RAPID SCREENING TECHNIQUE FOR ALTERNARIA BLIGHT RESISTANCE IN INDIAN MUSTARD (*BRASSICA JUNCEA* L.) USING COTYLEDONARY LEAF METHOD

P.D. Meena[1], S.J. Jambhulkar[2], R. Gupta[1], H.S. Meena[1] and D. Singh[1]

[1]*ICAR-Directorate of Rapeseed-Mustard Research, Bharatpur-321 303, Rajasthan, India*
[2]*Nuclear Agriculture & Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400 085, Maharashtra, India*

SUMMARY

Alternaria blight (AB) caused by *Alternaria brassicae* (Berk.) Sacc. is a devastating disease of oilseed Brassicas all over the world, and responsible for significant seed yield losses up to 47%. No reliable, resistant germplasm is available to develop AB resistant cultivars. Various screening techniques have been reported so far, but cotyledonary leaf method is not yet reported. Three methods were tested using one susceptible cultivar (Varuna): inoculation of seed, inoculation of cotyledons, and inoculation of both seed and cotyledons. Fungal conidia were inoculated directly onto the seedlings with 1.5×10^5, 2.5×10^5, 4×10^5 and 5×10^5 conidia ml^{-1} concentrations for standardization. Percentage AB severity increased with the increase in conidial concentration, therefore the highest concentration was used for final screening. Among the three screening methods, inoculation of both seed, and cotyledon method was found highly effective where mean AB severity on cotyledon was 84.6% in comparison to 49.3% in the inoculation of seed and 62.5% in the inoculation of cotyledon methods. The technique was validated by screening susceptible and putative tolerant genotypes. The severity of AB was 54% of susceptible cultivar and 16.4%-21.2% of tolerant genotypes. The conidia number per microscopic field was 21.5 in putative tolerant and 43.5 in susceptible genotypes. Thus, *in vitro* screening of AB using inoculation of both seed and cotyledon method was found most effective and could be used for rapid screening in early stages of plant growth. A new 0-7 rating scale was also devised to observe the AB pathogen interaction phenotype at the cotyledonary stage of oilseed Brassica.

Keywords: Alternaria blight, *Brassica juncea*, cotyledonary leaf, resistance, screening technique, rating scale.

Corresponding author: P.D. Meena
Email: pdmeena@gmail.com

INTRODUCTION

Indian mustard [*Brassica juncea* (L.) Czern & Coss.] is an important edible oilseed crop in India. Alternaria blight (AB), caused by *Alternaria brassicae* (Berk.) Sacc., has been reported throughout the world on various oilseed brassicas, particularly Indian mustard [*Brassica juncea* (L.) Czern & Coss.] being the most frequently reported host (Meena *et al*., 2010) in India. Alternaria blight is responsible for causing up to 47% seed yield losses to the crop (Kolte, 1985) and it is more in late sown condition (Meena *et al*., 2002). Most of the currently cultivated cultivars of Indian mustard are susceptible to AB. One of the foremost constraints in breeding Brassica for AB resistance has been the lack of resistance germplasm *vis-a-vis* rapid and reliable *in vitro* screening technique. Field screening though provides the information about the actual performance of genotype, it often varies in the diverse natural environment, and thus lacks authenticity. The occurrence of a disease depends on congenial environmental conditions like relative humidity, temperature, sunshine hours and pathogen inocula (Chattopadhyay *et al*., 2005). Conidium of Alternaria starts germination in free water and penetrates host tissues through stomata at 25°C temperature with minimum leaf surface wetness period of 6-16 h for initiation of the infection (Verma and Saharan, 1994). Among the four methods, including foliar spray, agarose gel method, soil application and seed inoculation, Khan *et al.* (2012) reported that the spray on foliage with spore suspension was found to be the most effective method of inoculation to achieve severe disease symptoms. Sporulation in *A. brassicae* on naturally-infected leaf discs of oilseed rape requires RH >90% (Humpherson-Jones and Phelps, 1989) and the small differences in infection of *A. brassicae* are difficult to perceive in the field. Limitations of petite disparity among test genotype in the field conditions cause impediment to the AB disease resistance breeding programme. Therefore, a reliable *in vitro* technique that can be utilized for screening a large number of genotypes at seedling stage is highly needed.

