DOI: http://dx.doi.org/10.22092/cbj.2011.100364

Reaction of Brassica species to *Sclerotinia sclerotiorum* applying inoculation techniques under controlled conditions

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Received: July 2011

ABSTRACT

Rahmanpour, S., D. Backhouse, and H. M. Nonhebel. 2011. Reaction of Brassica species to *Sclerotinia sclerotiorum* applying inoculation techniques under controlled conditions. **Crop Breeding Journal 1 (2): 143-149.**

Oilseed rape is economically affected by stem rot caused by *Sclerotinia sclerotiorum* worldwide. Glucosinolates are the specific secondary metabolites of Brassica plants that appear in different profiles of each species. Their hydrolysis products have biocidal activity and may play a role in resistance against plant pathogenic fungi. The resistance of oilseed rape (*Brassica napus*) cultivars and two other Brassica species (*B. nigra* and *Sinapis alba*) was evaluated employing leaf disc inoculation, and oxalic acid and fungal inoculums on leaves of intact plants under controlled conditions. By using leaf disc inoculation, three plant ages were used to compare their reactions against the pathogen. No significant differences between genotypes were observed in this method. However, results demonstrated significant differences in main effects of wounding and plant age. The two intact plant inoculation techniques (oxalic acid and fungal mycelium) resulted in significant differences between genotypes in reaction to the disease. Furthermore, the oxalic acid assay followed the same pattern as fungal inoculations. Among the oilseed rape cultivars, AV-Sapphire and AG-Castle were the most resistant and susceptible genotypes, respectively. Brassica species differed significantly in their reaction to disease, in both wounded and non-wounded leaves with fungal mycelium inoculation and oxalic acid. Overall, non-significant differences between Brassica genotypes showed the unreliability of the leaf disc assay, whereas leaf inoculation of intact plants by means of either oxalic acid or fungal mycelium demonstrated significant differences in lesion size among Brassica cultivars and species.

Key words: Brassicaceae, oilseed rape, stem rot, resistance, pathogenicity factor, glucosinolates.

INTRODUCTION

Stem rot of oilseed rape, caused by *Sclerotinia sclerotiorum* (Lib) de Bary, is one of the most serious diseases of the crop and leads to significant losses of seed yield worldwide (Zhao *et al.*, 2004). Stem rot has become one of the most serious disease problems in oilseed rape-growing areas in Australia (Hind-Lanoiselet and Lewington, 2004). The ability of *S. sclerotiorum* to attack over 400 species of plants in 75 different families (Boland and Hall, 1994) has made it one of the most non-specific and invasive plant pathogens (Purdy, 1979). Brassica cultivars and species demonstrate different types of reactions against fungal diseases.

Although several methods have been developed for controlling the disease, including cultural and chemical techniques, strategies for selecting resistant hosts are considered the most economic and sustainable control means (Garg *et al.*, 2008). According to Garg *et al.* (2008), resistance against stem rot will be most effective when used in conjunction with cultural practices such as crop rotation. To develop resistant or tolerant genotypes, oilseed rape breeders have focused on morphological (e.g., stem diameter, Li *et al.*, 2006; or epicuticular wax, Skoropad and Tewari, 1977) and physiological (e.g., phytoalexins, Toal and Jones, 1999; or oxalate oxidase enzyme, Dong *et al.*, 2008) traits of host genotypes to improve resistance to stem rot in oilseed rape.

Resistance can be assessed in the field, depending on sources of inoculum and weather conditions (Bradley *et al.*, 2006; Li *et al.*, 2007). Variable responses of oilseed rape germplasm to inoculation with *S. sclerotiorum*, especially in field evaluations, are one of the main limitations for assessing resistance. However, there is no clear reason for this variability (Li *et al.*, 2007; Garg *et al.*, 2008). Thus an efficient, reliable and inexpensive screening method that would allow large-scale screening of canola germplasm and cultivars for sclerotinia stem rot resistance is needed to accelerate the development of resistant canola cultivars (Bradley *et al.*, 2006). Several methods have been used to identify resistance to *S. sclerotiorum* in canola. They include detached leaf inoculation (Bradley *et al.*, 2006), screening

against oxalic acid, which is a fairly well-known pathogenicity factor for the pathogen (Cessna *et al.,* 2000), petiole inoculation (Zhao *et al.,* 2004; Bradley *et al.,* 2006), leaf inoculation, stem inoculation (Chaocai, 1995; Li *et al.,* 2006) and, more recently, cotyledon inoculations (Garg *et al.,* 2008). Variability of responses of oilseed rape germplasm to sclerotinia stem rot using different methods and experiments is common (Wegulo *et al.,* 1998).

