Resistance to *Sclerotinia sclerotiorum* in wild *Brassica* species and the importance of *Sclerotinia subarctica* as a *Brassica* pathogen

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Abstract

Brassica crops are of global importance with oilseed rape (*Brassica napus*) accounting for 13% of edible oil production. All *Brassica* are susceptible to Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, a generalist fungal pathogen causing disease in over 400 plant species. Generally, sources of plant resistance result in partial control of the pathogen although some studies have identified wild *Brassica* species that are highly resistant. The related pathogen *S. subarctica* has also been reported on *Brassica* but its aggressiveness in relation to *S. sclerotiorum* is unknown. In this study, detached leaf and petiole assays were used to identify new sources of resistance to *S. sclerotiorum* within a wild *Brassica* C genome diversity set. High level resistance was observed in *B. incana* and *B. cretica* in petiole assays, while wild *B. oleracea* and *B. incana* lines were the most resistant in leaf assays. A *B. bourgeai* line showed both partial petiole and leaf resistance. Although there was no correlation between the two assays, resistance in the detached petiole assay was correlated with stem resistance in mature plants. When tested on commercial cultivars of *B. napus*, *B. oleracea* and *B. rapa*, selected isolates of *S. subarctica* exhibited comparable aggressiveness to *S. sclerotiorum* indicating it can be a significant pathogen of *Brassica*. This is the first study to identify *B. cretica* as a source of resistance to *S. sclerotiorum* and to report resistance in other wild *Brassica* species to a UK isolate, hence providing resources for breeding of resistant cultivars suitable for Europe.

Introduction

Oilseed *Brassica* crops such as oilseed rape and mustard are important commodities in Europe, India, Australia, China and Canada, contributing 13% of the total world's production of edible oil (Carr, 1990) while other *Brassica* species such as cabbage, cauliflower, broccoli, and turnip, are major food crops which make a significant contribution to nutrition and health (Zhang *et al.*, 1992). All Brassicas are susceptible to Sclerotinia stem rot (SSR), caused by *Sclerotinia sclerotiorum*. As a generalist necrotrophic pathogen which causes disease on over 400 plant species (Boland & Hall, 1994), the fungus is also a serious threat to many other economically important crops worldwide including soybean, sunflower, peas, beans, carrot, lettuce and potatoes (Mei *et al.*, 2013, Uloth *et al.*, 2013, Derbyshire & Denton-Giles, 2016).

Oilseed rape (also known as canola; *Brassica napus*) is one of the most widely grown *Brassica* species where SSR routinely results in serious losses, with incidence in the range of 10-20% in Canada, Australia, USA and Europe (Derbyshire & Denton-Giles, 2016). Soilborne sclerotia of *S. sclerotiorum* germinate to produce apothecia and subsequent release of ascospores results in infected petals which initiate lesions on the stems, leading to lodging and significantly reduced yields (Derbyshire & Denton-Giles, 2016). In India, substantial losses due to SSR have been recorded for other *Brassica* species, particularly for mustard (*B. juncea*) which is widely grown, and where yield losses of 37-92% have been recorded in the Rajasthan region (Shivpuri *et al.*, 2000).

Currently, there are no *Brassica* crop varieties with high levels of resistance to SSR commercially available. Identifying sources of resistance in *Brassica* is challenging as there can be considerable variability in plant screening assays depending on conditions, plant growth stage and *S. sclerotiorum* isolate (Garg *et al.*, 2010b, Uloth *et al.*, 2013, Ding *et al.*, 2015, Taylor *et al.*, 2015). Despite these problems, some sources of partial resistance have been

identified and mapped in *B. napus* (Zhao *et al.*, 2006, Li *et al.*, 2009, Yin *et al.*, 2010, Taylor *et al.*, 2015, Gyawali *et al.*, 2016, Wu *et al.*, 2016). However, higher level resistance to SSR has been identified in more diverse cruciferous plants including wild species (Navabi *et al.*, 2010, Mei *et al.*, 2011, Uloth *et al.*, 2013). A study in India reported that stem lesions caused by *S. sclerotiorum* were eight times smaller in *B. napus* and *B. juncea* introgression lines derived from wild germplasm (*Erucastrum cardamanoides*, *B. fruticulosa*, *Diplotaxis tenuiisiliqua* and *E. abyssinicum*) compared to standard susceptible *B. napus* and *B. juncea* lines (Garg *et al.*, 2010a). The only recent resistance study carried out in Europe used a *B. napus* diversity set and identified lines partially resistant to SSR (Taylor *et al.*, 2015). Prior to this, a study in Ireland reported an increased level of *S. sclerotiorum* resistance in a mutagenized *B. napus* population (Mullins *et al.*, 1999).

Several different approaches have been used to screen for resistance to SSR, which in some cases have produced conflicting results. The main methods reported include stem inoculations with a toothpick (Zhao & Meng, 2003, Yin *et al.*, 2010), petiole inoculations with an agar plug (Zhao *et al.*, 2006) or infected wheat grain (Taylor *et al.*, 2015), detached leaf inoculations using agar plugs (Zhao & Meng, 2003) or mycelial fragments on attached cotyledons (Garg *et al.*, 2008) and detached stem inoculations using agar plugs (Mei *et al.*, 2012). Arguably the most robust test for field resistance in OSR which has been widely employed is the inoculation of mature plant stems using an agar plug (Buchwaldt et al 2005). Using this method, it was shown that lesion size is strongly correlated with plant death, and hence directly linked to yield (Li *et al.*, 2006). The problem with this assay (and others) is that it takes a very long time to carry out, especially if replicate experiments are required; hence it might take several years to complete a robust resistance screen. Therefore, the development of more rapid assays is desirable as long as they relate to mature plant / field resistance.

Although *S. sclerotiorum* is the major pathogen causing SSR on *Brassica*, the related species *S. subarctica* (originally termed *Sclerotinia* sp. 1), first identified on wild plants and potato in Norway (Holst-Jensen *et al.*, 1998), can cause identical symptoms to *S. sclerotiorum* but appears confined to northern latitudes (Clarkson *et al.*, 2017). *S. subarctica* has been identified on lettuce, cabbage, bean and potato in Alaska (Winton *et al.*, 2006), and was first reported in England on *Ranunculus acris* (meadow buttercup) where the same isolate was shown to be pathogenic on *B. napus* (Clarkson *et al.*, 2010). More recently, the pathogen has been found on further crop plants including carrot, celery root, Jerusalem artichoke, pea, swede, and turnip rape (*Brassica rapa* subsp. *oleifera*) in Scotland and Norway (Brodal *et al.*, 2016, Clarkson *et al.*, 2017). Hence, *S. subarctica* appears to have a similarly broad host range to *S. sclerotiorum* and is a significant pathogen in some northern countries. However, an initial study comparing the aggressiveness of a single *S. subarctica* isolate with *S. sclerotiorum* suggested that *S. subarctica* was a weaker pathogen on three *Brassica* spp. (Taylor *et al.*, 2015).