Different techniques have been suggested for screening against AB in oilseed Brassica (Carmody *et al*., 1985; Doullah *et al.,* 2006). These techniques are based on per cent infection on the leaf under field condition. Under laboratory study, Bansal *et al*. (1990) screened *Brassica*

Fig. 1. Different interaction phenotypes at cotyledonary stage of Indian mustard.

sp. against *A. brassicae* on the basis of the size of lesions caused on detached wounded leaves. Similarly, Prasad *et al.* (2008) reported that greenhouse assay gave more reliable and consistent measurement of resistance to *A. helianthi* in sunflower compared to detached leaf assay. So far no efforts were made to screen *in vitro* AB resistance on the cotyledonary stage of the seedlings. There is a need to develop the rapid and reliable screening technique to isolate AB resistant cultivar. Therefore, the objective of the study was to develop a reliable, rapid and economic screening technique to identify Indian mustard cultivars resistant to AB, and devise a disease rating scale based on lesion severity at the cotyledonary leaf stage.

MATERIALS AND METHODS

Plant material. Susceptible check cultivar Varuna was used for all the experiments. Seed was collected from germplasm unit of ICAR-Directorate of Rapeseed-Mustard Research, Bharatpur (Rajasthan), India. Ten seeds in each cell were planted on pores in the double layered folded moist germinating filter paper fixed on a 17-cell plastic stand placed in plastic trays (cell size: 20 length×5 cm height). Trays were placed under controlled sterilized condition at 25 ± 2°C with 8/16 h light/ dark cycle of fluorescent light controlled by a photoperiodic timer (SAVEER). Sterilized distilled water was used as growing medium, supplied periodically in the bottom of plastic trays for cotyledon growth. Relative humidity (RH) was maintained 90 ± 5% by the periodic spray of water and maintained water level into the bottom of the tray. Trays were covered with transparent polyethylene sheet of 0.004-inch thickness to maintain the high relative humidity for disease development.

Preparation of conidial suspension and assessment of inocula concentration. Conidia of a 10-day old single spore culture grown on PDA medium were filtered through two layers of sterile muslin cloth to remove residual mycelia for artificial inoculation. The inoculum concentration was determined using a hemocytometer and adjusted to 1.5×10^3, 2.5×10^4, 5×10^4 and 5×10^5 conidia ml^{-1}, respectively, by diluting a conidial stock solution with double sterile distilled water (Doullah *et al.*, 2006).

A common population of cotyledons from 7-day old *Brassica* seedlings of susceptible cultivar Varuna was used to assess the effect of the four different conidial concentrations. Cotyledons were individually evaluated for disease symptoms 7-days after inoculation. Spore concentration of 5×10^5 conidia ml^{-1} was rated as the most suitable for obtaining the highest AB severity and was used for all the different inoculation experiments.

Inoculation methods. The conidial concentration of the suspension was adjusted to 5×0^5 conidia ml^{-1} with the aid of a hemocytometer (particle counting chamber). The 7 day old seedlings were sprayed with sterilized distilled water to clean the surface of cotyledons 24 h prior to inoculation. Cotyledons were inoculated with a 5×10^5 conidia ml^{-1} suspension with the help of a hair brush to scrape the surface of the host.

Three inoculation methods were compared: inoculation of seeds, inoculation of cotyledons, and inoculation of both seeds and cotyledon. Trays were sprayed with sterilized distilled water to maintain RH and cotyledon wetness twice a day in morning and evening with 6 h interval. The experiment was arranged in a completely randomized design with three replications and repeated twice. In control, the pathogen was not inoculated.