The most sophisticated method for assessing resistance in the laboratory is using ascospores to infect aging petals, which are then placed on detached leaf or stem pieces; the rate of lesion expansion is then measured (Seguin-Swartz and Lefol, 1999). Most researchers avoid this method because it requires laborious procedures to produce the ascospores. In most cases, lesion length or diameter is measured. Sedun *et al.* (1989) differentiated Brassica species/cultivars using the rate of stem lesion expansion as an indicator of resistance. There does not appear to have been any systematic attempt to categorize components of resistance to sclerotinia stem rot in Brassica germplasm.

Use of *S. sclerotiorum* mycelium or the pathogenicity factor oxalic acid has formed the basis of several inoculation techniques for evaluating the reaction of Brassica genotypes in a controlled environment. Researchers have used oxalic acid extensively for screening oilseed rape genotypes in the greenhouse. Bolton *et al.* (2006) reviewed the role of oxalic acid secreted by the fungus during pathogenesis.

The relative importance of oxalic acid in pathogenesis has been reassessed by producing mutants of *S. sclerotiorum* specifically lacking the ability to synthesize oxalic acid. These ¯OA mutants were non-pathogenic in bioassays with *Phaseolus vulgaris* (Godoy *et al.,* 1990). Hypo-virulent isolates of *S. sclerotiorum* in one study differed from the virulent isolate in their reduced oxalic acid accumulation in potato dextrose broth, and their reduced pathogenicity on canola (Li *et al.,* 2003). In studies on Brassica species, oilseed rape cultivars and near-isogenic lines, a negative correlation was observed between tissue response and *S. sclerotiorum* derived oxalic acid (Mullins and Jones, 1995).

Wegulo *et al.* (1998), who tested greenhouse inoculation techniques, found that oxalic acid assays showed the most reliable results when immersing cut stems into oxalic acid solutions. They measured lesion lengths on treated stems or pink pigment levels in the solutions. The methods employed included mycelial inoculation of stems, detached leaves and foliage, and oxalic acid assays. In another method, resistance of sunflower (*Helianthus annuus*) leaf cells was evaluated to lysis in various concentrations of oxalic acid (Noyes and Hancock, 1981). Tu (1989) identified tolerant and susceptible cultivars of white bean (*Phaseolus vulgaris*) based on their differences in the rate of diffusion of leaf tissue oxalic acid. These studies revealed that oxalic acid is likely a useful tool for screening cultivars and genotypes for their reaction to *S. sclerotiorum* (Wegulo *et al.,* 1998).

Relationships between glucosinolates (GSLs) (brassicas' specific secondary metabolites) and the level of resistance of oilseed rape to sclerotinia stem rot have been discussed by researchers. Upon being wounded by infection or pest attack, or any mechanical injury, GSLs are hydrolyzed (Mithen, 2001). Glucosinolate degradation products, in particular the Isothiocyanates (ITCs), are known to have broad biocide activity including insecticide, nematicide, fungicide, antibiotic and phytotoxic effects (Brown and Morra, 1997).

The correlations between brassicas' GSL content and disease resistance levels have been studied (Tierens *et al.,* 2001), and some GSL-resistance interactions have been observed. For instance, the host range of *Plasmodiophora brassicae* depends on root concentrations of 2-OH-2-phenylethyl GSL (Ludwig-Muller *et al.,* 1999). Li *et al.* (1999) observed a positive correlation between *S. sclerotiorum*-induced production of indole GSLs and 2-phenylethyl GSL in moderately resistant line 014 (*B. napus* L.) and resistance to the pathogen. According to the dual inoculation system of Li *et al.* (1999), this induction (local and systemic) in pre-inoculated line 014 plants was associated with a reduction in lesion size of the second inoculum. The poor local and systemic induction of GSLs (lines 016 and 024) associated with susceptibility suggested that GSL induction may be an important marker of resistance to *S. sclerotiorum* in oilseed rape (Li *et al.,* 1999). In contrast, no correlation was observed between vegetative tissue GSL content and resistance to *S. sclerotiorum*.

The main objectives of this study were to determine the reaction of oilseed rape cultivars and brassica species to *S. sclerotiorum*, and to compare three inoculation techniques used for disease development.

