



Validation of Inoculation test and Screening for Resistance Sources of Mustard-Rapeseed Cultivars in Bangladesh Against *Alternaria brassicicola*

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Abstract

Alternaria leaf spot or black leaf spot caused by *Alternaria brassicicola* is the devastating disease of mustard-rapeseed in Bangladesh, and can cause yield loss up to 60% every year. A total of 27 cultivars of *Brassica* spp. where *Brassica rapa* (13), *B. juncea* (5) and *B. napus* (9) were selected for resistance screening against *A. brassicicola*. Two inoculation methods viz. detached leaf and seedling inoculation were assessed for checking the validation of inoculation technique using cultivated mustard-rapeseed varieties in Bangladesh a method to measure resistance to *A. brassicicola*. A significant positive correlation between the results of two inoculation methods was found in this study. The detached leaf technique was more suitable due to development of clear symptoms on the leaves within 36hrs, and suitable for screening large scale genotypes for resistance. The 3rd leaves of 30 day-old plants were more suitable for inoculation having severe symptoms than the 4th leaves. Among 27 Bangladeshi mustard-rapeseed cultivars all cultivars expressed susceptible reaction to *A. brassicicola*, except BINA Sharisha-8, as it had border line resistance.



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Introduction

Mustard-rapeseed is the important crop and presently ranked as the world's leading oilseed crop in terms of area and production. Among the species, *Brassica napus* and *Brassica rapa* are regarded as "Rapeseed" while *Brassica juncea* is "Mustard". In

Bangladesh mustard-rapeseed is the most important edible oilseed crop. However, the total annual production of rapeseed and mustard in worldwide is 68 million tons of seeds from an area of 34.33 million hectares (FAO STAT 2015-2016, faostat.fao.org). Approximately 0.302 million hectares of area

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were under cultivation of mustard-rapeseed out of 14.22 million hectares in Bangladesh. Bangladesh is one of the important mustard-rapeseed growing country in South Asia after India producing 359 thousand tons in 2014-15 (FAO STAT 2015-2016). Till to date, the most widely cultivated crop species in the crucifer family (*Brassicaceae*) is mustard-rapeseed. Many factors are associated with the poor yield of mustard-rapeseed in Bangladesh. Diseases have been identified as one of the major factor (Ahmed, 1992)¹. Because of an strong bottle-neck in selection for canola quality i.e. double-low (00) seed quality (zero erucic acid, low glucosinolate content) during breeding in the last 3 decades, cultivated mustard-rapeseed varieties in worldwide have a relatively narrow genetic basis and are lacking of a broad spectrum of disease resistance (Obermeier *et al.*, 2013)¹⁹. Brassica oilseeds are suffered from thirty diseases (Khan, 2011)¹¹. Common diseases of mustard-rapeseed are Aternaria leaf spot, Stem rot, Stem canker, Clubroot, White rust, Light leaf spot, Downy mildew, Turnip Mosaic Virus (TuMV) and Turnip Yellow Virus (TuYV) (Snowdon *et al.*,2006)²⁰. Among these diseases Alternaria leaf spot is considered as one of the major diseases due to their wide distribution and yield losses to Brassica crops worldwide (Khan *et al.*, 2010; Khan, 2011; Meah *et al.*, 2002)^{10,11,16} including Bangladesh. On the other hand, in India this disease causes an average yield loss of 40-70%(Vishwanath and Kolte, 1997)²¹ and in Bangladesh this yield loss up to 60 % (Ahmed and Ahmed, 1994; Meah and Hossain, 1988)^{2,15}.

Alternaria leaf spot disease is described by the formation of spot on the infected leaves and stems and also on siliquae during maturing stage of infected plants. This disease may reduce photosynthesis capacity as grey or black lesion developed on the affected leaves and siliquae and enhance immature ripening. Besides leaf infection, the infection also leads to deteriorate the quality of the seeds i.e. seed size, seed colour, oil contents and germination capacity (Khan *et al.*,2010)¹⁰. Moreover, *A. brassicae* and *A. brassicicola* are seed borne pathogens and these pathogen can be passed on to next generation by direct infection of developing seeds in siliquae, resulting severe damage in seedlings mostly in the nursery beds (Kubota *et al.*,2003)¹⁴. Yield loss of up to 46-47% in yellow sarson and 35-38% in mustard may occur by this Alternaria leaf spot or leaf blight

