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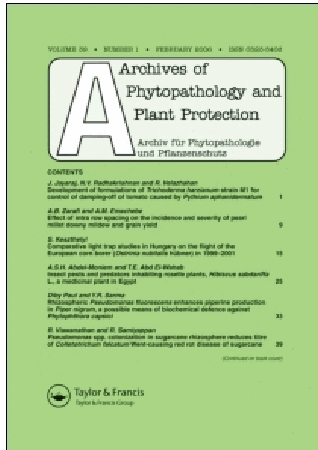

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Comparison of growthroom screening techniques for the determination of physiological resistance to sclerotinia stem rot in **Brassica napus**

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Comparison of growthroom screening techniques for the determination of physiological resistance to sclerotinia stem rot in *Brassica napus*

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Abstract
Screening *Brassica napus* L. lines for resistance to sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary in the field is usually unreliable. Six growthroom screening methodologies were evaluated and compared to accurately, reliably, and efficiently measure 10 *B. napus* lines for resistance to sclerotinia stem rot. These lines were planted in 2001 and 2002 in a nursery infested with *S. sclerotiorum* sclerotia and evaluated for disease incidence and tested in four growthroom experiments. The growthroom methodologies consisted of: (i) leaves of the *B. napus* lines inoculated with fragments of *S. sclerotiorum* mycelium suspended in a potato dextrose broth (Myspray technique); (ii) *B. napus* petals bearing *S. sclerotiorum* mycelium (Mypetal technique); (iii) *B. napus* petals bearing *S. sclerotiorum* ascospores (Ascpetal technique); (iv) leaf petioles dipped in a solution of oxalic acid (Oxalate technique); and (v, vi) both leaves and stems inoculated with agar plugs with *S. sclerotiorum* mycelium (Plug Leaf and Plug Stem techniques). Infection was low and highly variable in the field trials with CVs of 205.9% and 133.6%, respectively, and with low correlation between them ($R = 0.6164$, $p = 0.1405$). The Plug Leaf technique had low correlations among growthroom trials and resulted in inconsistencies among cultivar rankings between the trials. The Plug Stem technique had high levels of variation and was not considered to be accurate. The Ascpetal technique, although regarded as more accurate, was found to be highly variable and was cumbersome to use. The Myspray technique had the greatest variability of all the techniques with a CV of 57.3% and low correlation between the growthroom trials. Myspray technique was significantly correlated with field data ($R = 0.9150$, $p = 0.0039$) and with the Ascpetal technique ($R = 0.7945$, $p = 0.0060$). The Oxalate test was the most efficient of all techniques but did not correlate with any other technique. The Mypetal technique was chosen to best assess physiological resistance since it had the least variation in its results and cultivar ranks between growthroom trials. It was accurate in determining resistant lines observed in the field, and it was efficient to employ.

Keywords: Brassica napus, Sclerotinia sclerotiorum, *growthroom screening techniques, screening for resistance, physiological resistance determination*

Introduction

Sclerotinia stem rot (*Sclerotinia sclerotiorum* (Lib.) de Bary) is a difficult disease to manage in canola (*Brassica napus* L. and *Brassica rapa* L.). Fungicides are the only effective method of

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control available for producers, but these are costly and can have environmental concerns. The ideal control strategy is host resistance, but as *Sclerotinia sclerotiorum* infects more than 400 plant species (Boland & Hall 1994), finding and incorporating genetic resistance by conventional breeding practices has proved to be challenging. Field resistance to sclerotinia stem rot is the result of physiological resistance and escape mechanisms. Escape mechanisms in canola include lodging resistance, short open canopies, apetally (lack of flower petals), and short flowering duration (Jurke & Fernando 2002). Many of these escape mechanisms are polygenic and difficult to breed into elite germplasm. The use of a monogenic source of strong resistance has an appeal to plant breeders selecting for resistance for sclerotinia stem rot.

Transgenic sources of resistance for sclerotinia stem rot are available. Transgenes encoding for enzymes such as oxalate oxidase have been found to have some efficacy in canola, soybeans, and sunflowers (Thompson et al. 1995; Lefol 1998; Donaldson et al. 2001; Billings et al. 2003). To successfully determine whether a source of resistance is adequate at controlling sclerotinia stem rot infection, an effective screening methodology must be available.

Ideally, an indoor screening technique, which correlates well with field-level performance, is the most desirable method for screening for *S. sclerotiorum* resistance. However, disease nurseries in the field have their limitations. The development of sclerotinia stem rot is influenced strongly by environmental conditions, which are not always conducive for disease development. Furthermore, since field-level resistance in many species is influenced by the growth-habit of the crop, which includes avoidance and escape mechanisms, these factors can hamper the measurement of the physiological resistance. This can be better accomplished with a growthroom inoculation technique.

Researchers working in different crops have used a number of screening techniques for the determination of resistance to *S. sclerotiorum* (Table I). These techniques have tested various plant organs and used many different nutrient substrates, depending on the goals of the experiment. Some researchers have conducted studies comparing these inoculation methodologies to assess which might be most appropriate for their respective crop. Wegulo et al. (1998) compared five techniques for the determination of resistance in soybean and found that the use of soluble pigment determination and oxalic acid assays were more reliable methodologies than using mycelial disks to identify resistant lines. Kim et al. (2000) also compared inoculation techniques in soybeans, but found that mycelial disks could be used reliably to obtain preliminary information on white mold resistance. Fang (1993) examined four inoculation techniques in *B. napus* and found that the foliar applied mycelial disk method produced the most uniform results for the determination of sclerotinia stem rot resistance.