MATERIALS AND METHODS

Plant materials

Seed of the Australian commercial canola (*Brassica napus* L.) cultivars AG-Castle, AV-Sapphire, Dunkeld, Oscar, and Rainbow was provided by Dovuro Seeds, Horsham, Australia. The

other two species, black mustard (*Brassica nigra* L.) and white mustard (*Sinapis alba* L.) are condiment cultivars. Plants were grown in a greenhouse (in 140-mm diameter plastic pots filled with 1 sand: 1 loam: 1 peat moss mix) for one (33 and 35 days), two (50 and 54 days) and three months (105 days) at 20-25 ˚C and natural day light.

Leaf disc inoculation assay (LDIA)

Plant materials were inoculated following the method of Reglinski *et al.* (1997) with some modifications. Young, fully expanded leaves aged 33 days and 35 days, 50 and 54 days, and 105 days were detached from plants grown in the greenhouse and transferred to the laboratory. Using a 20-mm diameter cork borer and a cutting board, leaf discs were punched out and laid on moist filter paper in 55-mm diameter Petri plates (one disc per plate). Using a sharp scalpel blade, six small injuries were made in the middle of half the discs of each cultivar. Mycelial plugs (4 mm in diameter) taken from the growing edge of 3-day-old cultures on ¼ strength potato dextrose agar were used for inoculation. Inoculum plugs were placed with the mycelium side down in the middle of each leaf disc. Control leaf discs were treated with uninfected ¼ PDA plugs. The Petri dishes were then immediately sealed with two layers of Parafilm (American National Can, Greenwich, CT, USA) to maintain high humidity, and incubated at 25˚C in darkness.

A completely randomized experimental design with three replications was used. Leaf lesions were measured 24 h after inoculation. Lesion size was used as the measure of disease. Four treatments (including wounded with/without inoculum, and non-wounded with/without inoculum) with three replications for every cultivar/species were used.

Reaction of intact plants to the pathogen

The bioassay was based on the inoculation of leaves on intact plants. For this purpose, a fully expanded leaf of normal color and shape was selected from each plant and marked for inoculation. The treatments were wounded and non-wounded intact leaves with inoculum, and wounded leaves with ¼ PDA plugs as a control. The inoculation site for each selected leaf was the first one-third from the leaf tip. Wounded and non-wounded leaves were inoculated with 4-mm diameter agar plugs containing hyphae of *S. sclerotiorum* which had been transferred from the margins of 3-day-old cultures. The mycelium-containing surface of the plugs was laid on the adaxial side of leaves. The purpose of wounding was to provide an entry point for the pathogen.

Oxalic acid treatments were applied by attaching a small hydrophilic cotton wool ball soaked in 10 mM (pH = 4) solution of the acid to the leaf surface using a small stripe of adhesive tape. Plugs of ¼ PDA without the fungal mycelium were used on the control plants. All pots were immediately covered with transparent plastic bags to keep the humidity high. The experiment was established in the evening to provide dark conditions in the greenhouse. A completely randomized experimental design with three replications was used. Infection progress was measured as the diameter of necrotic areas at the inoculation points after 36 h and 60 h of incubation.

In another experiment, 105-day-old plants were inoculated without the oxalic acid treatment. A completely randomized experimental design with eight replications was used. To study the regular incubation and measurement intervals, lesion diameter was measured after 24, 48 and 72 h. Data were analyzed using SPSS software. The Student-Newman-Keuls test was used for mean comparison to classify the reaction of genotypes.

RESULTS

Leaf disc inoculation assay (LDIA)

Infection occurred in all fungus-inoculated treatments. Growth of *S. sclerotiorum* in infected tissues of wounded treatments, as soft and necrotic areas, was faster than in un-wounded ones. Data from all six experiments were combined to look for major effects. Individual experiments were not used in the analysis; instead, a fully factorial design with cultivar, wounding and age of plants was used. Results showed that although there was no significant main effect of genotypes, the main effects of wounding and age were significant. Lesions were larger on wounded leaf discs, and they were also larger on 50-54 day-old leaves (Fig. 1).

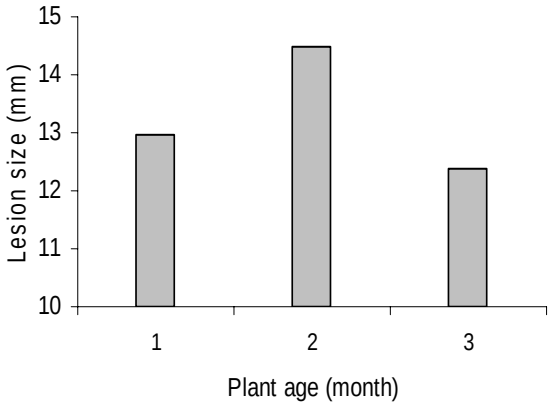

Fig. 1. Variation in lesion size on Brassica plants of different ages using wounded leaf disc inoculations by *S. sclerotiorum*. 1 = one month (33-35 days), 2 = two months (50-54 days), and 3 = three months (105 days).

