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# Reaction of *Brassica juncea* (Indian mustard) lines to Australian isolates of *Leptosphaeria maculans* under glasshouse and field conditions

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**Abstract**

*Brassica juncea* (Indian mustard) lines from diverse geographical locations around the world and from Australian breeding programs were screened for resistance to the blackleg fungus, *Leptosphaeria maculans*, in both glasshouse and field trials. The five Australian *L. maculans* isolates used in glasshouse trials could be classified into two groups; those that attacked all *B. juncea* lines, and those that attacked none. All these isolates caused lesions on cotyledons of *B. napus* cultivars including Westar, Glacier and Quinta, suggesting that they are in Pathogenicity Group 4 as described by Koch et al. (1991). The two isolates that attacked *B. juncea* also attacked *B. napus* lines to a similar extent, but did not attack the two *B. carinata* lines tested. *Brassica* lines were sown in a blackleg disease nursery at Lake Bolac, Victoria, Australia, and five indicators of blackleg disease were measured (survival rate, disease rating, disease incidence, external and internal lesion length). All 92 *B. juncea* lines developed blackleg symptoms. Although they displayed a high disease incidence in the field, almost all of the *B. juncea* lines were more blackleg-resistant than a *B. napus* cultivar, Dunkeld, which is amongst the most resistant cultivars in commercial production in Australia. Four *B. carinata* lines were more resistant than any of the *B. juncea* lines, suggesting that this species may be a useful source of blackleg resistance in *B. napus* breeding programs.

## Introduction

*Brassica napus* (canola) has become an important crop in Australia as a source of mono-unsaturated edible oil and animal feed, and as a break crop in rotations with cereals and legumes. Canola-quality *B. juncea* (Indian mustard) is being developed to extend *Brassica* production to the lower rainfall areas of Australia as this plant is better adapted to hotter, drier areas than *B. napus*. Blackleg, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not., is the most economically important disease of oilseed Brassicas worldwide and remains a constant threat to this industry in Australia (for review, see Salisbury et al., 1995).

U (1935) demonstrated the relationship between the cultivated *Brassica* species; *B. juncea* (AB) contains genomes from *B. nigra* (B genome) and *B. rapa* (A genome); *B. napus* (AC) has genomes from *B. oleracea* (C genome) and *B. rapa; B. carinata* (BC) has genomes from *B. nigra* and *B. oleracea*. Species with the B genome (*B. nigra, B. carinata* and *B. juncea*) are considered to have better blackleg resistance than species lacking it (for review, see Rimmer and van den Berg, 1992). *B. juncea* was used as a source of resistance in *B. napus* breeding strategies in Australia during the 1980s. However, this resistance appears to be unstable, presumably due to a lack of introgression of the B genome into the *B. napus* genome (Roy, 1984).

*L. maculans* consists of several strains or pathotypes that are morphologically similar but may be different species, as they do not interbreed and are distinct at a molecular level (for review, see Williams, 1992). Two of these pathotypes are termed 'highly virulent' and 'weakly virulent' based on whether or not they can form stem cankers on *B. napus*. Some isolates of the 'weakly virulent' pathotype infect the pith of

*B. juncea*, but do not produce stem cankers (Johnson and Lewis, 1994). Until recently there were no reports of isolates of the 'highly virulent' pathotype that could form stem cankers on *B. juncea*. In 1993 such isolates were found in the major *Brassica* growing areas of Australia (Ballinger and Salisbury, 1996). Genome analysis showed that these isolates belong to the 'highly virulent' pathotype (Chen et al., 1996).

The genetics of resistance in *B. juncea* to *L. maculans* is considered simple. Keri et al. (1997) reported the presence of two genes segregating in $F_2$ and $F_3$ families. Pang and Halloran (1996) described three genes in $F_3$ families derived from a cross between a *B. napus* line with *B. juncea* like resistance bred by Roy (1984), and a blackleg susceptible *B. napus* cultivar. Chevre et al. (1997), using a similar source of *B. juncea* germplasm to that used by Pang and Halloran (1996), described a single gene for resistance to *L. maculans* in the B genome of *B. napus–B. juncea* lines. Whilst the B genome is acknowledged as a good source of blackleg resistance, little is known about the range of resistance genes available in *B. juncea* and other B genome species. The aim of the present research was to identify lines of *B. juncea* from diverse geographical locations with different sources of resistance to Australian isolates of *L. maculans* under glasshouse and field conditions.

