Seedling Resistance to *Sclerotinia sclerotiorum* as Expressed Across Diverse Cruciferous Species

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Abstract

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Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is a serious disease of many cruciferous crops and frequently poses a threat to the sustainable and profitable production of these crops worldwide. Differences in seedling resistance to *S. sclerotiorum* across 46 diverse cruciferous genotypes from 12 different species were assessed by comparing the extent of pathogenesis on inoculated cotyledons under controlled conditions. Selections of *Brassica carinata*, *B. incana*, *B. juncea*, *B. napus*, and *B. napus* introgressed with *B. carinata*, *B. nigra*, *B. oleracea*, *B. rapa* var. *rosularis*, *B. rapa* var. *chinensis*, *B. tournefortii*, *Raphanus raphanistrum*, *R. sativus*, and *Sinapis arvensis* were tested. The average size of lesions on cotyledons 48 h post inoculation varied from 0.8 to 7.3 mm. The three most resistant genotypes with the smallest lesions were all from *B. oleracea* (viz., *B. oleracea* var. *italica* 'Prophet' and *B. oleracea* var. *capitata* 'Burton' and 'Beverly Hills'). Representatives of *R. raphanistrum*, *S. arvensis*, *B. juncea*, and *B. carinata* were the most susceptible to *S. sclerotiorum*, with the largest lesions. To our knowledge, this is the first report of high levels of resistance to *S. sclerotiorum* in *B. oleracea* at the cotyledon stage and also the first report of the host cotyledon reactions against *S. sclero-*

tiorum for all tested species except *B. napus* and *B. juncea*. The mean lesion size for *B. napus* introgressed with *B. carinata* was 5.6 mm, which is midway between the lesion size for the two parent species *B. napus* (5.1 mm) and *B. carinata* (5.8 mm). Separate genetic control for cotyledon versus mature plant resistance was demonstrated by the lack of correlation between lesion size from *S. sclerotiorum* on the cotyledon with the severity of disease initiated by stem inoculation or natural processes in a previous field test. On the most resistant genotypes, *B. oleracea* var. *italica* Prophet and var. *capitata* Burton, growth of *S. sclerotiorum* on the cotyledon surface prior to penetration was severely impeded, production of appressoria inhibited, and both cytoplasm shrinkage and protoplast extrusion in *S. sclerotiorum* hyphae prevalent. This is the first report of such resistant mechanisms in *B. oleracea*. Genotypes with cotyledon resistance identified in this study will be of great value not only in furthering our understanding of resistance mechanisms across different cruciferous species but also could be exploited for developing commercial crucifer cultivars with high-level resistance against *S. sclerotiorum*.

The family Brassicaceae contains important crop species used for broadacre oilseed, forage, and vegetable production. Species used for oilseed production include oilseed rape (*Brassica napus* and *B. rapa*) and oilseed mustards such as Indian mustard (*B. juncea*), Ethiopian mustard (*B. carinata*), and black mustard (*B. nigra*) (35). Important broadacre dual-purpose or straight forage-production species include oilseed rape (*B. napus*) and kale (*B. oleracea*) (2), while many variants of *B. oleracea* are used for vegetable production, along with Asian brassicas (e.g., *B. rapa* var. *rosularis*) and other species such as *Raphanus* spp., which can be important crops regionally (34). *B. napus*, *B. oleracea*, and *Raphanus* spp. can all be included in "mustard and cress" seedling mixtures grown for consumption as salad vegetable mixes (18).

Sclerotinia sclerotiorum causes serious endemic disease across oilseed brassicas, forage brassicas, and vegetable brassicas (2,21,36). *S. sclerotiorum* also causes damping off in a variety of plants, causing losses at the seedling stage and significantly affecting transplant production (23). This pathogen has a wide host range (5) and survives in soil in the absence of a host for several years as thick-walled resting sclerotia (1). *S. sclerotiorum* attacks plants in a variety of ways in the field. Sclerotia can germinate carpo-genically, releasing ascospores that infect petals, leaves, and

stems. It is generally accepted that the most common route for stem infection of oilseed brassicas is through the infection of petals, which then fall and adhere to the stem, providing a nutritional resource that allows *S. sclerotiorum* to penetrate and infect the stem (17). However, direct infection of leaves and stems by ascospores in the absence of flower petals also seems possible (38) and sclerotia can germinate myceliogenically, entering the stem at ground level, as commonly occurs on mustard crops (*B. juncea*) in India (36).

