SCREENING METHODS FOR RESISTANCE TO *SCLEROTINIA SCLEROTIORUM* IN RAPESEED*

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Abstract: Five leaf inoculation methods at seedling stage and match stick stem inoculation method at flowering stage for assessing oilseed rape variety resistance to *S. sclerotiorum* were compared. The results showed that there were more differences on disease lesions between varieties using mycelium plug (Method A), petals infected by ascospore suspension (Method C) or infected leaf tissue (Method D) as inoculum than ascospores PDA plug (Method B) or petals infected by "puffing" ascospores (Method E). Method C is recommended for assessing variety resistance in oilseed rape to sclerotinia stem rot because it is close to natural infection in the fields and easy to be handled. The optimal time for assessment was day 4 and day 5 after inoculation. Method A is also thought reliable and practical, and its optimal assessment time was day 3-7 after inoculation. Match stick stem inoculation method is also recommended as a reliable method for identifying resistance in oilseed rape to *Sclerotinia sclerotiorum*, because of its ease of use both in the field and in the greenhouse, less affected by environment.

Key words: method of resistance identification; oilseed rape; variety resistance; sclerotinia stem rot

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most important diseases on oilseed rape in the world. The disease has not been controlled consistently and economically[1,2] and there are no varieties possessing good resistance to sclerotinia stem rot. It has been reported that there were resistance differences among varieties[3], however. This means that there are promising sources of resistance in varieties of oilseed rape. So it is very important to screen resistance sources and develop hybrid varieties with resistance. Methods of variety identification in the fields with natural and artificial inoculation were mainly used to determine their resistance to sclerotinia stem rot. The disease severity on the varieties, however, was greatly influenced by environmental conditions and led to unstable results[4]. Therefore, it is essential to study practical, accurate and rapid methods for screening resistant sources and breeding programs.

There are two ways for *Sclerotinia sclerotiorum* to infect oilseed rape. One way is that the infection occurs from mycelium that originates from eruption germination of sclerotia in soil. Hypha germination of sclerotia causes infection by first invading nonliving organic matter and forms mycelium, which is an intermediate

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necessity for mycelial infection. The other way, the main way, the pathogen infects oilseed rape primarily through petals that infected by airborne ascospores deposited on leaves, nodes or other parts of plant. Apothecia developed from sclerotia locate either upon or buried in the soil, and eject ascospores to nonliving or senescent plant parts. Ascospores germinate, ramify the nonliving or senescent plant part, and invade healthy plant parts [5]. Five leaf inoculation methods at seedling stage and match stick inoculation method at flowering stage were tested according to the two ways of *Sclerotinia sclerotiorum* infection to oilseed rape.

The objectives of this research were to compare the six methods for assessing variety resistance to *Sclerotinia sclerotiorum*, to determine which methods could be reliable to distinguish differences in resistance among varieties

1 Materials and Methods

An isolate of *S. sclerotiorum*, Great Harpenden, used in the experiment was collected from Harpenden, UK. Wheat and Perlite medium were used for culturing sclerotia. Apothecia were produced in the laboratory from cultured sclerotia of isolate Great Harpenden[5]. Ascospores were collected onto millipore filter (TYPE Attp 0.8 μm) from apothecia by using a pump. Ascospore-laden filters were stored at -23°C in a freezer. Ascospore suspensions were prepared by soaking and shaking ascospore-laden filter in sterile distilled water.

Five varieties resistance to *S. Sclerotiorum* known were from China and European countries. Chinese varieties Zhongyou 821 and Y16 were resistance, European varieties Apex and RPC 681 were susceptible and PAUC 61 was intermadiate. Seedlings grown from seed germinated at 100% relative humidity (RH) flow-bench were transplanted to plastic pots (5 inch in diameter) containing soil-less compost with one plant per pot, and were placed into a greenhouse at average 15°C with supplementary lighting to maintain a 16-hour photoperiod. Plants were ready for leaf inoculation when they had five fully expended leaves (GS 1.5)[6], and for match stick stem inoculation when they were in full flowering stage (GS 4.5)[6].

The five leaf inoculation methods were (1) using mycelium plug as an inoculum (Method A): Surface sterilized sclerotia of Great Harpenden was placed in the center of Petri dish containing potato dextrose agar (PDA), then incubated at 20°C. Mycelium plugs (1 cm in diameter) were harvested from the edge of a 6-day old sclerotinia colony so that the mycelium was of the same age and virulence. (2) Ascospores PDA plug as an inoculum (Method B): Ascospore suspension was added to molten 3% PDA at 25-30°C (before it solidified). Ascospore concentration PDA was 2×10^4 ascospore/ml. Each plug was formed by 300 μl PDA into 1 cm in diameter. Plugs were placed in an incubator, 20°C, for 3 hours. (3) Petals infected by ascospore suspension (Method C): Petals that were collected from older pollinated flowers were soaked in 2×10^4 ascospore/ml ascospore suspension (10 petals per ml suspension) with soft shaking The petals then were incubated in incubator, 20°C, for 3 hours. Five-petal inocula were placed on the center of leaf surface in 1 cm diameter. (4) Infected leaf tissue as an inoculum (Method D): Infected leaf tissue was cut into pieces 1 cm in diameter. (5) Petals infected by "puffing" ascospores (Method E): Petals laid without overlapping were placed on the bottom of a big plastic box. The container with apothecia in it was opened in the box, followed with a puff of ascospores, and covered the box's lid immediately. Ascospores fell down slowly to the petals. The inoculation density was

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of Agricultural Sciences, major in oilseed rape breeding and diseases research.

about 190 ascospores on 1 cm^2 petal. Removed the petals to the incubator at 20°C for 3 hours. Five petals, as an inoculum, were placed on the center of leaf surface in 1 cm diameter.

