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Colonization of winter oilseed rape tissues by A/Tox$^+$ and B/Tox^0 *Leptosphaeria maculans* (phoma stem canker) in France and England

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The colonization of winter oilseed rape plants and epidemiology of phoma stem canker differed between A/Tox$^+$ and B/Tox^0 *Leptosphaeria maculans*. In France and England, where plant colonization was investigated during two and three growing seasons, respectively, there was a difference in timing of leaf infection; A/Tox$^+$ *L. maculans* was predominant on leaves in the autumn (October/November) but there was an increase in the incidence of B/Tox^0 in the winter (January/February). In May, June and July both species could be isolated from all external parts of the plant (root to the upper stem) and all crown (stem base) tissues, although they differed in their distribution. At the root and crown, A/Tox$^+$ *L. maculans* was predominant and was located throughout the cortex, wood and pith tissues, but the rarer B/Tox^0 was located mainly in the cortex. Approximately equal numbers of A/Tox$^+$ and B/Tox^0 isolates were obtained from the upper stem – there was a greater proportion of B/Tox^0 isolates than at the crown. In England, after harvest in 1999 and 2000, pseudothecia on the lignified tap root and crown tissues produced predominantly A/Tox$^+$ ascospores (94%), while pseudothecia higher up the stem produced more B/Tox^0 ascospores (60%) than A/Tox$^+$ ascospores (40%). The timing of the onset of leaf spotting, earlier in the season for A/Tox$^+$ than B/Tox^0 *L. maculans*, and the predominance of mycelium of A/Tox$^+$ at the crown are consistent with the assumption that A/Tox$^+$ is more likely to cause the most damaging stem cankers than B/Tox^0 *L. maculans*. Identification as A/Tox$^+$ or B/Tox^0 by cultural characteristics differed only slightly (2·3%) from identification by molecular techniques.

Keywords: blackleg, ISSR, ITS, *Phoma lingam*, rapeseed, systemic growth

Introduction

Phoma stem canker (blackleg), caused by *Leptosphaeria maculans*, is an important disease of oilseed rape (canola, rapeseed, *Brassica napus*) in Europe, including France and England (West *et al.*, 2001a). In these countries, epidemics on winter oilseed rape are initiated in the autumn (October–December), primarily by airborne ascospores produced on stem debris of crops from the previous season (West *et al.*, 2001a). The ascospores infect leaves to produce lesions (phoma leaf spot) from which the pathogen may grow biotrophically down the petiole to reach the stem (Hammond & Lewis, 1986; Hammond & Lewis, 1987; Hammond *et al.*, 1985). Infections on leaves early in the season are associated with the most damaging stem cankers at the crown (stem base), which are observed from April onwards, whereas later infections are thought to produce lesions higher up the stem (Zhou *et al.*, 1999; Sun *et al.*, 2000).

The pathogen *L. maculans*, which causes phoma stem canker on oilseed rape, is a complex that comprises at least two groups which have been described using a number of terms, including Tox$^+$ and Tox^0 (Rouxel *et al.*, 1994); and A-group and B-group (Johnson & Lewis, 1994; Williams & Fitt, 1999). Recently, isolates from these groups have been assigned to different species, *L. maculans* and *L. biglobosa*, respectively (Shoemaker & Brun, 2001). However, the B-group is highly polymorphic and probably includes more than one species (Rouxel *et al.*, 1994). In addition, the description of *L. biglobosa* is based on a few isolates from *B. juncea* (Somda *et al.*, 1996), so it is not yet clear whether the description of *L. biglobosa* corresponds to the B/Tox^0 subgroup NA1 (Rouxel *et al.*, 1994), which is the only B/Tox^0 subgroup isolated from oilseed rape in Europe to date (Gall *et al.*,

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1995; Balesdent *et al.*, 1998; Jedryczka *et al.*, 1999b). Therefore the two species of *L. maculans* are described as A/Tox$^+$ and B/Tox^0 in this paper.

Numerous methods to differentiate isolates of the two groups have been developed in recent years: B/Tox^0 isolates produce a water-soluble pigment in some agar media, whereas A/Tox$^+$ isolates do not (Williams & Fitt, 1999). One of the most convenient molecular methods for differentiating the groups is to use ribosomal DNA internal transcribed spacer region (ITS) size polymorphism following direct polymerase chain reaction (PCR) on conidia exuded from cirri on lesions (Balesdent *et al.*, 1998). Differences in the appearance of leaf symptoms caused by the two species can often be large, but are not always clear (Johnson & Lewis, 1994; Ansan-Melayah *et al.*, 1997; Brun *et al.*, 1997; Thürwächter *et al.*, 1999; West *et al.*, 1999). In field experiments in France, Ansan-Melayah *et al.* (1997) associated 'typical' large, fawn-coloured leaf lesions with many pycnidia with A/Tox$^+$ isolates, and 'atypical' small, dark leaf spots surrounded by a yellow halo bearing few small pycnidia with B/Tox^0 isolates. Similar observations were made in field and controlled environment experiments in England, but occasionally A/Tox$^+$ isolates were obtained from atypical lesions (Toscano-Underwood *et al.*, 2001). As A/Tox$^+$ is considered more damaging than B/Tox^0 *L. maculans* on winter oilseed rape in Europe (Williams & Fitt, 1999; West *et al.*, 2001a), it is important to know the relative frequencies of the two species on leaves and stems of crops, as a basis for devising appropriate strategies for control of phoma stem canker.

In two field experiments (cv. Cobra) in Norfolk, England in 1989/90, the proportions of plants with lesions attributed to A/Tox$^+$ and B/Tox^0 *L. maculans* increased between November and March (Johnson & Lewis, 1994), but the numbers of leaf spots of the different types were not reported. *In vitro* experiments have demonstrated that both the A/Tox$^+$ and B/Tox^0 *L. maculans* are able to move from lesions on leaves down the petiole towards the crown (Hammond *et al.*, 1985; Hammond & Lewis, 1987). On the stem, isolate Lm1 (A/Tox$^+$, IMI 254845) caused cortical lesions and penetrated to the pith, while isolate Lm3 (IMI 254848), obtained from *Thlaspi arvense* in Canada (Johnson & Lewis, 1990) and therefore probably in a different subgroup to European B/Tox^0 isolates (Balesdent *et al.*, 1998), was confined to the cortex (Hammond & Lewis, 1987). Similar results were obtained *in vitro* with three English B/Tox^0 isolates (Johnson & Lewis, 1994). However, in the Norfolk field experiments, B/Tox^0 isolates were predominantly found in the pith, in samples taken before and after harvest in 1990 (Johnson & Lewis, 1994). There are indications that isolates from crown cankers at the end of the season may be predominantly A/Tox$^+$ (Johnson & Lewis, 1994; Thürwächter *et al.*, 1999; Toscano-Underwood *et al.*, 2001) but little is known about the composition of infections on upper stems. On the basis of extensive sampling, verified by PCR-based identification, this paper reports the results of comparable experiments to compare the colonization by A/Tox$^+$ and B/Tox^0 *L. maculans* of winter oilseed rape root, crown and stem tissues during the growing season and after harvest, in France and England.