Currently, cultural and chemical controls provide only partial control and can be cost prohibitive, particularly for broadacre oilseed *Brassica* crops in countries such as Australia (3), and locating effective resistance to *S. sclerotiorum* remains a high priority across cruciferous crops worldwide. Resistance that reduces seedling losses would be valuable for improving crop establishment and survival and could be vitally important where *Brassica* seedlings are grown for consumption as salad, such as in "mustard and cress" mixtures. There have been recent successes in locating high levels of host resistance to the stem rot phase in oilseed rape and mustards, especially in *Brassica* genotypes following hybridization of *B. napus* or *B. juncea* with three wild crucifers (viz., *Erucastrum cardaminoides*, *Diplotaxis tenuisiliqua*, and *E. abyssinicum*) (11,26,27) and the testing of a diverse group of crucifers revealed very high levels of resistance to *Sclerotinia* stem rot (SSR) in selections of *Raphanus* spp., *B. carinata*, and *B. napus* (38).

Various methods have been developed for testing resistance to *S. sclerotiorum* in brassicas. Field evaluations of SSR, while generally considered the most appropriate and reliable technique for evaluating stem resistance against SSR, can still result in highly

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variable findings, particularly where fluctuating environmental conditions influence the response of individual genotypes (27,31). For this reason, controlled-environment screening methods have been widely used to assess physiological resistance to *S. sclerotiorum* in various crops. A cotyledon test has been developed for screening *B. napus* genotypes against *S. sclerotiorum* at the seedling stage (15) but its potential application across more diverse cruciferous species has not been evaluated. In addition, although the seedling cotyledon test on *B. napus* showed a significant positive correlation ($r = 0.62$) between SSR test results in the assay and in the field (15), some studies have suggested that different mechanisms govern the resistance of brassicas to *S. sclerotiorum* at different growth stages. For example, Zhao et al. (39) detected six quantitative trait loci associated with resistance to *S. sclerotiorum* in oilseed rape (*B. napus*), three of which were associated with cotyledon resistance at the seedling stage and the remaining three with stem resistance at the maturing stage. Our understanding of the expression of resistance to *S. sclerotiorum* could be increased by further comparisons between SSR at seedling and adult host stages, including observation of the growth of the fungus on the host.

The aim of this study was to define the range and extent of seedling resistance to *S. sclerotiorum* across a variety of oilseed, for-

age, and vegetable crucifers, including wild or weedy species, and *B. napus* containing B genome introgressions from *B. carinata*. In addition, we aimed to determine the correlation between resistance found at the seedling stage with a previous field assessment (38) and observe the growth of the pathogen on susceptible and resistant cotyledons.

Materials and Methods

Fungal isolate. *S. sclerotiorum* isolate MBRS-1, collected from infected tissue of *B. napus* in the Mount Barker region of Western Australia in 2004 (26), was used. Isolate MBRS-1 was chosen because it is highly pathogenic (12) and belongs to the prevailing pathotype (pathotype 76) occurring in Western Australia (16). This isolate has been extensively used for resistance screening of *B. juncea* and *B. napus* genotypes in both the glasshouse and the field (11,15,16,25–27).

Genotypes tested. In total, 46 cruciferous genotypes from 12 different species were tested (Table 1). The majority of genotypes tested were obtained from a seed collection held at the University of Western Australia, supplied by Dr. Bob Redden at the Australian Temperate Field Crops Collection at Department of Primary Industries, Horsham, Victoria, Australia, or purchased from commercial

Table 1. Average cotyledon width and lesion diameter on the cotyledons of 46 cruciferous genotypes 48 h after inoculation with *Sclerotinia sclerotiorum*