Leaf inoculation experiments were conducted at IACR-Rothamsted , UK. Plants were inoculated at growth stage GS 1.5. An inoculum (a mycelium plug, an ascospore PDA plug, five petals infected by ascospore suspension, a piece of infected leaf tissue or five petals infected by puffing ascospores) was placed on the central of the fourth leaf surface. After inoculation, plants were incubated in controlled growth chamber at 18°C, 100% relative humidity with 16-hour photoperiod. The temperature and the relative humidity were recorded by a data logger. Every treatment was assigned for 5-7 replicates. Lesion length along the vein of leaf and width were daily measured after inoculation until lesions expended to the edge of the leaves.

Analysis of variance (ANOVA) in the statistical package Genstat[7] was used to examine the effects of different methods of inoculation, varieties and dates of assessment to lesion length plus width.

Match stick stem inoculation method was conducted at three sites, where were at IACR- Rothamsted, UK (greenhouse), Anhui Academy of Agricultural Sciences (AAAS), China (field) and Rustica Prograin Genetique Company (RPG), France (greenhouse). In this method pieces of wooden match stick, about 2-3 cm long, were sterilsed and placed in Petri dishes containing PDA medium. A mycelia plug was placed in the center of each dish and then incubated. Mycelia were allowed to grow until the match stick were covered. Plants were inoculated during full flowering time by placing a mycelium coated match stick into a pre-drilled hole in the stem about 30 cm from the plant base. Lesion length along the stem was measured 8 days after inoculation[8].

2 Results

Leaf inoculation methods The development of lesion length plus width on all varieties tested over time using different inoculation methods was shown in the Figure 1. Among them, disease lesions of all five varieties appeared on the first day after inoculation, and afterwards increased quickly in Method A. There were significant differences between varieties ($p<0.001$) during day 3-7. The score of resistance of five varieties from susceptible to resistant, according to lesion length plus width, was RPG681, APEX, PAUC 61, Y 16 and Zhongyou 821. In Method B, tiny mycelium germinated from ascospores could be seen clearly on PDA on the first and the second day but no lesions occurred until the third day. Lesions developed more slowly than that in Method A. The score of resistance of the five varieties was APEX, RPC 681, PAUC 61, Zhongyou 821 and Y 16. Ascospore infected petals were used as inoculum in both methods C and E. Mycelium growing on the petals could be seen, but no lesions were found on the leaves on the first and the second day. Lesions appeared on four varieties except RPC681 on the third day in Method E, which was one day ahead of it in Method C. The score of the five varieties was APEX, RPC 681, PAUC 61, Y 16 and Zhongyou 821 from susceptible to resistant in Method C and it was RPC 681, APEX, PAUC 61, Zhongyou 821 and Y 16 in Method E. Lesions of all five varieties occurred on the first day and also developed rapidly in Method D. The score of resistance for the five varieties was changeable when assessed on different time course.

In general, lesion initially appeared within 3 days, in which Methods A and D were on day 1, Methods B and C on day 3, Method E on day 2-3. These indicated that mycelia invaded host leaf tissue earlier than ascospores because ascospores need to germinate and produce mycelia to infect host tissue. The resistance of varieties Zhongyou 821 and Y 16 was higher than that of PAUC 61, APEX and RPC 681, however, there were

some differences between method for the score of resistance of the five varieties.

(Figure is here)

Table 1 Analysis of variance for the effects of method, variety, assessment date on lesion length plus width (mean) of scerlotinia stem rot in controlled conditions

Source of variation	d.f.	SS	MS	F	p
Method	4	23701.797	5925.449	1171.59	<.001
Assessment date	5	30738.822	6147.764	1215.55	<.001
Variety	4	1569.544	392.386	77.58	<.001
Method×Assessment date	20	4948.672	247.434	48.92	<.001
Method×Variety	16	430.947	26.936	5.33	<.001
Assessment date×Variety	20	857.077	42.854	8.47	<.001
Method×Assessment date×Variety	80	338.865	4.236	0.84	<.001
Error	747	3778.039	5.058		0.840

Table 2 Analysis of variance for differences on disease lesion length plus width (mean) of sclerotinia stem rot between varieties by using different methods of inoculation at different assessment dates