Materials and methods

Field experiments in France and sampling procedures

Oilseed rape, cv. Capitol, was sown in adjacent field plots, 72 m × 72 m, at INRA, Versailles, on 4 September 1998 and 2 September 1999, after winter wheat. No fungicide sprays were applied to the plots in either season. Two groups of plants were tagged in each plot for sampling to examine the distribution of lesions of A/Tox$^+$ and B/Tox^0 *L. maculans* during November, January and March (the January sample was omitted in 1999/2000). One group comprised 12 plants located at random points within a radius of 2·5 m from the centre of the plot; the other comprised 12 plants located at defined points on a grid over the whole plot. On each plant, samples were taken from each phoma leaf spot visible on green or senescent leaves. Sampling was largely nondestructive, so that leaves sampled remained attached to the plant. One small piece (*c.* 4 × 4 mm) was excised from each lesion (including some pycnidia and the lesion margin with adjacent healthy tissue) and placed individually in sterile Eppendorf tubes. This technique left most of the lesion intact and allowed the pathogen to continue to develop in each leaf after sampling. The lesion samples were placed (unsterilized) in a humid chamber (50 mm diameter Petri dish with a layer of filter paper soaked in sterile water) to induce the pycnidia to exude cirri of conidia. Three days later, one cirrus per sample was collected with a sterile toothpick and transferred onto 20% (v/v) V8 juice agar plus antibiotics (V8 + A; antibiotics were streptomycin at 200 μg mL^{-1} plus ampicillin at 100 μg mL^{-1}; the V8 agar was modified for fungal growth by addition of 3 g L^{-1} $CaCO_3$).

Isolations of mycelium of A/Tox$^+$ and B/Tox^0 *L. maculans* were made destructively from crowns and stems of the 24 tagged plants, at the end of the growing season in June 1999, and from 12 plants in May 2000 and 12 plants in June 2000. Small pieces (*c.* 1·5 × 1·5 × 1·5 mm) were excised from tissues in different positions on each plant sampled (upper stem 100–300 mm above ground level, lower stem 10–100 mm above ground, crown from 5 mm below to 5 mm above ground, tap root from 5 mm to 70 mm below ground) under a stereo-microscope, and directly placed on V8 + A. At the crown, pieces were taken from the cortex/wood and pith tissues; this was not done for lesions higher up the stem which had generally not penetrated to the pith. After initial growth of colonies, any mycelium thought to be *L. maculans* was subcultured onto V8 agar.

Field experiments in England and sampling procedures

Changes in the distribution of A/Tox$^+$ and B/Tox^0 *L. maculans* infections on winter oilseed rape leaves in autumn/winter were investigated in unsprayed plots from field experiments at Rothamsted (south-east England) in 1999/

2000 and 2000/01. These field experiments were sown on 27 August 1999 (cvs Capitol and Lipton) and 23 August 2000 (cv. Apex). In 1999/2000, samples to study the distribution of A/Tox⁺ and B/Tox⁰ infections on leaves were taken from four untreated plots (15 × 3 m) in December, February, March and April. On each occasion, five plants were taken at random from each plot. In 2000/01, samples (10 plants per plot) were taken in October, November, December, January, February, March and April from three untreated plots (15 × 3 m). In both seasons, lesions on sampled plants were identified as A/Tox⁺ or B/Tox⁰ by visual appearance: large pale lesions with abundant pycnidia were classified as A/Tox⁺, and small dark lesions as B/Tox⁰ (Toscano-Underwood *et al.*, 2001). This classification by lesion type was confirmed by isolation (technique as for stems, below) and colony morphology/pigment production in culture for a subsample of 175 lesions of each type, taken over the course of the two seasons.

The distribution of A/Tox⁺ and B/Tox⁰ *L. maculans* mycelium in the stems of winter oilseed rape was investigated in the 1999/2000 and 2000/01 experiments, and in July 1999 (from similar untreated plots of cvs Capitol and Lipton grown during the 1998/99 season). In July 1999, samples were taken from visible crown cankers and stem lesions on 25 plants of each cultivar sampled from experimental plots. In both 2000 and 2001, isolations were made from crown cankers (associated with rosette leaf scars, i.e. < 50 mm above ground level); from lesions on the upper stem (100–300 mm above ground level); and from symptomless stems at 100 mm above ground level from 20 to 25 plants in April and May, and from 40 to 45 plants in June and July. A piece (3 × 3 × 3 mm) was taken from each crown canker or stem lesion, surface sterilized in NaOCl (*c.* 1% available chlorine) for 2 min, placed in a Petri dish containing 1% distilled water agar (DWA) and incubated in darkness at 20°C. Pieces were also taken from cortex/wood and pith tissues at the stem base. After 5 days, mycelium produced on the DWA was transferred aseptically to Petri dishes containing 4% w/v potato dextrose agar (PDA) and incubated at 20°C for 10 days.