Our objective was to evaluate six growthroom *S. sclerotiorum* inoculation techniques on canola for their efficacy in measuring physiological resistance. Accuracy, reliability, and efficiency were the determinants used for selecting the best technique(s).

Materials and methods

Trial design

Several trials were undertaken to evaluate *S. sclerotiorum* inoculation techniques on up to 10 *B. napus* lines (Table II). This included two field experiments conducted in an irrigated sclerotinia nursery in Carman in 2001 and 2002 and four indoor growthroom (GR) trials to evaluate the six inoculation techniques.

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Table I. Inoculum sources, target tissue, and references for growthroom techniques for resistance screening to *S. sclerotiorum* as described in the scientific literature.

Fungal structure/toxin	Nutrient substrate	Target tissue	Host crop	Author(s)
Mycelium	PDA[a] plugs	Leaf	*B. napus*	Fang (1993), Mullins et al. (1995), Zhao & Meng (2003)
			Sunflower	Mestries et al. (1998)
			Soybean	Kim et al. (2000) and Wegulo et al. (1998)
Mycelium	Autoclaved carrot pieces	Leaf	*G. max*	Cline & Jacobsen (1983)
Mycelium	PDA plugs	Stem	*B. napus*	Brun et al. (1987), and Buchwaldt et al. (2003)
			Cabbage	Dickson & Petzold (1996)
			Soybean	Arahana et al. (2001) and Vuong & Hartman (2003)
			Sunflower	Kohler and Friedt (1999)
Mycelium	Autoclaved barley or oat grains	Stem	*B. napus*	Scott (1984) and Thomson & Kondra (1983)
			Soybean	Cober et al. (2003) and Kim et al. (2000)
Mycelium LTI[b]	Autoclaved celery stick pieces	Stem	Bean	Hunter et al. (1981)
	Autoclaved celery sticks or green bean pods	Stem	Soybean	Boland & Hall (1986) and Cline & Jacobsen (1983)
Mycelium	Autoclaved carrot pieces	Stem	Soybean	Wegulo et al. (1998)
Mycelium	Autoclaved matchsticks	Stem	*B. napus*	Brun et al. (1987)
Mycelium	Blended carrot and water	Whole plant	Soybean	Wegulo et al. (1998)
Crumbled sclerotia	None	Stem base	*B. napus*	Frencel et al. (1987)
Ascospores	Petals	Leaf	*B. napus*	Brun et al. (1987) and Lefol & Seguin-Swartz (1998)
		Leaf axils	Bean	Hunter et al. (1981)
Ascospores	None	Leaf	*B. napus*	Thomson & Kondra (1983)
			Soybean	Cline & Jacobsen (1983)
Oxalate		Whole plant	Soybean	Kolkman & Kelly (2000)

[a]Potato Dextrose Agar; [b]Limited Term Inoculation.

The *S. sclerotiorum* isolate FR1 was used in all cases. It was isolated from a soybean crop in Homewood, Manitoba, Canada, in 1998 and maintained on potato dextrose agar (PDA). This isolate was chosen for its aggressiveness, its ease of culturing, and its consistency of infection. A number of preliminary trials were done to develop much of the technical aspects of each inoculation technique. Preliminary trials on age of inoculum, isolate, inoculum concentration, inoculum storage, plant age, and environmental conditions for infection were all completed before the test techniques were compared.

Three *B. napus* plants were seeded per pot – this represented the experimental unit for all growthroom tests. The growthroom diurnal cycle was 22°C for 16 h days and 18°C for 8 h

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Table II. List of sclerotinia stem rot inoculation techniques used in comparative trials and notes on trial performance.

		Growthroom inoculation techniques[a]									
Trial name	Field	Plug Leaf	Plug Stem	Mypetal	Myspray	Ascpetal	Oxalate	No. replications	CV (%)	No. *B. napus* lines	Note[c]
2001 field	X							3	205.9	7	No Oxox lines and No ZhongYou 821
2002 field	X							5	133.6	10	
GR trial[b] A		X		X	X	X		2	35.0	9	No ZhongYou 821
GR trial B		X		X	X			3	81.7	9	No ZhongYou 821
GR trial C		X		X	X	X		3	23.0	10	
GR trail D			X	X			X	3	31.4	10	

Note: An 'X' denotes that this techniques was tested in the trial; [a]'Plug Leaf' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Plug Stem' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* stems; 'Mypetal' *B. napus* petal bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Myspray' solution of PDB and *S. sclerotiorum* mycelium fragments sprayed on *B. napus* leaves; 'Ascpetal' *B. napus* petal bearing *S. sclerotiorum* ascospores on *B. napus* leaves; 'Oxalate' *B. napus* leaf petiole submerged in oxalic acid; [b]Growthroom trial; [c]Note explains what *B. napus* lines were not included in each experiment.

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nights. Leaves were the target tissues for five of the test techniques (Table III). For these techniques, the plants were inoculated at the pre-bolting stage (growth stages 2.6 – 2.8 [Harper & Berkenkamp 1975]) when leaf size was at a maximum prior to flowering. Three to seven leaves were inoculated per plant. Lesion size was determined by measuring the lesion diameter with a set of callipers. If the lesion was not circular, the mean of the length and width of the lesion was used. A humidity chamber was constructed to ensure consistent high humidity for the leaf inoculation techniques. Two residential ultrasonic cool mist humidifiers were attached on top of the chamber, continually delivering mist evenly and heavily to all plants.

