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# The Relative Performance of Different Inoculation Methods with *Alternaria brassicae* and *A. brassicicola* on Indian Mustard

[1,2]Mohd Mahmud Khan, [1]Mujeebur Rahman Khan and [1]F.A. Mohiddin
[1]Department of Plant Protection, Faculty of Agricultural Sciences,
Aligarh Muslim University, Aligarh, 202002, India
[2]Central Integrated Pest Management Centre, Directorate of Plant Protection Quarantine and Storage,
Department of Agriculture and Cooperation, Imphal, Manipur, 795001, India

**Abstract:** The relative performance of different inoculation methods viz., foliar spray, agarose gel method, soil application and seed treatment with *Alternaria brassicae* and *Alternaria brassicicola* was evaluated to standardize the methods of inoculation for screening. For *A. brassicae* two cultivars of Indian mustard namely Pusa Bold and Rohini were used to evaluate relative performance of inoculation methods, whereas for *A. brassicicola* the cultivars BS-2 and Kranti were tested. The foliar spray was found to be the most effective method of inoculation to achieve severe disease symptoms. Phylloplane population of the fungi was also recorded greater on the plants sprayed with spore suspension of *Alternaria* spp. The next in effectiveness in causing the disease and its further development was agarose gel inoculation. The plant length was decreased significantly due to inoculation with *A. brassicae* or *A. brassicicola* by foliar spray. Seed and soil inoculation methods resulted to mild blight symptoms but significant reduction in plant growth variables did not occurs. The regression analysis between the disease severity and yield decline has shown stronger relationship between the two variables for foliar inoculation followed by agarose gel method. The foliar inoculation caused the blight of severity that led to the yield decline greater than other methods. The study has demonstrated the spray of foliage with spore suspension of *A. brassicae* or *A. brassicicola* is highly effective method of inoculation.

**Key words:** Alternaria blight, agarose gel, foliar, seed, soil, inoculation, screening, Indian mustard

## INTRODUCTION

The productivity rate of rapeseed-mustard is considerably low in comparison to other countries such as Algeria, France and Canada. There are indeed, multitudes of factors which are responsible for a lower productivity and yield declines. Among major constraints, the occurrence of diseases and insect pests appears to be an important factor which have restricted fast expansion of its cultivation and abate productivity of these crops. There are about thirty diseases which are reported to occur on this crop and of these very few are of economic importance (Kolte, 1985; Rajak, 1999; Mukerji *et al*., 1999; Hegde, 2002; Khan *et al*., 2007a-c; Khan, 2011). Among these, Alternaria blight is considered as of major consequences on the basis of their wide distribution and yield losses caused to *Brassica* spp. world over including India (Kolte *et al*., 1987; Khan *et al*., 2010; Khan, 2011). This disease is caused by three species of *Alternaria* viz., *A. brassicae* (Berk.) Sacc, *A. brassicicola* (Schw.) Wilts. and *A. raphani* Groves and Skolko however, former two species are principal causal pathogens of the disease. They are widespread in occurrence and destructive in nature causing significant damage to rapeseed-mustard production throughout the country.

The disease is characterized by formation of lesion on leaves, stem and siliquae. Nevertheless, lesions produced by *A. brassicae* appear usually as gray compared to the black sooty velvety spots produced by *A. brassicicola*. Spots of *A. raphani* show distinct yellow halos around them (Kolte, 1985; Mukerji *et al*., 1999). Besides leaf infection reducing the photosynthetic area, the infection also leads to deteriorate the quality of the seeds i.e., seed size, seed colour, oil contents and germination capability (Randhawa and Aulakh, 1981; Khan *et al*., 2010). The disease may cause yield loss of up to 46-47% in yellow sarson and 35-38% in mustard but in susceptible cultivars the losses may be as high as up to 70% (Kolte, 1985; Saharan, 1991; Vishwanath and Kolte, 1997; Prasad *et al*., 2003; Khan *et al*., 2010; Khan, 2011). Present study was carried out to determine the different inoculation methods with *Alternaria* spp. on Indian mustard.