Experiments with stem debris in England

The distribution of A/Tox⁺ and B/Tox⁰ *L. maculans* on oilseed rape stems after harvest was examined by isolation of ascospores that were ejected from pseudothecia at different positions on the root, crown and stem, in both 1999/2000 and 2000/01 (i.e. on debris from 1998/99 and 1999/2000 seasons). Debris of winter oilseed rape (including the tap root, crown and stem up to 300 mm above ground) was collected from untreated plots of cvs Lipton and Capitol after harvest in July 1999 and 2000. The debris was incubated in natural conditions in freely draining plastic trays (0·45 × 0·75 m) lined with hessian sacking material. In early October 1999, mature pseudothecia (containing fully developed ascospores) were observed by microscopic examination of pseudothecia excised from the surface of the debris. On 21 October 1999, small pieces (*c.* 10 × 5 mm) bearing pseudothecia, were excised from the tap root, crown (–5 to 50 mm from soil surface level) or upper stem (100–300 mm height) of each of 10 lengths of stubble. Each piece was attached inside the lid of a Petri dish using petroleum jelly (Vaseline) and sprayed with distilled water (to induce ascospore release) i.e. a total of 30 Petri dishes. The lids were placed above DWA plates, incubated at room temperature (*c.* 20°C), and dishes were monitored every hour for ascospore release under a stereo-microscope. When ascospores had been released (after 1–3 h), an ascospore was taken from three different areas on each of the DWA plates and transferred to Petri dishes containing PDA, which were incubated at 20°C for 10 days. Sampling was repeated in December, February and March, from similar pieces, taken from each of 10 lengths of crown or upper stem debris (20 Petri dishes). The experiment was repeated in 2000/01, with samples taken in early October, mid-October, November, December, January, February and March from debris collected at harvest in July 2000. The same methods were used, except that four pieces were taken from each length of crown or upper stem used in each Petri dish, and 10 ascospores per Petri dish (instead of three) were assessed.

Experiments with potted plants in England

In both 1999/2000 and 2000/01, the stem debris (cvs Capitol and Lipton, harvested in July) used to study release of A/Tox⁺ and B/Tox⁰ ascospores was also used as a source of inoculum for an experiment with potted plants. In 1999/2000, 32 winter oilseed rape plants (cv. Lipton) with two leaves fully expanded, each growing in 130 mm plastic pots, were set in a bed of sand outdoors at Rothamsted on 6 September 1999. Debris of oilseed rape plants (*c.* 300 stems of cvs Capitol or Lipton), collected at harvest in July 1999 and incubated in natural conditions, were placed on the surface of the sand 1 m from the potted plants to supplement naturally occurring airborne ascospores of *L. maculans*. The number of leaves with lesions attributed to A/Tox⁺ or B/Tox⁰ *L. maculans* were assessed by visual appearance (Brun *et al.*, 1997; Toscano-Underwood *et al.*, 2001) each month from October to February. In 2000/01, oilseed rape (cv. Apex) was sown on 1 September 2000. A total of 25 plants, each with five leaves fully expanded and growing in a 200 mm diameter pot, were transferred to a bed of sand outdoors at Rothamsted on 20 September. Stem debris in 10 trays (0·45 × 0·75 m), each containing *c.* 50 stems, was placed at a distance of *c.* 50 m from the plants. The numbers of leaves with lesions attributed as A/Tox⁺ or B/Tox⁰ by visual appearance were assessed monthly.

Statistical analyses

To examine the effects on the ratios of A/Tox⁺ to B/Tox⁰ of country, month of sampling, growing season, and position on stem, a generalized linear model with an ln-link function was used to analyse the data, assuming the data were normally distributed. The higher order interactions

between country, month, season and stem position were generally incorporated into the residual error. The statistical software GENSTAT was used for these analyses (Payne *et al.*, 1993).

Identification of A/Tox$^+$ and B/Tox^0 *L. maculans* isolates

All English isolates (n = 872) of *L. maculans* taken from leaf or stem tissues were classified as A/Tox$^+$ or B/Tox^0 by an assessment of colony morphology and absence/presence of a yellow pigment in the PDA medium (Williams & Fitt, 1999). A visual classification as A/Tox$^+$ or B/Tox^0 on the basis of colony morphology (as no pigment was visible in the V-8 agar) was made on 621 out of the 945 French isolates to confirm the reliability of the morphological identification done in England. All French isolates (n = 945) were identified by PCR on the basis of size polymorphism of the ITS1-5·8S-ITS2 region using the PN3-PN10 primer pair, with fresh conidia as the PCR template (Balesdent *et al.*, 1998). A total of 37 English isolates were also identified using this PCR method, for comparison with the morphological classification.

Inter-simple sequence repeat (ISSR; Zietkiewicz *et al.*, 1994) primers were also used to produce discriminating fingerprints for 172 out of the 945 French isolates. ISSR primers, used alone or in combination, allow the amplification of sequences flanked by two microsatellites, without any knowledge of the target sequences. The three ISSR primers used were: $(TG)_8$: 5′-VHVHTGTGTGTGTGT-GTGTG-3′; $(T3G3)_3$: 5′-VHHGTTTGGGTTTGGGTT-TGG-3′; and $(ATG)_5$: 5′-BVHATGATGATGATGATG-3′. The ISSR-PCR was done with frozen suspensions of conidia (*c.* 10^8 spores mL^{-1}) used as the amplification template. Amplifications were done in 15 μL final volumes, containing 3 μL conidial suspension, 200 μM each dNTP, 2 μM primer (Invitrogen SARL, Cergy Pontoise, France), 1 U *Taq* polymerase (Appligene, Pleasanton, CA, USA) in 10 mM Tris–HCl pH 9·0, 50 mM KCl, 1·5 mM $MgCl_2$, 0·1% Triton X-100, and 0·2 mg mL^{-1} bovine serum albumin. PCR was done in a GeneAmp 9600 Perkin Elmer thermal cycler (Applied Biosystems, Foster City, CA, USA). Initial denaturation was for 4 min at 95°C to lyse the conidia, followed by 37 cycles of 30 s at 95°C, 45 s at either 64°C [$(TG)_8$ primer] or 49°C [$(T3G3)_3$ and $(ATG)_5$ primers], 2 min 30 s at 72°C, and a final 7 min extension step at 72°C. PCR products underwent electrophoresis on 1·4–1·8% agarose gels, and were detected by ethidium bromide staining. Control amplifications were made on 74 isolates out of the 172 analysed by ISSR fingerprinting, using mycelial fungal DNA as a PCR template (Balesdent *et al.*, 1998) to check the reliability of the amplification profiles with conidia used as a template.