disease (Vishwanath and Kolte, 1997; Khan *et al.*, 2010; Khan, 2011)^{21,10,11}. However, due to the lack of resistance variety against *A. brassicae/A. brassicicola* in *Brassicaceae* family, Alternaria leaf spot is considered the most destructive and widespread fungal disease of Brassica (Ghose *et al.*,2008)⁶. As the increase of area cultivated with mustard-rapeseed and lack of resistance in current Brassica cultivars, infection by pathogen such as *Alternaria* spp. has become a major constrain to mustard-rapeseed production worldwide including Bangladesh. Since Alternaria leaf spot is increasingly critical in oil production, resistant varieties need to be developed as to control this disease and to increase seed production and to enhance sustainable crop production without perilous chemical control. However, in any of the disease resistance breeding program, the first approach is to quickly screen all the available germ plasm including local races, improved variety and exotic genetic stocks through traditional techniques in controlled condition in the green house, or by field evaluation. Moreover, resistance breeding is the promising initiative for the long term control of diseases as fungicides could not provide effective field control of *A. brassicae/A. brassicicola*. Intriguingly, no comprehensive report on resistance screening for *Alternaria* leaf spot/ Black leaf spot of mustard-rapeseed in Bangladesh have been reported. In view of the facts, the present research work has been carried out to develop an effective inoculation method with *A. brassicicola* on mustard-rapeseed and to screen genotypes of mustard-rapeseed resistance against *A. brassicicola* through the developed techniques in Bangladesh.

Materials and Methods

Plant Materials

Twenty seven (27) mustard-rapeseed genotypes with control were used in this resistant screening study. The seeds were collected from Department of Genetics and Plant Breeding (GPB), Bangladesh Agricultural University (BAU), Bangladesh Agricultural Research Institute (BARI), Bangladesh Institute of Nuclear Agriculture(BINA) and Sher-E-Bangla Agricultural University (SAU). This screening populations comprised of *Brassica campestris /Brassica rapa*, *Brassica juncea*, and *Brassica napus* species which are commonly cultivated in Bangladesh (Table 1). The seeds were sown in soil using plastic pots in a net house of Seed Pathology Centre (SPC),

Bangladesh Agricultural University, Mymensingh-2202, Bangladesh and the entire experiments were

conducted during the period from October, 2015 to March, 2016.

Table 1: Genotypes of mustard-rapeseed used in this study

Name of the Brassica species	Name of the Brassica	Collected Organization genotypes	Number of genotypes
Brassica rapa	Shampad, Sonali, BARI sarisha-6,9,12,14,15, Kallaynia, Safal, Agrani, Tori-7, BINA Sarisha-6, SAU-1	BAU, BINA and SAU	13
Brassica juncea	Daulat, Rai-5, BARI Sarisha 10 & 11, BINA Sarisha-8	BARI and BINA	5
Brassica napus	BARI Sarisha-7,8,13, BINA Sarisha-4,5,9 & 10, GPB (BAU)-Line 1 & 2	BARI, BINA and GPB (BAU)	9

Collection, Isolation and identification of *Alternaria brassicicola* isolate

Isolate of *Alternaria brassicicola* was obtained from infected mustard seeds which were collected from farmers stored seeds. Standard blotter method (ISTA, 1996) was used for the isolation of this *Alternaria* pathogen. In this blotter method, 25 mustard seeds were placed on blotter paper (moistened with water drop) in plastic petridishes. Then the petridishes

were incubated at the incubation room at 20-25°C. After 7-10 days of incubation pathogenic structures (mycelia and conidia) on seeds were observed under stereo binocular microscope. Then the infected seeds were transferred on Potato Dextrose Agar (PDA) medium for 7-10 days in the incubation room to allow the fungus to grow and the concern pathogen was detected by preparing slide and comparing the morphological characters (Figure 1).

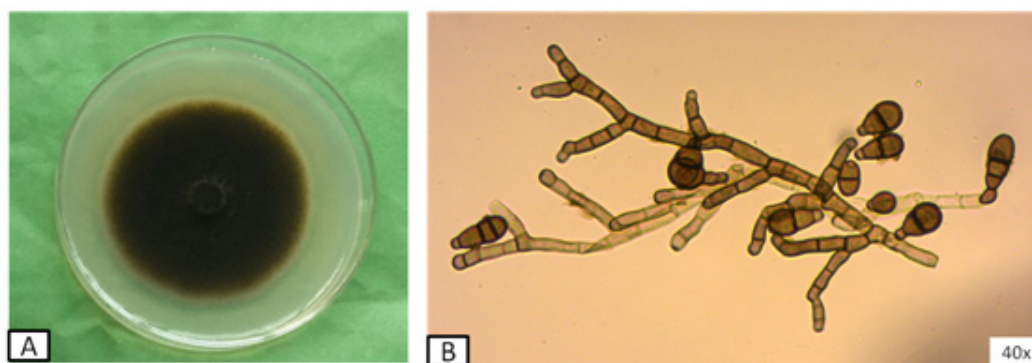


Fig. 1: Pure culture of *Alternaria brassicicola* (A) and different structures of *Alternaria brassicicola* (B)