Significant genotype × wounding interaction was observed. Wounding had less effect on the mustard genotypes than it did on the canola cultivars (Fig. 2). Genotype × age interaction was also significant. There was little effect of age on *S. alba*, and there was no difference between leaves 1 and 2 months old of *B. nigra* or *cv*. Rainbow (Fig. 3).

Based on a separate analysis of data, effects of genotype, wounding, and their interactions were significant for plants 1 month (33-35 days) old. The same results were obtained from leaf discs that were 2 months old (50-55 days). However, despite significant effects of genotype and wounding on 105-day-old plants in the first experiment, their interactions were not significant. Moreover, there were no significant genotypic effects on 105-day-old plants in the second experiment, despite significant effects of wounding and their interactions.

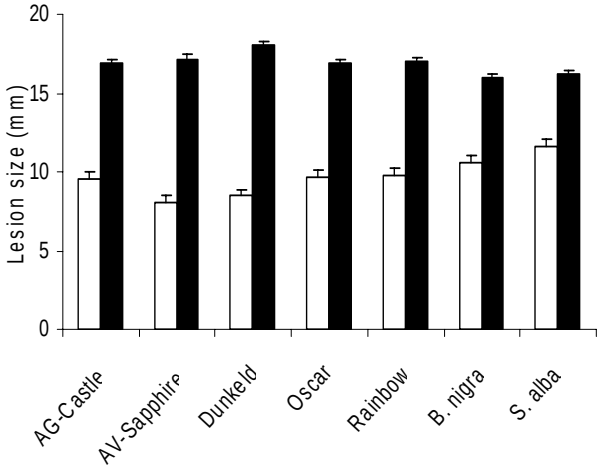

Figure 2. Lesion size on leaf discs (20 mm in diameter) of Brassica genotypes infected by *S. sclerotiorum* after 24 h incubation. Treatments: wounded (■) and non-wounded (□).

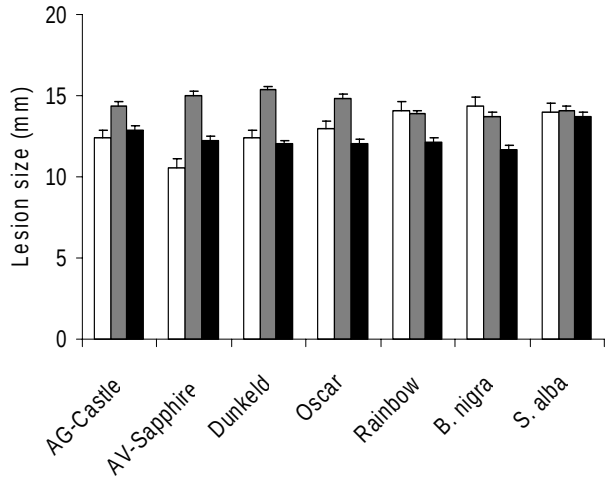

Fig. 3. Effect of age on lesion size in Brassica genotypes inoculated with *S. sclerotiorum* and incubated for 24 h using the leaf disc inoculation method. Ages: one month old (□), two months old (▒), and three months old (■).

Reaction of intact leaves to the pathogen

Leaves inoculated with *S. sclerotiorum* showed symptoms of necrosis and gray-colored lesions 60 h after inoculation. Some leaves also showed a dirty white-creamy cottony mycelium on the surface. Areas above the point of inoculation looked necrotic or wilted as a result of sclerotinia rot. In most cases, even in oxalic acid treatments, the lesions had a tendency to elongate towards the leaf petiole along the main vein. Fungus-caused lesions had a mostly necrotic soft and ovoid-shaped expansion, whereas those produced by oxalic acid looked pulled along the lateral main veins with dried necrotic areas.