**Corresponding Author:** Mujeebur Rahman Khan, Department of Plant Protection, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, 202002, India

## MATERIALS AND METHODS

Four indigenous cultivars of Indian mustard viz., BS-2, Kranti, Pusa Bold and Rohini were procured from local authorized seed dealer. The seeds were surface sterilized with 0.5% NaOCl (Sodium Hypochlorite) and sown in steam sterilized field soil and compost (3:1 ratio) at 15 cm diameter clay pots (10 seeds/pot). The germinating seedlings were thinned to maintain one seedling per pot after four weeks of sowing (Khan and Khan, 2010). Plants were harvested four months after sowing and leaf spot disease (0-5 scale) (Khan, 2011), plant growth, phylloplane population and yield (weight of grains/plant) were determined.

Inoculum of *A. brassicae* and *A. brassicicola* was prepared in Richard's liquid (Potassium nitrate-10.0 g, Potassium dihydrogen orthophosphate -5.0 g, Magnesium sulphate -2.5 g, Ferric chloride -0.02 g, Sucrose -50.0 g and Distilled water -1000 mL) medium in 500 mL conical flasks separately and were incubated in a BOD incubator for a week at 25±2°C after inoculation with the pure culture of fungus. Thereafter, the mycelial mat was collected from the flasks and blended in double distilled water to make a homogenous suspension of spores. The spore suspension was diluted to $10^{5-8}$ CFUs $mL^{-1}$ and counted with a haemocytometer.

To study the sporulation and colonization of *A. brassicae* and *A. brassicicola* each on the plant leaves, phylloplane population was determined by dilution plate method. Ten discs of 1 cm diameter were cut from the infected leaves with the help of cork borer. The discs were shaken in 100 mL sterilized double distilled water in a conical flask for 30 min. Thereafter, discs were separated and the suspension was diluted to $10^{-5}$ dilution. Petri plates containing solidified PDA were inoculated with $10^{-5}$ dilution suspension (0.3 mL $plate^{-1}$) and were incubated in a BOD incubator at 25±2°C for 72 h. Each colony of *A. brassicae* and *A. brassicicola* were counted in the plates to determine number of spores/unit leaf area of plants.

A set of two different cultivars viz., Alankar and Rohini (*A. brassicae*) and Pusa Bold and Kranti (*A. brassicicola*) were used to evaluate relative performance of seed, soil, foliar and agarose gel inoculation with the *Alternaria* spp. in causing the leaf blight. The experiment was conducted in polyhouse. The polyhouse was made up of iron frame and covered with UV resistant polysheet and it was divided into four cabins having independent entry through a corridor inside the house. Each cabin had a movable window and a low speed exhaust fan. Inoculation of *A. brassicae* and *A. brassicicola* on plants was done in four different ways using equal amount of inoculum (mL $plant^{-1}$) to determine and effective methods of inoculation for screening of fungus.

**Soil inoculation:** The pots were filled with 1 kg mixture of autoclaved soil and Farm Yard Manure (FYM) in the ratio 3:1. The 1 mL spore suspension ($10^5$ CFU's of *Alternaria* spp.) was added in the top pot soil and thereafter surface sterilized seeds of mustard were sown in the pots.

**Seed inoculation:** The pots were filled with 1 kg mixture of autoclaved soil and FYM. The seeds were first surface sterilized with 0.5% NaOCl and then inoculated with 1 mL spore suspension/10 g seeds. The seeds were first coated with 2% sucrose solution. A few hours later the seeds were applied with the spore suspension of *A. brassicae* and *A. brassicicola* separately.

**Agarose gel inoculation:** The surface sterilized seeds were sown in the pots filled with 1 kg mixture of sterilized soil and FYM. Micro inoculation was done on 1 month old plants by loading 1 μL spore suspension at a spot on upper surface temporarily positioned horizontally followed by covering with 4 drops of 0.5% melted agarose gel. A total of 1 mL spore suspension was inoculated on different leaves.

**Foliar inoculation:** One month old plants were inoculated by spraying with 5 mL spore suspension/plant ($10^5$ spores $mL^{-1}$).