The aim of this study was to screen wild *Brassica* species for resistance to *S. sclerotiorum* to determine if new and higher level sources of resistance could be identified compared to those identified previously in *B. napus* (Taylor *et al*., 2015). To achieve this, and overcome the problem of highly variable wild *Brassica* morphotypes, improved detached petiole and detached leaf assays were developed. In addition, to determine the importance of *S. subarctica* as a pathogen, the aggressiveness of 12 isolates was compared with three previously characterised *S. sclerotiorum* isolates on different *Brassica* species.

Materials and Methods

Sclerotinia isolates and ascospore production

The four *S. sclerotiorum* isolates used in this study were obtained from infected lettuce (L6, L44), pea (P7) and buttercup (*Ranunculus acris*, DG4) from different locations in England. These isolates also represented different genotypes as identified previously using microsatellite markers (Clarkson *et al*., 2017; Table 1). The pathogenicity of these isolates was tested previously against three *Brassica* spp. with L6 and P7 identified as aggressive, L44 as intermediate and DG4 as weak in terms of their virulence (Taylor *et al*., 2015). The 12 *S. subarctica* isolates were obtained from England, Scotland, Norway and Sweden from buttercup (six isolates), pea (two isolates), lettuce (two isolates), potato (one isolate) and swede (one isolate) and also represented different microsatellite genotypes (Clarkson *et al*., 2017; Table 1). Cultures of each isolate were initiated from stock sclerotia maintained at 5°C; a single sclerotium was bisected and placed on potato dextrose agar (PDA) or glucose rich medium (10 g peptone, 20 g glucose, 18 g agar, 0.5 g KH_2PO_4, 1 L H_2O, adjusted to pH 4.0) and incubated at 20°C for 3-4 days to produce actively growing colonies. These were then further subcultured onto PDA plates and grown for 2 days at 20°C to provide actively growing mycelium for petiole inoculations.

S. sclerotiorum ascospores for leaf inoculations were produced as described by Clarkson *et al.*, (2014). Briefly, this involved burying cold-conditioned sclerotia (isolate L6) in moist, pasteurised compost and incubating at 15°C to stimulate germination and production of apothecia. Ascospores were then collected onto a filter paper using a suction pump and stored at 4°C until use.

Brassica lines

All *Brassica* lines tested for resistance to *S. sclerotiorum* were derived from the Warwick Genetic Resources Unit and a wild *Brassica* 'C genome' diversity set (Table 2). The full diversity set comprises 89 founder accessions representing 14 different species and also includes fixed doubled haploid lines for 35 accessions which were crossed with a compatible rapid cycling line to overcome self-incompatibility (Pink *et al.*, 2008).

Detached leaf and petiole assays

Previously, *B. napus* lines were screened for resistance to *S. sclerotiorum* using both immature and mature plants assays (Taylor *et al.*, 2015). In the former test, plants at the 7-9 leaf stage were inoculated by placing a wheat grain colonised by *S. sclerotiorum* in a leaf axil and assessing severity of infection based on leaf wilting and lesion development while the latter involved inoculating the stem of mature flowering plants with an agar plug of mycelium and measuring lesion size over time (Taylor *et al.*, 2015). However, neither of these methods were suitable for the wild *Brassica* lines in this study because of their diverse morphology and differences in both their growth rate and ability to produce elongated stems. Therefore, two other assays were employed, comprising inoculation of detached petioles with agar plugs of mycelium and inoculation of detached leaves with *S. sclerotiorum* ascospores.

The detached petiole assay was based on the method of Mei *et al*., (2012). *Brassica* plants were grown in a glasshouse (20°C, 16 h photoperiod) until they had eight true leaves (approximately 14 weeks). Petioles from side stem branches (the three oldest non-senescent branches) were then excised at approx. 1 cm from the main stem and a 10 cm section of petiole prepared. Both ends were sealed with parafilm and the petioles placed on moist chromatography paper (Whatman 3MM, Fisher Scientific, UK) in a clear plastic box (three stems per box). An agar plug (4 mm) taken from the leading edge of an actively growing

colony of *S. sclerotiorum* isolate L6 on glucose rich medium was then placed mycelium side down in the centre of each of the detached petioles. Following incubation at 15°C for 3 days in a controlled environment room (12 h photoperiod), the length of the resultant lesions was measured. Mock (control) inoculations were set up using 4 mm plugs of clean glucose rich agar.

The detached leaf assay was based on the methods published by Garg *et al*., (2008) and Mei *et al*., (2011) with the exception that ascospores were used rather than mycelium. *Brassica* plants were grown in a glasshouse (20°C, 16 h photoperiod) until the first two true leaf stage (approx. 4 weeks) after which leaves 1 and 2 were detached from the test plant, blotted dry and placed on tap water agar (8 g L^{-1}) in a propagator (35 x 23 cm, Sankey, UK) containing 600 ml of agar, 24 leaves per propagator. Inoculum was prepared by placing a section (approx. 4.5 cm^2) of filter paper containing ascospores of *S. sclerotiorum* isolate L6 in a 50 ml tube containing 8 ml of sterile 50% potato dextrose broth (PDB; Formedium, UK). The tube was then shaken vigorously for approx. 1 min to break up the filter paper and the slurry filtered through Miracloth (Merck Millipore, UK) to remove paper fragments. The resultant spore suspension was adjusted to a concentration of 1 x 10^5 ml^{-1} using a haemocytometer. Two 15 µl drops of this ascospore suspension were pipetted onto the adaxial side of each *Brassica* leaf (one on each side of the mid vein), and incubated at 20°C for 3 days in a controlled environment room (12 hour photoperiod). Leaves were then photographed and the area of each lesion measured using ImageJ software (Schneider *et al.*, 2012). Mock (control) inoculations were set up using 50% PDB only.

178 **Screening wild *Brassica* lines for resistance to *S. sclerotiorum***

179

180 ***Resistance screen 1***

181 49 lines from the wild *Brassica* 'C genome' diversity set and seven *B. carinata* lines from the
182 Warwick Genetic Resources Unit were screened for *S. sclerotiorum* resistance using both leaf
183 and petiole assays as described above along with three previously tested *B. napus* lines (line
184 57, susceptible; line 58, highly susceptible; line 59, partially resistant; Table 2; Taylor *et al.*,
185 2015). For the petiole assay, four replicate experiments were carried out using two plants per
186 line and three inoculated petioles per plant for each experiment giving a total of 24
187 measurements per line for the statistical analysis. Each box contained all three petioles from a
188 single plant, boxes were positioned in the growth room using an alpha lattice design and data
189 were analysed by ANOVA using Genstat 18th Edition (Payne *et al.*, 2009) with replicate
190 experiment, position and replicate plant included as factors. For the leaf assay, there were four
191 replicate experiments, each consisting of eight inoculated leaves (from four plants) for each
192 line giving a total of 64 measurements per line for the statistical analysis. Each propagator
193 contained all eight leaves from three different lines, and positions within the growth room were
194 randomised using an alpha lattice design with data analysed by ANOVA using Genstat as
195 described for the detached petiole assay. Differences in lesion size (petiole assay) or lesion area
196 (leaf assay) between lines were considered significant if they were larger than the overall
197 calculated LSD value ($P < 0.05$).

