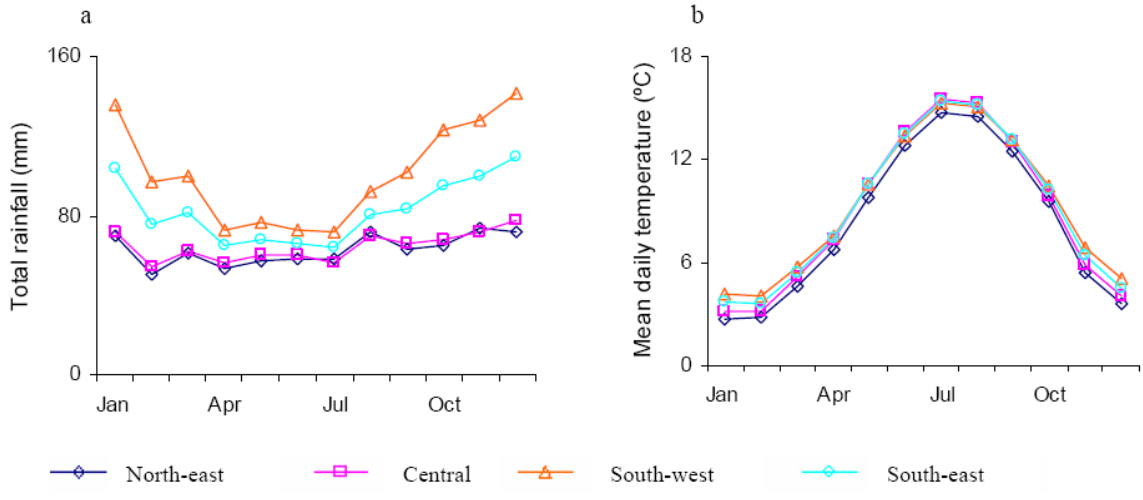

Figure 3: Regional monthly climatic data (www.metoffice.com), (a) total rainfall (1961-1990 mean), (b) mean daily temperature (1961-1990 mean).

These results suggest that *L. maculans* and *L. biglobosa* are able to exist in the same lesion in the UK, although there are differences between years in the proportion of lesions with the two species. The large difference in proportion of *L. maculans* and *L. biglobosa* between 2006 and earlier seasons may be due in part to the different PCR approaches used. In 2001, 2002 and 2003 multiplex PCR reactions were used, whereas in 2006 separate PCR reactions were done to detect *L. maculans* and *L. biglobosa.* The reason for this change in assay procedure was that recent evidence suggests that interference between levels of amplicons of both *L. maculans* and *L. biglobosa* in a single sample could lead to specific reaction inhibition through competition if the concentration of either species is below a threshold level. Thus it is possible that the less abundant species in lesions was not detected in the 2001, 2002 and 2003 surveys if present at a critically low concentration. Preliminary work has shown that by exclusion of a species if its proportion is very small the 2006 data are similar to the results obtained in the 2001, 2002 and 2003 surveys. Work is currently being done to determine the ratio of *L. biglobosa* to *L. maculans* in these samples using quantitative PCR.

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