## Materials and methods

### Brassica *lines and* L. maculans *isolates*

*B. juncea* lines from more than thirty countries were obtained from A. McIntyre, Australian Temperate Field Crops Collection (ATFCC), Victorian Institute for Dryland Agriculture, Horsham, Australia, as were *B. napus* cultivars. Most lines are referred to by their ATC number in the text and in the Figures; more information about them is available from the authors upon request. Several of the putative *B. juncea* lines were subsequently identified as *B. carinata*, *B. nigra* and *Sinapis arvensis*. Additionally two sets of lines from Australian *B. juncea* breeding programs were used. One set from Dr R. Oram was tested in both the glasshouse and the field and comprised lines which showed blackleg cankering in a field trial at Wagga Wagga, New South Wales in 1990 (389-19Bu, 449-2-5Bu, 449-26-1Bu, 397-23Bu and 187-15-3Bu); the other set (JE13, JK1, JL1, JK2, JK3, JM97.1, JM97.2, JM97.3, JM97.4, JM97.5) was from the Victorian Institute for Dryland Agriculture and had not been tested previously in the field. *L. maculans* isolates used in glasshouse experiments (M1, V4, C13, NC13 and MC2) were derived from single ascospores cultured from infected *B. napus* trash in Australia (Ballinger and Salisbury, 1996). Isolates M1 and V4 form cotyledonary and stem lesions on *B. juncea* cultivars Stoke and Zaria, whilst isolates C13 and MC2 do not (Chen et al., 1996). Isolate NC13 has not been tested.

### *Glasshouse trials*

Twenty nine putative *Brassica* lines were sown in potting mix (BJH/321, Propine Nursery Supplies, Kilsyth, Australia) in a growth cabinet at 22 °C, 12 h photoperiod. In each experiment, *B. napus* cultivars Midas and Westar were included as susceptible controls for all isolates, and *B. juncea* cultivars Stoke and ATC90281 were included as susceptible controls for isolates M1 and V4. Ten days after sowing, cotyledons of each seedling were punctured with a 25 gauge needle (four wounds per cotyledon) and inoculated by placing a droplet (10 μL of $10^6$ pycnidiospores/ml) over the wounded area. Fourteen plants of each line were inoculated with each isolate and kept for 72 h under high humidity. Two weeks after inoculation, the greatest length of the lesion on each cotyledon was measured. Also cotyledons of *B. napus* cultivars Westar, Quinta and Glacier were inoculated with the five isolates (M1, V4, C13, NC13 and MC2). Lesion length was determined as described above and also disease was scored from 0 (no darkening around wounds) to 9 (large grey-green lesions with profuse sporulation) as described by Koch et al. (1991).

For stem inoculations, plants at the four leaf stage of growth were inoculated by placing a droplet (10 μL of $10^6$ pycnidiospores/ml) onto the axil of the first leaf of plants and then piercing the stem with a 25 gauge needle through the droplet (Plummer et al., 1994). Fourteen plants of each line were inoculated with each isolate. Five weeks after inoculation, the lengths of the lesions were measured.

### *Field trials*

Each of 98 putative *B. juncea* lines were sown on 1 June 1997 in two rows (5 m long with a row spacing of 0.75 m) in a blackleg disease nursery at Lake Bolac, Victoria, Australia where *B. napus* stubble was present from the previous year's crop. *B. napus* cultivars Dunkeld, Monty and Westar were included.

Cultivar Dunkeld is amongst the most blackleg resistant *B. napus* cultivars in commercial production in Australia. Westar, the susceptible control, was planted in every tenth row. After five weeks (10 July 1997) and at maturity (12 December 1997) the number of plants for each row was counted and the percentage survival determined. At maturity, 25 plants from each row (50 plants of each line) were sampled and cut with secateurs at the base of the stem, and a segment approximately 30 cm long was assessed for blackleg symptoms in the laboratory. The lengths of external and internal blackened lesions from the base of the stem were measured. The lines were also rated for blackleg resistance using a 0–6 scoring system whereby ratings of 0, 1, 2, 3, 4 and 5 respectively are assigned to plants with 0, 1–5, 6–25, 26–50, 51–75 and 76–100% of the cross section infected, and a rating of 6 for dead plants (Ballinger and Salisbury, 1996). Disease incidence was determined as percentage of plants showing blackleg symptoms (eg. blackened lesions) out of the 50 plants sampled.