Genotype name	Species	Origin	Lesion diameter (mm) ^a	Cotyledon width (mm)
Prophet	<i>Brassica oleracea</i> var. <i>italica</i>	sAustralia	0.8 (1)	15.4
Burton	<i>B. oleracea</i> var. <i>capitata</i>	sAustralia	2.3 (2)	15.3
Beverly Hills	<i>B. oleracea</i> var. <i>capitata</i>	sAustralia	3.6 (3)	12.3
BTO 2	<i>B. tournefortii</i>	Australia	4.0 (4)	7.8
Choy sum	<i>B. rapa</i> var. <i>chinensis</i>	sAustralia	4.2 (5)	9.9
SAR22	<i>Sinapis arvensis</i>	Australia	4.3 (6)	7.7
YM16	<i>B. napus</i>	China	4.4 (7)	16.4
94697	<i>B. incana</i>	sAustralia	4.5 (8)	10.5
06-P71-2	<i>B. napus</i>	China	4.6 (9)	16.3
Tatsoi	<i>B. rapa</i> var. <i>rosularis</i>	sAustralia	4.7 (10)	8.9
ZY005	<i>B. napus</i>	China	4.7 (11)	9.9
YM04	<i>B. napus</i>	China	4.7 (12)	15.5
BTO 6	<i>B. tournefortii</i>	Israel	4.8 (13)	8.3
SAR 5	<i>S. arvensis</i>	Australia	4.8 (14)	9.9
ZY004	<i>B. napus</i>	China	4.8 (15)	15.1
Charlton	<i>B. napus</i>	Australia	4.9 (16)	11.0
NC2-6	<i>B. napus</i> with <i>B. carinata</i> introgression	India	5.0 (17)	16.0
PI 193459	<i>B. carinata</i>	Ethiopia	5.1 (18)	12.5
BRA 926/78	<i>B. carinata</i>	Ethiopia	5.1 (19)	15.5
NC5	<i>B. napus</i> with <i>B. carinata</i> introgression	India	5.1 (20)	14.5
BRA 926/81	<i>B. carinata</i>	Ethiopia	5.3 (21)	15.4
YM11	<i>B. napus</i>	China	5.3 (22)	18.5
Boss	<i>Raphanus sativus</i>	sAustralia	5.4 (23)	15.5
YM13	<i>B. napus</i>	China	5.4 (24)	16.9
Black mustard	<i>B. nigra</i>	sAustralia	5.4 (25)	10.9
054103	<i>B. carinata</i>	Ethiopia	5.4 (26)	16.8
JO006	<i>B. juncea</i>	Australia	5.4 (27)	11.7
054107	<i>B. carinata</i>	Ethiopia	5.5 (28)	15.0
YM07	<i>B. napus</i>	China	5.5 (29)	17.6
UPM6563	<i>B. incana</i>	sAustralia	5.6 (30)	12.9
JM06006	<i>B. juncea</i>	Australia	5.6 (31)	11.3
054106	<i>B. carinata</i>	Ethiopia	5.7 (32)	15.6
Mystic	<i>B. napus</i>	Australia	5.7 (33)	16.0
NC9	<i>B. napus</i> with <i>B. carinata</i> introgression	India	5.8 (34)	16.3
WARR 7	<i>R. raphanistrum</i>	Australia	5.8 (35)	12.4
BCA 1	<i>B. carinata</i>	Australia	5.8 (36)	17.3
NC11-2	<i>B. napus</i> with <i>B. carinata</i> introgression	India	6.0 (37)	17.4
NC3-4	<i>B. napus</i> with <i>B. carinata</i> introgression	India	6.0 (38)	16.9
054113	<i>B. carinata</i>	Ethiopia	6.1 (39)	15.0
YM10	<i>B. napus</i>	China	6.2 (40)	15.6
JM06018	<i>B. juncea</i>	Australia	6.3 (41)	12.8
054104	<i>B. carinata</i>	Ethiopia	6.4 (42)	16.9
WARR 20	<i>R. raphanistrum</i>	Australia	6.4 (43)	15.3
SAR 7	<i>S. arvensis</i>	Australia	6.6 (44)	11.0
Xinyou 9	<i>B. juncea</i>	China	6.9 (45)	14.8
SMP3-82	<i>B. carinata</i>	Pakistan	7.3 (46)	14.8

^a Lesion diameter was analyzed using analysis of variance ($P = 0.001$) and means were separated using Fisher's least significant difference (5%) = 1.14. Relative genotype ranking for lesion diameter is shown in parentheses, where 1 represents the most resistant genotype and 46 represents the most susceptible genotype. sAustralia indicates that seed was sourced in Australia.

seed companies. *B. oleracea* 'Prophet' was obtained from Fairbanks Seeds, 'Burton' and 'Beverley Hills' from Lefroy Valley Seeds, and *B. rapa* 'Choy sum' and 'Tatsoi' from Johnson's Seeds. The test genotypes were selected on the basis of their diversity, as well as performance in earlier field or cotyledon tests (15,38).