Inoculation of seed. Seeds were inoculated by immersing them in a spore suspension (5×10^5 conidia ml^{-1}) at 25 ± 2°C overnight. After 24 h, 10 seeds were planted in a row on pores of double folded moist germinating paper (Sonar, India) fixed in the plastic cell to hold filter paper

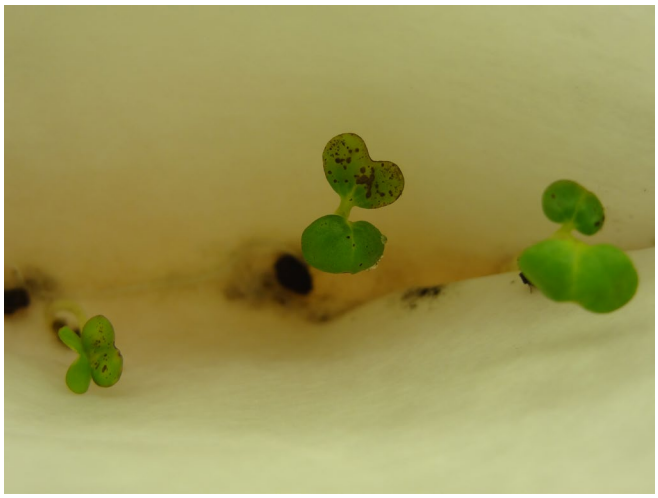

Fig. 2. Alternaria infection on cotyeldonary leaf of Indian mustard.

(5 cm height) placed in a plastic tray (30 × 40 × 8 cm). Trays were incubated at 25 ± 2°C under 8 h light/16 h dark cycle with 5.7 Klux intensity for 7 days. Cotyledons were evaluated individually 7 days after inoculation using the visual interaction phenotypic (IP) scale developed for the study presented in Fig. 1.

Inoculation of cotyledon. Cotyledons were inoculated in 5×10^5 conidia ml^{-1} suspension of *A. brassicae* with the help of a hair brush to facilitate scrape the surface. Seventy-five cotyledons of cultivar Varuna (three replicates consisting of 25 cotyledons per replicate) were used for cotyledon inoculation. Controls were 10 cotyledons inoculated with distilled water. The trays of inoculated cotyledons were then incubated in the 16/8 h dark/ light with 5.7 Klux light intensity for 7 days at 25 ± 2°C temperature.

Inoculation of seed and cotyledon. Seeds were soaked in a spore suspension (5×10^5 conidia ml^{-1}) at 25 ± 2°C overnight. After 24 h, 10 seeds were planted as in the above method. Inoculated trays were incubated at 25 ± 2°C under 8 h light/16 h dark cycle with 5.7 Klux intensity for 7 days. Cotyledons were inoculated with a 5×10^5 conidia ml^{-1} suspension of *A. brassicae* with the help of a hair brush. Seventy cotyledons of cultivar Varuna in three replications were used for cotyledon inoculation. The trays of inoculated cotyledons were then incubated under same conditions for other 7 days, then cotyledons were evaluated individually using the visual interaction phenotypic (IP) scale developed for the study.

Development of rating scale. Cotyledons were evaluated individually after 7 days of seeding and again after 7 days of inoculation using the scoring system to assess the host-pathogen interaction response. According to pathogenic symptomatic response of cotyledon infection, a rating scale was developed by using a 0-7 scoring scale and interaction phenotype based on visual evaluation of lesions on cotyledons (NR = No response; F = Light necrotic flecking; FN = Heavy necrotic flecking; ML = minute lesions, FL = Few lesions, NL = numerous lesions, LL = large scattered, and CL = coalescing lesions represents resistant/tolerant/susceptible classes were used, adapted with minor modification from the paper of Leckie *et al.* (1996).

Percent AB severity was calculated by using the formula as:

$$\text{Percent disease severity (PDS)} = \frac{\text{Total sum of numerical ratings} \times 100}{\text{Max. rating} \times \text{No. of samples observed}}$$

However, the AB disease score was given based on host response for different interaction phenotype.

Screening of genotypes. Putative tolerant genotypes of *Brassica juncea* namely PHR-2, EC-299399, PAB-9511, PAB-9534, EC299296, DRMR-2805, DRMR-2807 and one genotype of *Sinapis alba* (a wild *Brassica* commonly known as white mustard) were screened for *A. brassicae* infection along with susceptible cultivar Varuna. All the above three methods of inoculation were carried out to confirm the AB disease response under controlled experimental conditions. The experiment was arranged in a completely randomized design with three replicates (ten cotyledons per replicate). Control cotyledons were inoculated with distilled water only.

