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A screening test for Mycosphaerella brassicicola on Brassica oleracea

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Abstract

A greenhouse screening method for resistance to ringspot (*Mycosphaerella brassicicola*) in *Brassica oleracea* is described. High infection levels were achieved by spraying young plants by mycelial inoculum enriched with 3% sucrose. The screening method was tested on three Brussels sprouts, three cabbage and three cauliflower cultivars, with known reactions to ringspot in the field. Resistance was expressed both in cotyledons and true leaves by a lower number of lesions than the susceptible control and/or by hypersensitive reactions. Results of the seedling tests reflected differences in resistance in the field. Under controlled conditions the new test can be applied year-round to young plants, thus accelerating selection procedures.

Additional keywords: Brussels sprouts, cabbage, cauliflower, resistance, screening method, ringspot.

Introduction

In the Netherlands severe epidemics of ringspot in cabbage and Brussels sprouts in 1984 and 1988, caused by *Mycosphaerella brassicicola* (Duby) Lindau, emphasized the need for resistant cultivars. Sources of resistance are available (Dixon, 1981; Mulder, 1985; Zornbach, 1990), but progress is hampered by the time-consuming procedure of the current screening method. A test requires a complete growing season because fully grown brassica crops have to be tested in the field and disease symptoms develop slowly. Screening plants in the field is time-consuming and expensive, and results are confounded by many factors, including weather conditions and uneven distribution of the disease. For some pathogens, cabbage is screened in an early growth stage (Natti et al., 1967; Greenhalgh and Dickinson, 1975; Braverman, 1977; Williams, 1985; Sjödin and Glimelius, 1988; Bansel et al., 1990). The use of seedlings or young plants has an advantage since large populations of plants can be screened under controlled conditions, in a short period, economizing growth chamber or greenhouse space. The results of screening tests on seedlings or young plants are only reliable under the condition that resistance found in an early growth stage of the host is correlated with resistance in its adult stage (Sjödin and Glimelius, 1988).

Inoculations with *M. brassicicola* are mostly carried out with suspensions of mycelial fragments (Nelson and Pound, 1959; Zornbach, 1990), because mass production of spores in vitro is hardly possible. It is not known whether inoculations with myce-

lium give the same reactions on cultivars with different resistance levels as natural infection by ascospores (field situation). Temperature and leaf wetness are crucial factors in infection studies with *M. brassicicola* on cabbage. Optimum values of these parameters have been determined in other studies (Weimer, 1926; Nelson and Pound, 1959; Van den Ende, in preparation). From studies with *M. citri* it is known that sucrose increases the level of disease of citrus leaves after inoculation with ascospores (Whiteside, 1974). Glucose was found to be a weak stimulant for lesion formation of *Botrytis squamosa* and *B. cinerea* on onion (Clark and Lorbeer, 1977). Comparable results for *M. brassicicola* are not available.

The objective of the present study was to develop an inoculation technique on young plants of *B. oleracea* as part of a routine screening method for resistance against *M. brassicicola*.

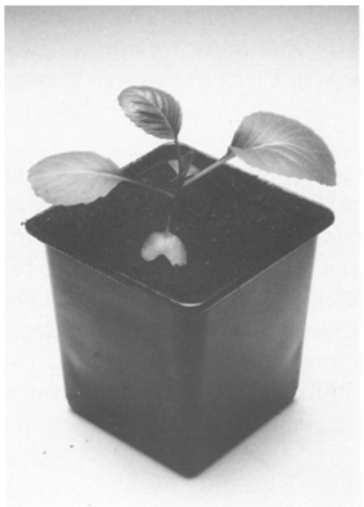
Materials and methods

Inoculum preparation. *M. brassicicola* was isolated from diseased leaves, collected in cabbage fields in the northern part of the Netherlands. Isolates were grown on V8 agar at 17 °C under alternating light: 12 h UV (380 nm) – 12 h dark. Four weeks before inoculation small pieces of mycelium (1–2 mm^2) were transferred to fresh V8 agar to start new colonies. After four weeks, eight of these colonies were suspended in 250 ml of distilled water by use of a microblender. An estimation of the number of units in the suspension, which can possibly act as infection units, was determined by counting individual mycelial fragments with a hemocytometer. For good results isolates should not be used when they are older than six months, or have been transferred more than four times (Van den Ende, unpublished). Therefore, every six months *M. brassicicola* was reisolated from diseased plant material.