Cotyledon inoculation experiment. Plants were grown in a pasteurized compost mixture in 35-by-29-by-6-cm trays, each having 30 individual cells. Five seeds of each genotype were sown in each cell and thinned to two seedlings per cell after emergence. Groups of two trays, together containing all 46 test genotypes, were placed in separate 30-liter clear plastic storage boxes with lids. The trays were incubated in a controlled environment growth room, set to $18 \pm 1^\circ\text{C}$ during the day and $14 \pm 1^\circ\text{C}$ at night, on a 12-h light-dark cycle, with light intensity of $220 \mu\text{m}^{-2} \text{s}^{-1}$. Seedlings were grown until their cotyledons were fully expanded, equivalent to growth stage 1.00 on the Sylvester-Bradley and Makepeace scale (37).

Production of inoculum and inoculations were based on the methods of Garg et al. (15). Five discs were excised from the actively growing margin of 3-day-old colonies of *S. sclerotiorum* grown on potato dextrose agar at 20°C and used to inoculate 150 ml of liquid growth medium (24 g of potato dextrose broth, 10 g of peptone, and 1 liter of H_2O) in 250-ml flasks. The cultures were shaken at 150 rpm at 20°C . After 3 days, actively growing mycelium was harvested by straining the broth through four layers of cheesecloth and washing with deionized water. The fungal mats obtained were then transferred to 125 ml of the same liquid medium and macerated using a hand-held blender for 3 min. The concentration of mycelia in the suspension was measured using a hemocytometer (Superior) and adjusted to 1×10^4 fragments/ml by diluting with fresh growth medium.

Cotyledon width (two lobes) was measured with a ruler immediately prior to inoculation. A single 10- μl droplet of mycelial suspension was spotted onto each of the four lobes of each seedling using a micropipette. The inoculum was shaken often to maintain a homogeneous mycelial suspension. It was noted that the initial droplet of inoculum spread to cover an area approximately 3 mm in diameter. Where a genotype had a small leaf size (<10 mm), only two droplets were applied to each plant (i.e., one droplet per cotyledon), and double the number of plants were inoculated to compensate.

Plants were saturated on the morning of their inoculation and a high-humidity environment attained by spraying a fine mist of water on the inside of the containers before closing the lids and placing the boxes in sealed, black plastic bags. Lesion diameter was measured in millimeters 48 h post inoculation (hpi), as described by Garg et al. (15), who presented this technique as a reliable means of comparing the level of resistance in *B. napus* genotypes. A complete randomized block design was used with four replications and eight measurements (four droplets on each of two seedlings) per replication. The entire experiment was conducted twice. Lesion diameter was analyzed separately for the two experiments using analysis of variance with Genstat (14th edition, Lawes Agricultural Trust). Fisher's least significant differences ($P < 0.05$) were used to compare genotype reactions. The relationship between the two experiments was assessed using a one-sided paired *t* test in Genstat. The paired *t* test detected no difference between experiments ($P > 0.05$); therefore, all data were consolidated and reanalyzed together. Regression analyses were undertaken using the regression function in Microsoft Excel to examine the relationship between the lesion diameters on cotyledons in this study and the length of stem lesions after inoculation with *S. sclerotiorum* MBRS-1 and incidence of natural leaf infection by *S. sclerotiorum* on the same genotypes in an associated field trial (38).

Anatomical studies on the infection process of *S. sclerotiorum*. Growth of MBRS-1 on cotyledon tissues of two genotypes previously determined to be resistant to *S. sclerotiorum* (*B. oleracea* var. *italic* Prophet and *B. oleracea* var. *capitata* Burton) and three genotypes found to be susceptible to *S. sclerotiorum* (*B.*

carinata 054104, *B. carinata* 054106, and *Raphanus raphanistrum* WARR 20) were sampled at 24 hpi and decolorized in acetic acid/ethanol/water (2:2:1) for 5 days at 25°C , with the solution replaced with fresh where required to complete the decolorization. At least four cotyledons of each genotype were examined in two repeated experiments. At the time of examination, cotyledons were washed with two changes of deionized water and stained with 0.05% (wt/vol) aniline blue for 90 s. Stained cotyledons were wet mounted whole on glass microscope slides, examined, and photographed (Olympus BX51 microscope and DP71 digital photographic system for bright field optics, or Olympus SZX10 stereo microscope and DP21 digital photographic system). After examination of the stained cotyledons, an attempt was made to remove the hyphal mat with tweezers, to determine how firmly it adhered to the surface of the cotyledon. Several seedlings from each genotype were observed 48 hpi to ensure that disease progress was consistent with the earlier experiments.

