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Development of an effective screening method for partial resistance to *Alternaria brassicicola* (dark leaf spot) in *Brassica rapa*

M. A. U. Doullah[1], M. B. Meah[2], and K. Okazaki[1,*]
[1]*Faculty of Agriculture, Niigata University, 2-8050, Ikarashi, Niigata, 950-2181, Japan;* [2]*Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh, 2202, Bangladesh;* **Author for Correspondence (Fax: +8125-262-6615; E-mail: okazaki@agr.niigata-u.ac.jp)*

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Abstract

In order to develop a method to measure resistance to *Alternaria brassicicola* (cause of dark leaf spot disease) in *Brassica rapa*, the effects of inoculum concentration, leaf stage, leaf age and incubation temperature of inoculation on infection were studied under controlled conditions using several *B. rapa* genotypes. Three inoculation methods (cotyledon, detached leaf and seedling inoculation) were evaluated for this purpose. The detached leaf inoculation test was the most suitable for screening *B. rapa* genotypes because clear symptoms were observed on the leaves in less than 24 h, and there was a significant positive correlation between the results from the detached leaf inoculation test and the seedling inoculation test, an established method considered to yield reliable results. In addition, it was very easy to screen plants for resistance on a large scale and to maintain standard physical conditions using detached leaves. For successful infection, inoculum concentration should be adjusted to 5×10^4 conidia ml^{-1}, and incubation temperature should be between 20 °C and 25 °C. The 3rd/4th true leaves from 30 day-old plants were optimal for inoculation. In a screening test using 52 cultivars of *B. rapa*, the detached leaf test effectively discriminated between various levels of partial resistance among cultivars. As a result, we identified two cultivars, viz Saori and Edononatsu, as highly resistant and five cultivars, viz Tokinashi Taisai, Yajima Kabu, Purara, Norin-F_1-Bekana and Tateiwa Kabu, as having borderline resistance.

Introduction

Dark leaf spot, caused by *Alternaria brassicicola* and *Alternaria brassicae*, is an important and severe disease of brassicas worldwide (King, 1994; Rotem, 1998; Meah et al., 2002). This disease appears on leaves and stems of seedlings and adult plants and also in siliquae during the ripening stage. Dark spots on the leaves and siliquae reduce the photosynthetic capacity and induce immature ripening, which causes reduced amount of quality seed production in both vegetable and oleiferous brassicas. Moreover, the pathogens can be transmitted to descendents by direct infection of developing seeds in siliquae, causing severe damage in seedlings, especially in nursery beds where large numbers of plug seedlings grow under high humidity (Kubota et al., 2003).

In general, *A. brassicicola* is more virulent and common than *A. brassicae* (Schimmer, 1953; Changsri and Weber, 1963; Humpherson-Jones, 1985), and seed surveys indicate that *A. brassicicola* is more common in commercial vegetable seeds (Schimmer, 1953; Humpherson-Jones, 1985).

Dark leaf spot is very difficult to control due to the numerous sources of inoculum and wide range of spore dispersal (King, 1994).

Resistant cultivars are of interest to plant breeders. Westman et al. (1999) reported a weedy crucifer, *Camelina sativa*, that has a promising level of disease resistance to *Alternaria* pathogens. *Sinapis alba*, a *Brassica* coenospecies, is resistant to dark leaf spot caused by *Alternaria* spp. (Hansen and Earle, 1997). However, interspecific gene transfer by conventional hybridization is difficult. Jasalavich et al. (1993) reported that the degree of susceptibility varies among *Brassica* species. Tewari and Mithen (1999) reported that nearly all commercial brassicas are susceptible to *A. brassicicola* and *A. brassicae* and also showed that *Brassica rapa* and *B. juncea* are more susceptible to *A. brassicae* than *B. napus* and *B. carinata*. Resistance to *A. brassicicola* has also been identified by screening genotypes of *B. napus* and *B. oleracea* (King, 1994).

Brassica rapa is an important source of oilseed in South Asia and vegetables in East Asia. Although resistant cultivars are required to stabilize seed production and to promote sustainable agriculture without hazardous chemical control, no screening test has been developed that establishes the degree to which cultivars of *B. rapa* are susceptible to *A. brassicicola*. In an epidemiological study of *A. brassicicola* and *A. brassicae*, factors such as plant age, wetness period, inoculum concentration and incubation temperature affected the severity of leaf spot disease (Mridha and Wheeler, 1993; King, 1994; Hong and Fitt, 1995; Hong et al., 1996). These studies have been conducted mainly in *B. napus* and *B. juncea* because these crops are important for oilseed and mustard seed production. Few studies have reported the optimal inoculation conditions of *A. brassicicola* in *B. rapa*, despite the agricultural importance of *B. rapa*. Our present study aimed to develop a test of inoculation conditions to easily screen genotypes of *B. rapa* for resistance to *A. brassicicola* by examining factors that affect disease symptoms. These factors include *A. brassicicola* inoculum concentration, leaf position, leaf age, incubation temperature and inoculation technique.