Conidial count in host tissues. Conidia of *Alternaria* were counted per microscopic field under the 20× objective of a compound microscope (Olympus) using lactophenol 0.1% staining, to confirm the level of resistance and pathogen growth per cotyledon by section cutting with a refrigerated microtome (Thermo, US). Conidia were counted on five sections from each cotyledon).

Statistical analysis. The experiment was conducted in a completely randomized design with three replicates (10 cotyledons per replicate) with appropriate controls. The values observed in percentage were converted using arc sin transformation. Data from the experiments were subjected to analysis of variance (ANOVA), and inter-mean differences between treatments were established using the least significant difference (LSD), and Student's t-test.

RESULTS

After 7 days of incubation, the cotyledons were evaluated with regard to disease symptoms using the interaction phenotype. The Alternaria blight disease symptoms were clearly visible on the inoculated cotyledons indicating that the experimental conditions were conducive for infection by the fungus. Symptoms did not appear on the cotyledons of uninoculated control. The disease appeared as minute flecking lesions on the upper surface of cotyledon that converted in large scattered or coalescing lesions with the

Table 1. Response of *B. juncea* cv. Varuna to *A. brassicae* after 7-days in seed inoculation method.

Cell no.	No. of plants	Mean IP	IP (n) 0-2, 3-5, 6-7	IP Range	% AB severity*
1	22	3.0	9, 12, 1	0-7	60.2 (50.9)
2	27	2.9	12, 14, 1	0-7	50.9 (45.5)
3	26	2.4	9, 16, 1	0-7	52.5 (46.4)
4	24	3.6	9, 12, 3	0-7	42.1 (40.4)
5	21	4.1	5, 12, 4	0-7	40.8 (39.7)
LSD (p=0.01)					4.4

*Figures in parenthesis are arc sine transformed values.

development of pathogen. Microscope analyses showed that once the epidermal cells are fully invaded within the host, and mycelia ramify through and between the mesophyll and palisade cells, the entire cotyledon leaf is almost instantly parasitized. Accordingly, the cotyledons with a light small to heavy necrotic flecking lesion on upper the surface of cotyledon were rated resistant, and those with numerous or large scattered coalescing lesions were rated susceptible. The result of inoculation of cotyledon method was compared with the inoculation of seed and inoculation of both seed and cotyledon methods.

Inoculation of seed method. Upon germination of seeds, disease development on cotyledon was observed in susceptible variety Varuna when cotyledon wetness was maintained for 5-8 h (Table 1). Seed inoculation method showed consistency in causing AB severity of 40.8 to 60.2% in fifteen rows having ten plants after 7-days of seeding. Lowest mean interaction phenotype (IP) was 2.4 and maximum of 4.1 with IP range of 0 to 7 although maximum IP (number) were between the range of 3-5.

Inoculation of cotyledon method. In 7-day old seedlings, the per cent AB severity on cotyledon was in the range of 58.7 to 66.7% with highest IP number in the range of 3-5 (Table 2). While per cent infection in check was less than 10.0% with IP range of 0-3 might be due to conidial movement during water spray or through infected seeds. Microscope analysis showed that the number of

Table 2. Response of *B. juncea* cv. Varuna in cotyledon inoculation test to *A. brassicae.*

Cell no.	Total plants	Mean IP	IP (n) 0-2, 3-5, 6-7	IP range	% AB severity*	No. of conidia/ microscopic field 20×
2	8	4.5	1, 5, 2	2-7	64.3 (53.3)	14.8
3	8	4.3	1, 5, 2	2-7	60.7 (53.2)	15.0
4	9	4.4	1, 6, 2	2-7	63.5 (52.8)	14.3
5	8	4.3	1, 5, 2	2-7	60.7 (51.2)	12.3
7	9	4.5	1, 5, 3	2-7	65.1 (53.8)	15.3
8	8	4.3	2, 3, 3	0-7	60.7 (55.0)	15.8
9	8	4.3	2, 4, 2	2-7	62.5 (52.2)	11.3
10	9	4.1	2, 4, 3	2-7	58.7 (53.9)	13.3
11	8	4.7	0, 4, 2	3-7	66.7 (54.8)	11.5
LSD (p=0.01)					4.8	

*Figures in parenthesis are arc sine transformed values.

pathogen spores in cotyledon tissues ranged from 11.3 to 15.8 conidia/ microscopic field at 20× magnification, while they were negligible in uninoculated cotyledons.