In the first experiment, all treatments showed necrotic lesions around the fungal inoculation sites. There were significant differences in lesion size among genotypes in both the 36 and 60 h measurements. Lesions on wounded leaves were significantly larger than those on non-wounded leaves. In addition, clear differences were observed in genotype × wound interactions. Based on lesion diameter 36 and 60 h after inoculation, cultivars AG-Castle and Oscar were the most susceptible in the wounded treatments. Meanwhile, lesion size of *B. nigra* was the smallest, indicating its considerable resistance to disease development. The genotypes did not show significant differences among non-wounded treatments 36 h after inoculation, whereas a difference was observed at 60 h incubation time, with AV Sapphire having significantly smaller lesions than Dunkeld or Oscar (Table 1).

Results revealed that effects of genotype and wounding were significant on the rate of lesion expansion between 36 h and 60 h in wounded and non-wounded treatments, but there was no significant interaction between genotype × wounding. Based on these results, *B. nigra* and AG-Castle were the most resistant and susceptible genotypes, respectively. Among the canola cultivars, AV-Sapphire and Rainbow were more resistant.

Lesions caused by oxalic acid were compared with those on wounded leaves inoculated with *S. sclerotiorum*. The necrotic areas caused by oxalic acid were smaller in comparison to those caused by the fungus. There were significant differences among genotypes in terms of lesion size (Table 1).

The effect of inoculation type (oxalic acid or fungus), at 36 and 60 h incubation, and on lesion expansion between 36 and 60 h was significant. There was no significant interaction between genotype × inoculation type with respect to lesion size after 36 h incubation, or to lesion expansion between 36 and 60 h. This indicated that genotypic response to the two types of inoculation was similar. However, there was significant genotype × inoculation type interaction after 60 h.

Table 1. Lesion sizes of wounded/non-wounded leaves of Brassica species and cultivars inoculated with *S. sclerotiorum* mycelium plugs or its pathogenicity factor, oxalic acid.

	Lesion size (mm)/Grouping					
	Mycelial plug				Oxalic acid	
	36 hours[a]		60 hours		36 hours	60 hours
Cultivar/Species	W[b]	NW[c]	W	NW	W	W
AG-Castle	16.3d	0	33.3e	8.5[d]	12.25ab	23c
AV-Sapphire	12cd	1.7a	24.35cde	5.3a	11.17ab	18.92abc
Dunkeld	12cd	5.2ab	20cde	17.35bc	10.75ab	17.42ab
Oscar	14.4d	5ab	28.5de	16.5bc	13.58b	21.67bc
Rainbow	10.7bcd	1a	21.15cd	8.35ab	9.5ab	15.67a
B. nigra	7.7bc	0.7a	17.35bc	6.85ab	8.58a	14.58a

Means followed by the same symbol do not differ by the SNK test.
[a] Incubation time [b] Wounded leaves [c] Non-wounded leaves
[d] Could not be included in multiple comparison because there was only one replication that did not have lesions after 36 h incubation.

Generally, *B. nigra* and Rainbow genotypes had the most resistant reactions to oxalic acid, while AG-Castle proved to be the most susceptible to oxalic acid; this reflected their relative reactions to *S. sclerotiorum* (Table 1).

In the second experiment, lesions were found on all wounded treatments at 24 h incubation, but non-wounded leaves did not show disease symptoms until 48 h incubation, except for one leaf of AG-Castle (Table 2). There were significant differences between cultivars/genotypes with respect to lesion size 48 h after inoculation ($P \leq 0.001$). Wounded and non-wounded treatments were significantly different. In addition, significant genotype × wounding interactions were observed at 48 h incubation (Table 2). Similar results, including significant effects of genotype, wounding, and genotype × wounding interactions, were obtained at 72 h incubation based on lesion diameter (Table 2).

Using the Student-Newman-Keuls test, AV-Sapphire and AG-Castle were found to be the most resistant and susceptible cultivars, respectively.

Table 2. Reaction of Brassica species/cultivars to *S. sclerotiorum* by wounding after 24 h, 48 h and 72 h.

	Lesion size (mm)/Grouping					
	24 hours[*]		48 hours		72 hours	
Cultivar/Species	W[**]	NW[***]	W	NW	W	NW
AG-Castle	8.4a	0.1	22.7e	12.6bc	32.9def	23.7cd
AV-Sapphire	6.4ab	0	18de	5.8a	26.2cde	15.8a
Dunkeld	6.6ab	0	21.9e	11.6bc	34.2f	24.1cd
Oscar	5.1b	0	17.4de	9.4b	30def	21.7c
Rainbow	8.3a	0	22.9e	4.7a	32def	12.6b
B. nigra	6b	0	16.25cd	11.1bc	-	-

Means in each column followed by similar letter (s) are not significantly different at the 5% probability level using the SNK test.
[*] Incubation time [**] Wounded leaves [***] Non-wounded leaves