Materials and methods

Plant material

Genotypes of *Brassica rapa* used in this study were collected from Japanese seed companies (Tables 1 and 2) and Gene Bank of the National Institute of Agrobiological Resources, Japan and Bangladesh Agricultural Research Institute, Bangladesh. Plants were grown from seeds in soil using 10-cell plastic trays (cell size: 5 cm × 5 cm) in a greenhouse (25 °C/15 °C day/night cycle). Liquid nutrient ($N:P_2O_5:K_2O$, 0.5:1.0:0.5 g/l; Hyponex, Osaka, Japan) was periodically supplied for plant growth.

Fungal isolates

Two isolates of *Alternaria brassicicola*, Akakura and Cl 0045, obtained from infected *Brassica* seeds from Japan and Bangladesh, respectively, were isolated by the standard blotter method (ISTA, 1996). Isolates were maintained on Potato Dextrose Agar (PDA) media at 4°C. For the production of inocula for artificial inoculations, a fresh culture of *A. brassicicola* Akakura grown on PDA media was prepared from stock culture at 22 ± 1°C in the dark. Conidia of a 10 day-old culture were washed off with distilled water and filtered through cheesecloth of 0.1 mm diam mesh size. The conidial suspension was then shaken and supplemented with 10 μl Tween-20 l^{-1} of suspension. The concentration of the conidial suspension was determined at least three times using a haematocytometer and adjusted to 5×10^4 conidia ml^{-1}.

Effect of inoculation conditions in the different inoculation methods

Detached leaf inoculation test

In this test, two leaves (3rd/4th true leaves) were collected from 30 day-old plants having approximately six leaves. A replicate for the detached leaf test consisted of three randomly selected leaves from a set of the 3rd/4th true leaves collected from several plants that were pooled. *Alternaria brassicicola* Akakura inoculum (5×10^4 conidia ml^{-1}) was sprayed on the upper surface of the collected leaves to run-off. Inoculated leaves were placed in a plastic box (length × width × height = 32 × 23 × 5 cm) where strips of moist tissue paper

Table 1. Evaluation of three different screening methods for leaf spot disease caused by *Alternaria brassicicola* in 14 cultivars of *Brassica rapa*

Cultivar	Origin[a]	Average disease severity index			P^b
		Detached[c]	Seedling[c]	Cotyledon[c]	
Edononatsu	Nih	2.56	2.22	3.20	ns
Purara	Ni	3.11	3.00	5.73	***
Norin F_1 bekana	Nih	3.22	3.44	5.26	ns
Harue	Ta	4.55	4.22	3.93	ns
Shirokuki Seusuzi	Ta	4.90	5.22	6.00	ns
Mustard (BARI 11)	BARI	4.56	4.68	4.60	ns
Kounan	Ni	4.78	4.56	5.80	ns
Mustard (Doulat)	BARI	5.00	5.22	5.93	ns
Mustard (Tori-7)	BARI	5.00	5.22	6.87	**
Nikanme taisai	Hok	6.22	7.99	5.40	**
Mustard (BARI 8)	BARI	6.67	6.55	5.00	*
Nozawana (Strain2)	Hok	6.78	6.89	7.40	ns
Akakura kabu	Kob	7.22	6.78	5.40	*
Satomaru	Kyo	7.97	7.33	5.00	*
	P	***	***	***	

$F_{cultivars}$ = 6.12**; $F_{methods}$ = 0.19 n.s.; $F_{cultivars \times methods}$ = 6.30**

[a]Nih = Nihonnorin Seed Co., Japan; Ni = Nitto Seed Co., Japan; BARI = Bangladesh Agricultural Research Institute; Ta = Takii Seed Co., Japan; Hok = Hokuetsu Seed Co., Japan; Kob = Kobayashi Seed Co., Japan; Kyo = Kyowa Seed Co., Japan.

[b]*P* = probability of *F*-test: *0.05, **0.01, ***0.001, n.s. = non significant.

[c]Detached: Detached leaf inoculation test; Seedling: Seedling inoculation test; Cotyledon: Cotyledon inoculation test.

were put on four vertical sides of the inoculation box to maintain humidity at ~98%. The plastic box was then incubated at 22 ± 1 °C for 3 days in the dark in an incubator or controlled-temperature room.

Seedling inoculation test

In this test, 30 day-old plants having approximately six leaves were used. A replicate for the seedling test consisted of three seedlings. *Alternaria brassicicola* Akakura inoculum (5×10^4 conidia ml^{-1}) was sprayed on the upper leaf surface of the seedlings to run-off. Inoculated plants were covered with polythene bags to create high humidity and maintained in a greenhouse (25 °C/15 °C at day/night) for three days.

These two inoculation tests were conducted using 52 *B. rapa* cultivars, of which 14 cultivars were used in the first experiment (Table 1) and 42 cultivars were used in the second experiment (Table 2). The experiments were arranged in a completely randomized design with three replicates. A susceptible cultivar (Nozawana) served as the positive control, and for the negative control, the leaves were sprayed with distilled water only. After three days of incubation, detached leaves or plants were rated using the disease severity index, which ranges from 1 to 10 of *Alternaria* leaf spot, as modified from King (1994), where 1 = no spots and no chlorosis on the leaf, 2 = a few pinpoint spots but no chlorosis, 3 = some spots but no large lesions and no chlorosis, 4 = some spots with a few lesions surrounded by light chlorosis, 5–9 = increasing number and size of lesions and chlorosis on the leaf, 10 = lesions with chlorosis on more than 90% of the leaf. Cultivars with a disease severity index of 1–3 were classified as highly resistant, those having an index of 3.1–4 were borderline resistant, and those having an index of 4.1–10 were classified as susceptible to the disease. In the seedling test, the 1st/2nd leaves were not evaluated for disease severity index because they were too old to be reliably rated.