198

199 ***Resistance screen 2***

200 The 20 *B. napus* lines (61-77; Table 2) which had shown a range of resistance / susceptibility
201 responses in previous mature plant tests (Taylor *et al.*, 2015) were screened for *S. sclerotiorum*
202 resistance using both leaf and petiole assays as well as five selected lines from resistance screen

1 (Line 3, partial resistance in both petiole and leaf assays; Lines 9 and 14, high level resistance in petiole assay; Lines 10 and 19, susceptible in both assays), seven fixed doubled haploid lines derived from Lines 3, 9, 14 through crossing with a rapid cycling *Brassica* line as well as this parent line itself (Table 2). Replication, experimental design and data analysis were as described for resistance screen 1.

Spearman's rank correlations were calculated using Genstat in order to examine the relationship between leaf and petiole lesion size for both *S. sclerotiorum* resistance screens. Similarly, the lesion sizes previously recorded for the 20 *B. napus* lines in the previous mature plant test (Taylor *et al.*, 2015) were compared with the data from the detached leaf and petiole assays of the same lines in resistance screen 2. Correlations between resistance screens 1 and 2 were also analysed in the same way.

Comparison of aggressiveness of *S. subarctica* and *S. sclerotiorum* isolates

The aggressiveness of 12 *S. subarctica* isolates was compared with three *S. sclerotiorum* isolates (Table 1) that were previously identified as being of high (P7), medium (L44) or low (DG4) aggressiveness on semi-mature plants of *B. napus* (oilseed rape cv. Temple), *B. oleracea* (broccoli cv. Beaumont), and *B. rapa* (turnip cv. Manchester; Taylor *et al*., 2015). This was done using the detached petiole assay and the same *B. napus*, *B. oleracea* and *B. rapa* host cultivars as used previously. Three replicate experiments were carried out using two plants per treatment and three petioles per plant for each experiment. Each box contained all three petioles from a single plant, boxes were positioned in the growth room using a randomised block design and data analysed using ANOVA in Genstat with replicate, block and position as factors. ANOVAs were carried out to assess the effect of isolate, *Brassica* type and any interaction. Differences in lesion size between isolates were considered significant if they were larger than

the overall calculated LSD value ($P < 0.05$). To generate means of *Brassica* type, isolate was removed as a factor from the ANOVA analysis.

Results

Screening wild *Brassica* lines for resistance to *S. sclerotiorum*

Resistance screen 1

In the detached petiole assay, significant differences in lesion size were identified between the 60 *Brassica* lines ($P < 0.001$, Fig. 1a; Fig. 2abc). The most resistant lines were 14 (*B. incana*) and 9 (*B. cretica*) which had mean lesion sizes of 3.1 mm and 11.4 mm, respectively. The most susceptible lines were 55 and 54 (both *B. carinata*) with mean lesion sizes of 94.4 and 88.1 mm, respectively. The majority of the wild *Brassica* lines (47 out of 56) were significantly more resistant to *S. sclerotiorum* than the elite winter OSR cultivar Temple (line 57, mean lesion size 71.9 mm, Fig. 1a). Of the top ten most resistant lines, five were *B. incana* and two were *B. cretica*.

In the detached leaf assay, significant differences in lesion areas were identified between the 60 *Brassica* lines ($P < 0.001$, Fig. 1b, Fig. 2 def). The most resistant lines in this assay were 39 (wild *B. oleracea*) and 17 (*B. incana*) with mean lesion areas of 32.2 and 50.4 mm^2, respectively (Fig. 1b). The most susceptible lines were 18 (*B. incana*) and 7 (*B. cretica*) with mean lesion areas of 221.2 and 212.6 mm^2, respectively. Only line 39 (wild *B. oleracea*) was significantly more resistant to *S. sclerotiorum* than the elite winter OSR cultivar Temple (line 57, mean lesion area 76.4mm^2, Fig. 1b). Of the top ten most resistant lines, four were wild *B. oleracea*.

There was no significant correlation between leaf and petiole resistance to *S. sclerotiorum* in the two assays ($r = -0.021$, $P = 0.87$, Fig. S1a). However, some lines performed well in both tests, in particular line 3 (*B. bourgeai*, lesion sizes 19.4mm and 62.2mm^2 in petiole and leaf assays, respectively, Fig. 1ab).

Resistance screen 2

In the detached petiole assay, significant differences in lesion size were identified between the 33 *Brassica* lines ($P < 0.001$, Fig. 3a). The most resistant lines were 14 (*B. incana*) and 81 (*B. cretica*, DH line) with mean lesion sizes of 21.3 and 29.4 mm, respectively. The data was consistent and significantly correlated with the results of resistance screen 1 for the eight lines that were evaluated in both tests ($r = 0.75$, $P = 0.012$, Fig. S1b), with lines 14, 3 and 9 identified as being more resistant to *S. sclerotiorum*. Line 14 showed a very high level of resistance in both resistance screens 1 and 2 with mean petiole lesion sizes of 3.1 mm and 21.3 mm, respectively. Overall lesion sizes were greater in screen 2 than in screen 1.

In the detached leaf assay, significant differences in lesion area were observed between the 33 *Brassica* lines ($P < 0.001$, Fig. 3b) with the most resistant lines identified as 63 and 59 (both *B. napus*) with mean lesion areas of 28.4 and 33.4 mm^2, respectively (Fig. 3b). Again, the data was broadly consistent with resistance screen 1 but the correlation fell just below the level of significance ($r = 0.48$, $P = 0.054$, Fig. S1c). Line 14, which showed a high level of petiole resistance in both resistance screens, was only partially resistant in the leaf assay (mean lesion area 64.4 mm^2) and quite susceptible in resistance screen 1 (mean lesion area 147.6 mm^2). Again, as for resistance screen 1, no significant correlation was found between leaf and petiole resistance ($r = -0.24$, $P = 0.051$, Fig S1d).

As the 20 *B. napus* lines used in resistance screen 2 had previously been evaluated for *S. sclerotiorum* resistance in a mature plant test (Taylor *et al.*, 2015), direct comparisons could be made with the detached leaf and stem assays reported here. A significant correlation was evident between lesion size in the previous mature plant data and the detached petiole assay ($r = 0.50$, $P = 0.009$, Fig. S1e), but not with those from the detached leaf assay ($r = 0.14$, $P = 0.15$, Fig. S1f).