To ensure that the lesions observed were caused by *L. maculans*, isolates were cultured from stem cankers of ten *B. juncea* lines onto 10% V8-juice agar plates containing Penicillin G (100 U/mL) and streptomycin sulphate (100 μg/mL). Hyphal tips were cultured and resultant pycnidiospores inoculated onto cotyledons of *B. napus* cultivar Westar and *B. juncea* cultivar Stoke.

*Statistical analysis*

To examine patterns of similarity between the lines, disease parameters from glasshouse trials (cotyledonary lesion length caused by isolates M1, V4, C13, NC13 and MC2, and stem lesion length caused by isolates M1 and V4) and field trials (survival rate, disease incidence, disease rating and length of internal and external lesions) were examined by Principal Components Analysis using the software PC-ORD (McCune and Mefford, 1997). The variance explained by each axis was calculated and used to judge the information represented by each axis. The lines were graphed in a rotated space, simplifying the information to a smaller number of dimensions, in which each axis is a linear combination of the disease parameters.

**Results**

*Glasshouse trials*

Two of 29 putative *B. juncea* lines were subsequently identified as *B. carinata*. All the *B. juncea* lines had cotyledonary lesions of more than 3 mm in length after inoculation with *L. maculans* isolates M1 and V4. However, none of these lines developed lesions after inoculation with isolates C13, NC13 and MC2 (Figure 1). These latter isolates often induced the formation of necrotic spots indicative of a hypersensitive response around the inoculation site. *B. napus* cultivars Westar and Midas developed lesions larger than 7 mm when inoculated with any of the five isolates, whilst cultivars Dunkeld and Monty developed lesions less than 6 mm. Although the five isolates could be discriminated by their reaction on *B. juncea* lines, they all caused lesions of between 9 and 11 mm and scores of above 7 on cotyledons of *B. napus* cultivars Westar, Quinta and Glacier (data not shown). In contrast, the two *B. carinata* lines did not develop cotyledonary lesions after inoculation with any isolate (Figure 1).

Nearly all *B. juncea* lines had stem lesions greater than 20 mm in length when inoculated with isolates M1 and V4; the exception being an Indian line, ATC 90295, which had lesions of 10 mm in length and also relatively small cotyledonary lesions (Figure 1). All four *B. napus* cultivars developed lesions of greater than 20 mm when inoculated with M1 and V4, whilst the two *B. carinata* lines developed extremely small stem lesions.

Principal Components Analysis showed that of the seven disease parameters measured, the proportions of total variation contributed by the first two are approximately 50% and 26% respectively, implying that two axes are sufficient to explain the majority of the variation amongst lines (Figure 2). This analysis showed that *B. napus* cultivars Midas and Westar (arrow) which are highly susceptible to all isolates are not clustered with the majority of lines, nor are the two *B. carinata* lines ATC 93184 and ATC 93188, and *B. juncea* line ATC 90295 which are relatively resistant to all isolates (Figure 2). The *B. juncea* lines from both Australian breeding programs grouped with those from other countries.

*Field trials*

Disease incidence in the blackleg nursery at Lake Bolac was even higher than usual in 1997 as a large amount of stubble was left from the previous crop, creating conditions conducive to severe disease development. Amongst 98 putative *B. juncea* lines tested in the field, four were subsequently identified as *B. carinata*, one as *B. nigra* and one as *Sinapis arvensis*. The responses of a representative sample of these lines to *L. maculans*, in