Host preparation. Inoculations were carried out on cotyledons and young plants in the third leaf stage. Plants are in the third leaf stage when the third leaf is unfolded and the fourth leaf is just visible (Fig. 1). Plants were grown on a potting mixture consisting of decomposed sphagnum peat to which some clay and marl were added (TRIO 17: pH 5.4; organic matter 74%). For the inoculation of cotyledons, plants were grown in small polyethylene pots (7 × 7 × 6 cm) in the greenhouse at 17–20 °C in daylight, supplemented with artificial light (Philips, HPIT, 400 W) when necessary. Cotyledons were inoculated at an age of 10 days. For inoculation of young plants, plants were grown in bigger polyethylene pots (10 × 10 × 12 cm). Starting when the two first leaves of the plants in the greenhouse were visible, fertilizer was added weekly (Kristalon blauw: 19% N, 6% P, 20% K, 3% Mg). Plants were inoculated in the third leaf stage.

Inoculation technique. Young plants were inoculated by spraying a mycelial suspension with a micro ulva (Micron Sprayers LTD, Bromyard, England), approximately 3 ml per plant. For the inoculation of cotyledons 3 ml per ten seedlings was used. After inoculation, plants were transferred to a growth chamber with a constant temperature (15 °C) and low light intensity (1000 lux) (16 h light – 8 h dark). Plants inoculated in the cotyledon stage were kept in closed plastic containers to maintain a high humidity. Plants inoculated in the third leaf stage were covered with plastic bags to

Fig. 1. Third-leaf stage of a cabbage plant.



ensure a high humidity. After a 6-days period of high humidity plants were transferred to the greenhouse (17–20 °C, daylight only). Symptoms could be read 18–24 days after inoculation. Isolations were made from lesions on the cotyledons and leaves to confirm the presence of *M. brassicicola*.

Effect of sugar. To test a possible influence of sucrose on the disease level of *M. brassicicola* on cabbage cotyledons of a susceptible cultivar, sucrose was added to standard mycelial inoculum (1.6×10^5 infection units per ml) in order to obtain three concentrations and a control (0, 2, 4, 6 g per 100 ml). Each concentration was applied to ten pots with five plants each, in two replications. Inoculation of cotyledons was carried out according to the standard procedures.

The effect of sucrose and glucose added to the mycelial inoculum of *M. brassicicola* when used in inoculation studies on young plants (third-leaf stage) was tested on eight cultivars with different levels of resistance to ringspot. Standard mycelial inoculum (1.4×10^5 infection units per ml) was divided in five parts. To each part different amounts of glucose or sucrose were added to obtain the following concentrations: 0% sugar (C), 1% sucrose (S1), 3% sucrose (S3), 1% glucose (G1) and 3% glucose (G3). Per treatment, two plants were inoculated in four replications according to the standard procedures.

Screening of cultivars. Cultivars of cabbage, Brussels sprouts and cauliflower which showed resistance, partial resistance or susceptibility in previous field trials were selected for the screening tests. The screening test was carried out on cotyledons (CO test) and on plants in the third leaf stage (YP test). In the CO test twelve pots of five plants each were used per cultivar in three replications. Cotyledons were inoculated with mycelial suspension (1.6×10^5 infection units per ml) to which 3 g sucrose per 100 ml was added. As a control twelve pots with five plants each were used, of which cotyledons were treated with a 3% sucrose solution. In the YP test two plants per

cultivar were inoculated in four replications by standard procedures. The inoculum consisted of standard mycelial inoculum (1.4×10^5 infection units per ml) to which 3 g sucrose per 100 ml was added. As a control two plants of each cultivar were treated with a 3% sucrose solution.

Screening of cultivars on location. To evaluate the practical use of the screening tests, both the CO test and the YP test were carried out on different locations. Six breeding companies (A, B, C, D, E, F) used the same nine cultivars from the preceding screening test. In both tests plants were grown according to the standard procedures of the companies, using their own potting mixtures and fertilizers. Circumstances in the greenhouse could vary per location due to differences in construction of the greenhouses. Inoculations were carried out after a fixed number of days from sowing. The different inoculations at the six locations took place within a 3-day period to minimize a possible time effect. Preparation of the inoculum was standardized, using the same isolate for all locations.