Results

Diameter of lesions caused by *S. sclerotiorum* on cruciferous genotypes. Lesion diameters following inoculation with MBRS-1 varied significantly ($P < 0.001$) among the cruciferous genotypes (Table 1). There was a significant relationship between the width of a genotype's cotyledons and the diameter of lesions resulting from *S. sclerotiorum* infection on that genotype ($P < 0.004$, $r = 0.41$). However, when the two genotypes with the largest cotyledons, *R. sativus* Krasnodar Market B (average cotyledon width = 22.6 mm, average lesion width = 8.51 mm) and *R. sativus* Minowase Nr2 (average cotyledon width = 23.5 mm, average lesion width = 7.5 mm) were excluded from the analysis, this relationship between cotyledon width and lesion diameter disappeared ($P = 0.14$, $r = 0.22$). *R. sativus* Krasnodar Market B and Minowase Nr2 were removed from all subsequent analyses, and the mean lesion diameter at 48 hpi for the remaining genotypes ranged from 0.8 mm for *B. oleracea* var. *italica* Prophet to 7.3 mm for *B. carinata* SMP3-82 (Table 1).

When lesion diameters on cotyledons under controlled environmental conditions in this study were compared with the length of stem lesions caused by *S. sclerotiorum* in the field in a previous study (38), there was no significant relationship between them ($P = 0.42$, $r = 0.14$). Likewise, there was no significant correlation between lesion size on cotyledons and the incidence of natural leaf infection in the field ($P = 0.21$, $r = 0.22$). In fact, the only selections with a reaction that was congruent between the cotyledon test and the stem test were *B. juncea* 'Xinyou 9' and *B. carinata* 'SMP3-82', which were highly susceptible in both studies. Of the other genotypes, *B. oleracea* var. *italica* Prophet was the most resistant in the cotyledon assay (mean lesion diameter = 0.8 mm) but was midway in genotype rankings for SSR in the field. Similarly, *B. rapa* var. *chinensis* Choy sum was highly susceptible to SSR in the field but ranked fifth most resistant in the cotyledon test (mean lesion width = 4.2 mm). Examples of genotypes that displayed resistance in the field but were highly susceptible in the cotyledon test included *B. carinata* 054104 (mean lesion diameter = 6.4 mm), *B. carinata* 054113 (mean lesion diameter = 6.1 mm), *B. napus* 'Mystic' (mean lesion diameter = 5.7 mm), and *R. raphanistrum* WARR 20 (mean lesion diameter = 6.4 mm). *B. napus* 'Charlton' and Mystic were both in the midrange for mean lesion diameter in this experiment, in spite of the fact that Mystic was highly resistant in a previous cotyledon evaluation (15) and had the smallest stem lesions in the field trial.

Variation in lesion diameter caused by *S. sclerotiorum* within a species. There was apparent variation between different genotypes within a single species in terms of lesion diameter (Table 2). The two species with the largest number of representatives (*B. carinata* and *B. napus*) did not have the widest range in lesion width. In fact, *B. oleracea* was the species with the largest variation, in spite of being represented by only three genotypes. The five selections of *B. napus* introgressed with *B. carinata* were close to one another in terms of cotyledon lesion size from *S. sclerotio-*

orum, with a maximum range of only 0.9 mm. The mean lesion size for these latter five selections (5.6 mm) was midway between the lesions observed on the two parent species, *B. napus* (5.1 mm) and *B. carinata* (5.8 mm).

Infection process of *S. sclerotiorum*. In all genotypes, the droplets of inoculum applied to the cotyledon surface initially remained discrete from the leaf surface, with surface tension maintaining a rounded shape. In all instances, the mycelial fragments of *S. sclerotiorum* grew using the nutrients in the droplet, forming a hyphal mat on the cotyledon surface. In most cases, by 24 hpi, hyphae had extended beyond the limits of the droplet and appressoria were forming at the edge of the hyphal mat (Fig. 1A and B).