Inoculation of seed and cotyledon method. In 7-day old seedlings, AB infection on cotyledon was 49.0 to 66.5%, when caused by seed inoculation, while it reached 78.6-88.9% in 15-day old seedlings when cotyledons were inoculated after 7-days of seeding (Table 3). Interaction phenotype ranged from 1 to 7 even after inoculation of cotyledons. The highest IP range of 4-7 was observed after inoculation of cotyledon. The number of conidia counted in tissues of cotyledons was in the range of 16.0 to 21.5 conidia per microscopic field at 20× magnification, proving the infection efficiency of this method.

Screening of genotypes. Eight genotypes of *B. juncea* including susceptible/tolerant and one genotype of *B. alba* were screened to assess the performance of screening methods. Table 4 indicated that susceptible genotype Varuna showed highest 54% AB severity with maximum 3.5 IP mean. Lowest mean IP was recorded in *B. alba* (0.7) with minimum 16.4% AB severity. Conidia were counted in tissues of cotyledons under the microscope to confirm the infection level. A maximum of 43 conidia/microscopic field at 20× magnification was found in susceptible cultivar

Table 3. Response of *B. juncea* cv. Varuna to *A. brassicae* in seed and cotyledon inoculation method.

Cell no.	Total plant	Before inoculation				After inoculation				
		Mean IP	IP (n) 0-2,3-5,6-7	IP range	% AB	Mean IP	IP (n) 0-2, 3-5, 6-7	IP range	% AB severity*	No. of conidia/ microscopic field 20x
1	10	1.7	3, 4, 0	(0-3)	59.0 (50.2)	4.6	0, 3, 5	(3-7)	82.1 (64.8)	18.5
2	10	2.7	2, 3, 1	(2-6)	52.5 (46.5)	5.4	0, 4, 6	(4-7)	85.7 (67.8)	21.5
3	10	3.8	2, 4, 1	(1-5)	62.5 (52.3)	5.0	0, 4, 6	(4-7)	87.1 (69.2)	19.3
4	10	2.0	2, 2, 0	(1-3)	61.5 (51.7)	2.8	0, 5, 5	(4-7)	78.6 (62.4)	18.3
5	10	2.7	2, 4, 1	(1-6)	66.5 (54.7)	4.7	0, 4, 6	(4-7)	84.3 (66.7)	16.0
6	10	2.7	2, 4, 1	(1-6)	53.5 (47.1)	3.3	0, 2, 4	(4-7)	85.7 (67.8)	19.5
7	10	2.8	2, 2, 1	(1-6)	49.0 (44.5)	5.6	0, 3, 6	(4-7)	88.9 (70.5)	20.5
LSD (p=0.01)					3.6				3.3	3.6

*Figures in parenthesis are arc sine transformed values.

Table 4. Response of *Brassica* genotypes to *A. brassicae* in seed inoculation test.

Genotypes	Total plant	AB infected	Mean IP	IP (n) 0-2, 3-5, 6-7	IP range	% AB severity*	No. of conidia/ microscopic field 20×
Varuna	20	10	3.5	4,5,1	0-7	54.0 (47.3)	43.5
PHR-2	20	15	1.4	12,3,0	0-5	21.2 (27.4)	12.5
PAB-9511	20	18	1.2	13, 5, 0	0-4	22.2 (28.1)	13.8
PAB-9534	20	18	1.0	16, 2, 0	0-3	23.0 (28.7)	17.0
S. alba	20	13	0.7	12, 1, 0	0-3	16.4 (23.9)	14.5
DRMR-2805	20	14	1.1	12, 2, 0	0-4	23.9 (29.3)	17.5
DRMR-2807	20	18	2.1	14, 3, 1	0-6	27.1 (31.4)	18.8
EC-399000	20	19	2.3	11, 6, 2	0-6	31.7 (34.3)	17.8
EC-399296	20	15	1.4	12, 3, 0	0-4	33.8 (35.5)	15.0
LSD (p = 0.01)						5.0	3.6

*Figures in parenthesis are arc sine transformed values.