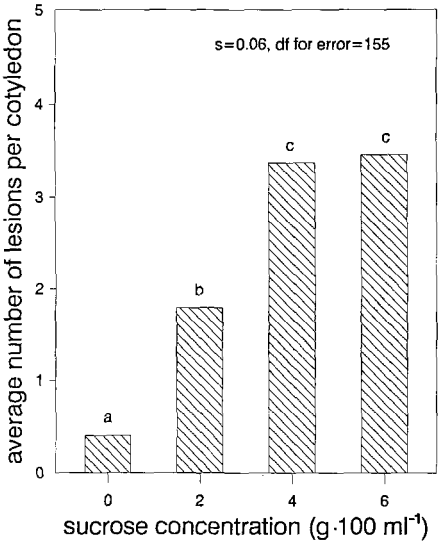
The CO test was carried out in April–May 1989. On each location twelve pots with five plants each were used per cultivar in three replications. Cotyledons were inoculated 10 days after sowing, with an inoculum containing 1.7×10^5 infection units per ml to which 3 g sucrose per 100 ml was added. As a control twelve pots with five plants each were used, of which cotyledons were treated with a 3% sucrose solution. After inoculation plants were kept at high humidity in closed plastic containers at 15 °C in the dark during 6 days. After this incubation period they were transferred to the greenhouse.

The YP test was carried out in April–May 1990. On each location two plants of each cultivar were inoculated in nine replications. Plants were inoculated 28 days after sowing. The inoculum consisted of 1.4×10^5 infection units per ml to which 3 g sucrose per 100 ml was added. As a control two plants of each cultivar were treated with a 3% sucrose solution. Before transfer to the greenhouse, plants were kept at high humidity in closed plastic containers at 15 °C under low light intensity (± 800 lux) for 6 days.

Results

Effect of sucrose. After 24 days the number of lesions per cotyledon was counted, and the average number of lesions per cotyledon was determined for each sucrose level (Fig. 2). The results were analyzed with ANOVA (LSD, $P = 0.05$). The inoculum without sucrose resulted in a significantly lower number of lesions than the inocula with sucrose. A concentration of 2% sucrose in the inoculum gave a significantly higher number of lesions per cotyledon than the control (0% sucrose), but a significantly lower one than the concentrations of 4 and 6% sucrose.

The number of lesions per leaf on young plants was counted 26 days after inoculation. Per treatment the average number of lesions per leaf per plant was determined. The results were analyzed by means of ANOVA (LSD, $P = 0.05$). Fig. 3 shows the results on the most susceptible cultivar, CA01. Inoculation with the standard mycelial inoculum enriched with 3% sucrose (S3) resulted in a significantly higher number of lesions per leaf than the other treatments (Fig. 3). This was true for all the susceptible cultivars tested. A low concentration of sucrose (S1) resulted in a low number of lesions



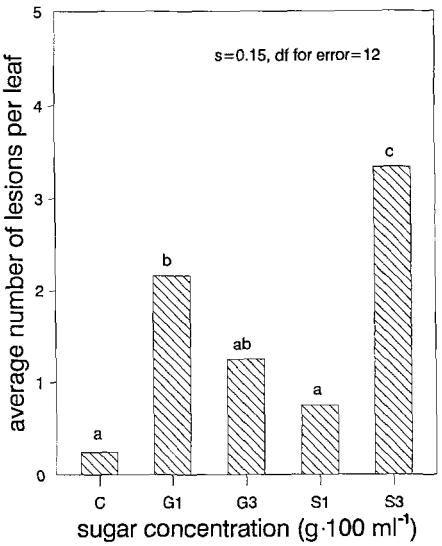


Fig. 2 (left). Average number of lesions per cotyledon (CO test) per concentration of sucrose (0, 2, 4, 6 g per 100 ml). Bars with different letters are significantly different (LSD, $P = 0.05$)

Fig. 3 (right). Average number of lesions per leaf (YP test) in relation to sugar concentration in mycelial inoculum. 0 = sugar-free control, G1 = 1 g glucose per 100 ml, G3 = 3 g glucose per 100 ml, S1 = 1 g sucrose per 100 ml, S3 = 3 g sucrose per 100 ml. Bars with different letters are significantly different (LSD, $P = 0.05$).

per leaf not significantly different from the control (C). A low concentration of glucose (G1) resulted in a significantly higher number of lesions per leaf than the control (C) and the S1 treatment. Increase of the glucose concentration (G3) in the inoculum led to a low number of lesions per leaf which was not significantly different from the control (C).