DISCUSSION

Brassica species (*B. nigra* and *S. alba*) and cultivars (*B. napus*) demonstrated non-significant differences in lesion sizes in the leaf disc assays. The effect of wounding on disease establishment and progress was obvious in all experiments. The lack of significant differences between cultivars and species showed that the leaf disc assay is inconsistent for comparing oilseed rape cultivars, which gave variable results between experiments in reaction to *S. sclerotiorum*. All test genotypes (except *S. alba*) demonstrated significant differences in lesion size in the three-month-old intact plant assay. The effect of wounding on disease establishment and progress was obvious in all experiments and in 3-month-old plants; the non-wounded treatments did not show disease symptoms by 24 h incubation. Brassicas' reactions in both 105-day-old intact plant assays were consistent for determining resistance and susceptibility of cultivars/species. Similar results were observed in 105-day-old plants in the oxalic acid treatments.

Rosa *et al.* (1996) reported that the concentration of GSLs as ITCs production resources, is expected to be higher in three-month-old plants than in two- or one-month old plants. However, larger lesions caused by the fungus in 2-month-old plants were inconsistent with expected GSL concentrations (Rosa *et al.,* 1996) and profiles (Sang *et al.,* 1984; Bellostas *et al.,* 2007). This confirms the hypothesis of Mithen (1992) on the lack of relationship between leaf GSL profiles and pest and disease resistance in oilseed rape. Rahmanpour *et al.* (2009) have demonstrated that significant toxic volatiles are produced during infection of brassica leaf discs by *S. sclerotiorum*. Therefore, it seems that despite variation in GSL profiles and subsequent production of inhibitory chemicals, the pathogen can infect the host cultivar and species. These toxic volatiles are related to GSL hydrolysis, as Doughty *et al.* (1996)

also reported the release of volatile ITCs and other similar chemicals following infection of *Brassica rapa* seedlings by *Alternaria brassicae*.

However, the phloem mobility of GSLs is assumed to have been destroyed due to the damage inflicted on the leaves when cutting leaf discs (Brudenell *et al.,* 1999). In addition, the role of salicylic acid in the expression of systemic resistance to the pathogen may have also been damaged (Uknes *et al.,* 1992). Therefore, GSL accumulation, which is induced by the salicylic acid system during injury (Kiddle *et al.,* 1994), will not occur and thus will not affect the pathogen through the hydrolysis of toxic products.

Based on results obtained using intact leaf inoculation techniques, *B. nigra* and Rainbow were determined to be the most resistant, and AG-Castle and Oscar the most susceptible groups, respectively. This indicated that there may be some relationship between fungus and oxalic acid. Application of 10 mM oxalic acid as physiological concentration (Rahmanpour *et al.,* 2010) resulted in correlations between the pathogenicity factor of *S. sclerotiorum* and disease appearance. Meanwhile, different concentrations and different conditions may affect the response of brassica plants to disease. However, more genotypes should be evaluated through this method to determine the strength of the relationship.

Bradley *et al.* (2006) detected significant differences among 19 cultivars for *S. sclerotiorum* using the oxalic acid assay method (excised canola plants in an oxalic acid mix), but not in the detached leaf assay method. The results of the oxalic acid inoculation method in the present study support their findings. Interestingly, AV-Sapphire was among the genotypes that performed best following inoculation by mycelial spray or myceliogenic germination of sclerotia (Li *et al.,* 2007), and also in the intact plant leaf inoculation by mycelium plugs. Significant differences between brassica cultivars and species at measurement times of > 48 h after inoculation were evident. This phenomenon suggests that to determine the differences between cultivars/genotypes, the time of evaluation should extend to > 48 h after inoculation. Li *et al.* (2007) suggested that by delaying time of assessment of disease development, the impact of the time of inoculation on stem lesion length could be minimized.

In conclusion, oilseed rape cultivars and Brassica species reacted differently to *S. sclerotiorum* inoculations. The leaf disc assay gave variable results in separate experiments and was not reliable for evaluating brassica resistance to the pathogen. Brassica plants responded similarly to the oxalic acid inoculation method and the fungal mycelium technique, which implies a relationship between them. Furthermore, time of evaluation and type of mycelium inoculation affected disease progress during incubation in resistance assays against white mold rot of brassicas.

ACKNOWLEDGMENTS

The financial support of the Agricultural Research, Education and Extension Organization (Iran) during my Ph.D. studies at the University of New England, Australia, is acknowledged and highly appreciated.

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