Cotyledon inoculation test

In this test, seeds were immersed in gibberellin solution (100 mg l^{-1}) at 4 °C overnight to overcome dormancy. Next, 15 seeds were plated on a sheet of moist filter paper (Advantec, Japan) in a 6 cm plastic Petri dish, incubated at 23 °C under a 12 h light/12 h dark cycle for 4–5 days. After incubation, 10 vigorous seedlings from each plate were retained. Thirty seedlings of each cultivar (three replicates consisting of 10 seedlings per replicate) were used for cotyledon inoculation.

Table 2. Mean disease score using two *Alternaria brassicicola* inoculation methods in 42 *Brassica rapa* genotypes

Origin[a]	Cultivar	Average disease severity index[b]		Origin	Cultivar	Average disease severity index	
		Detached[c]	Seedling[c]			Detached	Seedling
To	Saori	2.84	3.11	GB 26134	Yamashiona	5.33	5.33
Nih	Edononatsu	2.89	2.22	GB 26844	Yamauchi	5.39	4.45
GB 26061	Tokinashi Taisai	3.00	3.67	GB 25875	Nishikino Zairai	5.39	4.78
GB 26874	Yajima Kabu	3.05	3.22	To	Ayumi	5.39	5.56
Ni	Purara	3.17	3.00	GB 26863	Owari	5.44	6.33
Nih	Norin F_1 bekana	3.17	3.44	GB 25868	Ishikawa Zairai	5.45	4.89
GB 26824	Tateiwa Kabu	3.67	4.56	Wa	Maruchan	5.45	5.67
Ta	Soten	4.22	3.00	GB 26064	Yayoi Komatsuna	5.56	4.67
GB 26689	Bansei Tougou	4.44	4.67	Asa	Shigatsu Shirona	5.56	5.33
GB 26712	Shiodome	4.78	4.55	Ma	Tsugarubeni	5.61	3.78
GB 26801	Shimofusa Kabu	4.78	5.78	Ta	Suwan	5.61	5.9
GB 26070	Irakabu	4.84	4.89	Sa	Hanakazaki	5.72	5.33
GB 26090	Wase Mibuna	4.89	4.11	Asa	Saishin	5.78	5.56
GB 26870	Tennouji Kabu	5.00	4.67	Asa	Osakashirona	5.83	5.56
GB 26720	Kurihara Kashin	5.00	5.11	GB 26692	Nozaki Harumaki	6.00	5.67
No	Ootakana	5.11	5.78	GB 25846	Wasena	6.11	4.78
Sa	Kuromizuki	5.17	4.89	Hok	Nozawana(S-2)	6.34	6.11
Ta	Osome	5.17	5.78	GB 26818	Kisobeni Kabu	6.67	6.00
Ya	Eigyoku 65	5.27	5.45	Ta	CR Saitaikai	6.78	6.11
Asa	Nozawana(S-1)	5.28	5.33	Asa	Houfuhaksai	7.11	4.22
GB 25857	Kato Zairai	5.33	5.11	Fu	Kimuchiasu	7.39	6.78
					LSD[d]	0.34	0.32

[a]To = Tohuku Seed Co., Japan; Nih = Nihonnorin Seed Co., Japan; Ni = Nitto Seed Co., Japan; Ta = Takii Seed Co., Japan; No = Nojaki Seed Co., Japan; Sa = Sakata Seed Co., Japan; Hok = Hokuetsu Seed Co., Japan; Ya = Yamata Seed Co., Japan; Asa = Asahinouen Seed Co., Japan; Ma = Marutane Seed Co., Japan; Fu = Fukudane Seed Co., Japan; Wa = Watanabe Seed Co., Japan; and GB = Gene Bank of National Institute of Agrobiological Resources, Japan.

[b]Cultivars with a disease severity index of 1–3 are classified as resistant, those having an index of 3.1–4 are borderline resistant, and those having an index of 4.1–10 are classified as susceptible to the disease.

[c]Detached: Detached leaf inoculation test; Seedling: Seedling inoculation test.

[d]LSD ($P = 0.05$): Least Significant Difference.

Cotyledons were dipped in 5×10^4 conidia ml^{-1} suspension of Akakura isolate, and the inoculated seedlings were then incubated in the dark for 3 days at 22 °C. Seedlings were evaluated individually 3 days after inoculation using the visual disease severity index, which ranges in even numbers from 0 to 10; i.e., 0 = no spots, 2 = a few small pinpoint spots but no extensive lesions, 4 = small spots and one or two restricted large spots, 6 = spots with extensive lesions, 8 = spots with lesions and yellowing, and 10 = spots and lesions with mycelial growth, including yellowing and rotting (dead). Fourteen cultivars used in the detached leaf inoculation and seedling inoculation tests were also used in this test. The experiment was arranged in a completely randomized design, maintaining appropriate controls.