Results of the screening test. In both tests observations were made 26 days after inoculation. In the CO test the average number of lesions per cotyledon was used as a measure for resistance. In the YP test the average number of lesions on the second leaf was used as a measure for resistance, because the second leaf of the plants in the third-leaf stage is the most susceptible one (Van den Ende, unpublished). Data were tested by means of ANOVA. Considering the fact that the data from these experiments were used to gain insight in slight differences between cultivars and not to select cultivars, a rather discriminative multiple range test was chosen (LSD, $P = 0.05$). Table 1 shows the results of both tests. No symptoms were found in the control treatments. The second column of Table 1 shows the field observations according to the breeding companies. No quantative data on the resistance under field conditions are available. In the screening tests resistance was either expressed as a lower number of lesions per leaf than the susceptible cultivar and/or as hypersensitive reactions. On cotyledons high levels of resistance were identified in CA02 and BS05, a result corresponding with

Table 1. Differences in resistance to *M. brassicicola* between nine cultivars of *Brassica oleracea*, cabbage (CA01, CA02, CA03), Brussels sprouts (BS04, BS05, BS06) and cauliflower (CF07, CF08, CF09).

	Cultivar	Field data[a]	Average number of lesions	
			cotyledon	leaf
Cabbage	CA01	S	3.4 a	3.3 a
	CA02	R	0.2 c	0.0 d
	CA03	PR	0.3 c	0.0 d
Brussels sprouts	BS04	PR	1.4 b	0.3 cd
	BS05	R	0.5 c	0.0 d
	BS06	S	1.9 b	1.0 b
Cauliflower	CF07	R	1.2 b	0.1 d
	CF08	S	1.7 b	0.7 bc
	CF09	PR	1.2 b	0.4 bcd

[a] S = susceptible, PR = partially resistant, R = resistant.
Means followed by the same letter are not significantly different (LSD, $P = 0.05$).

the field observations. CA03 showed a high level of resistance, corresponding with the high level of partial resistance found in the field. No significant difference was found between the cauliflower cultivars, which disagrees with the field observations (CF07). Results of the YP test were in agreement with field observations. No symptoms were found on the second leaf in the cultivars CA02, CA03 and BS05. Even in cauliflower (CF07) some resistance could be distinguished. Differences between the partially resistant and resistant cultivars were not significant. As in the seedling test, the partially resistant cabbage cultivar (CA03) showed a high level of resistance.

Results of the screening test on location. At four locations (A, B, C, D) the CO test resulted in satisfactory disease levels on cotyledons of the susceptible cultivar (85–100% of the cotyledons of the susceptible cultivar were infected). At one location plants died before observations could take place, while at the sixth location disease levels were too low for analysis (10% of the cotyledons of the susceptible cultivar were infected). Considering the four locations as replications of one experiment, data can be analyzed by ANOVA. Although the results show high variability in numbers of lesions per cotyledon between the four locations (Fig. 4), no significant difference in overall disease level could be determined between the locations. Analysis of variance showed interaction between cultivars and locations. Three statistically different reaction types could be distinguished on each location: resistance or low susceptibility (CA02, CA03, BS05), partial resistance (CF07, BS04, CF09) and high susceptibility (BS06, CF08, CA01).

At two locations the instructions for the YP test were not followed. This resulted in poor plant growth. At one location plants died because of drought, while at the other location (the same as in the CO test) the disease level was too low for analysis.