Comparison of aggressiveness of *S. subarctica* and *S. sclerotiorum* isolates

Using the detached petiole assay, 11 of the 12 *S. subarctica* isolates were pathogenic on broccoli, turnip and OSR (Fig. 4a) with isolate ENG34 (from buttercup) failing to initiate lesions. This isolate was also noted to be slow-growing in culture. There were significant differences in lesion size between the different crop types ($P < 0.001$) and pathogen isolates ($P < 0.001$) and a crop type x isolate interaction was also observed ($P < 0.001$). Overall, the majority of the *S. subarctica* isolates were significantly less aggressive than the *S. sclerotiorum* isolates (Fig. 4a). On broccoli, *S. sclerotiorum* P7 was the most aggressive isolate resulting in a mean lesion size of 64.1 mm but this was not significantly different from *S. subarctica* isolates SC25 (mean lesion size 61.9 mm) and SC61 (mean lesion size 51.6 mm, Fig. 4a). On OSR, *S. subarctica* SC61 was the most aggressive isolate, resulting in a significantly greater mean lesion size (55.1 mm) than any other isolate (Fig. 4a). On turnip, *S. sclerotiorum* isolate P7 was again the most aggressive isolate resulting in a significantly greater mean lesion size (93.9 mm) than any other isolate (Fig. 4a). The most aggressive *S. subarctica* isolate was SC61 (mean lesion size 74.3mm), significantly greater than any other *S. subarctica* isolate but not significantly different from *S. sclerotiorum* isolates L44 and DG4 (Fig. 4a). Across all crop types, *S. subarctica* ENG10 from buttercup was consistently the least aggressive isolate. Comparing susceptibility to *S. subarctica* across crop types, significant differences were observed ($P < 0.001$) with turnip being the most susceptible (Fig. 4b). The order of susceptibility between OSR and broccoli however varied between isolates.

Discussion

There have been few studies examining wild *Brassica* species as sources of resistance to *S. sclerotiorum* and none have investigated resistance to a UK or European isolate. In this study, two rapid and reproducible assays identified a high level of resistance to a UK isolate within a variety of wild *Brassica* lines, particularly line 14 (*B. incana*) and line 81 (*B. cretica*; DH of line 9) which both showed a high level of petiole resistance. Line 14, also exhibited partial leaf resistance in resistance screen 2. As line 81 is a DH line, the resistance should be genetically fixed, which should allow a more straight-forward route for introgression into *B. napus*. In resistance screen 2, lines 14 and 81 exhibited a significantly higher level of resistance in petiole tests compared with *B. napus* line 62, a line which was previously identified as the most resistant within 96 lines from a *B. napus* diversity set (line 69; Taylor *et al.*, 2015). To our knowledge, this is the first report of *S. sclerotiorum* resistance in *B. cretica*, which hence provides another potential source of useful breeding material. *B. cretica* has not been widely studied and is lacking in genomic information although it has been reported that this species did not demonstrate any resistance to *Verticillium* wilt (Happstadius *et al.*, 2003). Furthermore, 47 lines (in resistance screen 1) were significantly more resistant than the elite winter OSR cultivar Temple, hence providing a range of potential sources of resistance.

In this study, lesion sizes in the petiole tests ranged from 0.3 cm in the most resistant line to 9.4 cm in the most susceptible. By comparison, studies using a similar method resulted in lesion sizes of 2.2 to 6.6 cm for cultivated and wild species of *B. rapa, B. oleracea, B. napus, B, juncea* and *B. carinata* (Mei *et al*., 2012), 2.5-10 cm in *B. oleracea* (Mei *et al.*, 2013), 3.1-13.0 cm in *B. napus* (Wei *et al.*, 2014) and 3.5-8.2 cm in *B. napus* lines with resistance introgressed from *B. oleracea* (Ding *et al.*, 2013). These results suggest that firstly, the most resistant lines identified here are comparable to, if not more resistant than, those reported by

other researchers. Secondly the results confirm previous reports that higher levels of resistance can more often be found in wild *Brassica* compared to cultivated species (Mei *et al.*, 2011, Uloth *et al.*, 2013, Ding *et al.*, 2015, You *et al.*, 2016). Five of the ten most resistant lines in the petiole test were *B. incana* which was also identified as a source of SSR resistance by Mei *et al.*, (2011) following toothpick inoculation of mature plant stems from a wide range of *Brassica* species. In another study, where stems of mature plants were inoculated with agar plugs of *S. sclerotiorum*, *B. incana* was again shown to have good resistance to SSR although higher levels were found in *Raphanus raphanistrum, B. carinata* and *R. sativus* (Uloth *et al*., 2013). Using the same method, good resistance has also been identified in lines of *B. nigra* and *B. carinata* (Navabi *et al.*, 2010). Overall therefore, there is strong evidence that *B. incana* lines can provide useful sources of resistance against *S. sclerotiorum* while added value may also be gained through its resistance to cabbage whitefly (Pelgrom *et al.*, 2015). In contrast to some of the above studies however, none of the *B. carinata* lines used exhibited resistance to SSR in the tests reported here. This may be due to differences between the *S. sclerotiorum* isolates from the UK and Canada or differences between *B. carinata* accessions. It has been observed previously that different accessions of *B. carinata* and other wild species can be either highly resistant or highly susceptible to SSR (Uloth *et al.*, 2013).

Whilst resistance to SSR has been found in wild *Brassica* species previously, this is the first report of resistance to a UK isolate of *S. sclerotiorum*, hence confirming that such wild sources of resistance could be suitable for development for *Brassica* crops in the UK and potentially the rest of Europe. It has been suggested previously that it is critically important to identify resistance to 'local' isolates of *S. sclerotiorum* (Taylor *et al*., 2015). This may be because the pathogen is highly diverse and although a few genotypes are widespread within countries and very occasionally between countries, the majority are confined to specific fields or growing areas (Clarkson et al., 2017). The importance of using local isolates in resistance

screening programmes was demonstrated in a previous study where Mystic, a *B. napus* cultivar shown to be consistently resistant to *S. sclerotiorum* isolates from Australia (Garg *et al.*, 2008, Garg *et al.*, 2010a, Uloth *et al.*, 2013) was highly susceptible to isolates from the UK (Taylor *et al.*, 2015). Moreover, *S. sclerotiorum* isolates from different regions may also vary in their response to environmental conditions under which the resistance test is performed. This effect was demonstrated by one study where lines which had previously been shown to be resistant were found to be highly susceptible to the same *S. sclerotiorum* isolate (You *et al*., 2016, Uloth *et al*., 2013).

In addition to the detached petiole tests, the same set of *Brassica* lines was assessed for *S. sclerotiorum* resistance using a detached leaf assay. In contrast to all previous detached leaf studies which have used either an agar plug (Mei *et al.*, 2011) or macerated mycelial fragments on attached cotyledons as inoculum (Garg *et al.*, 2008), ascospores were used which are normally the primary source of *S. sclerotiorum* infections in the field. Although ascospores take a significant amount of time to produce, they can be stored for several years on dry filter paper at 4°C. Using this test, only a single wild *Brassica* line (39, wild *B. oleracea*) was significantly more resistant than the commercial *B. napus* variety Temple (line 57). *B. napus* line 59 which showed consistent resistance over the two leaf assays also showed partial resistance in previous work using stem inoculation of mature plants (line 83; Taylor *et al.*, 2015). As observed in the petiole tests, some *S. sclerotiorum* resistance was also evident in *B. incana* lines using the leaf test although a different line (line 17) was the most resistant. This again indicates the value of this species as a source of resistance, further supporting the results of previous work (Mei *et al.*, 2011, Mei *et al.*, 2013, Ding *et al.*, 2015). This is also the first study to identify partial leaf resistance to *S. sclerotiorum* in wild *B. oleracea*, *B. macrocarpa*, *B. vilosa* and *B. bourgeai* and to our knowledge, this is also the first study to investigate leaf or stem resistance in *B. hilarionis*, *B. macrocarpa* and *B. atlantica*. Whilst no high level

resistance was observed in these species, some moderate stem resistance was observed in *B. atlantica* and *B. macrocarpa*, potentially presenting alternative sources of resistance for future breeding programmes. This resistance would need to be introgressed into *B. napus*, something which has been done successfully for resistance from wild crucifers (Garg *et al*., 2010a).