Effect of conidial concentration

An inoculum concentration series of 1.5×10^3, 2.5×10^4, 5×10^4 and 5×10^5 conidia ml^{-1} was produced by diluting a conidial stock solution with sterile distilled water. A mixed population of 3rd/4th true leaves detached from 30 day-old plants were used to assess the effect of four different conidial concentrations for disease development 3 days after inoculation. The cultivars used were Nozawana, BARI 11, Norin-F_1-bekana and Edononatsu, which have different susceptibilities to infection, as determined in preliminary tests. The experiment was arranged in a completely randomized design with three replicates (three leaves per replicate). Control leaves were sprayed with distilled water only.

Effect of leaf age and plant age

To obtain leaves of different ages at the same leaf position, seeds were sown at 10-day intervals and two leaves (3rd/4th true leaves) were collected from 30, 40 and 50 day-old plants. To examine disease severity among young leaves (10 days after emergence) in plants of different ages, two fully expanded leaves attaching at the first and second, third and fourth, fifth and sixth, and seventh and eighth leaf positions were collected from 20, 30, 40 and 50 day-old plants, respectively, and inoculated as described above. Cultivars Edononatsu, Saori, Yokkaichi-Marubashi and Nozawana were used to determine the effect of leaf age and plant age.

Effect of incubation temperature

The effect of incubation temperature (15, 20, 25 and 30 °C) on disease development was evaluated using third and fourth true leaves of 30 day-old plants of the same cultivars used in the leaf age and plant age experiments. Leaves were inoculated using the detached leaf test with 5×10^4 conidia ml^{-1} of *A. brassicicola* (Akakura). The experiment was arranged in a completely randomized design with three replicates (three leaves per replicate), maintaining appropriate controls.

Comparison between different inoculation methods

Fourteen cultivars of *B. rapa* (Table 1) were used to evaluate the three different inoculation methods under the conditions described above. After 3 days of incubation, the detached leaves and the plants were evaluated with regard to leaf spot disease symptoms using the disease severity index. Forty-two cultivars of *B. rapa* (Table 2) were examined to confirm the correlation between the detached leaf test and seedling test. The detached leaves were inoculated separately with the two isolates (Akakura and Cl 0045) of *A. brassicicola*. Results of the detached leaf test using the two isolates were combined and compared with the seedling test. In the seedling test, seedlings were inoculated with a mixture of the two isolates.

Statistical analysis

Data from the experiments were subjected to analysis of variance (ANOVA), and inter-mean differences between treatments were established using the Tukey, least significant difference (LSD), Student's *t*-test, using statistics computer software (ESUMI Co. Ltd., Japan). Linear and quadratic regression analyses were performed using statistics computer software to understand effect of temperature on disease severity (ESUMI Co. Ltd., Japan). Coefficient of correlation analysis was used to evaluate relationships using Microsoft Excel.

Results

Effect of conidial concentration

In the detached leaf test, symptoms began appearing on leaves 24 h after inoculation, even at the lowest conidial concentration (1.5×10^3 conidia ml^{-1}) (data not shown), and 3 days after inoculation, the symptoms were rather variable between cultivars. In the seedling test, most of the inoculated plants showed symptoms, except cultivars BARI 11, Norin-F_1-bekana and Edononatsu, which had no symptoms at the lowest inoculum concentration 3 days after inoculation (Figure 1). When the inoculum concentration was increased, disease severity increased in both the detached leaf and seedling tests. There was a significant positive correlation between conidial concentration and disease severity in the detached leaf inoculation test ($r = 0.95$; $n = 9$; $P < 0.001$) as well as in the seedling symptom inoculation test ($r = 0.91$; $n = 9$; $P < 0.001$), but there was no significant difference between the symptoms obtained at the two highest conidial concentrations (5×10^4 and 5×10^5 conidia ml^{-1}) in each the cultivars used (Figure 1).

Comparison of disease severity between the two fungal isolates

Forty-two cultivars of *B. rapa* were assessed for disease severity using the detached leaf test. The detached leaves were inoculated independently with the *A. brassicicola* Akakura and the Cl 0045 isolate. The mean ($\pm$SE) disease severity indices of the leaves exposed to isolates Akakura and Cl 0045 were 4.97 ± 0.18 and 5.26 ± 0.18, respectively, with no significant difference between the two ($t = 1.16$; $n = 42$; $P = 0.25$). The responses