B. napus *lines*

Brassica napus cultivars and lines were chosen on the basis of potential susceptibility and resistance to sclerotinia stem rot (Table IV). Six of these lines were transformed with genes believed to confer some level of physiological resistance to *S. sclerotiorum* infection. The parent canola cultivar, Drakkar, was transformed using an Agrobacterium-mediated technique to produce these transgenic events. The specific transgenic lines used in this study were selected from a screening program completed prior to this experiment, which

Table III. Inoculation techniques, life stage of *S. sclerotiorum*, nutrient substrate used, host target tissue, and reference source of technique used in evaluation trials to determine physiological resistance in *B. napus* to sclerotinia stem rot.

Technique	Fungal structure	Nutrient substrate	Target tissue	Reference
Plug Leaf	Mycelium	PDA[a] plug	Leaf	Fang (1993)
Mypetal	Mycelium	*B. napus* petal	Leaf	
Myspray	Mycelium	PDB[b]	Leaf	Boland & Hall (1986)
Ascpetal	Ascospores	*B. napus* petal	Leaf	Lefol & Seguin-Swartz (1998)
Oxalate	None	None	Leaf	Kolkman & Kelly (2000)
Plug Stem	Mycelium	PDA plug	Stem	Buchwaldt et al. (2003)

[a]Potato Dextrose Agar; [b]Potato Dextrose Broth.

Table IV. *Brassica napus* lines used to evaluate sclerotinia stem rot inoculation techniques and their resistance.

B. napus line	Level of resistance[a]	Resistance gene	Source of resistance
Drakkar	None	None	
HyLite 225RR	None	None	
HyLite 201	Very good	Apetally	*B. napus*
678-22	Minor	Rs-AFP2 (Anti-fungal protein)*	*Raphanus sativus*
479-84	Minor	Ox-ox (Oxalate-oxidase)*	*Triticum aestivum*
783-182	Minor	Chi/glu (Chitinase and β-1,3-glucanase)*	*Nicotiana tabacum*
375-144	Minor	Ox-dec (Oxalate decarboxylase)*	*Collybia velutipes*
375-112	Moderate	Ox-dec*	*C. velutipes*
375-53	Good	Ox-dec*	*C. velutipes*
ZhongYou 821	Good	Unknown	

[a]Level of resistance determined from previous field and growthroom screening trials; *Transgenic genes.

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identified potential physiological resistance. ZhongYou 821 is a Chinese rapeseed cultivar reported to have moderate levels of sclerotinia stem rot resistance (Li et al. 1999; Buchwaldt et al. 2003). HyLite 201 is an apetalous canola cultivar that has been shown to effectively escape sclerotinia stem rot infection (Jurke et al. 1998; Jurke & Fernando 2002). HyLite 225RR is a canola cultivar with no physiological resistance or escape mechanisms to sclerotinia stem rot.

Mycelium-infested agar plug technique (Plug Leaf)

Sclerotinia sclerotiorum mycelium was grown on PDA (Becton Dickson Co., Sparks, MD, USA) plates. Mycelium-bearing agar plugs, 6 mm in diameter, were cut with a cork borer. Since the age of the culture was found to be important, plugs were cut only within 1 cm of the advancing margin of a new culture (about 2 days old). If the mycelium was allowed to reach the margins of the plate (older cultures), its infection ability was found to decline substantially. The plugs were then inverted, mycelium side down, onto a large canola leaf and pressed with the fingers to keep it in place. If these plugs were not pressed onto the leaf they often would roll or slide off the leaf before leaf infection would occur. Plants were inoculated at the pre-bolting stage (growth stages 2.6 – 2.8 [Harper & Berkenkamp 1975]) prior to flowering when leaf size was at a maximum. Three to seven leaves were inoculated per plant. Infected plants were placed in the humidity chamber for 48 – 72 h. Severity was rated on the diameter of the lesion upon removal from the chamber. A mean score of all lesions was calculated for each pot.

Stem inoculation technique (Plug Stem)

This technique was modified from Buchwaldt et al. (2003). Four mm diameter plugs from *S. sclerotiorum* mycelium growing on PDA were placed mycelium side down against canola stems and gently wrapped with parafilm to prevent desiccation. Plants were not placed in the humidity chamber. Plants were at the bolting to early flower stage and were inoculated up the length of the stem every 10 cm. Seven days after inoculation, the parafilm was removed and lesion length was measured. A qualitative description of the lesion also was assessed at the same time: C for a collapsed lesion, S for a soft lesion, F for a firm lesion, and B for a firm lesion with a black outline.

Mycelium-infested petal technique (Mypetal)

A potato dextrose agar plug containing mycelium of *S. sclerotiorum* was transferred to potato dextrose broth (PDB, Sigma-Aldrich, St Louis, MO, USA), and allowed to grow for one week at room temperature with 16 h days and 8 h nights (when the broth appeared to be thick with mycelium). The mycelium was strained from the PDB, weighed and added to sterile distilled water at a concentration of 0.15 g of mycelium per ml of water. It was then macerated using a hand-held blender/food processor. Canola petals of a similar age, which were previously collected and stored at -15°C, were submerged in this slurry for 10 min. A single petal, infested with *S. sclerotiorum* mycelium, was placed on a leaf, and minimum of three leaves per plant were infected in this way. The inoculated plants were placed in the humidity chamber for at least 72 – 96 h when lesions were large enough to differentiate between *B. napus* lines. Infection was evaluated by measuring the diameter of the lesion. A mean score of all lesions was calculated for each pot. Figure 1 illustrates a typical lesion resulting from this technique.