Method of inoculation	Parameter of statistics	Assessment date after inoculation								
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Method A	F	4.48	4.93	7.96	8.52	7.43	10.85	12.80	-	-
	p	0.010	0.006	<.001	<.001	<.001	<.001	<.001	-	-
Method B	F	-	-	1.80	2.15	2.16	2.35	2.30	2.35	-
	p	-	-	0.16	0.11	0.10	0.08	0.087	0.081	-
Method C	F	-	-	5.92	8.49	8.06	5.71	4.48	3.66	3.44
	p	-	-	0.003	<.001	<.001	0.003	0.010	0.022	0.027
Method D	F	1.29	3.30	5.69	6.42	6.97	6.96	-	-	-
	p	0.297	0.023	0.002	<.001	<.001	<.001	-	-	-
Method E	F	-	-	0.85	5.38	6.50	3.36	-	-	-
	p	-	-	0.50	0.002	<.001	0.022	-	-	-

Notes: Method A: using mycelium plug as an inoculum; Method B: using ascospores PDA plug as an inoculum; Method C: using petals infected by ascospore suspension as an inoculum; Method D: using infected leaf tissue as an inoculum; Method E: using petals infected by "puffing" ascospores as an inoculum.

ANOVA variance analysis for the effects of three-factors, the methods, the varieties and the assessment date to disease lesions under controlled conditions, was shown in Table 1. The results indicated that there were highly significant differences on disease lesions between the different inoculation methods, varieties or assessment dates. There were also highly significant effects of interactions between method and variety, method and assessment date, or variety and assessment date to disease lesions. These suggested that disease lesion was obviously related to methods of inoculation, varieties and assessment date. Therefore, further variety analysis was done for investigating the differences on disease lesion between varieties by using different methods of inoculation at different assessment dates (Table 2). The results demonstrated that there were more differences on disease lesions between varieties using Method A, Method C and Method D than using Method B and Method E. The optimal assessment date were 3-7 days ($p<0.001$) after inoculation for Method A, 4-5 days ($p<0.001$) for Method C, 4-6 days ($p<0.001$) for Method D and the fifth day for Method E.

Match stick stem inoculation method The results showed that there were different levels of resistance to infection by *S.slerotiorum* present in the varieties tested (Table 1), and that Chinese varieties Zhongyou 821 and Y 16 appeared to be more resistant than the European varieties APEX and RPC 681, PAUC 61 to be intermediate.

Table 3 The levels of resistance to infected by *S. slerotiorum* present in the varieties tested using match stick stem inoculation method at IACR-Rothamsted, UK (greenhouse), AAAS, China (field) and RPG, France (greenhouse) in 1999

Varieties	Sites		
	IACR	AAAS	RPG
Y 16	R	I-R	R
Zhongyou 821	I-R	I	-
PAUC 61	I-R	I-S	-
RPC 681	S	-	S
APEX	S	-	-

Note: R-resistant, I-intermediate, S-susceptible.), IACR-IACR-Rothamsted, UK, AAAS- Anhui Academy of Agricultural Sciences RPG-Rustica Prograin Genetique Company, France

3 Discussion

The results of all experiments reveal that resistance of Y16 and Zhongyou 821 were stronger than PAUC 61, APEX and RPC 681. There were, however, differences between the resistance scores of the five varieties to *Sclerotinia sclerotiorum* for different inoculation methods. Authors argue that these differences were related to the evaluation method used for variety resistance.

Leaf inoculaton methods Method A is easy to obtain inoculum with the same age and virulence to plants, and the results showed significant ($p<0.01$) differences on disease lesions between varieties on all assessment

dates using ANOVA variance analysis. The optimal assessment dates were between 3-7 days after inoculation and its initial assessment date was earlier (the third day). The period of assessment (3-7 days) was longer than those in other methods. Therefore, this method is the simplest and fastest for assessing variety resistance. It was difficult for using Method B to make uniform ascospores in PDA because PDA began to solidify when ascospores were put into PDA under 30^{o}C (lower than this temperature could keep ascospores survive). This led to different concentration of ascospore. Thus, method B was not suggested as a screening method. The score of the five varieties in relative susceptibility was changeable on different assessment dates when using Method D. So this method is unlikely to be a reliable method for identifying variety resistance to *Sclerotinia sclerotiorum*. The results of ANOVA variance analysis showed that there were significant differences on disease lesions between varieties at day 3-9 for Method C and day 4-6 for Method E. This indicates that both methods could better identify the differences of resistance between varieties. Both methods used ascospore infected petals as inoculum although Method E was closest to natural infection to host plants in the fields, it was difficult for method E to get petals with uniform ascospores landed and to control the ascospore density on the petals. It was not only easy for method C to get petals with uniform ascospores landed and the ascospore density could be controlled, but also more closer to natural infection in the fields. Therefore, using ascospore infected petals as inoculum is recommended as a reliable method for identifying resistance in oilseed rape to *Sclerotinia sclerotiorum*.

Match stick stem inoculation method is also recommended as a reliable method for identifying resistance in oilseed rape to *Sclerotinia sclerotiorum*, because of its ease of use both in the field and in the greenhouse, less affected by environmental conditions compared with leaf inoculation methods.

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