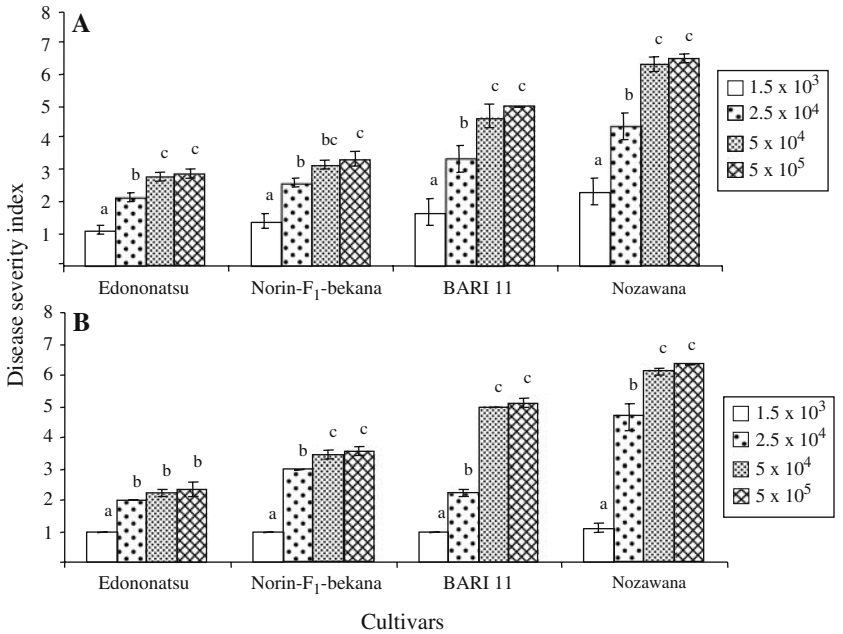

Figure 1. Effect of *Alternaria brassicicola* conidial concentration on disease development in *Brassica rapa* using two inoculation methods. Symptom severity was judged in plants 3 days following inoculation using: (A) the detached leaf inoculation test and (B) the seedling inoculation test. Different letters indicate a significant difference at the 5% level by Tukey test. Error bars indicate standard errors of the mean ($n = 9$).

of the plants to the two isolates correlated strongly ($r = 0.89$; $n = 42$; $P < 0.001$) (data not shown).

Effect of leaf age and plant age

Older leaves exhibited more symptoms than younger leaves in both resistant and susceptible cultivars. Disease development was significantly different among leaves of different ages at the 3rd/4th leaf position by the Tukey test (Figure 2), indicating that detached leaf inoculation success was affected by the age of the leaf. This result was also confirmed by the 2-way ANOVA in which cultivar and leaf age were significant ($P < 0.01$)

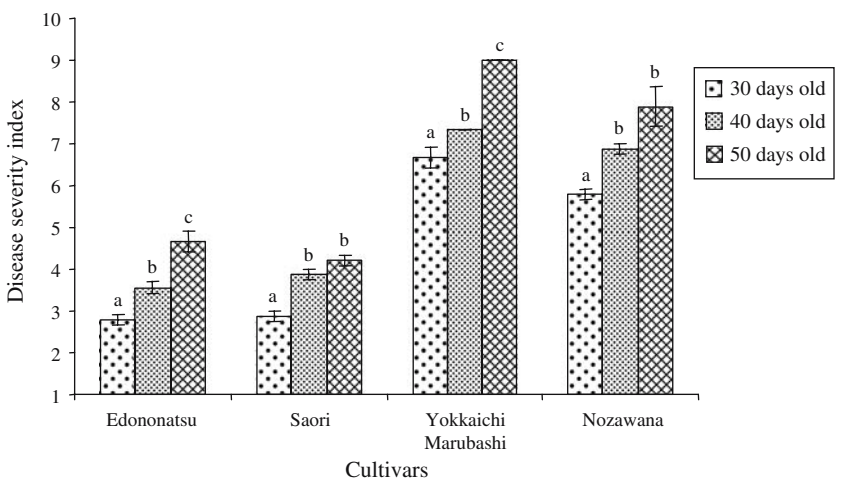

Figure 2. Effect of leaf age on disease development in four cultivars of *Brassica rapa* inoculated with *Alternaria brassicicola*. Third and fourth true leaves were collected from 30, 40 and 50 day-old plants. Different letters within a cultivar indicate a significant difference at the 5% level by Tukey test. Error bars indicate standard errors of the mean ($n = 9$).

and the cultivars × leaf age interaction was significant ($P < 0.05$). Highly resistant cultivars had strongly resistant 30 day-old leaves but their 40 day-old leaves were only borderline resistant.

To examine disease severity among young leaves (10 days after emergence) in plants of different ages, plants were sown at 10-day interval and leaves were collected from different leaf positions. In cvs Saori and Yokkaichi Marubashi, disease severity did not significantly change on the young leaves obtained from different ages, whereas the resistant cv. Edononatsu became borderline resistant, and the susceptible cv. Nozawana showed more severe disease symptoms at the 5th/6th true leaf positions, compared to the 3rd/4th true leaves (Figure 3). The 2-way ANOVA revealed significant differences ($P < 0.01$) on cultivars and plants of different ages and a significant difference ($P < 0.05$) on the cultivar × plant age interaction.

Effect of incubation temperature

For the resistant cultivars, there was a significant linear relationship between disease severity and incubation temperature ($R^2 = 0.96$; $F = 50.3$; $P = 0.019$, for cv. Edononatsu and $R^2 = 0.96$; $F = 53.7$; $P = 0.018$ for cv. Saori) (Figure 4), but such was not the case for the susceptible cultivars in both linear (Figure 4) and quadratic regression analysis (data not shown). The 2-way ANOVA showed significant differences ($P < 0.01$) on cultivar, temperature and the cultivars × temperature interaction. In all cultivars, the greatest differences ($P < 0.01$) were observed between 15 °C and 20 °C. In the susceptible cultivars, disease severity did not differ significantly between 20 °C and 30 °C. In the resistant cultivars, disease severity indicated a change to borderline resistance at 25 °C, and there was no significant difference between symptom severity at 25 °C and 30 °C by the Tukey test.