Table 5. Effect of different conidial concentration on Alternaria infection.

Conidial concentration	Total plant	AB infected	Mean IP	IP (n) 0-2,3-5,6-7	IP range	% AB severity*	Conidia/ microscopic field 20×
1.5×10^3	30	27	3.1	7, 19, 2	(0-7)	65.0 (53.7)	12.3
2.5×10^4	30	28	4.3	1, 13, 14	(2-7)	68.4 (55.9)	17.5
5×10^4	30	27	5.5	0, 13, 14	(3-7)	78.0 (62.0)	29.8
5×10^5	30	25	6.1	0, 9, 16	(4-7)	86.4 (68.5)	35.3
Check	40	20	0.2	20, 0, 0	(0-2)	12.5 (20.5)	4.3
LSD (p = 0.01)						5.1	5.7

*Figures in parenthesis are arc sine transformed values.

Varuna and a minimum of 12.5 conidia/microscopic field was in tolerant genotype PHR-2 followed by 13.8 in PAB-9511.

Effect of conidial concentration. Conidial suspension of four different concentration showed that percentage Alternaria blight significantly increased with the increase in conidial concentration (Table 5). Highest AB severity was 86.4% with IP range 4-7 and 35.3 conidia/microscopic filed at 20× magnification, observed on cotyledons inoculated with a conidial concentration of 5×10^5, Consistently, least AB severity was 65.0% with IP range 0-7 and 12.3 conidia/microscopic field at 20× magnification in 1.5×10^3 conidial concentrations. However, the uninoculated control also showed infection less than 10% which might be due to conidial movement during water spray or through infected seeds (Table 5). An increasing conidial concentration applied on host surface may perhaps adequately infect the host tissues. However, Doullah *et al.* (2006) used conidial concentration of 5×10^4 conidia ml^{-1} for successful infection of *A. brassicicola* on a $3^{rd}/4^{th}$ leaf of *Brassica rapa.*

Alternaria blight rating scale. The interaction phenotype (IP) of individual cotyledon for Alternaria blight pathogen was recorded as rating scale for that particular cotyledon (Fig. 1). A rating scale was developed, by using a 0-7 scoring scale and interaction phenotype based on visual evaluation of lesions on cotyledons (Table 6). Accordingly, cotyledons were categorized into eight IP classes, ranges in numbers from 0 to 7; i.e., 0 = no response (NR), 1 = light small necrotic flecking (F), 2 = heavy necrotic flecking (FN), 3 = minute lesions on upper surface of the cotyledon (ML), 4 = few lesions on cotyledon (FL), 5 = numerous lesions' on cotyledon (NL), and 6 = large scattered lesions' cotyledon (LL), and 7 = coalescing lesions' (CL) on cotyledon including yellowing and rotting (dead), represents resistant/tolerant/susceptible classes were used, adapted with minor modification from the paper of Leckie *et al.* (1996).

Table 6. Host response, pathogen growth and scores for different interaction phenotype classes.

Interaction phenotype	Host response	Pathogen growth	Disease score
NN	No response	No sporulation	0
F	Light necrotic flecking	No sporulation	1
FN	Heavy necrotic flecking	No sporulation	2
S_1	Any host response	Minute lesions on upper surface of cotyledon (ML)	3
S_2	Any host response	Few (FL) or numerous lesions (ML) on upper surface of the cotyledon	4 5
S_3	Any host response	Large scattered (LL) or coalescing lesions (CL) on upper surface of cotyledon	6 7