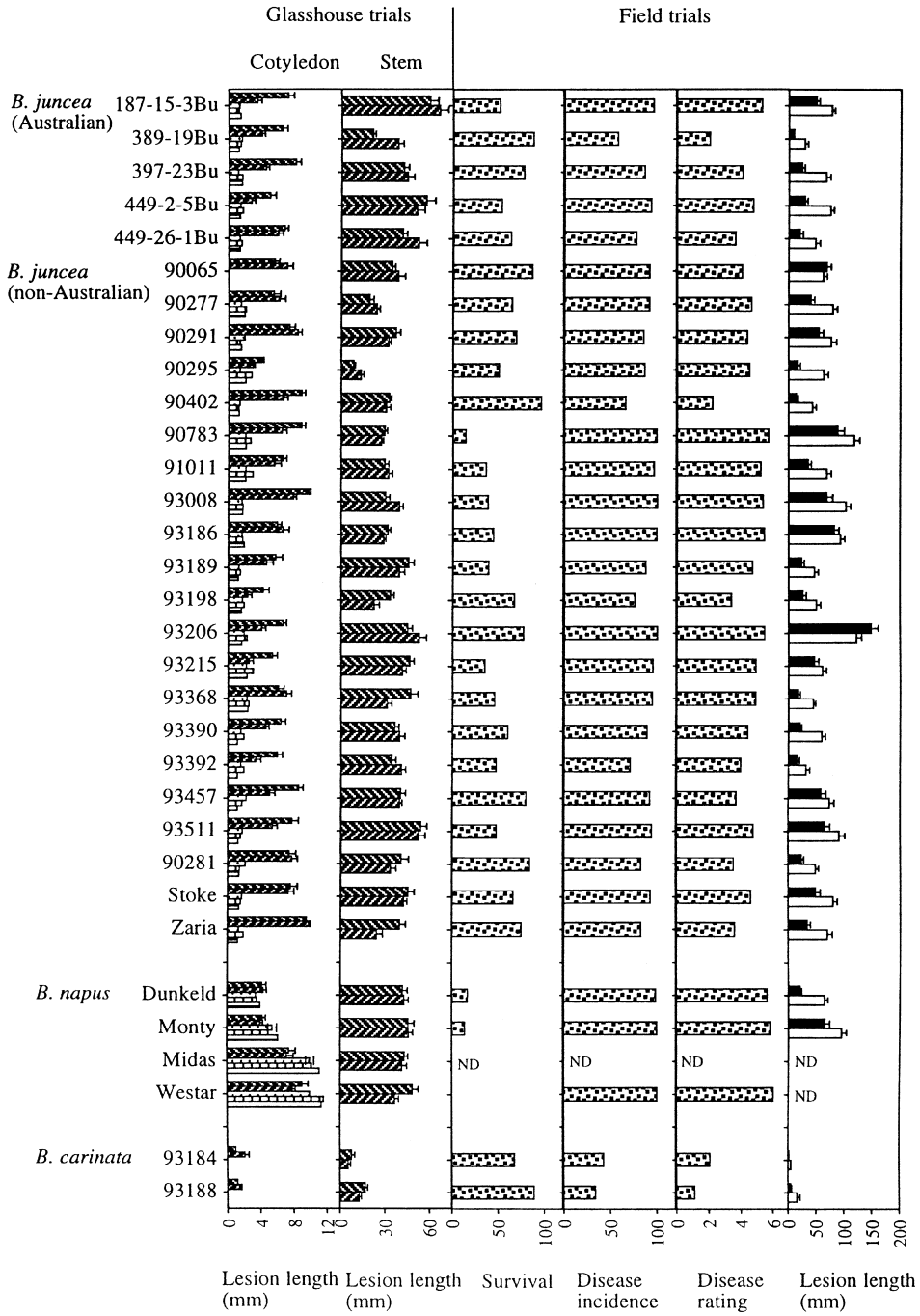


*Figure 1.* Responses of *B. juncea*, *B. napus* and *B. carinata* lines to *L. maculans* in the glasshouse (expressed as cotyledon and stem lesion length) and the field (expressed as survival rate, disease incidence, disease rating, length of internal and external lesions). For the glasshouse trials, 14 replicates of each line were assayed. Cotyledons were inoculated with *L. maculans* isolates M1(▨), V4(▧), C13(▩), NC13(▤) or MC2(▥); and stems were inoculated with either M1 or V4. For field trials, 50 replicates of each line were assayed for disease incidence, disease rating and length of internal (□) and external (■) lesions. B. napus cultivar Midas was not included in the field trials. All cultivar Westar plants died in field trials. ND not done. Bars represent standard errors.