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Figure 1. Lesion produced by the Mypetal inoculation technique on a *B. napus* leaf in the growthroom.

Macerated mycelium spray technique (Myspray)

Sclerotinia sclerotiorum mycelium was grown in PDB for about one week at room temperature with 16 h days and 8 h nights. The solution of mycelium and PDB was macerated using a hand-held blender and diluted to a concentration of 0.15 g mycelium per ml PDB. The mycelial solution was then sprayed on to the plants using a hand-held misting bottle until the leaves were wet with the solution. An effort was made to ensure that all leaves of the plants were evenly covered. The inoculated plants were then placed in the humidity chamber for 5 – 6 days. Disease severity was scored in two ways: (i) percentage of leaf area covered with lesions; and (ii) diameter of the largest lesion on a single leaf. A mean score for each scoring method was calculated for each pot. Figure 2 illustrates the types of lesions resulting from this spray technique.

Ascospore-infested petal technique (Ascpetal)

Ascospores were collected from sclerotia using a modified protocol described by Lefol (1998). Sclerotia harvested from PDA plates were placed on moist sand and incubated in 24 h darkness at 15°C for 1 – 2 months. When stipes were produced, the sclerotia were transferred to a 1% agar medium with 16 h of daylight at room temperature. Apothecia were soon formed, and ascospores were collected with a vacuum filter (Model MF 75, Nalgene, Rochester, NY, USA). Ascospores were collected on filter paper with a pore size of 0.2 μm and stored at -15°C. Canola petals, which were previously detached and stored at -15°C, were dipped for 10 min in a solution of ascospores (8.0×10^5 spores/ml sterile distilled water).

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Figure 2. Lesions produced by the Myspray inoculation technique on a *B. napus* leaf in the growthroom.

Single infested petals were placed on a minimum of three leaves per plant, and the plants kept in the humidity chamber for 5 – 6 days. Disease severity was rated by the diameter of the lesion. A mean lesion diameter was calculated for each pot.

Oxalic acid technique (Oxalate)

Inoculation of excised canola leaves was done in a large tub containing 4 litres of a 20 mMol oxalic acid solution. Two leaves, with a minimum leaf blade length of 10 cm, were detached at the base of the petiole, labelled, and suspended above this solution on inverted Petri dishes with the petioles submerged. Progression of wilt symptoms up the petiole and along the main vein of the leaf was measured 24 h following immersion.

Plant age

Two experiments were conducted examining the effect of plant age on growthroom inoculation techniques. The first experiment was a split-split plot design used the Mypetal and the Myspray techniques as main plots, the 6 leaf and flowering developmental stages as sub plots, and six *B. napus* lines as sub-sub plots. The second experiment was a split plot design. The Mypetal technique was used to inoculate the four main plot 4 growth stages (cotyledon, 3 leaf, 6 leaf, and flowering) of the six *B. napus* sub plots.

Field trials

For comparative purposes, field trials with the same lines were conducted in 2001 and 2002 at Carman, MB. The transgenic lines used in this experiment restricted experimentation to only

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one location per year. A randomized complete block design with three replicates of single row plots 3 m in length was used. Trials were seeded in late May into soil that had 100 sclerotia/ m^2 incorporated into the soil. Tents were erected over the site and a misting system was installed to ensure high humidity. Disease assessments were made shortly before swathing (Harper & Berkenkamp [1975] growth stage 5.3). The total numbers of infected and non-infected plants were counted. A plant was considered to be infected if it showed any level of infection, whether it was a basal stalk infection or an upper canopy infection. The percentage of infected plants (disease incidence, DI) was calculated for each plot.

Data analysis

Statistical analysis was carried out using Agrobase 20 (Agronomix Inc. Winnipeg, MB) and Statistical Analysis System 8.2 (SAS Institute Inc. Cary, NC) at $p = 0.05$. Analysis of variance was performed using a general linear model. Tukey's HSD (Honestly Significantly Different) test was used to compare treatment means. Mean values for each *B. napus* line for each inoculation technique were calculated by averaging all of the individual replications from the six experiments involved in this study. Additionally, the values of each *B. napus* line were normalized to the untransformed parent line, Drakkar for each replication. These normalized values were averaged from all six experiments. The means and normalised values were used in correlation analysis and rank correlation analysis. Pearson correlation coefficients among inoculation techniques were calculated on the mean values and the normalized mean values of the *B. napus* lines. Spearman's coefficients of rank correlation were used to measure the relationship among the ranks of cultivars among inoculation techniques.

Choice of the most effective inoculation technique was based on accuracy, reliability, and efficiency criteria. Accuracy was defined as the ability of an inoculation technique to correctly identify physiological resistance in a *B. napus* line to *S. sclerotiorum*. It was examined in two ways: (i) correlation of inoculation technique with field trial data; and (ii) a qualitative assessment of the representation of an inoculation technique to the natural infection processes. Reliability was defined as the repeatability of an inoculation technique to consistently identify resistance or susceptibility of a *B. napus* line. This criterion was quantified by an examination of the correlation of results and ranks between growthroom trials, the variance of each technique, and frequency of failed inoculations. Efficiency was determined by the amount of plant material needed, the basis of length of time to obtain results and the simplicity in the application the inoculation technique. For each criterion, quantification by rank of these techniques was used to determine the most effective inoculation technique.