**Statistical analysis:** Each experiment was performed over two consecutive years. The general trend in the effect of treatments on the considered variables was more or less identical during year replication but the effect of years was frequently significant at $p \leq 0.05$. Hence, the data obtained from five replicates maintained each year were analyzed separately. During repetition of experiments, the methods were used more precisely as a result of experience gained from the previous year; hence, results described in this study are based on the experiments conducted during second year. The data on different inoculation methods were subjected to a single factor analysis of variance (ANOVA) and Least Significance Differences (LSD) were calculated for each variable at one probability levels, $p \leq 0.05$ and Duncan's multiple range test was employed to identify significantly different clonal responses (Dospekhov, 1984).

## RESULTS

Two cultivars namely Pusa Bold and Rohini were used to evaluate the performance of different inoculation methods viz., foliar spray, agarose gel method, soil inoculation and seed treatment with *Alternaria brassicae* and another two cultivars i.e., BS-2 and Kranti with *A. brassicicola*. The disease intensity, phylloplane population, plant length and crop yield were recorded

after 3 months of inoculation. The two cultivars used in the evaluation had varied response, the first one was highly susceptible to the fungal species while the second being moderately susceptible. This was done to know whether different methods of inoculation do affect the clonal reaction to the pathogen.

**Symptoms:** Typical symptoms of Alternaria blight caused by *A. brassicae* appeared on Indian mustard cultivar Pusa Bold irrespective of the method of inoculations (Fig. 1). Concentric lesions yellow to brown coloured developed on the leaves, later on stem and siliquae (Fig. 1). The uninoculated (control) plants did not show any symptom of the disease. Highest disease intensity was recorded in the cultivar Pusa Bold (76%) due to foliar spray, followed by the agarose gel inoculation method, in which the disease intensity was 61% (Pusa Bold) and 20% (Rohini). With soil and seed inoculation, the disease intensity was 45 and 53% in the cultivar Pusa Bold whereas 15 and 18% in the cultivar Rohini, respectively (Table 1).

Cultivar BS-2 was found highly susceptible to *A. brassicicola* and developed characteristic symptoms of concentric lesions, zonate spots of 1-10 mm in diameter, dark brown to almost black coloured on the leaves and later on the stem and siliquae. Much greater disease intensity was noticed in the cultivar BS-2 (71%) than Kranti (21%) due to foliar inoculation with the fungus (Fig. 2, Table 1). Disease intensity caused by the agarose gel inoculation method was 48% in BS-2 and 15% in Kranti (Fig. 2). With soil and seed inoculation method, the disease intensity was 29 and 39% (BS-2) and 10 and 12% (Kranti), respectively.

**Plant growth and yield:** Inoculation of *A. brassicae* with foliar spray method resulted to significant decrease in the plant length of Pusa Bold (10.3%, $p \leq 0.01$) and Rohini (8.4%, $p \leq 0.05$). With agarose gel inoculation method, the plant length of Pusa Bold and Rohini was reduced at $p \leq 0.05$ in comparison to the controls (Table 2). Inoculation of *A. brassicae* in the soil significantly decreased plant length in the cultivar Pusa Bold (9.6%, $p \leq 0.01$) and Rohini (6.4%, $p \leq 0.05$) where as with seed inoculation method, the significant decreased in plant length was 9.4 (Pusa Bold, $p \leq 0.01$) and 6.1% (Rohini, $p \leq 0.05$) (Table 2).

Foliar inoculation of Indian mustard cultivars with *A. brassicicola* caused a significant decrease in the plant length, i.e., 11.8 (BS-2, $p \leq 0.01$) and 9.6% (Kranti, $p \leq 0.05$) in comparison to uninoculated plants. With agarose gel, the plant length reduction was 10 (BS-2, $p \leq 0.01$) and

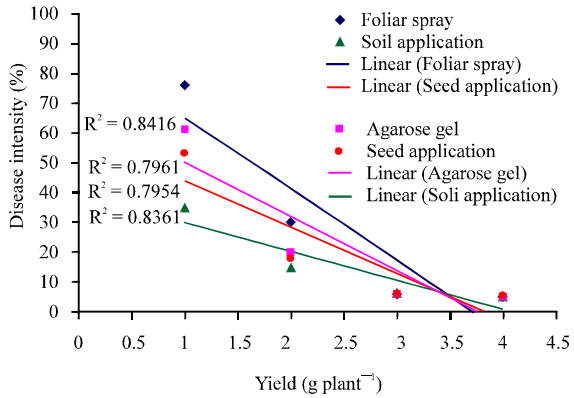


Fig. 1: Correlation between disease intensity and yield of mustard against *Alternaria brassicae* on different inoculation methods