In *B. oleracea* var. *italica* Prophet at 24 hpi, the hyphal mat was confined to the area of the inoculation droplet and no appressoria were formed (Fig. 1C). If the hyphal mat was removed at 24 hpi, there was little macroscopic evidence of the inoculation having ever occurred, except for small necrotic flecks on the surface of the cotyledon in the area of the inoculation droplet, indicative of a hypersensitive response (HR). The hyphae present on the cotyledon showed cytoplasmic shrinkage and protoplasmic extrusion and, with the latter symptom, extensively diffuse cytoplasmic contents were evident around the hyphae (Fig. 1D).

Macroscopically, the initial site of infection on the cotyledons was frequently at the edge of the hyphal mat, often giving the appearance of “pitting” (Fig. 1E). In most cases, the infection was so advanced by 48 hpi that the extensive hyphal growth in the lesion had destroyed the structure of this section of the cotyledon. Because the majority of seedlings were very susceptible to attack by *S. sclerotiorum*, the test was concluded at 48 hpi, prior to the more susceptible seedlings being completely consumed by the pathogen. In *B. oleracea* var. *italica* Prophet at 48 hpi, however, almost no *S. sclerotiorum* hyphae were observed on any part of the cotyledon and the infection process had ceased, while discoloration and spotting typical of an HR was evident on the cotyledon surface (Fig. 1F). Hyphae growing on *B. oleracea* var. *capitata* Burton also showed clear evidence of both cytoplasmic shrinkage and protoplasmic extrusion (Fig. 1G) but, by 48 hpi, more substantial lesions were visible on the cotyledons than the HR observed with *B. oleracea* var. *italica* Prophet.

Browning of epidermal and upper cortical tissues was sometimes observed at high magnification directly underneath appressoria by 24 hpi; for example, on *R. raphanistrum* WARR 20 (Fig. 1H) and *B. carinata* 054106. However, for both of these genotypes, no HR was observed on the inoculated cotyledons and no shrinkage of cytoplasm away from cell walls or protoplasm extrusion from hyphae was observed.

Discussion

These experiments, involving diverse cruciferous genotypes, including genotypes carrying introgression from *B. carinata*, highlight the enormous potential for incorporating high levels of seedling resistance to *S. sclerotiorum* into commercial germplasm of cruciferous oilseed, forage, and vegetable crops. This is the first time that cotyledon seedling reactions to *S. sclerotiorum* have been reported on *B. carinata*, *B. incana*, *B. napus* introgressed with *B. carinata*, *B. nigra*, *B. oleracea*, *B. rapa* var. *rosularis*, *B. rapa* var. *chinensis*, *B. tournefortii*, *R. raphanistrum*, *R. sativus*, and *Sinapis arvensis*.

We observed a high level of resistance to *Sclerotinia sclerotiorum* in the cotyledons of *B. oleracea*, compared with partial resistance reported in other tissues of *B. oleracea* and its wild relatives (8,10,32). Further, the black “flecking” on the surface of the cotyledon in the area of the inoculation droplet indicated an HR on *B. oleracea* var. *italica* Prophet, similar to that previously reported in *B. napus* Charlton challenged with *S. sclerotiorum* (14). We believe this to be the first report of an HR to *S. sclerotiorum* on cotyledons of *B. oleracea*. Cytoplasmic shrinkage and protoplasmic extrusion for *S. sclerotiorum* hyphal cells, as observed on both *B. oleracea* var. *italica* Prophet and var. *capitata* Burton, were previously reported on crucifer seedling cotyledons for *B. napus* Charl-

ton (14). It is noteworthy that, in our study, we readily observed these symptoms at 1 day post inoculation (dpi) compared with 4 dpi. Our study is the first protoplast extrusion from hyphal cells reported in the *B. oleracea*–*S. sclerotiorum* pathosystem. A similar phenomenon was also observed in *B. napus* by Garg et al. (14). We concluded that the contents emerging from the end or at the center of the fungal hyphae were protoplast extrusions and not appressoria because of the extensive shrinkage of the cytoplasmic content of the hyphal cells, while appressoria of *S. sclerotiorum* on the surface of a susceptible host genotype always arose from dichotomous branching of the terminal hyphae (30), not from the intercalary hyphal cells, as we observed here. The occurrence of protoplast extrusion from hyphal cells, and only on the resistant genotypes, points to the possibility of an as-yet-undefined defense mechanism in *B. oleracea* and suggests that it may be similar to that found in *B. napus* Charlton (13,14).