Fungal block was transferred to the centre of a fresh PDA plate using a sterilized block cutter. The plates were sealed with parafilm and all the isolates were then grown at 24°C with alternate 12/12 h UV light and darkness for 10 days. Sequential culturing from fungal stock was done for 4-6 times to get a pure culture which was used for inoculation (Figure 1).

All of these process was done within laminar air-flow cabinet.

Inoculum Preparation

A pure culture of *A. brassicicola* grown on PDA medium was developed from stock culture for artificial inoculation. Conidia of 10 days old

culture were collected by washing the pure culture of *A. brassicicola* with double distilled water and the filtered through cotton cloth. The concentration of the conidial suspension was determined using a hemocytometer and adjusted to 5×10^4 conidia ml^{-1} .

Methods of Inoculation

Two different inoculation techniques were followed in the present study.

Detached leaf Inoculation

The detached leaf inoculation was performed according to Doullah *et al.*, (2006)³, in brief, 3rd and 4th true leaves i.e. two different leaves were collected from each of 30 days old plants. Three replications were maintained for each of the genotypes including one control. Leaves were then washed with distilled water and remove moisture by tissue paper, and placed on petridishes having three layers of moistened blotter paper with distilled water. Then the conidial suspension sprayed on the detached leaf using hand sprayer and the petridishes were wrapped with parafilm. The concentration of the conidial suspension was maintained at 5×10^4 conidia ml^{-1} . After inoculation, the petridishes containing inoculated leaves were marked and incubated for 3 days at 24°C in incubator with alternate cycle of light/darkness. One Japanese *B. rapa* resistance variety, Edononatsu was used as resistant control. BARI-Sharisha-7 was used as susceptible check. Then the disease severity was observed after 72 hrs of inoculation.

Seedling Inoculation Test

In case of seedling inoculation test, 30 day-old plants were used and the inoculum of *A. brassicicola* was sprayed on the foliar surface of the plants according to Doullah *et al.*, (2006)³. All the genotypes received same amount of suspension and covered with polythene bag having small pores for aeration. After that the inoculated plants were kept in a net house after covering with polythene bags for creating high humidity within the micro climate. Japanese *B. rapa* variety, Edononatsu and BARI-Sharisha-7 were used as resistant and susceptible control, respectively as before. The inoculum suspension contained 4×10^5 conidia/ml throughout the test. Distance between control and inoculated seedlings maintained in the net house.

Then the disease severity was observed after 72 hrs of inoculation.

Assessment of Disease Symptoms

Development of *Alternaria* spot/black leaf spot on inoculated detached 3rd & 4th leaves as well as seedlings were assessed by a disease severity scale consisting of 10 classes according to King, (1994)¹³. Disease symptoms on detached leaves or seedlings were evaluated after 72 hrs of inoculation. Leaf area infection for each of the inoculated plant was considered for assessment of disease severity.

Statistical Analysis

The experiment was performed in a completely randomized design (CRD) with three replicates for both detached leaf and seedling inoculation. In case of seedling inoculation one detached leaf either it is 3rd or 4th for one plant has been considered one replication. In case of seedling inoculation one pot containing 2-3 plants considered as one replication in this study. Statistical analysis for phenotypic data was carried out with SPSS statistical software.

Results

Detached Leaf Inoculation

In case of detached leaf inoculation, 3rd and 4th leaf of 30 days old were collected from each of the 27 mustard-rape seed varieties. Then the detached leaves were inoculated with the conidial suspension (5×10^4 conidia ml^{-1}) of *A. brassicicola* and then incubated them at 24°C for 72 hrs. After 72 hrs of inoculation, the inoculated susceptible mustard variety BARI Sharisha-7 produced typical black spot caused by *A. brassicicola* on the leaf blade (Figure 2). On the other hand, the resistant Japanese *B. rapa* variety Edononatsu produced no spot on the leaf blade after 72 hrs of inoculation by *A. brassicicola* suspension. This result indicates that detached leaf-inoculation was successful.