No correlation was found between leaf and stem resistance to *S. sclerotiorum* in this study, suggesting that resistance in these two different tissue types may be controlled by different genes or pathways. This confirms the results of previous work where results using a toothpick inoculation method on mature *B. napus* stems were different from those using an agar plug method on detached leaves (Zhao & Meng, 2003). Similarly, Uloth *et al.*, (2013) and You *et al.*, (2016) observed different responses between stems of field grown mature *Brassica* plants inoculated with agar plugs of *S. sclerotiorum* and leaves of the same plants which had been naturally infected by ascospores, and also concluded that genetic control of leaf and stem resistance is probably different. In contrast, two studies have demonstrated weak correlations between the size of lesions produced on detached leaves inoculated with agar plugs, compared with those resulting from inoculation of mature stems using a toothpick method (Mei *et al.*, 2011) or detached stems inoculated with an agar plug (Mei *et al.*, 2013).

Although the detached stem/petiole inoculation method has been widely used in screening for *S. sclerotiorum* resistance, few studies have compared this approach with stem inoculation of intact mature plants. Using a set of 20 *B. napus* lines, a significant correlation was observed between results from the detached petiole test here and those from the stem inoculation of mature plants in a previous study (r = 0.50; Taylor *et al*., 2015) while the only previous comparison reported correlations of 0.21 or 0.29 (Wei *et al.*, 2014). The detached petiole method is therefore a valid, and rapid resistance screening approach which could be employed as a primary screen for large numbers of breeding lines in order to pre-select a smaller number of lines for testing using the mature stem inoculation method. The detached

petiole assay is also particularly applicable to screening for resistance relevant to OSR where infections are generally initiated in the stem while the detached leaf assay may be more applicable to leafy *Brassica* crops.

This is also the first study to evaluate the relative aggressiveness of a range of *S. subarctica* isolates and used the same *Brassica* species and cultivars employed previously to compare aggressiveness of *S. sclerotiorum* isolates (Taylor *et al*., 2015). This involved inoculation of stems of immature plants rather than detached petioles, but results were consistent in that P7 was generally the most aggressive of the three *S. sclerotiorum* isolates included in both studies and *B. rapa* (turnip) was the most susceptible of the three *Brassica* species. It was shown for the first time that *S. subarctica* isolates exhibit a range of aggressiveness across *Brassica* species as observed previously in studies with *S. sclerotiorum* on different host plants (Ekins *et al.*, 2007, Otto-Hanson *et al.*, 2011, Taylor *et al.*, 2015). The reasons for this are unclear although for *S. sclerotiorum* it has been suggested that differences between isolates in oxalic acid production may be responsible (Durman *et al.*, 2005). Although it is not known whether oxalic acid is produced by *S. subarctica,* as indicated above, populations are similarly genetically diverse as reported for *S. sclerotiorum* (Clarkson *et al*., 2017) which could account for this biological variation. Overall however, the majority of the *S. subarctica* isolates were moderately or weakly aggressive compared to *S. sclerotiorum*, especially those isolated from wild buttercup and this was also a trend observed previously for *S. sclerotiorum* isolates (Taylor *et al*., 2015). Again, in studies with *S. sclerotiorum* it has been suggested that isolates from different hosts may vary in their ability to produce oxalic acid; for instance, isolates collected from lettuce produced less oxalic acid resulting in smaller lesions compared with isolates collected from sunflower or soybean (Durman *et al*., 2005). However, further studies with a greater number of isolates from different hosts would be required to test this hypothesis for *S. subarctica*. In contrast, two *S. subarctica* isolates (SC25, SC61) from

swede and potato were consistently highly aggressive across the *Brassica* hosts and were comparable with *S. sclerotiorum* isolates. This suggests that some *S. subarctica* isolates at least constitute a similar threat to crop plants and hence this pathogen should be included in plant resistance screening for crops grown in northern latitudes where *S. subarctica* is most prevalent.

In conclusion, wild *Brassica* lines showing high level resistance to *S. sclerotiorum* have been identified using rapid detached leaf and petiole assays, which constitute a useful resource for future breeding programmes of relevance to the UK and potentially Europe. It was also shown that although isolates of *S. subarctica* vary in their aggressiveness, some can cause significant disease on *Brassica* that is comparable to the most aggressive *S. sclerotiorum* isolates.

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564 **Table 1:** List of *Sclerotinia* isolates used in this study.

Isolate code	**Origin[1]**	**Host**	**Year isolated**	**Haplotype[2]**
Sclerotinia sclerotiorum				
DG4 (ENG 91)	Warwickshire, England (DG2)	Buttercup	2009	176
L6 (ENG 189)	West Sussex, England (LE1)	Lettuce	2005	3
L44 (ENG 185)	West Sussex, England (LE1)	Lettuce	2005	78
P7 (ENG 254)	Herefordshire, England (PE1)	Pea	2009	1
Sclerotinia subarctica				
HE1 (ENG 20)	Herefordshire, England	Buttercup	2009	1
ENG10	Herefordshire, England	Buttercup	2011	4
ENG8	Herefordshire, England	Buttercup	2011	8
ENG34	Herefordshire, England	Buttercup	2009	6
SC25	Isla Bend, Scotland	Potato	2012	2
SC52	Fife, Scotland	Buttercup	2012	5
SC58	Fife, Scotland	Buttercup	2012	9
SC63	Perthshire, Scotland	Pea	2012	3
SC70	Perthshire, Scotland	Pea	2012	11
SC61	East Lothian Scotland	Swede	2012	68
LST3	Tranägen, Sweden	Lettuce	2012	ND
NOR41	Rogaland, Norway	Lettuce	2012	42

565

566 [1] Locations followed by codes in brackets refer to populations characterised by Clarkson *et al*.,

567 (2013).