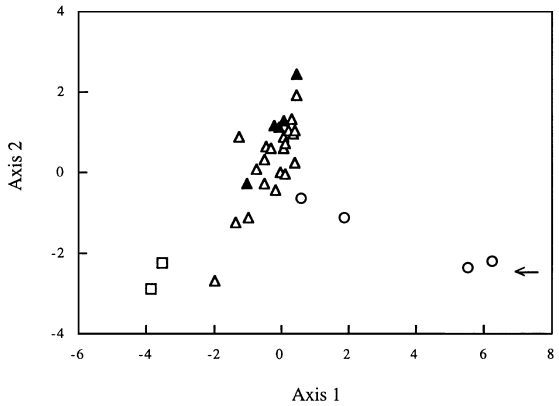


*Figure 2*. Principal Components Analysis ordination of *Brassica* lines from glasshouse trials using seven disease parameters: cotyledonary lesion length caused by isolates M1, V4, C13, NC13 and MC2, and stem lesion length caused by isolates M1 and V4. *B. juncea* (Australian)(▲), *B. juncea* (non-Australian)(△), *B. napus* (○) and *B. carinata* (□). The ordination depicts axes 1 and 2 (eigenvalue $\lambda_1 = 0.504$, eigenvalue $\lambda_2 = 0.258$).

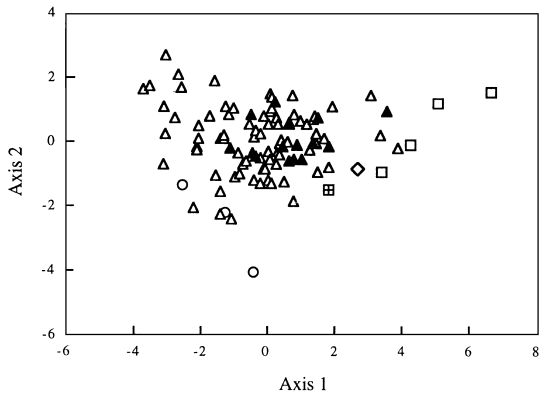


*Figure 3*. Principal Components Analysis ordination of *Brassica* lines from field trials using five disease parameters (survival rate, disease incidence, disease rating and length of internal and external lesions). *B. juncea* (Australian)(▲), *B. juncea* (non-Australian) (△), *B. napus* (○), *B. carinata* (□), *B. nigra* (⊞) , *S. arvensis* (◇). The ordination depicts axes 1 and 2 (eigenvalue $\lambda_1 = 0.675$, eigenvalue $\lambda_2 = 0.240$).

terms of survival rate, disease incidence, disease rating, and length of internal and external lesions, are shown in Figure 1. Data about the responses of remainder of the lines (66 *B. juncea*, 2 *B. carinata*, 1 *B. nigra*, 1 *S. arvensis*) are available from the authors upon request. Disease incidence of all except one *B. juncea* line (ATC 93550) was more than 50%. Only five *B. juncea* lines had disease ratings of less than 3; i.e. between 26 and 50% cross-section infected. Generally, the internal lesions were longer than the external lesions, and often stems with internal lesions did not show any external lesion. This is not surprising as the fungus grows in the vascular tissue of *B. napus* and *B. juncea* (Hammond et al., 1985; Chen and Howlett, 1996). The lesions or discoloration originated from petiole scars close to the crown, and then developed up and down the stem, suggesting that many of these plants may have been infected via cotyledons or primary leaves. The survival rate of almost all *B. juncea* lines was much higher than that of *B. napus* cultivars. Monty and Dunkeld had survival rates of 17 and 14%, and disease ratings of 5.8 and 5.6, respectively; normally in blackleg nurseries they have average survival rates of 50–80% (P.A. Salisbury, unpublished). All *B. napus* cultivar Westar plants died, most within two months of sowing (Figure 1).

The four *B. carinata* lines developed extremely small lesions and had high survival rates (ranging from 53–97%) and low disease ratings (0.3–2.9). The only *B. nigra* line tested, ATC 93452, had a survival rate of 42%, and a disease rating of 3.9, and appeared more susceptible than any of the *B. carinata* lines. The *S. arvensis* line had a survival rate of 56%, disease incidence of 63% and disease rating of 3.2. Principal Components Analysis of data from field trials (five parameters) showed that the first two components account for 91% of the total estimated variation. The lines were not clustered in any distinct pattern (Figure 3), suggesting that there is little variation amongst the lines in major disease resistance gene(s) to the *L. maculans* isolates at Lake Bolac, and that *B. juncea* lines from Australian breeding programs behave similarly to lines from other countries.