Seedling Inoculation

In case of seedling-inoculation method, 30 days old seedlings of 27 mustard-rape seed genotypes were inoculated with *A. brassicicola* suspension (5×10^4 conidia ml^{-1}) and the disease symptoms was observed after 72 hrs of inoculation. The inoculated susceptible mustard variety BARI Sharisha-7 produced typical *Alternaria* leaf spot symptom. However, the seedlings of resistance Japanese B.

rapa variety Edononatsu produced no spot on the leaf surface after inoculation. This result indicates

that seedling-inoculation technique also successful for screening assessment.

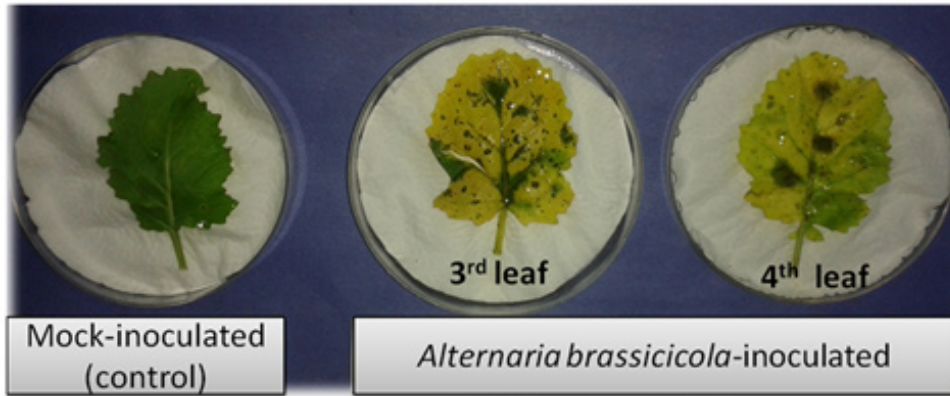


Fig. 2: Detached leaves of BARI Ssrisha-7 showing typical symptoms of Black leaf spot disease on *A. brassicicola*-inoculated leaves (3rd and 4th leaves; right) and no symptoms on mock-inoculated leaf (left)

Effect of Leaf Age on Disease Development

After inoculation with *A. brassicicola*, older leaves of mustard-rapeseed showed severe symptoms compared to the younger leaves for both tolerant and susceptible varieties in most of the cases. Variation in disease development was evident among

leaves at the 3rd or 4th leaf position (Figure 3). This result indicates that the success of detached leaf inoculation was affected by the age of leaf. Here, the disease severity index of 3rd leaf and 4th leaf of the inoculated mustard-rapeseed genotypes showed a strong positive correlation ($R^2=0.956$) (Figure 4).

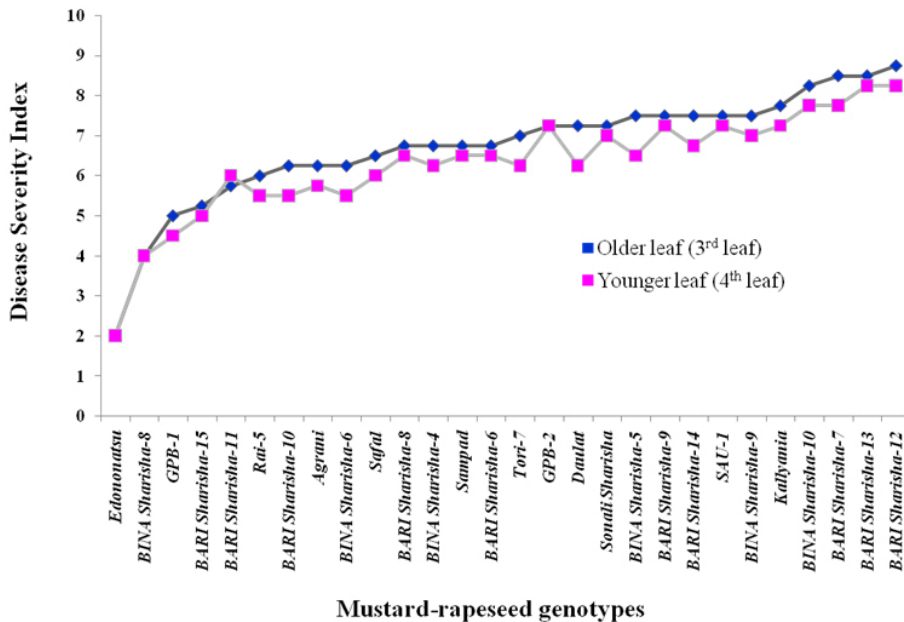


Fig. 3: Older leaf (3rd leaf: Blue line) showed more disease severity index than the younger leaf (4th leaf: Pink line) with *A. brassicicola* infection on mustard-rapeseed genotypes