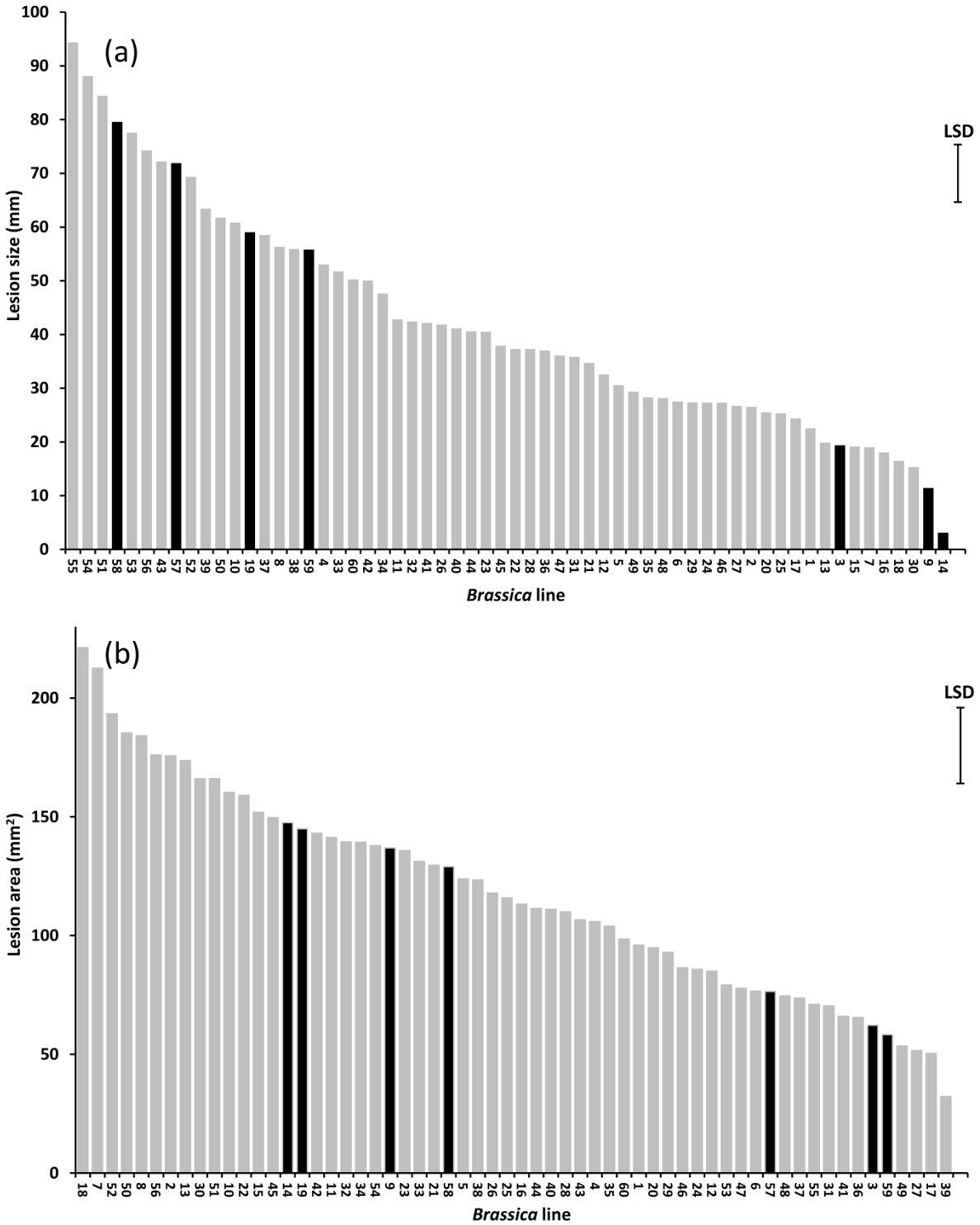
568 [2] Microsatellite haplotype as designated by Clarkson *et al*. (2013) for *S. sclerotiorum* and

569 Clarkson et al. (2017) for *S. subarctica*.

570 **Table 2:** List of *Brassica* lines used in this study.