Effect of different inoculation methods on disease development

Disease development was compared in 14 cultivars of *B. rapa* with the cotyledon test, detached leaf test and seedling test (Table 1). There was a significant difference between cultivars ($F = 47.0$; $P < 0.01$). The severity indices of six cultivars were significantly different (by ANOVA) between the three inoculation methods. However, there was no significant difference between the methods in 2-way ANOVA within 14 cultivars ($F = 1.69$). The cultivar × method interaction was statistically significant ($F = 8.93$; $P < 0.01$). Results from the detached leaf test were highly correlated with the seedling test ($r = 0.94$; $n = 14$; $P < 0.001$) (Figure 5). The cotyledon test showed the poorest correlation with the detached leaf test ($r = 0.34$; $n = 14$; $P > 0.05$) and seedling test ($r = 0.41$; $n = 14$; $P > 0.05$) (Figure 5b, c).

Forty-two cultivars of *B. rapa* were examined to test the correlation between the detached leaf and seedling tests (Table 2). In these tests, the mean (±SE) disease severity indices were 5.12 ± 0.17

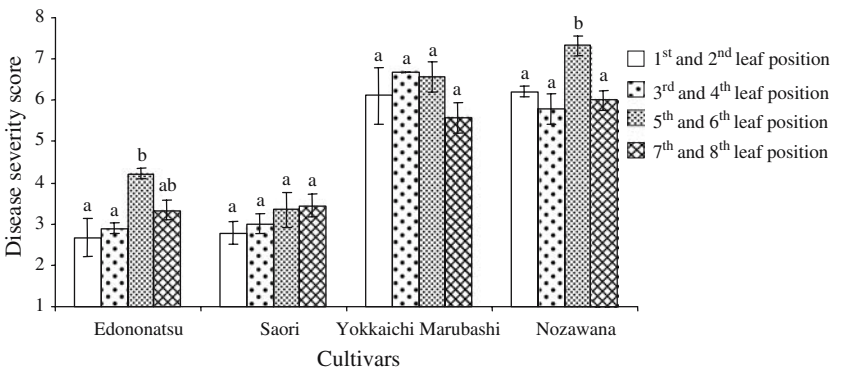

Figure 3. Effect of plant age on disease development in four cultivars of *Brassica rapa* inoculated with *Alternaria brassicicola*. Young leaves (10 days after emergence) attached at the 1st/2nd, 3rd/4th, 5th/6th and 7th/8th leaf positions were collected from 20, 30, 40 and 50 day-old plants, respectively. Different letters within a cultivar indicate a significant difference at the 5% level by Tukey test. Error bars indicate standard errors of the mean ($n = 9$).

(combined data of the two fungal isolates) and 4.88 ± 0.16 (data from a mixture of the two fungal isolates), respectively. The Student's *t* test revealed that the mean disease severity did not differ significantly ($t = 0.99$; $n = 42$; $P = 0.32$) between the two inoculation tests. The disease indices of the two tests were positively correlated ($r = 0.76$; $n = 42$; $P < 0.001$).

Evaluation of resistance

A total of 52 *B. rapa* cultivars were screened for resistance to *A. brassicicola*. Among them, 14 cultivars were screened using three inoculation methods (Table 1) and 42 cultivars were screened using two inoculation methods (Table 2), of which four cultivars were included from the 14 cultivars used to compare all three-inoculation methods. Significant differences in resistance were found among these cultivars. Only two cultivars, Edononatsu and Saori, were resistant, five were borderline resistant, and most of the *B. rapa* genotypes displayed various degrees of susceptibility to *A. brassicicola* in the detached leaf and seedling tests (Table 2).

Discussion

There were significant differences in symptom development in plants sprayed with the various inoculum concentrations used in the study, with the most severe disease symptoms achieved at 5×10^4 conidia ml^{-1}. This result is consistent with previous studies by Hong and Fitt (1995), who reported a maximum disease severity in pods at 5×10^4 *A. brassicae* conidia ml^{-1} in *B. napus*. Similarly, King (1994) reported no difference in disease severity between 2.3×10^4, 3.7×10^4 and 5×10^4 *A. brassicicola* conidia ml^{-1} in *B. oleracea* var. *capitata* and *B. napus*. In the cases of *B. nigra* and *B. juncea*, 4×10^3 to 5×10^4 conidia ml^{-1} were used in screenings for response to *A. brassicicola* and *A. brassicae* infection (Westman et al., 1999; Sharma et al., 2002). Ideally, use of the optimal inoculum concentration will not only reduce the