Results

Field trials

Infection levels were low in the field trials in both 2001 and 2002. Sclerotinia stem rot symptoms were present but only occurred in random patches. In both years, the flowering period coincided with three weeks of maximum temperatures averaging 30°C and low relative humidity. As a result, the coefficient of variation (CV) for the 2001 and 2002 trials were 206% and 134% respectively (Table II) and the mean DI of the check line, Drakkar, was only 5.2% and 8.8% respectively (Table V). Combining and analysing the two field trials together increased the CV to 155% and decreased Drakkar's mean DI to 7.4%. Given the high

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Table V. Sclerotinia stem rot infection evaluations in *B. napus* lines from field trials at Carman, Manitoba and growthroom inoculation trials.

	Field trials				Growthroom inoculation techniques[a]											
	2001		2002		Plug Leaf		Plug Stem		Mypetal		Myspray		Ascpetal		Oxalate	
B. napus lines	DI[b]	Rank	DI	Rank	Lesion size[c]	Rank	Lesion size	Rank	Lesion size	Rank	Lesion size	Rank	Lesion size	Rank	Wilt length	Rank
Drakkar	5.2%	6	8.8%	8	37.3	10	19.0	3	21.2	10	14.6	8	18.6	3	76.9	2
HyLite 225 RR	7.3%	7	15.6%	9	27.2	5	31.8	10	15.1	4	15.6	10	25.3	10	87.8	9
HyLite 201	2.1%	5	0.0%	1	29.1	4	31.6	9	19.8	8	11.3	5	19.3	6	76.1	4
678-22	3.4%	4	0.0%	1	36.2	9	31.4	8	20.3	9	9.6	4	19.8	8	93.3	8
479-84			5.9%	5	32.7	7	20.8	4	14.4	3	13.3	6	20.5	9	75.6	3
783-182			2.0%	4	24.0	3	30.2	7	13.8	2	12.5	7	19.4	7	60.4	1
375-144	2.1%	3	11.6%	10	31.5	8	26.9	6	15.2	5	8.9	2	18.3	2	91.9	10
375-112	0.0%	2	6.3%	6	22.8	2	22.8	5	18.5	7	8.7	3	18.8	4	92.2	6
375-53	0.0%	1	0.0%	1	22.2	1	16.4	2	10.6	1	5.7	1	9.0	1	89.8	7
ZhongYou 821			6.4%	7	31.1	6	11.7	1	16.2	6	11.0	9	18.9	5	87.2	5
No. replications	4		6		8		3		11		8		4		3	
Mean	2.9%		6%		29.4		24.3		16.5		11.1		18.8		83.1	
HSD[d]	5.1%		27.8%		15.08		21.59		10.00		12.86		27.52		74.07	

[a]'Plug Leaf' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Plug Stem' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* stems; 'Mypetal' *B. napus* petal bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Myspray' solution of PDB and *S. sclerotiorum* mycelium fragments sprayed on *B. napus* leaves; 'Ascpetal' *B. napus* petal bearing *S. sclerotiorum* ascospores on *B. napus* leaves; 'Oxalate' *B. napus* leaf petiole submerged in oxalic acid; [b]Disease Incidence (percentage of infected plants); [c]Lesion size and wilt length measured in millimetres; [d]Tukey's Honestly Significantly Different test.

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variability in these trials and the overall low infection rates, their reliability and usefulness is questioned.

Plug Leaf technique

The Plug Leaf technique produced the largest lesions in the shortest amount of time. Small lesions were already apparent 36 h after inoculation and lesions were large enough for rating by 72 h. significant differences in lesion size were found between *B. napus* lines (Table V). There was no significant growthroom trial effect, nor was there a significant line by trial interaction. The correlations of line performance between trials were low ($R = 0.134 - 0.563$). The ranks of the lines varied between GR trials, but lines 375-53 and 375-112 had consistently less disease than the other lines, and these two lines were only marginally different from one another.

Plug Stem technique

The appearance of lesions occurred after 4 days after inoculation as incipient brown necrosis. After seven days, the lesions had progressed up and down the stems from the inoculation point. There were no significant differences among the lines, nor was there a replication or interaction effect with this technique (Table V). Ranks from this technique did not reflect the ranks from the previous techniques, although line 375-53 had the second smallest amount of lesion development. Zhongyou 821 had the smallest lesions and ranked first, which is contrary to its ranking with all other inoculation techniques.

Mypetal technique

Lesions formed more slowly on *B. napus* lines inoculated with the Mypetal (Figure 1) than the Plug Leaf technique. Small lesions were observed 48 h after inoculation and by 96 h these were large enough for rating. Significant differences in lesion size were found between *B. napus* lines (Table V). There was no significant growthroom trial effect, nor was there a significant line by trial interaction. Correlations of line performance between trials were high for most trials (Table VI), indicating the repeatability of results was good with this technique. Like the Plug Leaf method, ranks of lines varied from one GR trial to another. Only line 375-53 was consistent in its ranking having the smallest lesion size using this technique.

Myspray technique

Lesions on plants inoculated with this mycelial mist appeared as small black speckles 96 h after inoculation. These spot-like lesions often covered the margins of the leaf and would grow

Table VI. Matrix of Pearson correlation coefficients for sclerotinia stem rot disease evaluations for the Mypetal inoculation technique between the growthroom trials.