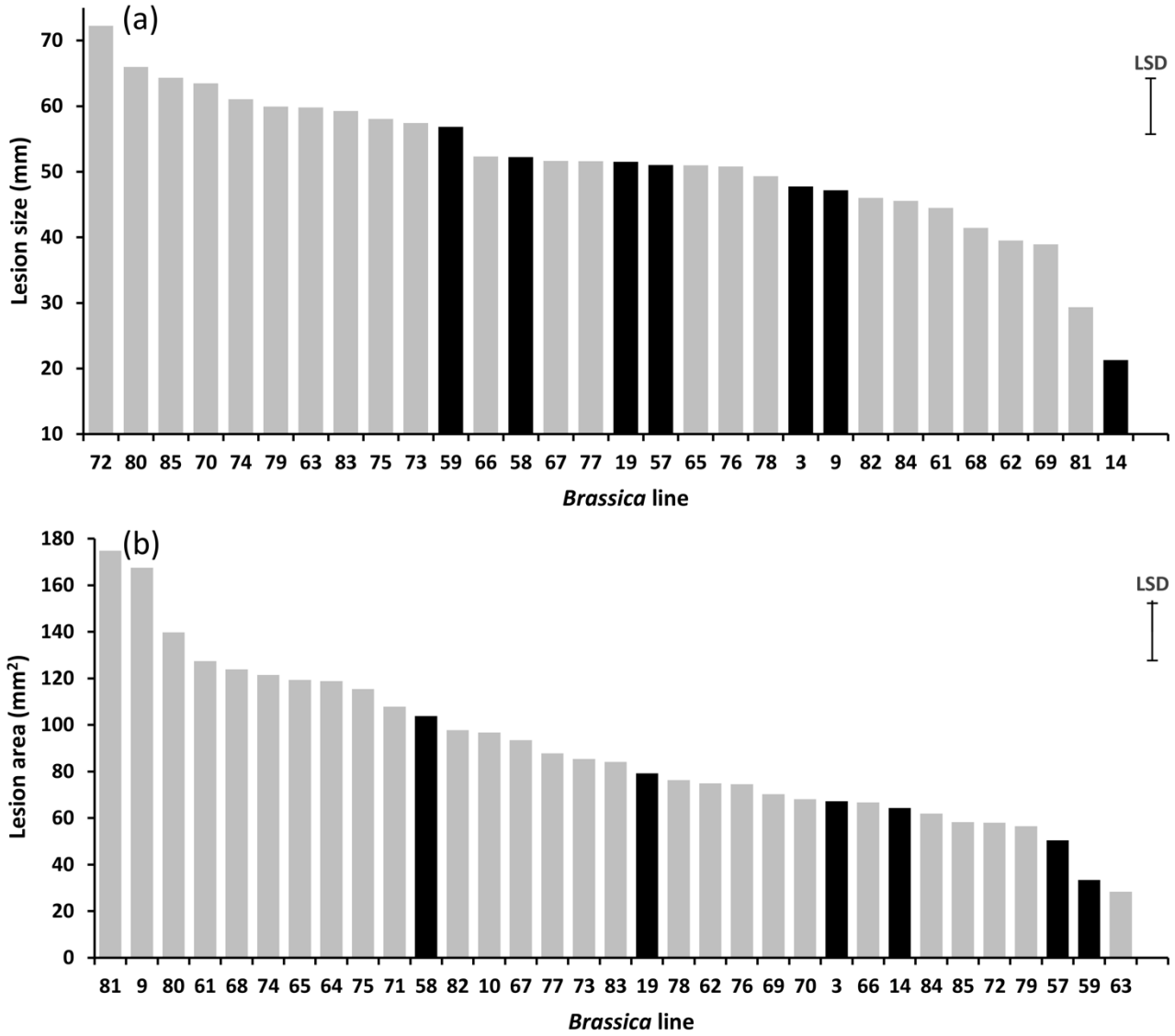
Line No.	Brassica species[1]	Resistance screen no.	Line No.	Brassica species[1]	Resistance screen no.
1	*B. atlantica*	1	45	*B. rupestris*	1
2	*B. atlantica*	1	46	*B. vilosa*	1
3	*B. bourgeai*	1&2	47	*B. vilosa*	1
4	*B. cretica*	1	48	*B. vilosa*	1
5	*B. cretica*	1	49	*B. vilosa*	1
6	*B. cretica*	1	50	*B. carinata*	1
7	*B. cretica*	1	51	*B. carinata*	1
8	*B. cretica*	1	52	*B. carinata*	1
9	*B. cretica*	1&2	53	*B. carinata*	1
10	*B. hilarionis*	1&2	54	*B. carinata*	1
11	*B. hilarionis*	1	55	*B. carinata*	1
12	*B. incana*	1	56	*B. carinata*	1
13	*B. incana*	1	57	*B. napus* (27, cv. Temple)	1&2
14	*B. incana*	1&2	58	*B. napus* (41)	1&2
15	*B. incana*	1	59	*B. napus* (83)	1&2
16	*B. incana*	1	60	*B. napus*	1
17	*B. incana*	1	61	*B. napus* (18)	2
18	*B. incana*	1	62	*B. napus* (69)	2
19	*B. insularis*	1&2	63	*B. napus* (87)	2
20	*B. macrocarpa*	1	64	*B. napus* (36)	2
21	*B. macrocarpa*	1	65	*B. napus* (8)	2
22	*B. macrocarpa*	1	66	*B. napus* (91)	2
23	*B. macrocarpa*	1	67	*B. napus* (20)	2
24	*B. macrocarpa*	1	68	*B. napus* (33)	2
25	*B. macrocarpa*	1	69	*B. napus* (60)	2
26	*B. macrocarpa*	1	70	*B. napus* (3)	2
27	*B. macrocarpa*	1	71	*B. napus* (37)	2
28	*B. macrocarpa*	1	72	*B. napus* (56)	2
29	*B. macrocarpa*	1	73	*B. napus* (74)	2
30	wild *B. oleracea*	1	74	*B. napus* (11)	2
31	wild *B. oleracea*	1	75	*B. napus* (89)	2
32	wild *B. oleracea*	1	76	*B. napus* (17)	2
33	wild *B. oleracea*	1	77	*B. napus* (19)	2
34	wild *B. oleracea*	1	78	*B. bourgeai* (DH of line 3)	2
35	wild *B. oleracea*	1	79	*B. bourgeai* (DH of line 3)	2
36	wild *B. oleracea*	1	80	*B. cretica* (DH of line 9)	2
37	wild *B. oleracea*	1	81	*B. cretica* (DH of line 9)	2
38	wild *B. oleracea*	1	82	*B. cretica* (DH of line 9)	2
39	wild *B. oleracea*	1	83	*B. cretica* (DH of line 9)	2
40	wild *B. oleracea*	1	84	*B. incana* (DH of line 14)	2
41	wild *B. oleracea*	1	85	*B. oleracea* (rapid cycling line)	2
42	*B. montana*	1	45	*B. rupestris*	1
43	*B. montana*	1	46	*B. vilosa*	1
44	*B. rupestris*	1	47	*B. vilosa*	1

571 [1] Numbers in brackets refer to previous line number designation in Taylor *et al*., (2015)

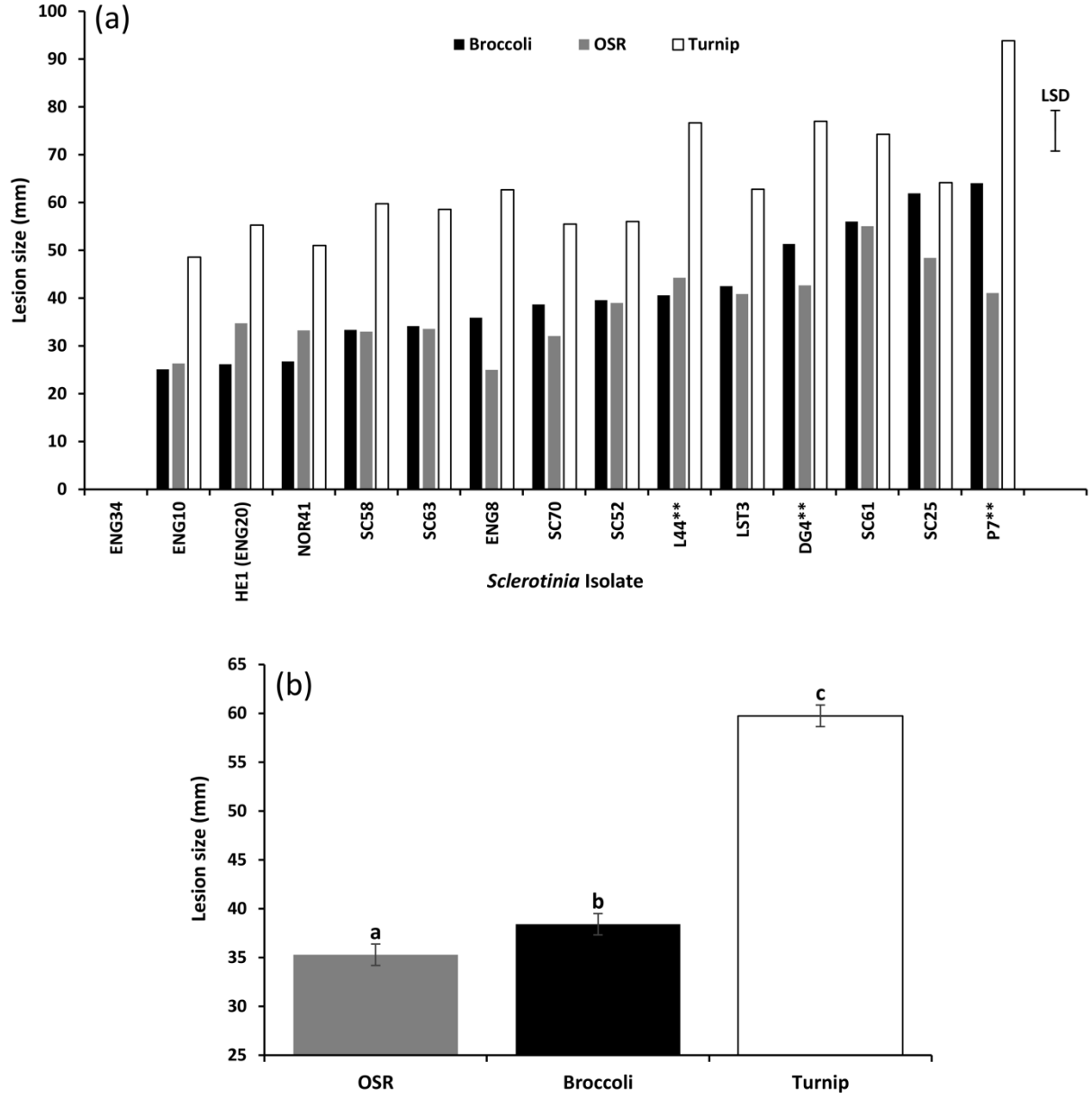


572 **Figure 1**: Mean lesion size/area for 60 *Brassica* lines following inoculation with *S.*
573 *sclerotiorum* for (a) detached petiole and (b) detached leaf assays in resistance screen 1.
574 Error bars represent the least significant difference (LSD, 5% level) following ANOVA
575 analyses. Black bars indicate lines used in both resistance screens 1 and 2.