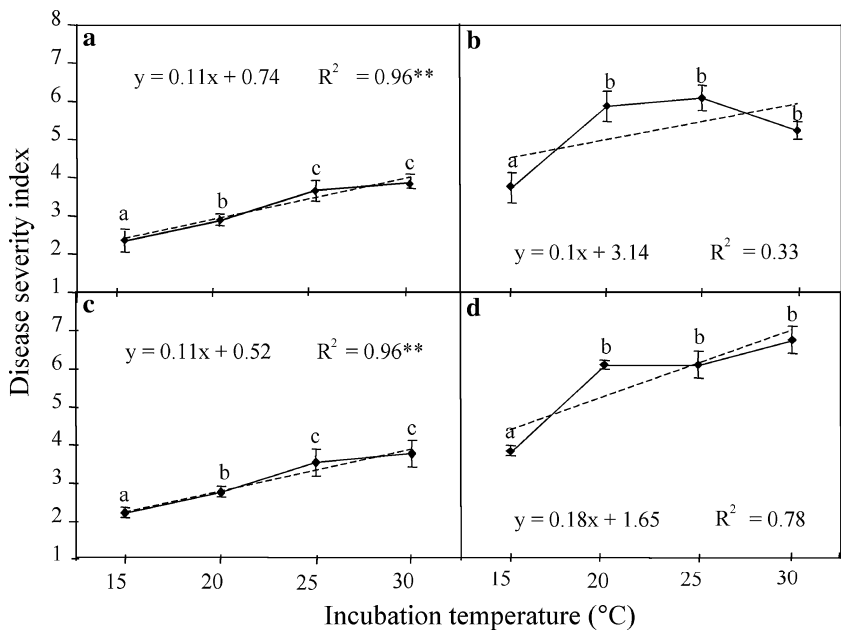

Figure 4. Effect of incubation temperature on disease development following *Alternaria brassicicola* inoculation of detached *Brassica rapa* leaves. Incubation at 15, 20, 25 and 30 °C using four cultivars of *Brassica rapa* (a) Edononatsu, (b) Nozawana, (c) Saori and (d) Yokkaichi Marubashi inoculated with *A. brassicicola* is shown. Different letters within a cultivar indicate a significant difference at the 5% level by Tukey test. The dotted lines were calculated by linear regression for effect of incubation temperature and the resultant line equations are shown. Asterisks (**) indicate significant fit at the 1% level. Error bars indicate standard errors of the mean ($n = 9$).

chance of overlooking susceptible plants, but will also discriminate between various levels of resistance (King, 1994; Surujdeo-Maharaj et al., 2003). Thus, the present study suggests that 5×10^4 *A. brassicicola* conidia ml^{-1} is the optimal inoculum concentration at which various levels of resistance in *B. rapa* genotypes can be effectively distinguished (Table 2).

The severity of dark leaf spot increased with increasing incubation temperature, and symptom severity between 25 °C and 30 °C did not differ significantly in both the resistant and susceptible cultivars (Figure 4). These results indicate a temperature optimum of approximately 25 °C. This finding agrees with that of Mridha and Wheeler (1993), who reported that most *A. brassicae* infections in *B. napus* occur at 25 °C, and fewer are seen at 15, 20 and 29 °C. Researchers have reported different optimal temperatures (19–30 °C) for infection due to differences in inoculation techniques, rating criteria and genotypes (Degenhardt et al., 1982; Bassey and Gabrielson, 1983; Rotem, 1998). It is likely that the optimal temperature for inoculation of *A. brassicicola* is around 25 °C. The present study suggests using an incubation temperature between 20 °C and 25 °C to screen resistance under controlled conditions. A temperature in this range should avoid high-temperature stress and promote greater symptom distinction between resistant and susceptible genotypes.

Susceptible plants showed different levels of susceptibility within their life cycles. Young leaves were less susceptible to infection compared with aged leaves because of the opportunistic parasitic behaviour of *A. brassicicola*, which requires a weakened plant or plant tissue for infection. These findings are in agreement with Mridha and Wheeler (1993), who found that more *A. brassicae* infections occurred in older *B. napus* plants. Greater susceptibility of aged leaves has been reported in nearly all *Alternaria*-host systems (Rotem, 1998). However, in the resistant cultivars of *B. oleracea*, both the youngest and oldest plants were less susceptible to *A. brassicicola* (King, 1994).

The 2-way ANOVA revealed significant effect ($P < 0.01$) of plant age on infection with *A. brassicicola*. Two cultivars, Edononatsu and Nozawana, were exceptions, in that at the 5th/6th leaf positions, the resistant cultivars became borderline resistant and the susceptible cultivars showed more severe disease symptoms. These results may reflect experimental error in the collection of these leaves at about 10 days after emergence. These leaves were probably a little older than other leaves collected for this study, because the trend did not continue at the 7th/8th leaf positions and the overall response to infection in the young leaves was similar among cultivars and in plants of different ages. Dueck and Degenhardt (1975) suggested that variation in disease severity within all leaf positions might be due to the age of leaves (i.e., older leaves are more susceptible to *Alternaria* spp.).