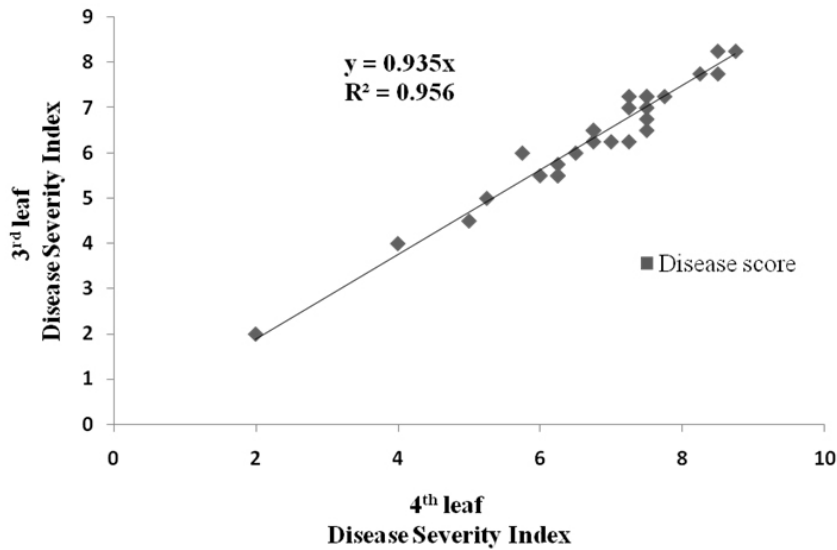


Fig. 4:Correlation between the disease severity of 3rd leaf and 4th leaf of 27 genotypes showed a strong positive correlation

Effect of Different Inoculation Methods on Disease Development

Disease development was observed in 27 cultivars of mustard-rapeseed following the detached leaf and seedling inoculation test. In the present study, the disease expression on detached leaf was higher among the genotypes compared to the seedling

inoculation technique (Figure 5; Table 2). However, there was a correlation was present between the disease expression of detached leaf inoculation and seedling inoculation method (Figure 6). This result indicating that detached leaf inoculation will be more effective and reliable for resistance screening of mustard-rapeseed genotypes.

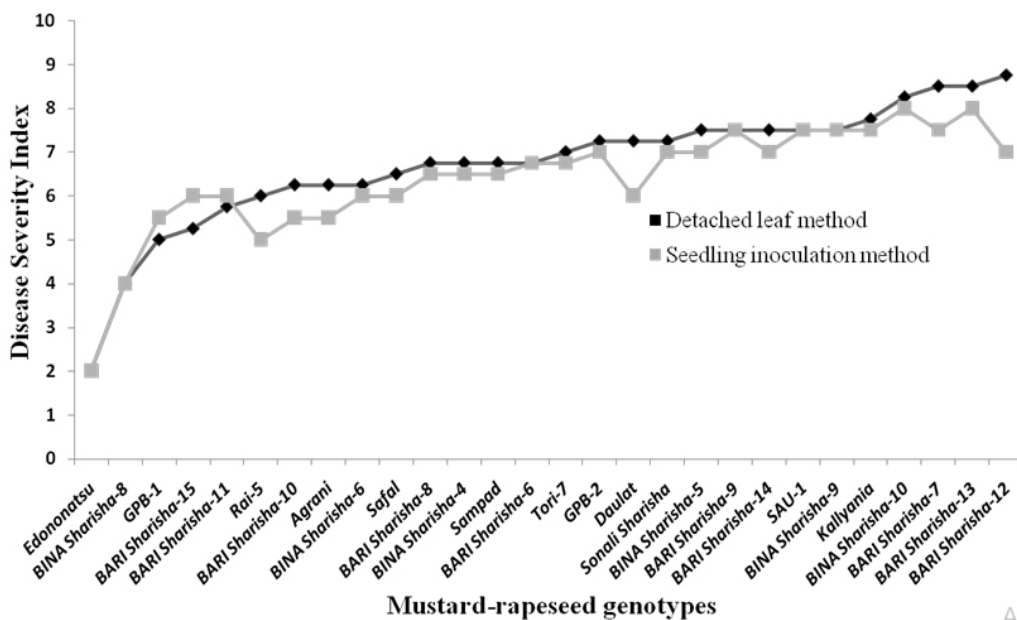


Fig. 5: Detached leaf-inoculation test showing high disease pressure compared to seedling-inoculation test among the mustard-rapeseed genotypes