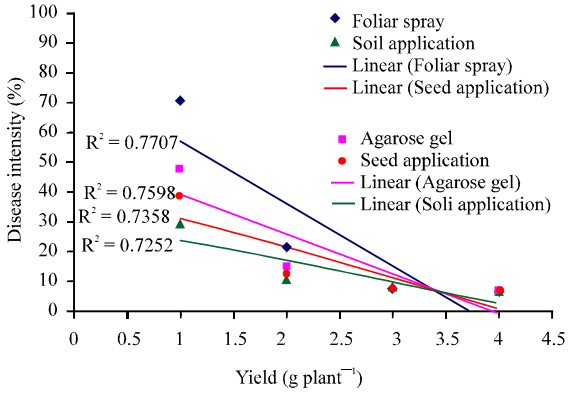


Fig. 2: Correlation between disease intensity and yield of mustard against *Alternaria brassicicola* on different inoculation methods

Table 1: Effect of different methods of inoculation with *Alternaria brassicae* and *A. brassicicola* on the development of blight and phylloplane population of *Alternaria* spp. on Indian mustard

| | Disease intensity (%) | | | | Phylloplane population | | | |
|---|---|---|---|---|---|---|---|---|
| | *Alternaria brassicae* | | *Alternaria brassicicola* | | *Alternaria brassicae* | | *Alternaria brassicicola* | |
| Treatment | Pusa bold | Rohini | BS-2 | Kranti | Pusa bold | Rohini | BS-2 | Kranti |
| Foliar spray | $76^{a}$ | $30^{a}$ | $71^{a}$ | $21^{a}$ | $12^{a}$ | $3^{a}$ | $10^{a}$ | $3^{a}$ |
| Agarose gel | $61^{b}$ | $20^{b}$ | $48^{b}$ | $15^{b}$ | $8^{b}$ | $2^{b}$ | $7^{b}$ | $2^{b}$ |
| Soil application | $35^{d}$ | $15^{d}$ | $29^{d}$ | $10^{d}$ | $5^{c}$ | $1^{c}$ | $5^{c}$ | $1^{c}$ |
| Seed application | $53^{c}$ | $18^{c}$ | $39^{c}$ | $12^{c}$ | $7^{b}$ | $2^{b}$ | $6^{b}$ | $2^{b}$ |
| LSD $p \leq 0.05$ | 6.63 | 3.54 | 5.61 | 2.37 | 1.81 | 0.34 | 1.39 | 0.42 |
| F-value treatment (df = 3) | $656.06^{z}$ | $264.06^{z}$ | $595.11^{z}$ | $143.75^{z}$ | $54.17^{z}$ | NS | $55.65^{z}$ | NS |

Values are mean of five replicates, Values followed by different alphabets are significantly different at $p \leq 0.05$ according to DMRT, F-values are significant at $^{x}p \leq 0.05$, $^{y}p \leq 0.01$, $^{z}p \leq 0.001$ and nonsignificant (NS) at $p \leq 0.05$

Table 2: Effect of different methods of inoculation with *Alternaria brassicae* and/or *A. brassicicola* on the plant length and yield of Indian mustard