It is noteworthy that the inhibition of *S. sclerotiorum* infection by *B. oleracea* var. *italica* Prophet occurred prior to any obvious visual damage to the host. Initial growth of *S. sclerotiorum* in a hyphal mat was evident prior to infection on both resistant and susceptible selections and protoplasmic extrusion was observed in this phase on *B. oleracea* var. *italica* Prophet. Together, these results strongly suggest the production of antifungal compounds by the host in response to infection. *S. sclerotiorum* is known to produce elicitors such as oxalic acid and a variety of polygalacturonases that move into host tissue ahead of its infection hyphae (6,29). These or similar compounds produced in the hyphal mat may have stimulated an active defense by *B. oleracea* var. *italica* Prophet. A complex mixture of defense metabolites with antifungal activity are produced by crucifers when they are attacked by pathogens, including phytoalexins such as brassilexin and sinalenin (33). Further work to fully understand the mechanism by which *S. sclerotiorum* is destroyed on the surface of resistant cotyledons is clearly warranted.

Why cotyledons of *B. napus* Charlton and Mystic were not highly resistant to *S. sclerotiorum* in our experiments, in contrast to their previous performance in a similar test, remains unexplained; however, it is possible that the use of a different seed lot was responsible (14). Seed source is known to affect expression of resistance to other diseases in brassicas, such as blackleg (*Leptosphaeria maculans*; M. J. Barbetti, unpublished data). The intermediate status of cotyledons between embryo and leaf (7) may mean that the metabolic fitness of the seed impacts the ability of the cotyledons to respond to challenge by a pathogen. Both *B. oleracea* var. *italica* Prophet and var. *capitata* Burton were F1 hybrid seed obtained fresh from commercial seed sources, whereas seed for many of the other selections, including *B. napus* Charlton and Mystic, had been stored for varying lengths of time and up to 8 years at 4°C. Further examination of the effect of seed age and

Table 2. Number of genotypes tested, average lesion width and range in lesion widths caused by *Sclerotinia sclerotiorum* for each species

Species	N ^a	Lesion (mm) ^b	Range (mm) ^c
<i>Brassica rapa</i> var. <i>chinensis</i>	1	4.2	...
<i>B. rapa</i> var. <i>rosularis</i>	1	4.7	...
<i>Raphanus sativus</i>	1	5.4	...
<i>B. nigra</i>	1	5.4	...
<i>B. tournefortii</i>	2	4.4	0.8
<i>B. incana</i>	2	5.0	1.1
<i>R. raphanistrum</i>	2	6.1	0.6
<i>B. oleracea</i>	3	2.2	2.7
<i>Sinapis arvensis</i>	3	5.3	2.3
<i>B. juncea</i>	4	6.1	1.4
<i>B. napus</i> with <i>B. carinata</i> introgression	5	5.6	1.0
<i>B. carinata</i>	10	5.8	2.1
<i>B. napus</i>	11	5.1	1.8

^a Number of genotypes tested.

^b Average lesion diameter.

^c Range of variation in lesion diameter.

production conditions on the expression of cotyledon resistance to *S. sclerotiorum* would be useful.

There appeared to be greater uniformity in reaction to *S. sclerotiorum* within representatives of a particular species in this experiment testing reactions on cotyledons than occurred in field trials for assessing resistance to SSR (38). This may be a consequence, in part, of the much tighter control over environmental conditions for the cotyledon screening studies compared with the stem infection studies under more variable field conditions, par-

ticularly because expression of intermediate levels of host resistance is particularly influenced by changes in environmental conditions (20).

Inoculating with droplets of fungal mycelium in potato dextrose broth containing peptone mimicked the natural infection processes. The growth of the mycelium through the droplet and formation of a hyphal mat prior to infection of the leaf mirrors the natural process of *S. sclerotiorum*, which uses infected petals as a source of nutrition prior to penetration of the leaf or stem (17). However, there