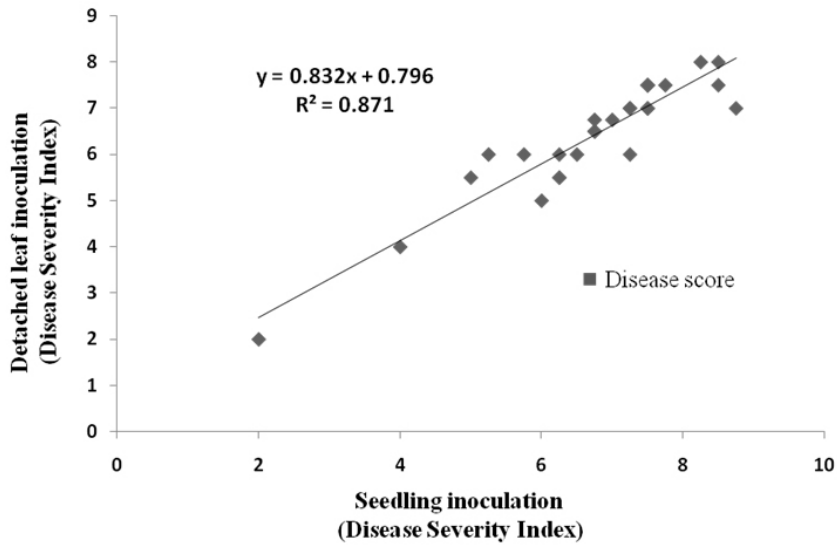


Fig. 6: Correlation between the disease severity of detached leaf inoculation and seedling inoculation test using 27 mustard-rapeseed genotypes showed a medium strong positive correlation

Evaluation of Genotypes for Resistance

A total of 27 cultivars of mustard-rapeseed were screened for resistance to *A. brassicicola* by two different inoculation techniques, of which only one

cultivar belong to *Brassica juncea* species showed border line resistance and significant differences in resistance were found among these cultivars. (Figure 7, Table 2).

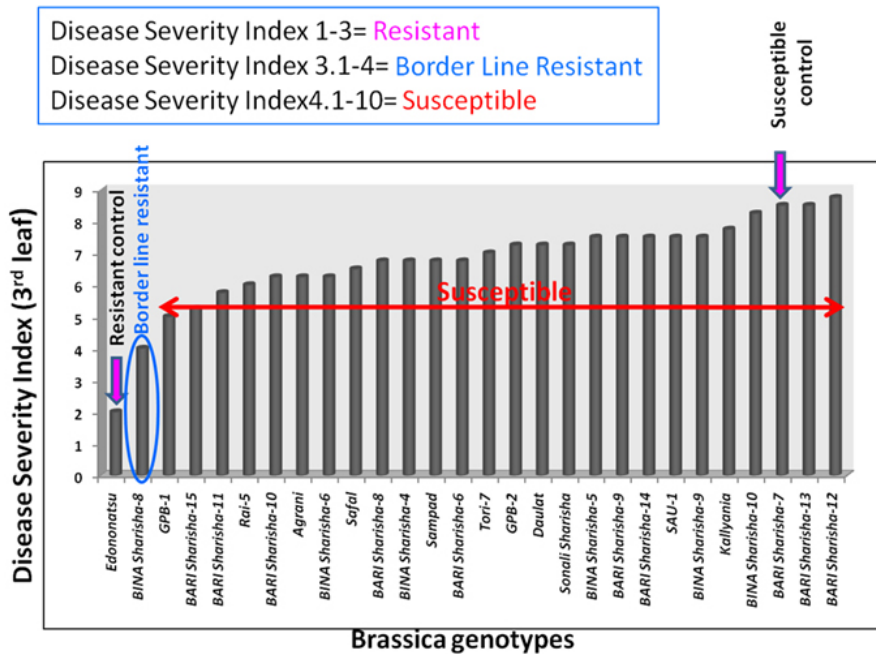


Fig. 7: Resistance scenario of 27 mustard-rapeseed genotypes including control against *Alternaria brassicicola*

Table 2: Mean disease score of two different screening techniques for *Alternaria* leaf spot disease caused by *Alternaria brassicicola* in 27 mustard-rapeseed varieties