| | Plant length (cm) | | | | Yield (g plant$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | *Alternaria brassicae* | | *Alternaria brassicicola* | | *Alternaria brassicae* | | *Alternaria brassicicola* | |
| Treatment | Pusa bold | Rohini | BS-2 | Kranti | Pusa bold | Rohini | BS-2 | Kranti |
| Control | 68.0 | 65.5 | 73.0 | 69.0 | 6.8 | 6.0 | 7.3 | 6.8 |
| Foliar spray | 61.0$^{c}$ | 60.0$^{b}$ | 65.0$^{c}$ | 62.4$^{b}$ | 5.2$^{c}$ | 5.2$^{b}$ | 5.8$^{c}$ | 6.2$^{a}$ |
| Agarose gel | 62.0$^{b}$ | 61.0$^{a}$ | 66.0$^{c}$ | 63.0$^{b}$ | 5.6$^{c}$ | 5.3$^{a}$ | 6.5$^{b}$ | 6.3 |
| Soil application | 61.5$^{b}$ | 61.3$^{a}$ | 67.0$^{b}$ | 64.0$^{b}$ | 6.1$^{a}$ | 5.4$^{a}$ | 6.6$^{a}$ | 6.4 |
| Seed application | 63.0$^{b}$ | 61.5$^{a}$ | 68.0$^{b}$ | 65.0$^{a}$ | 6.0$^{b}$ | 5.6 | 6.5$^{b}$ | 6.3 |
| LSD p≤0.05 | 3.09 | 3.09 | 3.09 | 3.09 | 0.52 | 0.52 | 0.52 | 0.52 |
| p≤0.01 | 4.51 | 4.51 | 4.51 | 4.51 | 0.75 | 0.75 | 0.75 | 0.75 |
| p≤0.001 | 6.76 | 6.76 | 6.76 | 6.76 | 1.13 | 1.13 | 1.13 | 1.13 |
| F-value Treatment (df = 4) | 8.94$^{y}$ | NS | 10.78$^{y}$ | 7.57$^{x}$ | 10.60$^{y}$ | NS | NS | NS |

Values are mean of five replicates, Values followed by different alphabets are significantly different at p≤0.05 according to DMRT, F-values are significant at $^{x}$p≤0.05, $^{y}$p≤0.01, $^{z}$p≤0.001 and nonsignificant (NS) at p≤0.05

8.7% (Kranti, p≤0.05) (Table 2). Soil inoculation with *A. brassicicola* caused significant decrease in plant length, i.e., 8.2% (BS-2) and 7.3% (Kranti) which was significant at p≤0.05, whereas the seed application significantly reduced the plant length in the cultivar BS-2 (6.8%, p≤0.05) and Kranti (5.8%, p≤0.05) over the respective controls (Table 2).

In uninoculated plants, the yield of Pusa Bold and Rohini was 6.8 and 6.0 g/plant, respectively. The yield of both cultivars significantly declined with all the methods of inoculation of *A. brassicae* over control. The significant yield decline with foliar inoculation was 23.5% (Pusa Bold, p≤0.001) and 13.3% (Rohini, p≤0.01) (Table 2). The decrease in the yield with agarose gel method was recorded as 17.7% (Pusa Bold) and 11.7% (Rohini) which were significant at p≤0.01 and p≤0.05, respectively. With soil inoculation, decrease in the yield was 13.3 (Pusa Bold) and 10% (Rohini, p≤0.05), respectively and. Seed inoculation resulted to the yield decline of 11.8% in cultivar Pusa Bold (p≤0.05) (Fig. 1, Table 2).

The two cultivars inoculated with *A. brassicicola* exhibited significant decrease in the yield with all the methods of inoculation in comparison to the control. The foliar inoculation with *A. brassicicola* caused significant yield decline in BS-2 (20.5%, p≤0.001) and Kranti (8.8%, p≤0.05) in comparison to respective control (Table 2). With agarose gel, soil and seed inoculation method, the percent decrease in the yield of BS-2 was 11.6 (p≤0.01), 9.6 (p≤0.05) and 11.0% (p≤0.01), respectively over respective control (Fig. 2, Table 2).

**Phylloplane population:** Greatest phylloplane population of *A. brassicae* was recorded with foliar spray method ($12\times10^3$ CFU cm$^{-2}$ leaf surface) followed by agarose gel ($8\times10^3$ CFU cm$^{-2}$ leaf surface), soil application ($5\times10^3$ CFU cm$^{-2}$ leaf surface) and seed treatment ($6\times10^3$ CFU cm$^{-2}$ leaf surface) (Table 1). The phylloplane population with the foliar spray was $12\times10^3$ CFU cm$^{-2}$ leaf surfaces in the cultivar Pusa Bold and $3\times10^3$ CFU cm$^{-2}$ leaf surface in the Rohini. The phylloplane population with agarose gel was recorded $8\times10^3$ CFU cm$^{-2}$ leaf surface. With soil and seed inoculation method, the phylloplane population of Pusa Bold was $5\times10^3$ and $7\times10^3$ CFU, respectively (Table 1).