DISCUSSION

Disease appeared on cotyledon after 4 days of inoculation, indicating the incubation time of the pathogen, although the incubation period of *A. brassicae* was 6–10 days on leaves of *Brassica* species under field conditions (Saharan & Kadian, 1983). The length of incubation period depends on the *Alternaria-Brassica* combination, with the stage of development of the host, and with the temperature and humidity in the environment of the infected plant. Since *A. brassicae* established contact with susceptible cells or tissues of the host, to procure nutrients from them, following black spot infection, *Alternaria* grows, multiplies, and invades the plant to a lesser or greater extent. The successful infection results in the appearance of black spot symptoms of the disease. For a successful infection to occur, several other conditions including the pathogenic stage of *A. brassicae*, the susceptibility of host and optimum temperature as well as relative humidity must also favor the growth and multiplication of the pathogen. Our results indicated that the technique is appropriate for the development of both pathogen and host. Therefore, the emphasis was placed on the results of *in vivo* assays as preliminary evidence, but simultaneously *in vitro* assays should also be used to obtain the supportive evidence of resistance.

The appearance of disease on cotyledonary leaves gives the better option for the screening of AB as it is the earliest stage of plant development, whereas Vishwanath and Kolte (1999) reported that detached true leaf inoculation was the most efficient and reliable method for the screening for resistance to AB in rapeseed-mustard. Table 2 revealed that cotyledon inoculation was the best method for massive screening germplasm for the search of resistance against the pathogen in comparison to inoculation of seed method.

Among the three methods, inoculation of both seed and cotyledon method was found superior in comparison to seed inoculation and cotyledon inoculation methods, respectively. In fact, in this method the germplasm is adequately challenged with the pathogen at a realistic inoculums dose to allow disease development in which difference in host response could be identified simply. The appearance of disease on cotyledonary leaf stage which is the earliest stage of plant growth development has the advantage of saving considerable time and also helps to plan the breeding strategies. It appeared to be the better screening technique instead of the field and inoculation of detached leaf techniques (Vishwanath and Kolte, 1999), which are performed on the fully developed plant and therefore takes more time. We concluded that the seed and cotyledon inoculation method could be the better option for screening the huge amount of breeding material of oilseed Brassica at cotyledonary leaf stage which may be further improved for other factors influencing the disease development.

The results showed consistent interaction phenotype response with resistance and susceptibility in the host. Because for foliar pathogens, the germplasm must be adequately challenged with pathogen at a realistic inoculum dose to allow disease development. Our results related to sporulation are supported by the study of Saharan and Kadian (1983), who found large differences in the number of lesions, the size of lesions, incubation (latency?) period, sporulation capability and infection rate while analyzing different components of horizontal resistance in *Brassica* genotypes against *A. brassicae*. Even if the difference within species for disease response is well recognized, it is preferable to collect and screen large numbers of accessions from each species. The number of conidia in host tissues proved the infection efficiency of different methods and response of host against pathogen growth. The number of conidia increases with the increase in disease severity. *Alternaria brassicae* penetrated and infected the host *B. juncea* and caused reduction of cell constituents by the production of toxin, which is mainly responsible for necrosis of leaf tissue.

Different concentrations of conidial suspension showed that percentage Alternaria blight was significantly increased with the increase in conidial concentrations, according to Prasad *et al.* (2008), who found that increasing inoculum concentration significantly increased the infection of *A. helianthi* in sunflower, whereas Doullah and Okazaki (2015) used the concentration of conidial suspension at 5×10^4 conidia/ml for inoculation of *B. rapa* leaf with *A. brassicicola*.

The interaction phenotype (IP) of each individual cotyledon to Alternaria blight pathogen was recorded as disease index for individual genotype (Fig. 1). Accordingly, cotyledons were categorized into eight IP classes, ranges in numbers from 0 to 7. Since there was a lack of suitable evaluation procedure for Alternaria blight infection at the cotyledonary stage, a new rating scale was developed for the first time. In conclusion,
the inoculation of both seed and cotyledon method, together with the 0-7 rating scale could be useful tools to observe Alternaria blight disease severity at cotyledonary leaf stage of oilseed for rapid and preliminary screening of *Brassica juncea* germplasm.