Variety name	Brassica species	Average Disease Severity	
		Detached leaf (3 rd leaf)	Seedling
BARI Sharisha-7	Brassica napus	8.50	7.50
BARI Sharisha-8		6.75	6.50
		8.50	8.00
BARI Sharisha-13		6.75	6.50
BINA Sharisha-4		7.50	7.00
BINA Sharisha-5		5.00	5.00
GPB-1		7.25	7.00
GPB-2		7.50	7.50
BINA Sharisha-9		8.25	8.00
BINA Sharisha-10			
Daulat	Brassica juncea	7.25	6.00
Rai-5		6.00	5.00
BARI Sharisha-10		6.25	5.50
BARI Sharisha-11		5.75	6.00
BINA Sharisha-8		4.00	4.00
Sampad	Brassica rapa	6.75	6.50
Tori-7		7.00	6.75
Sonali Sharisha		7.25	7.00
BARI Sharisha-6		6.75	6.75
BARI Sharisha-9		7.50	7.50
BARI Sharisha-12		8.75	7.00
BARI Sharisha-14		7.50	7.00
BARI Sharisha-15		5.25	6.00
Kallyania		7.75	7.50
Safal		6.50	6.00
Agrani		6.25	5.50
SAU-1		7.50	7.50
BINA Sharisha-6		6.25	6.00
Edononatsua		2.00	2.00
LSD ^a 0.48		0.62	

^aJapanese Brassica rapa cultivars; bLSD (0.05): Least Significant Difference

Discussion

Alternaria leaf spot or black leaf spot caused by *Alternaria brassicicola* is one of the vital diseases of Brassica crops worldwide including Bangladesh. The disease has been an increasing threat to

mustard-rapeseed production for the last couple of decades in Bangladesh. Owing to that disease resistance breeding is the promising approach for the durable and effective control of this disease. Moreover effective field control with fungicides is

not feasible as significant number of alternate host present during the growing season. No complete resistance is known in the available cultivated varieties against *A. brassicicola* in Bangladesh. Owing to that, development of inoculation techniques and the screening of mustard-rapeseed germplasm exist in Bangladesh will be a promising approach for the identification of resistant variety against *A. brassicicola* in Bangladesh.

In the present study, two inoculation techniques such as detached leaf and seedling inoculation techniques have been used for validation of the test using cultivated varieties in the country and to screen for resistance to *A. brassicicola* for 27 mustard-rapeseed genotypes. The aim of this study was to develop an effective inoculation technique with *A. brassicicola* and to identify resistant genotypes in the cultivated mustard-rapeseed. In this experiment, disease expression level developed by artificial inoculation following two methods considering all cultivars was positively correlated ($R^2 = 0.871$; $n = 27$) meaning that these artificial inoculation techniques are enable to screening various levels of resistance. In addition, incubation environment in the detached leaf inoculation technique do not affect the disease expression. These findings are in conformity with the results of Doullah *et al.*, (2006)³ where they also showed a positive correlation between the disease expression level of detached leaf and seedling inoculation techniques using 14 & 42 *Brassica* varieties. In this experiment detached leaf inoculation showed clear visible black spot symptoms on inoculated leaf within 36 hours and the results from the detached leaf technique are come reliable under consistent physical environment. Similar type of results reported by Doullah *et al.*, (2006)³ where they mentioned the detached leaf inoculation technique as suitable for screening of mustard-rapeseed genotypes because of having clear symptoms on the leaves within 24 hrs. Doullah and Okazaki (2015)⁵ used detached leaf and seedling inoculation test for the resistance screening of 281 *B. rapa* cultivars and detached leaf inoculation showed consistent results in two years compared to the seedling inoculation test. Nowakowska *et al.*, (2016)¹⁸ used several parameters like leaf age, leaf position, inocula concentration and incubation temperature under controlled conditions with two methods (detached leaf and seedling inoculation)

to screen resistant variety of mustard against *Alternaria* blight. Their results showed that either of the method can be used for the *A. brassicicola* resistance breeding considering crucial response to the pathogen. They also mentioned that the detached leaf bio-assays for *Alternaria* resistance as more effective than the field tests and two inter specific hybrids were identified that might be used in resistance breeding programs. Khan *et al.*, (2012)¹² evaluated the relative performance of several artificial inoculation techniques such as foliar spray, soil application, agarose gel method, and seed treatment with *A. brassicae* and *A. brassicicola* for screening of resistance genotypes of Indian mustard. In their study they evaluated that the foliar spray was the most promising and effective method of inoculation to developed distinct and severe disease symptoms on host plant. Doullah *et al.*, (2009)⁴ used spray method for the artificial inoculation of *B. rapa* leaves and pods for resistance screening against *A. brassicicola* which was more effective method and easy to assess the results. Giri *et al.*, (2013)⁷ described five artificial inoculation techniques on detached leaf viz. spraying, wounding, infiltration, spore suspension drop and spore suspension drop along with agarose for the establishment of disease symptoms on Indian mustard (*Brassica juncea*) plants by *Alternaria brassicae* and they suggested that spore suspension drop along with agarose inoculation method was ideal as this ensured the inoculum on the target site. However, the detached leaf inoculation technique showed clear symptoms rapidly and easy to use for disease resistance screening on a large scale maintaining standard physical condition within shortage period of time. Therefore, the detached leaf inoculation method is recommended for screening test to minimize the number of mustard-rapeseed genotypes required for the field test.