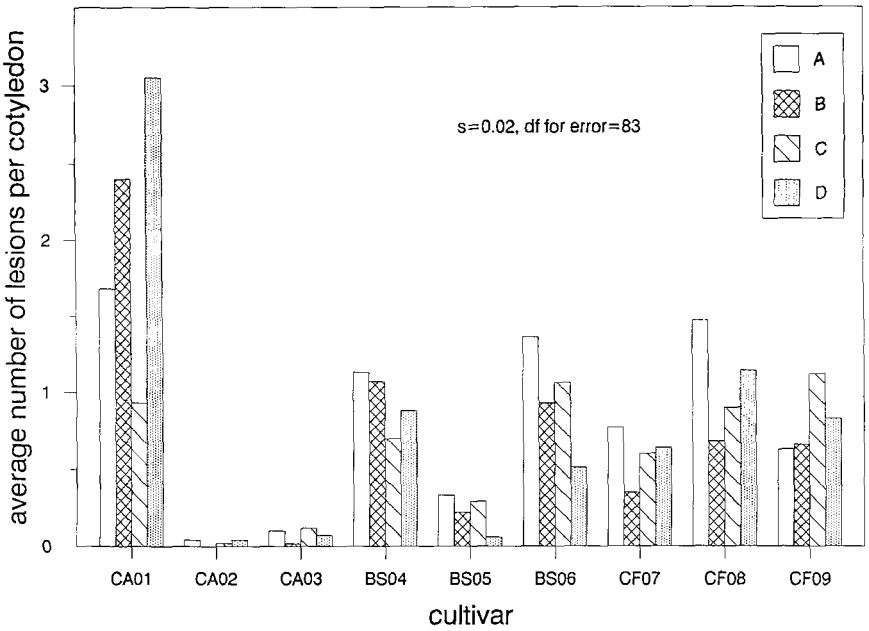


Fig. 4. Results of the CO test carried out at four locations (A, B, C, D). Differences in resistance to *M. brassicicola* between nine cultivars of *Brassica oleracea*, cabbage (CA01, CA02, CA03), Brussels sprouts (BS04, BS05, BS06) and cauliflower (CF07, CF08, CF09). Bars represent average numbers of lesions per cotyledon.

The remaining two locations can be considered as replications, and were statistically analyzed with ANOVA. Because of the rather high number of cultivars in the experiment a conservative multiple range test (Scheffé, $P = 0.05$) was used to discriminate between levels of resistance. Variability in numbers of lesions per leaf (Fig. 5) was not as high as in the CO test. No interaction between location and cultivar was found. CA02, CA03, BS05 and CF07 showed a significantly lower number of lesions per leaf than CF08, BS06 and CA01. Partial resistance was found on the cultivars BS04 and CF09, which showed a significantly lower number of lesions per leaf than CA01, but significantly higher than CA02 and CA03.

Discussion

For most diseases of cabbage, screening tests on plants are carried out by application of spore suspensions of the pathogen (Williams, 1985). Due to the difficulty of producing high numbers of ascospores of *M. brassicicola* in vitro, screening of cultivars by means of inoculation with ascospores is hard to accomplish on a large scale. In 1958, Nelson found no difference in disease level after inoculation of 8–16-week-old plants of a susceptible cultivar with mycelial and ascospore suspensions. The results of present inoculation studies on much younger plants of different cultivars indicated that inoculation with a mycelial suspension can be used to screen cultivars under controlled conditions. Data from the indoor tests, obtained with mycelial

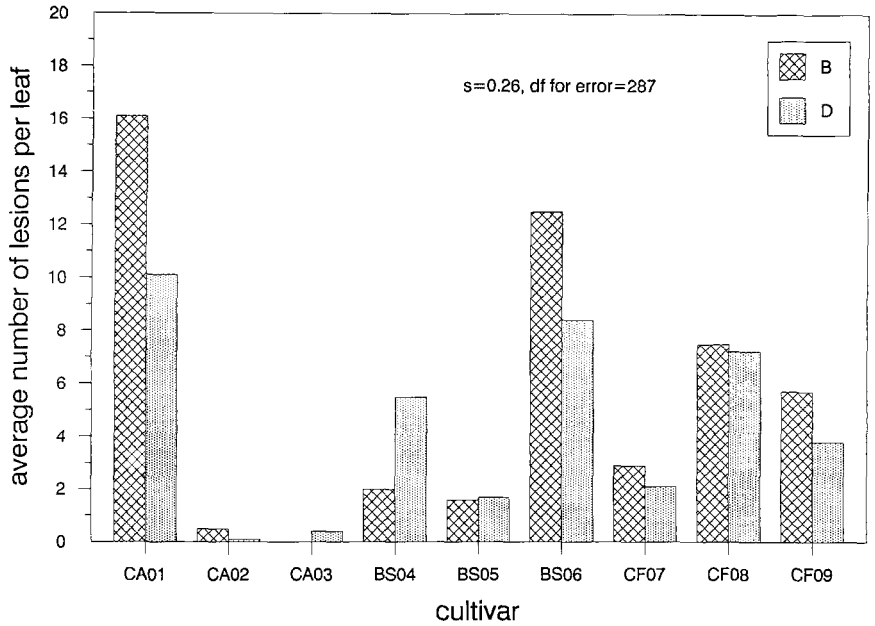


Fig. 5. Results of the YP test carried out at two locations (B, D). Differences in resistance to *M. brassicicola* between nine cultivars of *Brassica oleracea*, cabbage (CA01, CA02, CA03), Brussels sprouts (BS04, BS05, BS06) and cauliflower (CF07, CF08, CF09). Bars represent average numbers of lesions per leaf.

inoculum, corresponded to field data, resulting from natural infection by ascospores.