All of ten hyphal tip cultures derived from *B. juncea* lines from the field showing cankers, displayed morphology typical of *L. maculans*. Pycnidiospores were harvested from eight of these cultures (the other two did not sporulate sufficiently) and inoculated onto cotyledons of *B. napus* cultivar Westar and *B. juncea* cultivar Stoke. Lesions of $> 8$ mm developed in all cases (data not shown).

Relationships between disease parameters were determined for 26 *B. juncea* lines in glasshouse and field trials (Table 1). In the glasshouse there was a significant correlation ($p < 0.05$) between lesion lengths on cotyledons caused by isolates V4 and M1, between lesion lengths on cotyledons caused by isolates C13, NC13 and MC2, and between lesion lengths on stems caused by V4 and M1. In the field there were significant correlations ($p < 0.05$) between all pairs of disease parameters, except between survival rate and

*Table 1*. Pearson's correlation coefficients of blackleg disease variables in twenty six *Brassica juncea* lines in glasshouse and field trials

| Variable | Cotyledon lesion length (M1) | Cotyledon lesion length (V4) | Cotyledon lesion length (C13) | Cotyledon lesion length (NC13) | Cotyledon lesion length (MC2) | Stem lesion length (M1) | Stem lesion length (V4) | Survival rate | Disease incidence | Disease rating | External lesion length | Internal lesion length |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cotyledon lesion length (M1) | 1 | | | | | | | | | | | |
| Cotyledon lesion length (V4) | **0.625** | 1 | | | | | | | | | | |
| Cotyledon lesion length (C13) | 0.142 | −0.012 | 1 | | | | | | | | | |
| Cotyledon lesion length (NC13) | −0.180 | −0.282 | **0.673** | 1 | | | | | | | | |
| Cotyledon lesion length (MC2) | −0.098 | −0.108 | **0.675** | **0.852** | 1 | | | | | | | |
| Stem lesion length (M1) | 0.154 | −0.069 | 0.076 | −0.228 | −0.168 | 1 | | | | | | |
| Stem lesion length (V4) | 0.161 | −0.259 | −0.116 | −0.323 | −0.305 | **0.765** | 1 | | | | | |
| Survival rate | 0.109 | 0.171 | −0.338 | **−0.599** | **−0.581** | −0.050 | 0.171 | 1 | | | | |
| Disease incidence | 0.069 | 0.112 | 0.203 | 0.280 | 0.341 | 0.309 | 0.184 | **−0.552** | 1 | | | |
| Disease rating | −0.050 | −0.015 | 0.230 | 0.413 | 0.464 | 0.233 | 0.180 | **−0.754** | **0.919** | 1 | | |
| External lesion length | 0.230 | 0.138 | 0.128 | 0.034 | 0.046 | 0.160 | 0.238 | −0.143 | **0.644** | **0.587** | 1 | |
| Internal lesion length | 0.346 | 0.198 | 0.177 | 0.186 | 0.247 | 0.111 | 0.144 | −0.335 | **0.770** | **0.719** | **0.864** | 1 |

Values in bold represent coefficients which are significantly correlated ($p < 0.05$, $n = 26$).

external or internal lesion length. Similar relationships were seen when the disease parameters in the field for 92 *B. juncea* lines were assayed (data not shown). There were no significant correlations between any glasshouse and field parameters, except between survival rate and cotyledon lesion length caused by isolates NC13 and MC2. The most resistant *B. juncea* lines in the field (ATC 90300, 90301, 93454, 93462, JL2) developed lesions of similar size to those on other lines such as *B. napus* cultivar Westar, after inoculation of cotyledons and stems with isolates V4 and M1 in the glasshouse (data not shown).

## Discussion

Our results show that a range of *B. juncea* lines from diverse geographical locations develop blackleg symptoms in response to infection by Australian isolates of *L. maculans* under artificial conditions in the glasshouse, and natural conditions in the field. All *B. juncea* and the six *B. napus* lines tested were similarly susceptible to two *B. juncea* attacking isolates in the glasshouse; to our knowledge such a high degree of blackleg susceptibility has not been seen previously with *B. juncea*. The relatively high infection level of disease in all *B. juncea* lines tested suggests that there is no variation in major resistance genes in these lines to Australian isolates of *L. maculans*. As stated previously, other studies have described variation in major genes for blackleg resistance in *B. juncea* to particular *L. maculans* isolates (Pang and Halloran, 1996; Chevre et al., 1997; Keri et al., 1997). This discrepancy may be due to the nature of the *L. maculans* isolates present in Australia.