In this experiment, the older leaves (3rd leaves) of one month-old susceptible mustard-rapeseed plants were optimal for inoculation and showed severe diseased symptoms than the younger leaves (4th leaves). In most cases older leaves were more susceptible for infection compared to younger leaves due to the opportunistic parasitic behavior of the pathogen *A. brassicicola*, which requires comparatively a weakened host or plant tissue for infection. This finding also corroborates the findings of earlier

researchers. Doullah *et al.*, (2006)³ described that 3rd or 4th true leaves from one month-old plants are most favorable for inoculation and 3rd leaf showed more disease severity compared to the 4th leaf after 24 hrs of inoculation with *A. brassicicola*. Mridha and Wheeler (1993)¹⁷ showed that more *A. brassicae* infections happened in older *B. napus* plants. King (1994)¹³ found that both the youngest and oldest plants of *B. oleracea* resistant genotypes were less susceptible to *A. brassicicola*.

Among the 27 Bangladeshi mustard-rapeseed cultivars, all most all the cultivars expressed susceptible reaction to *A. brassicicola* except one cultivar, BINA Sharisha-8, as having border line resistance in this study. Doullah and Okazaki (2015)⁵ found that in a resistance screening of 281 genotypes of mustard-rapeseed in two different years by detached leaf and seeding inoculation techniques, two cultivars 'Edononatsu' and 'Saroi' showed high levels of resistance in both the years following the mentioned inoculation techniques, whereas some genotypes exhibited borderline resistance and most of them were susceptible to the black spot disease. In a resistance screening using 52 *Brassica rapa* genotypes, the detached leaf inoculation method effectively distinguished between different levels of resistance amongst genotypes and screened two *B. rapa* cultivars namely Saroi and Edononatsu as highly resistance and five cultivars have borderline resistance to *A. brassicicola* among the genotype used (Doullah *et al.*, 2006)³. Humauan *et al.*, (2013)⁸ investigated among 31 varieties of mustard-rapeseed and found 1 Resistant, 12 moderately resistant, 17 moderately susceptible and rest 1 showed susceptible against *Alternaria* leaf spot disease.

Conclusion

Alternaria leaf spot or black leaf spot caused by *Alternaria brassicicola* is the most important diseases of mustard-rapeseed in Bangladesh, and causing enormous yield loss every year. But the chemical fungicide is still the sole management practice at the farmers' level as none of the high yielding variety is completely resistant against this pathogen. Owing to that a total of 27 mustard-rapeseed cultivars were selected for resistance screening test. Two inoculation techniques *viz.* detached leaf and seedling inoculation were

evaluated in this resistance screening program. A significant positive correlation was found between the results from the seedling inoculation and the detached leaf inoculation method. Moreover, the detached leaf inoculation techniques was the most suitable for disease resistance screening of mustard-rapeseed cultivars as it was very easy to screen plants for resistance on a large scale within shortage period of time and to maintain standard physical environment. The older leaves (3rd leaf) of 30 day-old plants were showed severe disease symptoms than the younger leaves (4th leaf) of mustard-rapeseed. All most all the mustard-rapeseed cultivars used in this study expressed susceptible reaction to *A. brassicicola* except BINA Sharisha-8, as it showed border line resistance.

Based on the results of the this study it may be summarized that for resistance breeding program for this disease, there is no potential resistant cultivar exist in Bangladesh and consequently it will be necessary to develop resistant or tolerant cultivar against *A. brassicicola* through traditional as well as molecular breeding programme by using our high yielding varieties and wild races or resistant genotypes collected from international oilseed research organizations. However, this resistance screening experiment also needs to perform under field conditions or in nursery beds where large numbers of seedlings of mustard-rapeseed genotypes can be accommodate at a time.

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Conflict of Interest

No potential conflict of interest was reported by the authors.

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