ACKNOWLEDGEMENTS

Author's are grateful to Board of Research in Nuclear Sciences (BRNS) for financial support in the form of BRNS project entitled "Induced mutagenesis for isolation of Alternaria blight resistant mutant in *Brassica juncea*" bearing sanction No. 35/14/44/2014-BRNS and Director, ICAR-Directorate of Rapeseed-Mustard Research, Bharatpur for providing laboratory facilities.

REFERENCES

Bansal V.K., Sequin S., Rakow G., Petrie G.A., 1990. Reaction of *Brassica* species to infection by *A. brassicae*. *Canadian Journal of Plant Science* **70**: 1159-1162.

Carmody B.E., Miller M.E., Grisham M.P., 1985. A technique to screen muskmelons for resistance to Alternaria leaf blight. *Plant Disease* **69**: 426-428.

Chattopadhyay C., Agrawal R., Kumar A., Bhar L.M., Meena P.D., Meena R.L., Khan S.A., Chattopadhyay A.K., Awasthi R.P., Singh S.N., Chakravarthy N.V.K., Kumar A., Singh R. B., Bhunia C.K., 2005. Epidemiology and forecasting of Alternaria blight of oilseed *Brassica* in India - a case study. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz (Journal of Plant Disease Protection)* **112**: 351-365.

Doullah M.A.U., Meah M.B., Okazaki K., 2006. Development of an effective screening method for partial resistance to *Alternaria brassicicola* (dark leaf spot) in *Brassica rapa*. *European Journal of Plant Pathology* **116**: 33-43.

Doullah M.A.U., Okazaki K., 2015. Evaluation of resistance to dark leaf spot (*Alternaria brassicicola*) in *Brassica rapa*. *International Journal of Experimental Agriculture* **5**: 5-14.

Humpherson-Jones F.M., Phelps K., 1989. Climatic factors influencing spore production in *Alternaria brassicae* and *Alternaria brassicicola*. *Annals of Applied Biology* **114**: 449-458.

Khan M.M., Khan M.R., Mohiddin F.A., 2012. The relative performance of different inoculation methods with *Alternaria brassicae* and *A. brassicicola* on Indian mustard. *Plant Pathology Journal* **11**: 93-98.

Kolte S.J., 1985. *Diseases of Annual Edible Oilseed Crops Vol. II. Rapeseed Mustard and Sesamum Diseases.* CRC Press, Boca Raton, Florida, pp 135.

Leckie D., Astley D., Crute I.R., Ellis P.R., Pink D.A.C., Boukema I., Monterio A.A., Dias J.S., 1996. The location and exploitation of genes for pest and disease resistance in European gene bank collection of horticultural Brassicas. Proceedings of the International Symposium on Brassicas 9th Crucifer Genetics Workshop, Eds. Dias JS, Crute IR, Monterio AA, *Acta Horticulturae* **407**: 95-101.

Meena P.D., Chattopadhyay C., Singh F., Singh B., Gupta, A., 2002. Yield loss in Indian mustard due to white rust and effect of some cultural practices on Alternaria blight and white rust severity. *Brassica* **4**: 18-24.

Meena P.D., Awasthi R.P., Chattopadhyay C., Kolte S.J., Arvind Kumar, 2010. Alternaria blight: a chronic disease in rapeseed-mustard. *Journal of Oilseed Brassica* **1**: 1-11.

Prasad M.S.L., Sujatha M., Rao S.C., Sarada C., Singh H., 2008. A new technique for evaluating sunflower germplasm against Alternaria leaf blight. *Journal of Mycology and Plant Pathology* **38**: 39-46.

Verma P.R., Saharan G.S., 1994. Monograph on Alternaria Diseases of Crucifers. Research Branch. Agriculture and Agri-Food Canada, 162 pp.

Vishwanath, Kolte S.J., 1999. Methods of inoculation for resistance to Alternaria blight of rapeseed and mustard. *Journal of Mycology and Plant Pathology* **29**: 96-99.

Saharan G.S., Kadian A.K., 1983. Analysis of components of horizontal resistance in rapeseed and mustard cultivars against *Alternaria brassicae*. *Indian Phytopathology* **36:** 503-507.

Received February 9, 2016
Accepted May 9, 2016