Adult plant resistance against *M. brassicicola* could already be detected in the cotyledons of seedlings. Resistance expressed at all developmental stages of the host was also found for *Phoma lingam* on *Brassica oleracea* (Mithen and Lewis, 1988; Sjördin and Glimelius, 1988). Results from cauliflower were not as clear as from cabbage and Brussels sprouts (hybrids), probably because the cauliflower cultivars were not genetically uniform.

The standard inoculum resulted in rather low numbers of lesions on leaves of susceptible cultivars. To increase the number of lesions per leaf the mycelial suspension can be enriched with a sugar, which stimulates leaf penetration partly because of nutritional value and partly because of hygroscopic properties (Whiteside, 1974). A concentration of 2% sucrose in the mycelial inoculum increased the number of lesions on cotyledons significantly. If mycelial inoculum was enriched with 3% sucrose and applied on plants in the third leaf stage, the number of lesions was over six times that of the control. Enrichment of the inoculum by glucose can have a similar effect as by sucrose, but only when glucose is used in a lower concentration (1%). A 3% glucose treatment decreases the number of lesions per leaf in comparison to the sucrose 3% treatment. Whether differences in disease level might be explained by differences in osmotic value between sugar concentrations is not known.

Development of a screening test by research workers should be followed by evalua-

tion of the test on different locations carried out by potential users. Unexpected variability and other problems will provide more insight in the practical use of the test. High variability in the results of the CO test on the four locations demonstrated the importance of standardized circumstances during growth of the cotyledons. Differences in potting soil, fertilizer use, temperature and radiation can affect the growth of the plant. The developmental stage of the plant can affect the susceptibility for ringspot as shown in other studies (Van den Ende, in preparation). It is important to screen cultivars uniform in growth stage at the time of inoculation. Although much variability existed in disease level at the different locations, resistant cultivars and highly susceptible cultivars could be easily distinguished. Results of the YP test also showed clear differences between resistant cultivars and highly susceptible cultivars. Failures of the YP test at the different companies were caused in three cases by lack of standardization in plant handling during growth. The low disease levels in both tests at location E cannot be explained. High temperatures in May 1990 may have affected the results of the YP test. Optimum growth of the fungus occurs between 15 and 22 °C (Nelson and Pound, 1959). When temperatures rise above 25 °C, the fungus cannot survive in leaf tissue.

In the present study inoculation of plants with *M. brassicicola* was followed by a 5-day period of high humidity. Longer periods of high humidity gave higher numbers of lesions, as shown in a previous study (Van den Ende, unpublished). However, results obtained from experiments with very long periods of high humidity may not be representative for natural conditions. Plants in an early stage of development cannot survive a long period of high humidity, which is a disadvantage for screening seedlings. Seedlings of cauliflower could not withstand a period of 6 days of high humidity, and died before the symptoms of ringspot could develop. Another disadvantage of the CO test is the senescense of cotyledons before disease assessment. At high temperatures (> 20 °C) cotyledons of some cultivars show early senescence which makes reliable assessment difficult. Such problems do not arise when plants are tested in the third leaf stage. Testing young plants in the third leaf stage will give the best results. With a YP test, resistant and highly susceptible cultivars can be distinguished, but partial resistance is still difficult to assess. Screening *B. oleracea* in the greenhouse for resistance against *M. brassicicola* should therefore be considered a preliminary step to field screening. The ability to screen *B. oleracea* for resistance to *M. brassicicola* under controlled conditions offers the potential for rapid determination of suitability of *B. oleracea* selections for inclusion in a breeding and selection program. Physiological specialization in *M. brassicicola* (Dixon, 1981) is not (yet) known. The present study was carried out with isolates of *M. brassicicola* from the northern part of the Netherlands. The existence of more races of the fungus would complicate the interpretation of the results of the screening test.

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