The phylloplane population of *A. brassicicola* on BS-2 received inoculation through foliar spray, agarose gel, soil and seed treatment method was $10\times10^3$ CFU, $7\times10^3$ CFU, $5\times10^3$ CFU and $6\times10^3$ CFU cm$^{-2}$ leaf surface, respectively (Table 1). In Rohini the phylloplane population of *A. brassicicola* was $3\times10^3$ CFU, $2\times10^3$ CFU, $1\times10^3$ CFU and $2\times10^3$ CFU cm$^{-2}$ leaf surface with foliar spray, agarose gel, soil application and seed treatment, respectively (Table 1).

## DISCUSSION

Characteristic leaf spot symptoms caused by *Alternaria* spp. developed on the plants inoculated through soil, seed, foliage or agarose gel have indicated that the methods used were successful in initiating the infection by *A. brassicae* as well as *A. brassicicola*. However, degree of disease severity varied with the method and significant differences in symptom development were recorded with the four methods of inoculation tested in the study.

The *Alternaria* spp. are basically a foliar pathogen and attacks foliar parts (Rotem, 1994; Kohl *et al*., 2010). Foliar spray with the fungus suspension gave direct access to the spores to susceptible part and tissue resulting to infection in the leaves and latter in stem and pods (Humpherson-Jones and Ainsworth, 1982; Rotem, 1994; Singh and Singh, 2004). For this reason, the foliar spray was found to be the most effective method of inoculation to achieve severe disease symptoms among the four different modes of inoculations. Phylloplane population of the fungi was also recorded greatest on

the plants sprayed with spore suspension of *Alternaria* spp. Humpherson-Jones and Ainsworth (1982) have also reported highest population of *A. brassicae* and *A. brassicicola* spores and also symptoms on the plants inoculated by foliar spray. The next in effectiveness in causing the disease and its further development was agarose gel inoculation. Ryan and Clare (1974) have reported agarose gel method effective for precise inoculation and limited inoculation as the method is much time taking although is able to inoculate a very small amount of inocula, just a few spores. Different inoculation methods have been used by many researchers for the screening for resistance to *Alternaria* spp., with the goal to identify resistant genotypes (King, 1994; Vishwanath and Kolte, 1999). Among them foliar spray inoculation method has been found most effective method and hence is recommended for screening the rapeseed mustard genotype against Alternaria blight.

There were significant differences in the plant growth parameters of plants received *Alternaria* inocula through different methods indicating that the methods were effective for infection by the pathogen. Foliar inoculation with *A. brassicae* and *A. brassicicola* has been found highly suppressive for plant growth and significantly reduced the yield of *Brassica* spp. (Humpherson-Jones and Ainsworth, 1982). Significantly greater reduction in the plant growth was recorded with foliar inoculation of *A. brassicae* and *A. brassicicola*, followed by agarose gel method. Seed and soil inoculation methods were not so effective in comparison with foliar spray inoculation or agarose gel method. The regression analysis between the disease severity and yield loss in plants inoculated with different methods has shown stronger relationship in foliar inoculation followed by agarose gel method (Fig. 1, 2). This has shown that the foliar inoculation caused disease of the severity that led to the yield decline greater than other methods. Moreover, this effect was observed in both highly susceptible and moderately susceptible cultivars with both species of *Alternaria*. This has also proved that mode of inoculation did not affected the varietal reaction as the coefficient of regression ($r^2$) was lower in both highly and moderately susceptible cultivars for seed and soil inoculation method.

## CONCLUSION

The study has shown that foliar inoculation method is much handy and was found relatively more effective in causing higher disease severity and resulted to greater reduction in plant growth and yield and increase in the phylloplane population of the fungus in comparison to other methods. The different methods used did not influence the varietal/clonal reaction to the fungus as the cultivar Pusa Bold and BS-2 exhibited blight symptoms and yield reductions greater than cultivars Rohini and Kranti irrespective of mode of inoculation. Hence foliar spray method can be used in screening programmes.

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