Results

Characterizing isolates

Colonies of French isolates on V8 agar classified as B/Tox^0 had white fluffy mycelium and pycnidia produced mainly in central, older parts of the colony which, with age, often developed a yellow pigmentation of the mycelium. Under a stereo-microscope the hyphae produced by these colonies were straight with few short branches. These colonies usually had yellow or brown drops of liquid on the aerial part of the mycelium. Colonies classified as A/Tox$^+$ had white mycelium, short aerial hyphae, a relatively fast radial growth rate, relatively few pycnidia, and sometimes patches of beige-brown mycelium. Under a stereo-microscope the hyphae produced by these colonies showed much more branching than B/Tox^0 hyphae, and numerous mycelial loops or spherical aggregates of mycelium were visible. The relationship between this morphological classification and that obtained by PCR ITS amplification was generally good, as only 11 isolates (six A/Tox$^+$ and five B/Tox^0) out of 621 isolates were assigned to the wrong *L. maculans* species by morphology. In addition, 20 isolates of uncertain morphology were shown by PCR to comprise 13 A/Tox$^+$ and seven B/Tox^0 isolates, and three isolates identified as B/Tox^0 by colony morphology were shown by PCR not to be *L. maculans*. Therefore only, 2·3% of isolates were wrongly identified, and a further 3·2% could not be identified by colony morphology alone. ISSR profiling was also highly consistent with ITS size polymorphism, as only three out of 172 isolates (1·7%) were identified as B/Tox^0 by ITS amplification, but as A/Tox$^+$ by ISSR profiling. In one case the difference was linked with ISSR profiles, indicating the presence of both species (Fig. 1). This may suggest that the sample was not purified enough, which cannot be judged solely on colony morphology, nor on ITS amplification using fresh conidia from one single pycnidium as a PCR template. In contrast, ISSR amplification from a suspension of conidia, or DNA extracted from the whole colony, is likely to identify such mixtures whenever they occur.

Colonies of English isolates classified as B/Tox^0 produced a yellow pigment in the colourless PDA and had white, fluffy mycelium, occasionally with yellow or brown drops of liquid. Colonies classified as A/Tox$^+$ did not produce an intense pigment in the medium and had less aerial mycelium and a greyer colour. For the 37 English isolates (31 A/Tox$^+$, six B/Tox^0) analysed by molecular methods in France, the classification by ITS PCR gave the same result as the morphological classification.

Proportions of A/Tox$^+$ and B/Tox^0 infections on leaves in France and England

Generalized linear model analyses suggested that there were differences in the ratio of B/Tox^0 to A/Tox$^+$ infections on leaves between months of sampling (P = 0·05; 7 d.f.) but not between growing seasons or between France and England. In France, in both 1998/99 and 1999/2000, no or very few B/Tox^0 infections were detected on leaves in November, although there were many lesions from which A/Tox$^+$ *L. maculans* was isolated (Table 1a). In November 1999, most isolates from infected senescing (57 out of 61) and green (62 out of 63) leaves were A/Tox$^+$. The proportion of B/Tox^0 infections increased by January in 1999 to

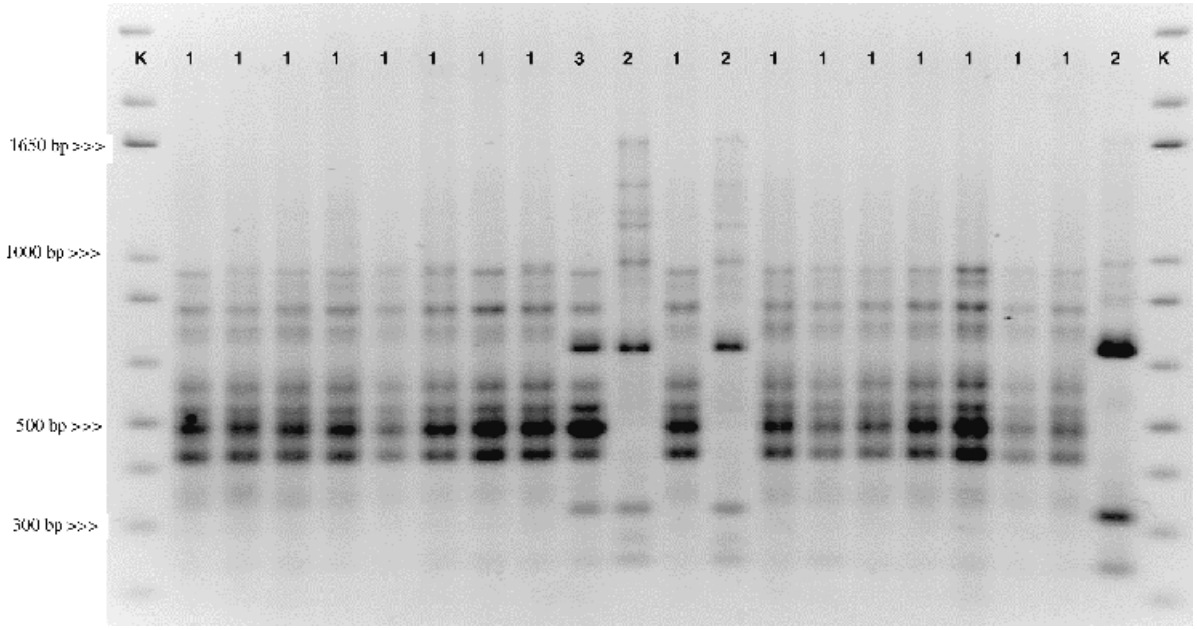

Figure 1 Profiles generated by ISSR fingerprinting on extracted DNA of *Leptosphaeria maculans*. The primer used was $(T3G3)_3$. 1, isolates identified as A/Tox⁺ on the basis of morphological traits and ITS size; 2, isolates identified as B/Tox⁰ on the basis of morphological traits and ITS size; 3, one isolate identified as B/Tox⁰ on the basis of morphological traits and ITS size, and showing a profile including major bands from both A/Tox⁺ and B/Tox⁰ isolates; K, 1 kb-plus size standard. An inverted photograph of the ethidium bromide agarose gel migration is presented.