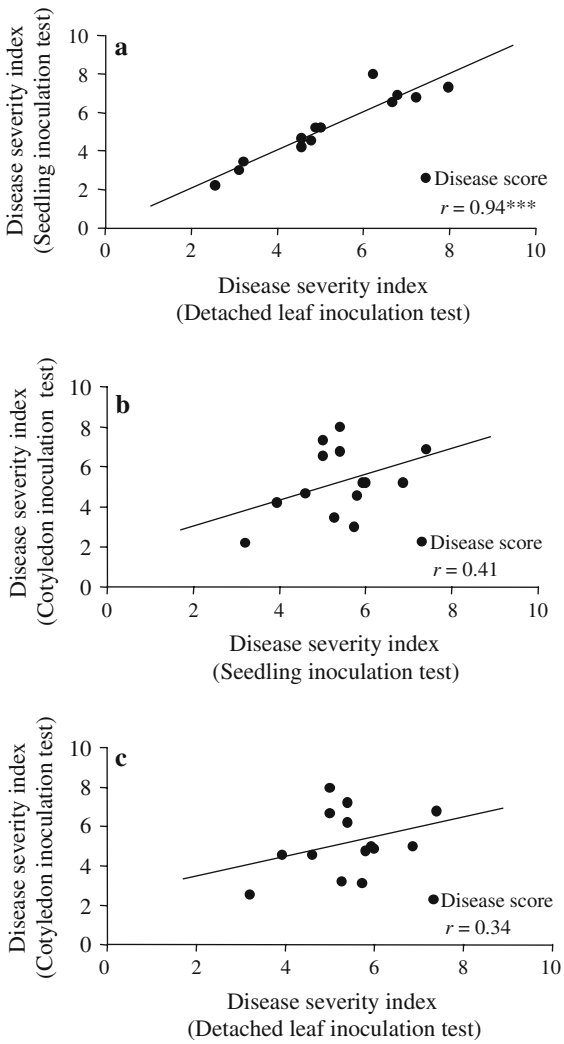

Figure 5. Correlation between three different *Alternaria brassicicola* inoculation methods using 14 cultivars of *Brassica rapa*: (a) detached leaf inoculation and seedling inoculation tests, (b) seedling inoculation and cotyledon inoculation tests, (c) detached leaf inoculation and cotyledon inoculation tests. Asterisks (***) indicate significant correlation r at the 0.1% level.

In the detached leaf inoculation test, there was no significant difference between two isolates (Akakura and Cl 0045) and correlation between the responses to the two isolates was very strong ($r = 0.89$; $n = 42$; $P < 0.001$), indicating similar virulence of the isolates. This result is in agreement with Changsri and Weber (1963) and King (1994), who reported that there is no race identified according to the pathogenicity of individual strains of *A. brassicicola* on specific host genotypes. Based on the results, seedling inoculation tests with 42 cultivars were done using a mixture of the two isolates because a large amount of inoculum was needed.

Several inoculation methods have been used to screen for resistance to *Alternaria* spp., with the goal of identifying resistant genotypes (King, 1994; Vishwanath and Kolte, 1999). In the present study, we found a very strong positive correlation between the results from the detached leaf inoculation test and the seedling inoculation test using 14 cultivars (Figure 5a) and 42 cultivars ($r = 0.76$; $n = 42$; $P < 0.001$) (data not shown), suggesting that these inoculation tests are equivalent with regard to screening different levels of resistance and that the artificial incubation conditions in the detached leaf test do not affect the reliability of the results. Results from the detached leaf test are more consistent under standardized physical conditions, and it is very easy to manipulate plants for resistance screening on a large scale, as compared to the seedling test. In addition, the detached leaf test offers the advantage that symptoms can be clearly assessed within 24 h. Therefore, the detached leaf test is recommended as the primary screening test to limit the number of *B. rapa* accessions required for the field test to assess dark leaf spot caused by *A. brassicicola*.

On the other hand, results show that the cotyledon test did not correlate with the detached leaf test and the seedling test. This was confirmed by the result of the 2-way ANOVA in which the cultivar × method interaction was statistically significant. The lack of correlation result from the cotyledon test and those involving true leaves may be due to physical and physiological differences between cotyledonary and true leaves. In *Brassica* species, leaves contain anti-pathogenic substances such as glucosinolates (Meah et al., 1988; Milford et al., 1989). The total glucosinolate content in cotyledons of seedlings varies between cultivars of rapeseeds (Ishida et al., 2003), and there is no correlation between the total glucosinolate content in true leaves and cotyledons (Kim et al., 2001). It may be necessary to investigate the inhibitory effect of anti-pathogenic substances in the two different leaf types in order to understand the lack of correlation of disease response between the detached leaf test and the cotyledon test.

No *B. rapa* cultivars have been identified with complete resistance to dark leaf spot caused by *A. brassicicola*. In screening for partially resistant cultivars, 3rd/4th true leaves were collected from 30 day-old plants because these leaves reliably display differences in resistance and susceptibility among *B. rapa* genotypes. As a result, we identified cvs Edononatsu and Saori as highly resistant to *A. brassicicola*, and several more cultivars (Tokinashi Taisai, Yajima Kabu, Purara, Norin-F_1-bekana and Tateiwa Kabu) were identified as borderline resistant. Although these cultivars could potentially be used in breeding programmes to develop strains for dark leaf spot resistance, the highly resistant cultivars became borderline resistant in the inoculation test using relatively older leaves. Thus, it will be necessary to examine the usefulness of these resistance levels under field conditions or in nursery beds where large numbers of seedlings grow under high humidity conducive to dark leaf spot disease.

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