Growthroom trial	GR trial A	GR trial B	GR trial C
GR trial B	0.5006		
GR trial C	0.7815*	$0.6453^{\dagger}$	
GR trial D	0.4224	$0.7451^{\dagger}$	0.6102

Note: * denotes significance at $p < 0.05$ and † indicates significance at $p < 0.10$.

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into larger distinguishable lesions after 5–6 days in the humidity chamber (Figure 2). Significant differences between lines were not demonstrated using this technique (Table V). Correlations of lines between trials was low (R = 0.248–0.546). Line ranks between trials varied. Line 375-53 had the smallest lesions in two of three trials but was 6th in the third, and line 375-112 ranked second in two trials and was fourth in the third. There were significant GR trial and replication effects using this technique (data not shown).

Ascpetal technique

Lesions first appeared 96 h after inoculation and were large enough to rate by 5–6 days. There were no significant differences in lesion size among lines, nor were there any significant trial, replication, or interaction effects (Table V). Correlation of line performance between the two GR trials was low (R = −0.01794). Ranks between the two trials varied significantly. Line 375-53 had the lowest lesion size in GR trial C and ranked third in GR trial A. HyLite 201 ranked first in GR trial A but consistently ranked low with all other inoculation techniques.

Oxalate technique

The appearance of wilt symptoms progressing up the petiole was visually different from the lesions of the other indoor techniques used. The wilting progressed with time from the base of the severed petiole, and after 24 h was evident along the veins in the leaf blade. There were no significant differences between lines using this technique, even when employing a less conservative measurement of difference such as Fisher's LSD (Table V). There was a significant replication effect. The ranks obtained with this technique did not reflect the trends observed with the previous techniques; line 375-53 ranked seventh.

Comparison of inoculation techniques

To compare inoculation techniques, mean scores across all growthroom trials were tabulated (Table V) and Pearson's correlation coefficients (R) were calculated (Table VII). Significant relationships were found between the Myspray and the Ascpetal techniques and between

Table VII. Matrix of Pearson correlation coefficients of sclerotinia stem rot disease evaluations for various inoculation techniques from the growthroom and the field trials at Carman, MB.

Inoculation technique	2001 field	2002 field	Plug Leaf[a]	Plug Stem	Mypetal	Myspray	Ascpetal
2002 field	0.6164						
Plug leaf	0.5287	0.1477					
Plug stem	0.4113	−0.0073	−0.0056				
Mypetal	0.2666	−0.0204	0.6077†	0.2448			
Myspray	0.9150**	0.5147	0.4002	0.2371	0.2938		
Ascpetal	0.7384†	0.5594†	0.3332	0.5037	0.4033	0.7945**	
Oxalate	−0.2986	0.2244	0.0543	−0.1693	0.0739	−0.4996	−0.1482

Note: * indicates significance at $p < 0.05$, a double asterix indicates significance at $p < 0.01$, and † indicates significance at $p < 0.10$; [a]'Plug Leaf' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Mypetal' *B. napus* petal bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Myspray' solution of PDB and *S. sclerotiorum* mycelium fragments sprayed on *B. napus* leaves; 'Ascpetal' *B. napus* petal bearing *S. sclerotiorum* ascospores on *B. napus* leaves; 'Oxalate' *B. napus* leaf petiole submerged in oxalic acid; 'Plug Stem' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* stems.

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Myspray and the 2001 field trial. Spearman's rank correlation analysis found only a significant relationship between the Myspray technique and the 2002 field trial.

Using the data set normalized to the untransformed parent line, Drakkar, the correlations remained the same. Correlations were also tabulated without the data from the apetalous cultivar, HyLite 201, and the relationships remained the same. Of the leaf inoculation techniques, the Plug Leaf technique consistently produced the largest lesions and had the least number of misses (Table VIII) across all GR trials. Although the Mypetal technique had a greater overall CV in lesion size and had a greater frequency of misses than the Plug Leaf technique, its repeatability between GR trials was the highest for all growthroom techniques. The Oxalate technique was the quickest to perform and required the fewest number of plants.

Quantification of the accuracy, reliability, and efficiency of each technique was done (Table IX). The techniques were ranked according to these criteria and summarized. The Plug Leaf and Mypetal techniques had the lowest, i.e., best scores.

Plant age

An experiment examining the effect that plant age might have on these growthroom techniques was carried out using the Mypetal technique. Plant age was found to have little

Table VIII. Comparisons of aspects on the efficiency and reliability of the inoculation techniques used in this experiment.

Technique[a]	Number of plants required	Number of days from planting to rating	Mean lesion size (mm)	Frequency of misses (%)	Coefficient of variation (%)
Plug Leaf	12	31	29.2 b	10	31.2
Plug Stem	12	80	24.3 bc	1	27.5
Mypetal	12	33	17.7 d	16	36.4
Myspray	12	35	11.1 e	43	57.3
Ascpetal	12	35	19.0 cd	42	37.0
Oxalate	6	29	83.1 a	0	27.6
Field	400	110	n/a	?	155.0

Note: Means followed by a letter (a, b, c, etc.) differ significantly using $p = 0.05$ with Tukey's HSD, which controls Type 1 experiment-wise error; [a]'Plug Leaf' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Plug Stem' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* stems; 'Mypetal' *B. napus* petal bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Myspray' solution of PDB and *S. sclerotiorum* mycelium fragments sprayed on *B. napus* leaves; 'Ascpetal' *B. napus* petal bearing *S. sclerotiorum* ascospores on *B. napus* leaves; 'Oxalate' *B. napus* leaf petiole submerged in oxalic acid.