Table 1 Changes in occurrence of A/Tox⁺ and B/Tox⁰ *Leptosphaeria maculans* infections on leaves of winter oilseed rape during autumn/winter at (a) Versailles, France (1998/99, 1999/2000) and (b) Rothamsted, UK (1999/2000, 2000/01)

	Number of each group				Total (%) of each group	
Sample date	**A/Tox⁺**	**B/Tox⁰**	**A/Tox⁺**	**B/Tox⁰**	**A/Tox⁺**	**B/Tox⁰**
(a) Versailles[a]	1998/99		1999/2000			
November	47	0	119	5	97	3
January	14	15	–[c]	–	48	52
March	42	8	30	40	60	40
(b) Rothamsted[b]	1999/2000		2000/01			
October	–	–	41	14	75	25
November	–	–	292	89	76	24
December	146	65	329	145	69	31
January	–	–	503	155	76	24
February	251	124	171	90	66	34
March	235	67	255	77	77	23
April	236	32	129	62	80	20

[a]Numbers obtained by isolation from pieces sampled from lesions on leaves of 24 winter oilseed plants (cv. Capitol); the same tagged plants were analysed at each sampling date. All isolates classified by ITS size polymorphism.
[b]Numbers obtained by classifying appearance of lesions on leaves of plants sampled from unsprayed plots of winter oilseed rape crop (1999/2000, cv. Lipton, 20 plants); (2000/01, cv. Apex, 30 plants) and confirmed by isolation and colony morphology/pigment production in culture for 125 of the large pale lesions and 175 of the small, dark lesions (on a subsample of the plants taken on each date in both seasons).
[c]No sample taken.

a level approximately equal to that of A/Tox⁺, then decreased by March. In 1999/2000 there was an increase in the proportion of B/Tox⁰ infections on leaves between November and March (to 57%) (Table 1a). In total, 79% of isolates taken from leaves in France were identified as A/Tox⁺. In England, the overall occurrence of the two groups on leaves of winter oilseed rape was similar to that in France, with 75% of lesions assessed in autumn/winter

Table 2 Changes in occurrence of A/Tox$^+$ and B/Tox^0 *Leptosphaeria maculans* mycelium in stem tissues of winter oilseed rape sampled during spring/summer at (a) Versailles, France (1999, 2000) and (b) Rothamsted, UK (2000, 2001)

Sample date	Tissue	Number of isolates of each group				Total (%) of each group	
		A/Tox$^+$	B/Tox^0	A/Tox$^+$	B/Tox^0	A/Tox$^+$	B/Tox^0
(a) Versailles[a]		1999		2000			
May	Crown[c]	–[d]	–	92	7	93	7
	Stem	–	–	3	8	27	73
June	Crown	316	36	99	3	91	9
	Stem	30	21	7	3	61	39
(b) Rothamsted[b]		2000		2001			
April	Crown	4	2	11	3	75	25
	Stem	0	0	0	0	0	0
May	Crown	6	7	21	3	73	27
	Stem	6	6	2	1	53	47
June	Crown	35	13	22	3	78	22
	Stem	16	15	5	13	43	57
July[e]	Crown	38	7	46	17	79	21
	Stem	17	8	10	30	39	61

[a]Numbers obtained by isolation from pieces of stem from winter oilseed rape cv. Capitol; from 24 plants (June 1999) or 12 plants (May 2000 and June 2000); isolates classified by ITS size polymorphism.
[b]Numbers obtained by isolation from pieces of stem from samples of 20–50 plants (2000, cv. Lipton; 2001, cv. Apex).
[c]At Versailles, samples were taken from crown and tap root (10 mm above ground to 70 mm below ground) or stems (> 10 mm above ground level); at Rothamsted, England, samples were from stem bases and tap root (50 mm above ground to 70 mm below ground) or upper stems (100–300 mm above ground level).
[d]No sample taken.
[e]At Rothamsted, samples were also taken in July 1999 (cvs Lipton and Capitol, 25 plants each) from the stem base (32 A/Tox$^+$, seven B/Tox^0) and upper stem (19 ATox$^+$, 34 B/Tox^0); these figures are incorporated in the July total percentages.

of 1999/2000 and 2000/01 classified as A/Tox$^+$ (Table 1b). From the subsamples of lesions observed at each sampling date, a total of 171 A/Tox$^+$ and four B/Tox^0 colonies were isolated from 175 large pale lesions; 166 B/Tox^0 and nine A/Tox$^+$ colonies were isolated from 175 small dark lesions.

Distribution of A/Tox$^+$ and B/Tox^0 mycelium within crown and stem tissues

There were differences in the ratio of A/Tox$^+$ to B/Tox^0 isolates obtained between crowns and upper stems ($P < 0{\cdot}01$, 13 d.f.) but not between growing seasons, months of isolation or countries (Table 2). The percentage of isolates identified as A/Tox$^+$ changed very little between May (86% out of 110 isolates) and June (88% out of 515 isolates). Generally, A/Tox$^+$ *L. maculans* was predominant in both root and crown regions (89 and 92%, respectively; Fig. 2). There was an increase in the proportion of B/Tox^0 isolates taken from the lower and upper stem regions, although there were still more A/Tox$^+$ than B/Tox^0 isolates (B/Tox^0 comprised 48 and 35%, respectively; Fig. 2). There were considerably more A/Tox$^+$ than B/Tox^0 isolates obtained from crowns in both countries, but the proportion of B/Tox^0 isolates was smaller in France (10% in 1999 and 5% in 2000; Table 2a) than England (18% in 1999, 25% in 2000, 12% in 2001; Table 2b). In both

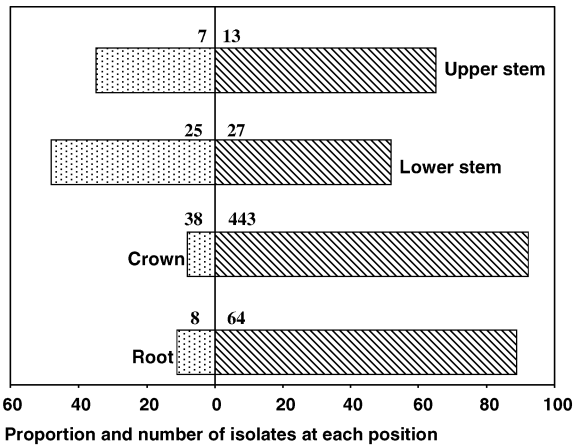

Figure 2 Proportions (numbers) of B/Tox^0 (dotted bars) and A/Tox$^+$ (hatched bars) *Leptosphaeria maculans* isolates obtained from different stem tissues in Versailles, France in 1999 and 2000.