576 **Figure 2**: Photographs of petioles (a-c) and leaves (d-f) of *Brassica* lines inoculated with *S.*
577 *sclerotiorum* illustrating the range of phenotypes. (a) line 14, highly resistant; (b) line 29,
578 intermediate resistance; (c) line 43, highly susceptible; (d) line 39, highly resistant; (e) line
579 23, intermediate resistance; (f) line 52, highly susceptible.



580 **Figure 3**: Mean lesion size/area for 20 *B. napus* lines, 12 selected wild *Brassica* lines and a
581 rapid cycling *B. oleracea* line following inoculation with *S. sclerotiorum* for (a) detached
582 petiole and (b) detached leaf assays in resistance screen 2. Error bars represent the least
583 significant difference (LSD, 5% level) following ANOVA analyses. Black bars indicate lines
584 used in both resistance screens 1 and 2.



585 **Figure 4**: Mean lesion size for *B. napus* (oilseed rape cv. Temple), *B. oleracea* (broccoli cv.
586 Beaumont), and *B. rapa* (turnip cv. Manchester) inoculated with three *S. sclerotiorum* and 12
587 *S. subarctica* isolates (a) for all isolates; (b) for 12 *S. subarctica* isolates across each crop
588 type in detached petiole tests. Error bars in (a) represent the least significant difference
589 (LSD, 5% level) following ANOVA analyses. Letters in (b) indicate a significant difference
590 based on LSD values following ANOVA analysis. ** indicates *S. sclerotiorum* isolates.

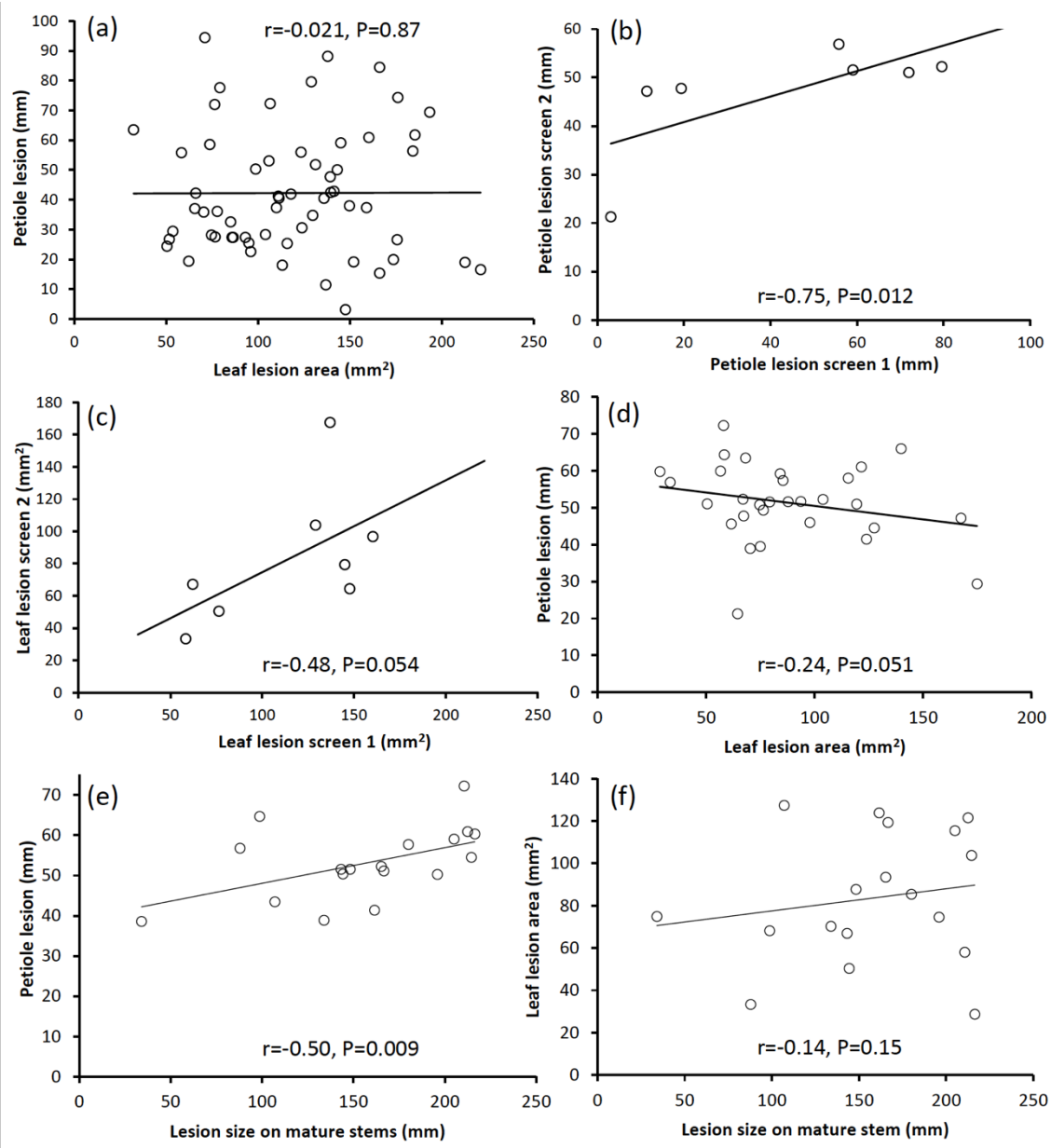


Figure S1: Correlation plots for traits measured in this study. (a) *S. sclerotiorum* lesion size on petioles vs lesion area on leaves for resistance screen 1; (b) comparison of *S. sclerotiorum* lesion size on petioles in resistance screens 1 and 2; (c) comparison of *S. sclerotiorum* lesion area on leaves between resistance screens 1 and 2; (d) *S. sclerotiorum* lesion size on petioles vs lesion area on leaves for resistance screen 2; (e) comparison of *S. sclerotiorum* lesion size on petioles and mature plant stems (Taylor et al., 2015) for 20 *B. napus* lines; (f) comparison of *S. sclerotiorum* lesion area on leaves and lesion size on mature plant stems (Taylor *et al*., 2015) for 20 *B. napus* lines.