Table IX. Comparison by rank of growthroom inoculation techniques on the basis of their efficiency, reliability and accuracy.

Technique	Accuracy	Reliability	Efficiency	Summary
Plug Leaf	3	2	2	7
Plug Stem	3	4	6	13
Mypetal	2	1	4	7
Myspray	3	6	3	12
Ascpetal	1	5	5	11
Oxalate	6	3	1	10

Note: Lower number indicates higher rank.

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effect with the inoculation technique (Table X). Lesion size increased significantly with plant age. The *B. napus* line rankings were similar in the cotyledon, 6 leaf, and flowering plant stages but were significantly different at the 3 leaf stage. An earlier experiment using the Mypetal and Myspray techniques at the 6 leaf and flowering plant stages had the same rankings for both techniques (data not presented).

Discussion

Sclerotinia stem rot is a difficult disease to investigate in the field. The ability to achieve consistently high levels of infection is hampered by this fungus's sensitivity to environmental conditions (in a disease nursery). Since measurements of physiological resistance in the field can be confounded by escape mechanisms, the use of a growthroom technique for sclerotinia stem rot screening is preferred for the sake of accuracy. Reliability and efficiency also are important in a breeding program. For this reason the Mypetal technique appears to be the best overall choice for accuracy, reliability and efficiency of selecting lines for sclerotinia stem rot resistance in *B. napus*.

The question of accuracy needs elaboration since a technique is not useful unless it is representative of resistance that can be observed in the field. Since the field trials had very low levels of infection and very high levels of variation, good correlations among growthroom and field results were not expected. Furthermore, since escape mechanisms due to the plant's morphology may be involved in field results, even with consistent levels of infection, field results would not correlate well with a growthroom technique. For example, HyLite 201, lacking petals, had no infection in the field trials but was quite susceptible in all growthroom trials. Nevertheless, field trials are still useful to verify susceptibility and to verify the accuracy of an inoculation technique. As an example, the Plug Stem technique is not considered to be very accurate since Zhongyou 821 was identified as the most resistant line, but in the field trials it ranked 7th, being one of the more susceptible lines tested. All foliar growthroom techniques found that the *B. napus* line 375-53 had the smallest lesions, and no infection was seen in this line in the field.

The Mypetal technique proved to be the most repeatable and therefore the most reliable of the inoculation techniques judging by the correlation results and the ranks among GR trials. The Mypetal technique was relatively simple to employ and efficient. However, production of inoculum require the extra step of growing *S. sclerotiorum* in liquid medium compared to

Table X. Effects of plant age on relative lesion sizes in *B. napus* lines caused by *S. sclerotiorum* using the Mypetal technique (infesting *B. napus* petals with *S. sclerotiorum* mycelium).

	B. napus growth stage							
	Cotyledon		3 leaf		6 leaf		Flowering	
B. napus line	Lesion mean	Rank	Lesion mean	Rank	Lesion mean	Rank	Lesion mean	Rank
Drakkar	14.4	5	16.8	4	28.9	3	43.4	4
HyLite 225RR	13.8	4	15.2	5	38.1	6	48.7	6
479-08	8.8	2	20.1	6	33.5	4	42.3	3
783-182	15.7	6	14.6	2	33.9	5	46.2	5
375-144	9.8	3	14.8	3	22.4	2	24.7	2
375-53	7.9	1	10.5	1	17.6	1	20.5	1

Note: Lesion means are recorded in mm.

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the agar medium only for the Plug Leaf technique. In preliminary trials, individual petals were removed from *B. napus* flowers for storage, which was quite tedious. Later flowering racemes were cut and stored, which took considerably less time. The Mypetal technique appears to be an accurate system. The Ascpetal technique is believed to best represent the actual mode of infection in the field. The Mypetal technique uses the same substrate, or the same nutrient source as what is seen in the field. It differs in it bypassing the ascospore germination phase.

The Plug Leaf technique also appeared useful and was a close second. It has been used successfully in canola by Fang (1993), Li et al. (1999), Liu et al. (2001), and Mullins et al. (1995). It has also been used in soybeans (Arahana et al. 2001; Hoffman et al. 2002; Kim et al. 2000; Vuong et al. 2001), in sunflowers (Noyes & Hancock 1981; Bert et al. 2002) and in beans (Zhou & Boland 1999). The reason for ultimately selecting the Mypetal technique over the Plug Leaf technique is reliability. The Plug Leaf technique had the lowest CVs among the foliar techniques, but the correlations between GR trials were low. The Plug Leaf technique was more efficient than the Mypetal technique because of its ease in inoculum production, ease in application, and fewer days from planting to rating. This technique uses PDA as a nutrient source, which may give the fungus a nutritional boost to facilitate infection compared to the nutrients available in petals. This speculation is reflected by the fact that it initiated infection the quickest and produced the largest lesions of the foliar techniques. Sensitivity with this technique is also a concern. In preliminary trials, barley leaves were successfully infected with the Plug Leaf technique. This raises the concern that this technique might be overly aggressive and unrepresentative.