France and England the proportion of B/Tox^0 isolates obtained from lesions or from symptomless tissue higher up the stems was much greater (40–70%) than at the stem base, although fewer isolates were obtained. Examination of the distribution of *L. maculans* within the tissues of the crown showed that A/Tox$^+$ *L. maculans* was present throughout the cortex, wood and pith, while B/Tox^0 was rarely found in the pith and tended to be more

Table 3 Occurrence of A/Tox⁺ and B/Tox⁰ *Leptosphaeria maculans* mycelium in the cortex/wood or pith tissues of crowns[a] of winter oilseed rape during spring/summer in (a) Versailles, France (1999, 2000) and (b) Rothamsted, UK (2000, 2001)

	Number of each group				Total (%)	
Tissue	**A/Tox⁺**	**B/Tox⁰**	**A/Tox⁺**	**B/Tox⁰**	**A/Tox⁺**	**B/Tox⁰**
(a) Versailles[b]	1999		2000			
Cortex/wood	149	28	110	9	88	12
Pith	114	2	71	3	97	3
(b) Rothamsted	2000		2001			
Cortex/wood	70	27	38	18	71	29
Pith	13	2	30	2	91	9

[a]Lesions on stems above the crown had generally not penetrated to the pith and were not included.
[b]Samples taken in May and June in Versailles and in April to July in Rothamsted (see Table 2).

frequently isolated from the cortex ($P < 0{\cdot}01$, 4 d.f.; Table 3).

Positional and temporal changes in proportions of A/Tox⁺ and B/Tox⁰ ascospores ejected from oilseed rape debris and occurrence of leaf lesions on potted plants in England

In October 1999, 89 and 100%, respectively, of viable (germinating) ascospores from tap-root and crown regions produced A/Tox⁺ colonies. By contrast, only 44% of ascospores from the stem (>50 mm above ground level) produced A/Tox⁺ colonies. Due to the similarity in ascospore production between the tap-root and crown regions, pieces from these regions were combined for later assessments of ascospore release (referred to as 'crown' material). In general, there were differences in the ratio of A/Tox⁺ to B/Tox⁰ isolates obtained from ascospores between crowns and upper stems ($P < 0{\cdot}001$, 13 d.f.); between seasons ($P < 0{\cdot}001$); and between months of isolation ($P < 0{\cdot}001$) (Table 4). In total, 93% of viable ascospores from the crown were A/Tox⁺, while only 36% from the upper stem were A/Tox⁺. In 1999/2000 and 2000/01, the differences between the crown and upper stems (debris from the previous season) in the proportions of A/Tox⁺ and B/Tox⁰ *L. maculans* ascospores released were greater (Table 4) than the differences between them in the proportions of A/Tox⁺ and B/Tox⁰ *L. maculans* obtained by isolating from mycelium colonizing these tissues prior to harvest (Table 2). The proportion of B/Tox⁰ ascospores released from crown pieces was often <10% (and sometimes 0%), and there was no evidence that this proportion changed with time during the autumn/winter period (October to March). However, the proportion of B/Tox⁰ ascospores released from stem pieces increased with time from October/November onwards in both seasons. A linear regression of the percentage of B/Tox⁰ ascospores released from the stem against time (slope, +0·4% per day) accounted for 86% of the variance. In most cases, all the cultures obtained from ascospores

Table 4 Changes during autumn/winter in numbers of isolates of A/Tox⁺ and B/Tox⁰ *Leptosphaeria maculans* obtained from single ascospores subsampled from ascospores ejected from pseudothecia on the crown or stem[a] of untreated crops (cvs Capitol and Lipton) harvested in July 1999 and 2000 at Rothamsted and subsequently sampled during exposure under natural conditions

		Number of each group[b]					
		1999/2000		2000/01		Total (%)	
Sample date	**Tissue**	**A/Tox⁺**	**B/Tox⁰**	**A/Tox⁺**	**B/Tox⁰**	**A/Tox⁺**	**B/Tox⁰**
Early October	Crown	–[c]	–	40	0	100	0
	Stem	–	–	40	10	80	20
Mid-October	Crown	57	3	94	6	94	6
	Stem	12	15	60	40	57	43
November	Crown	–	–	100	0	100	0
	Stem	–	–	46	54	46	54
December	Crown	23	7	83	17	87	13
	Stem	15	12	40	60	43	57
January	Crown	–	–	93	7	93	7
	Stem	–	–	20	80	20	80
February	Crown	48	0	100	0	100	0
	Stem	16	32	3	97	13	87
March	Crown	54	4	89	11	91	9
	Stem	20	40	19	81	24	76

[a]Crown (stem base and tap root) < 50 mm above ground level; stem 100–300 mm above ground level. Ten pieces of each stem tissue were sampled on each occasion and ascospores ejected (if mature pseudothecia present). Isolates were produced from three (1999/2000) or 10 (2000/01) ascospores per stem piece.
[b]Single-ascospore isolates from a single piece of crown or stem were generally all A/Tox⁺ or all B/Tox⁰ (1999/2000; 60 crown pieces, 54 all A/Tox⁺, three B/Tox⁰, three mixed; 50 stem pieces, 18 A/Tox⁺, 28 B/Tox⁰, four mixed); (2000/01; 62 crown pieces, 55 all A/Tox⁺, 0 B/Tox⁰, seven mixed; 63 stem pieces, 21A/Tox⁺, 39 B/Tox⁰, three mixed). Some pieces sampled did not produce ascospores.
[c]No sample taken.

Table 5 Changes in the occurrence of A/Tox$^+$ and B/Tox^0 *Leptosphaeria maculans* infections on leaves of winter oilseed rape plants in pot experiments during autumn/winter in 1999/2000 and 2000/01 at Rothamsted

Assessment month	Number of leaves with one or more lesions of each group[a]					
	1999/2000		2000/01		Total (%)	
	A/Tox$^+$	B/Tox^0	A/Tox$^+$	B/Tox^0	A/Tox$^+$	B/Tox^0
October	49	7	77	13	86	14
November	27	6	37	11	79	21
December	42	16	32	9	75	25
January	28	25	20	0	66	34
February	30	14	15	8	67	33

[a]On 32 potted plants, cv. Lipton, in 1999/2000 and 25 potted plants, cv. Apex, in 2000/01; numbers obtained by classifying appearance of lesions on leaves.

released from a single crown or stem piece (10 × 5 mm) belonged to the same group (Table 4). Thus, of 122 crown pieces which released ascospores, 89% produced only A/Tox$^+$, 3% produced only B/Tox^0, and 8% produced mixtures of A/Tox$^+$ and B/Tox^0 isolates. Of 113 stem pieces producing ascospores, 35% produced only A/Tox$^+$, 59% only B/Tox^0, and 6% mixtures of A/Tox$^+$ and B/Tox^0 isolates.

In the autumn of 1999, the first spots were observed on leaves of potted plants on 7 October (Table 5). Most of the leaves had lesions attributed to A/Tox$^+$ *L. maculans*. However, the proportion of leaves with B/Tox^0 lesions gradually increased between October and January. In the autumn of 2000, the first phoma leaf spots were observed on the potted plants on 12 October, and were predominantly A/Tox$^+$ lesions. A/Tox$^+$ lesions remained predominant throughout the autumn and winter, with a maximum proportion of 35% leaves with B/Tox^0 lesions in February.