Fig. 1. **A**, *Brassica carinata* 054106 with hyphal mat at 24 h post inoculation (hpi). Appressoria are visible at the edge of the lesion (arrows). **B**, Higher magnification of **A** showing appressoria at edge of hyphal mat in *B. carinata* 054106 at 24 hpi, with repeated dichotomous branching of the terminal hyphae (arrows) leading to the formation of appressoria (arrows). **C**, *B. oleracea* var. *italica* 'Prophet' with hyphal mat at 24 hpi. **D**, Higher magnification of **C** showing *B. oleracea* var. *italica* Prophet with protoplast extrusion (longer arrows) and diffused cytoplasmic contents surrounding affected mycelium (shorter arrows). **E**, Example of infection starting from the edge of the hyphal mat on *B. napus* introgressed with *B. carinata* NC11-2 at 48 hpi. **F**, Typical appearance of *B. oleracea* var. *italica* Prophet at 48 hpi. **G**, *B. oleracea* 'Burton', $\times 100$ magnification showing typical symptoms of cytoplasm shrinkage (shorter arrows) and cytoplasmic extrusion (longer arrows). **H**, Brown discoloration observed on *Raphanus raphanistrum* WARR 20 directly underneath the location of appressoria at 24 hpi.

was a lack of correlation between the results of tests for a given genotype at the cotyledon stage in this study with either adult plant stem resistance following artificial inoculation or naturally occurring leaf infection across a similar diverse range of cruciferous genotypes in field studies (38). Differences in the expression of resistance in juvenile and adult plants has already been reported in brassicas for other disease–host combinations such as *L. maculans* in oilseed rape (*B. napus*) (28), downy mildew (*Hyaloperonospora parasitica*) in broccoli (9), and *Cauliflower mosaic virus* and *Pseudomonas syringae* in *Arabidopsis* (22,24). More specifically, in reactions to *S. sclerotiorum*, several studies have indicated that there are differences between juvenile and adult plant reactions in *B. napus* (4,19) and that the mechanisms of resistance are different in brassicas of different ages (39). The only genotypes in our study with reactions which were congruent between adult stem and cotyledon tests were the two most susceptible selections, *B. juncea* Xinyou 9 and *B. carinata* SMP3-82. Kim et al. (20), looking at three different methods of assessing the resistance of soybean cultivars to *S. sclerotiorum*, also found that highly susceptible genotypes were consistent between tests, whereas cultivars with intermediate reactions were not. This topic needs further investigation in relation to cotyledon seedling resistance in crucifers. It is now evident that, outside of *B. napus*, field resistance cannot be predicted from cotyledon resistance and should be tested separately to locate the seedling or adult plant resistance needed.

Preliminary tests in our study soon revealed the challenges in expanding the use of the technique proposed by Garg et al. (15) as a method of assessing the susceptibility of a variety of genotypes of *B. napus* to be across a more diverse range of cruciferous species. For example, it was evident that cotyledons of *Sisymbrium irio*, *D. tenuifolia*, and *Camelina sativa* had lobes smaller than the diameter of the inoculation droplet applied to *B. napus* (approximately 3 mm in diameter); therefore, crucifers with an average cotyledon width less than 7 mm were excluded from our study. Where cotyledon width is between 7 and 10 mm, an adjustment can be made by applying the inoculum to only one lobe instead of two, enabling realistic comparisons between small-lobed genotypes and genotypes with larger cotyledons. Genotypes with very large cotyledons were also excluded to remove a slight positive correlation between cotyledon size and lesion size where genotypes with cotyledons greater than 22 mm were included. Although the need to exclude genotypes with cotyledons less than 7 mm or greater than 22 mm restricts the application of this technique to assessing the physiological susceptibility of some crucifer species to *S. sclerotiorum* at the cotyledon stage, there was excellent differentiation between the reactions to *S. sclerotiorum* across species as varied as *R. raphanistrum*, *B. carinata*, *B. oleracea*, and *S. arvensis*. Thus, the expansion of the cotyledon screening technique to a wider range of new genera and species was successful and provides the first new information on cotyledon reactions against *S. sclerotiorum* for these previously untested species.

Because current management of *S. sclerotiorum* is often suboptimal (3), there is an urgency to develop and commercially utilize new host resistances such as those identified in this study. To our knowledge, this is the first report of high levels of resistance in *B. oleracea* at the cotyledon stage. The results also provide evidence for a disparity in resistance reactions between juvenile and mature plants of the same host genotype. Although most studies of seedling resistance to *S. sclerotiorum* seem to have been undertaken with the hope of developing a quick and efficient estimate of adult plant resistance, the inherent value of resistance at the seedling stage should not be ignored because it would improve crop establishment and survival. It is vital that assessment of resistance to *S. sclerotiorum* should be undertaken at all important stages of plant growth to ensure commercial relevance.

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