The Plug Stem technique was largely rejected on the basis of the difficulty in inoculation and the length of time to retrieve data. Concern about the variation of this technique has been raised by other researchers. Fang (1993) found that the variability with this technique was too high to be of any use in *B. napus* screening suggesting that this might because of plant age. Cline and Jacobsen (1983) suggested plant age as well in soybeans might be responsible for this variation. But it has been indicated that this variability might be caused by variation in stem size, which can be accounted for by selecting appropriate stems (Buchwaldt et al. 2003). However, accuracy of this technique, like the Agar Leaf technique could not be assessed fully (Kim et al. 2000).

The Ascpetal technique can be regarded as the most accurate measure of physiological resistance since it uses the same propagules and nutrient substrate as the pathogen occurs naturally in the field. But it was rejected as a useful growthroom technique for high-throughput disease screening because of its low efficiency and high variability. This technique was cumbersome, requiring a constant and viable source of ascospores, which can take up to four months to produce in the laboratory. This technique also had a longer lag period between inoculation and the time lesions were large enough for disease assessment, compared to the Plug Leaf or Mypetal techniques. This could cause logistical problems for a large-scale screening program. Also, there was high variability between replications and GR trials, which might be because of variability in viability caused by the storage of ascospores for longer periods of time.

The Myspray technique correlated well with the Ascpetal technique, but the variability between replications and GR trials was very high. This technique had the greatest frequency of misses, questioning its reliability. It could not be determined if a plant with no lesions was truly resistant or whether it was an escape. This suggests that this technique might measure some form of foliar avoidance mechanisms such as leaf angle, leaf orientation, or cuticular wax structure and composition.

The Oxalate technique was the most efficient of the growthroom techniques. Oxalic acid tests have been done on detached leaves with success in soybeans (Kolkman & Kelly 2000),

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but in canola this technique does not appear to be effective. As it did not correlate with any other technique, its results were deemed not to accurately represent physiological resistance. Furthermore, since many of the sources of resistance in the test lines used mechanisms other than oxalate-degrading enzymes, the usefulness of this test may not go beyond those lines with an oxalate-resistant gene.

These findings are in agreement with comparative studies conducted by Fang (1993) and Kim et al. (2000) and that foliar applied mycelium-bearing substrates are effective for determining physiological resistance. Fang found that the Plug Leaf technique was the best assessment method. However, the scope of Fang's experiments was smaller and did not include any other foliar techniques. By using four foliar techniques, and comparing these, the Plug Leaf technique, while it was efficient and relatively reliable, was found to be of questionable accuracy. As well, Fang found that the Plug Leaf technique did not correlate well with field results. The findings by Kim et al. (2000) were that the Plug Leaf technique was not superior to other foliar techniques in soybeans, and that any of these would be useful in screening for white mold resistance.

Critical to development of a screening methodology is the availability of susceptible and resistant plant material. The difficulty with sclerotinia stem rot is that most *B. napus* material is largely susceptible. This study included two cultivars reported to have resistance in the field and lines with four transgenes that were believed to confer resistance. HyLite 201 possesses an avoidance mechanism but has no physiological resistance (Jurke et al. 1998; Jurke & Fernando 2002). Zhongyou 821 from China is reported to have field tolerance to sclerotinia stem rot (Li et al. 1999). The transgenic canola lines contained the Ox-ox gene from wheat, Ox-dec from the basidiomycete *Collybia velutipes*, Rs AFP2 (anti-fungal protein) encoding gene from *Raphanus sativus* and chitinase and β-1,3-glucanase genes from tobacco (Table IV). The enzyme oxalate oxidase (Ox-ox) oxidizes oxalic acid and has been shown to provide some level of resistance in transformed soybean (Billings et al. 2003; Donaldson et al. 2001). Mehta and Datta (1991) have shown that the oxalate decarboxylase enzyme from *C. velutipes*, which is inducible by the presence of oxalic acid, is able to decarboxylize oxalic acid effectively. Kesarwani et al. (2000) using tobacco and tomato transformed with the oxalate decarboxylase gene found good levels of control against *S. sclerotiorum*. Anti-fungal proteins have been found to have an effect on a number of filamentous fungi, including *S. sclerotiorum* (Terras et al. 1992). Chitinase and β-1,3-glucanase, have been found to be involved in sclerotial degradation (Gigzey et al. 2001) and found to provide some control of *S. minor* (El-Tarabily et al. 2000).

This study would have been more productive if better levels of resistance were available. Of the transgenic lines, only 375-53 had a moderate level of physiological resistance, while the remainder had similar susceptible reactions. Since these lines did not differ significantly from each other or from the untransformed parent, the correlations between GR trials and between techniques were not particularly strong. Rank correlation analysis was less useful in this regard, since a small difference between lines changed the ranks to a larger degree.

The experiments on plant age were done to confirm that these techniques could be used at any plant developmental stage. The general agreement in results indicates that the differences detected between lines are not age-mediated and that the resistance genes are expressed throughout the plant's life. This provides a large window for inoculation and enhances the value of the foliar inoculation techniques.

Nelson et al. (1991) found that growthroom evaluations had limited value in a breeding program for soybeans. But like Kim et al. (2000), it is believed that growthroom inoculation techniques can be employed to obtain useful preliminary data on the physiological resistance of genotypes to *S. sclerotiorum*. The Mypetal technique was assessed to be the best growthroom inoculation technique for determining physiological resistance to *S. sclerotiorum*

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in *B. napus*. Lines identified to have resistance in the growthroom will need to have this verified in field trials. Nevertheless, susceptible lines could be identified and discarded in growthroom tests. Ultimately field data is what will be most important for canola growers.

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