A system for classification of *L. maculans* isolates based on their ability to form lesions on cotyledons of *B. napus* cultivars Westar, Quinta and Glacier has been developed (Pathogenicity Groups 1–4) (Koch et al., 1991). The five Australian isolates used in the current study attack all three *B. napus* cultivars and accordingly belong to Pathogenicity Group 4, yet can be classified into two groups – those that attack all *B. juncea* lines (isolates M1 and V4), and those that attack none (isolates C13, MC2 and NC13). This classification supports previous testing of cultivars Stoke and Zaria with larger numbers of Australian isolates (Chen et al., 1996). All lines in the field displayed blackleg symptoms, suggesting that isolates with similar pathogenicity to that of M1 and V4 are present at Lake Bolac. This was confirmed by the observation that all sporulating isolates cultured from cankers from *B. juncea* lines in the field attacked *B. juncea* cultivar Stoke, as well as *B. napus* cultivar Westar.

In general Australian blackleg isolates may be more virulent on *B. juncea* than those found in other countries. Kutcher et al. (1993) described an Australian *L. maculans* isolate that attacked all *B. napus* and *B. rapa* lines tested, as well as one of two *B. juncea* lines tested. A more recent publication from this research group states that two blackleg isolates from Manitoba, Canada cause disease on only 3 of 296 *B. juncea* lines tested from a collection at the University of Manitoba (Keri et al., 1997). These results suggest that the Manitoba isolates are less virulent than two of the Australian isolates (V4 and M1) used in our study. Climatic conditions in Australia are optimum for the development of large numbers of pseudothecia on stubble over summer which may allow *L. maculans* to evolve rapidly and overcome resistance of the host. Harsher environmental conditions in Canada, such as snow cover, would minimise the formation of pseudothecia on stubble and the subsequent release of ascospores leading to less diversity and a decreased ability of the fungus to overcome host resistance.

Although in the glasshouse nearly all *B. juncea* lines developed large cotyledonary and stem lesions after inoculation with isolates M1 and V4 that were as large as those on *B. napus* lines, in the field these lines were more resistant than the *B. napus* lines. Throughout the growing season there were many fewer leaf lesions on *B. juncea* than on *B. napus* (data not shown) which indicates that leaves of *B. juncea* are more resistant to infection and/or disease development. Superior blackleg resistance in the field of *B. juncea* lines such as Zaria and Stoke, compared to that of *B. napus* lines has also been observed in France by Chevre et al. (1997). However, in contrast to our results, these authors observed that the *B. juncea* lines were much more resistant than the *B. napus* lines to a particular 'highly virulent' *L. maculans* isolate in glasshouse studies. These authors concluded that cotyledon tests provide an efficient method to select for blackleg resistance in *B. juncea* lines; this is not the case with Australian isolates.

The significant correlations between disease rating, survival rate, length of external and internal lesions justify the use of any of these assays to assess disease in *B. juncea* in the field. However, disease rating based on the cross section at the base of the stem is the most rapid and convenient measure and is currently used in Australia when germplasm is selected to

enhance blackleg resistance, whilst survival rate is used for screening large number of breeding lines (P.A. Salisbury, unpublished). Although we have shown that *B. juncea* lines in the field have relatively high disease ratings and all have disease incidences greater than 50%, it is unlikely that this will significantly affect their yield when grown commercially, as *B. napus* cultivars such as Monty and Dunkeld show acceptable levels of blackleg resistance during commercial production where disease pressure is lower than in the blackleg nursery.

Recently there have been extensive efforts to characterise and exploit blackleg resistance from *B. nigra*. Addition lines of *B. napus-B. nigra* have been developed and loci for blackleg resistance have been identified on particular B chromosomes (Chevre et al., 1996). Another B genome containing Brassica, *B. carinata* is known to have good blackleg resistance (Cohen and Knowles 1983; Gugel et al., 1990). In the present study, this species displayed a high degree of blackleg resistance both in the glasshouse and the field suggesting that it may provide a useful source of resistance for future *B. napus* breeding programs.

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