Discussion

This investigation of the temporal and positional distribution of *L. maculans* on winter oilseed rape in France and England suggests that there are epidemiological differences between A/Tox$^+$ and B/Tox^0 *L. maculans* (Fig. 3). The good agreement between the classification of isolates as A/Tox$^+$ or B/Tox^0 by colony morphology on agar media, PCR on conidia from cirri on lesions, and ISSR profiles suggests that all three methods can provide comparable results. The small error in the morphological classification of isolates on agar as compared with molecular identification is therefore acceptable for assessing large numbers of isolates from different populations for these comparative epidemiological studies. Furthermore, the comparisons between methods suggest that it is often sufficient to classify leaf lesions by their appearance, as concluded from results of experiments in France (Ansan-Melayah *et al.*, 1997; Brun *et al.*, 1997) and England (Johnson & Lewis, 1994; Toscano-Underwood *et al.*, 2001). However, it may sometimes be difficult accurately to classify small, dark leaf lesions as B/Tox^0, especially in the presence of dark leaf spot (*Alternaria brassicae*), light leaf spot (*Pyrenopeziza brassicae*), or hypersensitive resistance reactions to any agent, including avirulent A/Tox$^+$ isolates for which gene-for-gene interactions with *B. napus* cultivars, leading to hypersensitive resistance, have been described (Ansan-Melayah *et al.*, 1998).

The experiments in France suggest that the onset of leaf spotting differs between A/Tox$^+$ and B/Tox^0 *L. maculans*. Most early leaf spots (October/November) were caused by A/Tox$^+$ *L. maculans*, and the proportion of B/Tox^0 leaf spots increased with time during autumn/winter. Overall, there were similar proportions of A/Tox$^+$ to B/Tox^0 infections on leaves in France and England (79 and 75% A/Tox$^+$, respectively), although the ratio of leaf lesions attributed to the two species during autumn and winter did not vary as much in England as in France. However, the French data are supported by other experiments in England (Johnson & Lewis, 1994) and the studies on potted plants at Rothamsted, which showed that early leaf infections were attributed predominantly to A/Tox$^+$ *L. maculans*. As there are similar periods from ascospore germination (Huang *et al.*, 2001) and infection until the appearance of leaf spot symptoms (Biddulph *et al.*, 1999; Toscano-Underwood *et al.*, 2001) for the two species, differences between them in number of lesions in autumn were not due to differences in the incubation period. The predominance of A/Tox$^+$ in early leaf infections in France and England may contribute to the difference in populations between western Europe, where the A/Tox$^+$ predominates and autumn-sown oilseed rape is grown, and central/eastern Europe, where the B/Tox^0 is more common and the severe winter may remove the initial leaves of autumn-sown oilseed rape, cultural practices are different and spring-sown rape has been widely grown in the past (Jedryczka *et al.*, 1999a; Volke, 1999).

Since the thermal time (degree days) between appearance of leaf spots in autumn and appearance of cankers in spring is consistent for the same cultivar in different seasons (Sun *et al.*, 2000; Sun *et al.*, 2001), and cankers produced from early infections are most damaging (Zhou *et al.*, 1999; Sun *et al.*, 2001), the greater effect of A/Tox$^+$ *L. maculans* on yield (West *et al.*, 2001a) could be explained simply by its tendency for earlier infection. The difference in onset of leaf infections attributed to the two species can explain why, in both France and England, B/Tox^0 was found more frequently in the upper stem than in the root/crown region, while A/Tox$^+$ was predominant in the root/crown. Similarly, in Germany over 80% of isolates from stem bases (precise locations not indicated) were A/Tox$^+$ (Thürwächter *et al.*, 1999). Stem extension generally starts in February in England (Sun *et al.*, 2001) and uppermost leaves are raised above the positions of the rosette leaves. There were fewer isolations made from the upper stem than other areas, suggesting that fewer leaf infections occurred at this position. This is probably because fewer ascospores are released in winter than in autumn in western Europe (West *et al.*, 2001b). Hence

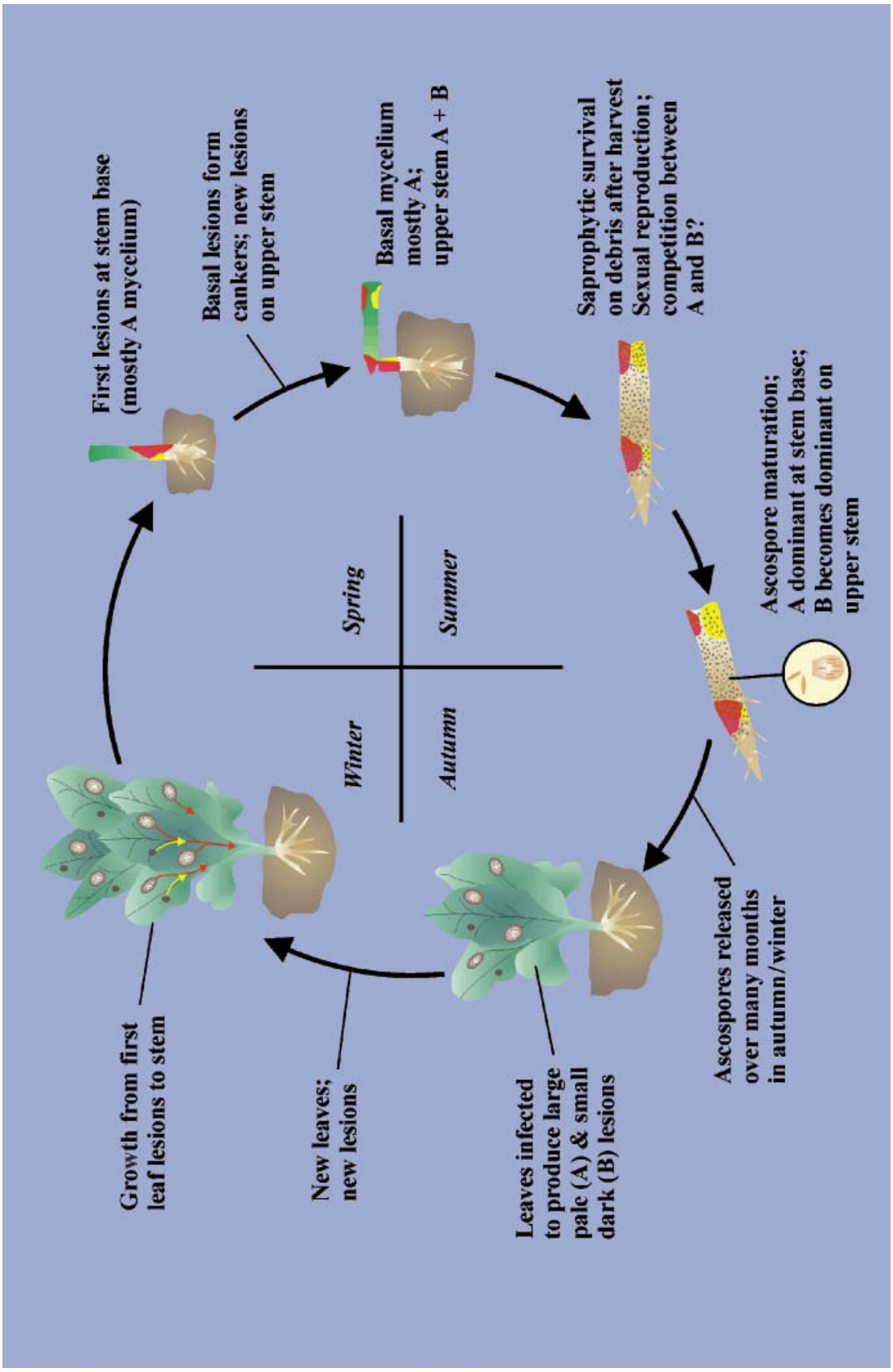

Figure 3 Seasonal changes in A/Tox⁺ and B/Tox⁰ *Leptosphaeria maculans* on winter oilseed rape in France and England, as lesions on leaves in autumn/winter, mycelium in stems in spring/summer, and ascospores produced on stem debris after harvest. Based on Figure 1 of West *et al.* (1999), reproduced with permission.

these experiments indicate that both A/Tox$^+$ and B/Tox^0 *L. maculans* can spread from leaves to colonize all parts of the tap root, crown and stem, but with marked differences in their distribution throughout the plant.

In addition to differences in infection timing, differences in stem colonization between the two species appear to affect the distribution within tissues of the crown, which may affect canker severity. The small number of B/Tox^0 isolates obtained from the pith at the crown in May/June, with a higher proportion of B/Tox^0 isolates in the cortex/wood, confirms evidence from controlled-environment experiments (Hammond & Lewis, 1987; Johnson & Lewis, 1994) that A/Tox$^+$ penetrates living crown tissues more effectively than B/Tox^0 *L. maculans*. The observed predominant colonization of the tap root and crown tissues by A/Tox$^+$ *L. maculans* in France and England (92 and 78% of isolates were A/Tox$^+$) may explain the relatively high severity of epidemics in western Europe, where a widespread, high prevalence of A/Tox$^+$ isolates on winter oilseed rape has been reported compared with central European countries, where B/Tox^0 predominates (Jedryczka *et al.*, 1999a; West *et al.*, 2001a).

The general distribution of the two species at harvest appeared to be sustained over the summer so that ascospores produced on root and crown debris in October yielded predominantly A/Tox$^+$ isolates, in contrast to upper stem debris which yielded more B/Tox^0 isolates. However, the ratio of B/Tox^0 isolates from ascospores released from the upper stem region (which was relatively sparsely colonized compared with the crown) increased during autumn and winter. The linear increase in the proportion of B/Tox^0 isolates from ascospores ejected from upper stem debris between October and March in the Rothamsted experiments in 2000/01 could indicate earlier maturation and release of ascospores by A/Tox$^+$ *L. maculans*; the ratio changing as predominantly A/Tox$^+$ pseudothecia become spent while later-maturing B/Tox^0 pseudothecia begin to release ascospores. Such a change in ascospore production in the upper stem could also indicate a greater saprophytic ability of B/Tox^0 *L. maculans* by increased colonization of substrate. There is some evidence that competition for resources may occur between the two species during the parasitic, saprophytic and reproductive phases. There were differences between the proportions of A/Tox$^+$ and B/Tox^0 isolates taken from mycelium in leaves and crown cankers/stem lesions before harvest, and isolates from ascospores produced on debris after harvest. The proportion of A/Tox$^+$ *L. maculans* was 69% on leaves in December 1999 and 78% on root/crown tissues in June/July 2000 (mycelial isolates), but increased to 96% on crown debris in October 2000 (ascospore isolates). Meanwhile the proportion of the B/Tox^0 infections on leaves, which was 17% in March/April 2000, increased to 41% on upper stem tissues in June/July 2000 (mycelial isolates) and then increased from 33% in October to 97% on upper stem debris in February 2001 (ascospore isolates). Johnson & Lewis (1994) showed that B/Tox^0 only rarely entered the stem *in vitro*, but reported that B/Tox^0 isolates were predominantly obtained from pith tissues of their field samples taken just before and after harvest in 1990. This suggests that B/Tox^0 may be a more effective saprophyte than A/Tox$^+$ *L. maculans*. However, the long-term survival of A/Tox$^+$ may be greater than that of B/Tox^0 *L. maculans* because A/Tox$^+$ isolates were found to predominate in the lignified root/crown tissue, which degrades more slowly than upper stems where B/Tox^0 predominates. Additionally, as old root and crown tissues remain largely in contact with soil, compared with upper parts of the standing debris after harvest, this moister environment may favour earlier formation and maturation of pseudothecia than on upper parts of the stem, which would promote early spore release. Hence the cycle would be completed with early infections of the next crop, predominantly by A/Tox$^+$ *L. maculans*.

This study not only confirms differences in the colonization of winter oilseed rape stems between A/Tox$^+$ and B/Tox^0 *L. maculans*, previously shown by *in vitro* experiments using a relatively small number of isolates (Hammond & Lewis, 1987; Johnson & Lewis, 1994), but also indicates further epidemiological differences in behaviour of the two species during the survival and sexual reproductive phases of their life cycles, with consequences for timing of ascospore release, disease severity, yield loss and even regional pathogen population structures. Furthermore, it suggests that control strategies to minimize losses from phoma stem canker need to be targeted at A/Tox$^+$ *L. maculans* because this species is predominantly responsible for the early leaf spots associated with severe, damaging basal stem cankers.

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