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Comparison of artificial inoculation methods for studying pathogenesis of *Alternaria brassicae* (Berk.) Sacc on *Brassica juncea* (L.) Czern. (Indian mustard)

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Establishment of disease by artificial inoculation is essential for studies of various aspects of plant pathology. Keeping this in mind, five inoculation methods were compared namely spraying, infiltration, wounding, spore suspension drop and spore suspension drop along with agarose method. The findings of the present study suggest that out of five inoculation methods used, spore suspension drop along with agarose inoculation method was most ideal as this fixed the inoculum on the target site. The mean value of number of initial disease lesions in drop plus agarose method was highest in all of the time intervals of observation namely 312.2, 484.2, 664.2, 734.2 and 799.2 at 24, 48, 72, 96 and 120 h after pathogen inoculation compared to other methods respectively. Statistical analysis software (SAS) was used to find out the significant comparison among the different methods. The spore suspension drop along with agarose method has the advantage of being accurate and precise, and it was also easy to handle the inoculated plants.

Key words: Artificial inoculation, *Brassica*, *Alternaria*, pathogenicity, screening.

INTRODUCTION

India is one of the largest rapeseed-mustard growing countries in the world, occupying the first position in area and second position in production after China. *Brassica* (rapeseed-mustard) is the second most important edible oilseed crop in India after groundnut and accounts for nearly 32% of the total oilseeds produced in the country (Meena et al., 2010). In spite of these achievements, the last couple of years, the low production of rapeseed and mustard in India has consequently resulted in drastic decrease in its per capita availability and has compelled India to import large quantity of edible oils. The main cause of low productivity is due to various diseases and pests associated with *Brassica. Alternaria* blight is one of the important diseases of *Brassica* responsible for average yield loss of 10 to 70% at different parts of Northern India depending upon the severity (Kumar, 2001). Due to the lack of availability of the sources of resistance against *Alternaria brassicae* within the family Brassicaceae, *Alternaria* blight is considered the most damaging and widespread fungal disease of *Brassica* (Ghose et al., 2008). Since *Alternaria* Blight is increasingly destructive in oil production, ways of controlling the disease need to be developed. The first step in any of the resistance breeding programme is to rapidly screen all the available genetic stocks, including the local land races, improved cuttings and exotic

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germplasms using empirical techniques in glass houses, or by field tests. For a successful screening, an adequate amount of inoculum is necessary. The artificial inoculation of test genotypes is necessary to obtain a more uniform disease; moreover establishment of disease by artificial inoculation is also essential for studies of various aspects of plant pathology, including epidemiology, etiology, disease resistance, host-parasite interaction and disease control (Xu and Ko, 1998). Jie et al. (2009) also reported that the artificial inoculation method provided a foundational understanding of ecological enrichment to control banana wilt disease in future.

Among few methods developed, three main procedures of artificial inoculation were generally employed; spraying the spore suspension on test genotypes using an atomizer; injection of spore suspension into the plants surface or into the intercellular air spaces of a leaf with the help of a hypodermic needle and immersion of seedlings of test genotypes in a spore suspension before transplanting them into fields. The conventional method like spraying has the disadvantage of causing considerable variation in spore distribution (Tuite, 1969). Although accuracy and precision were improved by applying a drop of inoculum with a modified hypodermic needle (Lapwood and Mckee, 1966) or capillary pipette (Toussoun et al., 1960). Xu and Ko (1998) used agarose for fixing the position of the inoculum drop. In many experiments, wounding is also used for artificial inoculation, but at the molecular level it might be confusing that whatever response is coming whether it is due to fungal invasion or it is due to wounding. Syringe infiltration is also one of the promising way, but not yet used for fungus. In this report, a comparison was carried out among five different artificial inoculation methods namely foliar spraying, syringe infiltration, wounding, spore suspension drop and spore suspension drop along with agarose method for pathogenesis studies of *A. brassicae* on *Brassica juncea* (Indian mustard).

MATERIALS AND METHODS

The culture *A. brassicae* (Berk.) Sacc. was derived from a conidium produced on a diseased leaf of *B. juncea* at Crop Research Centre (CRC) Pantnagar Uttarakhand India. *A. brassicae* Pantnagar isolate was maintained on potato dextrose agar (PDA) in the Department of Molecular Biology and Genetic Engineering, Pantnagar India. Conidiation of *A. brassicae* was induced by growing the fungus on V-8 agar (10% V-8 juice, 0.02% $CaCO_3$ and 2% agar) at 24°C for three to four days under cool white fluorescent light (2,000 lux) followed by 2 days incubation at 18°C (Aragaki, 1964). A conidial suspension was prepared by scraping mycelia and spores from plates of actively growing fungal cultures into autoclaved water and filtering the suspension through four layers of cheese cloth to remove most of the mycelia. The filtered spore suspension was centrifuged at 2000 x *g* for 5 min and resuspended in deionized water. This centrifugation was repeated one more time in order to ensure a clear spore suspension free of metabolites. After the final wash, supernatant was discarded and spores were resuspended in water containing 0.05% Tween-20. The spores in this suspension were counted using a haemocytometer and the concentration was adjusted to 10^4 spores/ml.

Seeds of brown mustard *B. juncea* were sown in plastic inserts (7.5 x 5 cm; 2 seeds per insert) containing mixture consisting of soil, sand and vermicompost in the ratio of 2:1:1. Plants were grown in the greenhouse (22/18°C day/night; 16 h photoperiod), watered at appropriate amount and time. Pathogenicity test were carried out with spore suspension on detached leaves of *Brassica juncea* seedling. Equal inoculums (500 µl) were inoculated with a sterile micropipette in each inoculation method. Replicates of 10 plants were used for each method.

In spore suspension drop method, inoculum were placed on each detached leaf in the form of drop whereas in spore suspension drop along with agarose method, agarose (Himedia laboratories private limited India) was used to stabilize the inoculum drops on target area to prevent any adverse effect on spores due to high temperature. 5 ml of 0.08% agarose in a test tube was heated in a microwave oven at high power for 40 s to melt the agarose. The test tube was then placed in 150 ml water bath at about 42°C in a 250-ml beaker to keep the agarose in the liquid state at this temperature. Each inoculum site on the leaf was covered with agarose. The agarose drop appeared as a clear dome on the plant surface. The agarose solidified and fixed the inoculum on the target site within few minutes. In the wounding method, inoculation was performed by gentle wounding the detached leaves with a pipette tip. In the spraying method, inoculum was sprayed on detached leaves with the help of atomizer. In syringe infiltration method, the detached leaf was carefully inverted, exposing the abaxial side. A 1-mL needleless syringe containing a spore suspension was used to pressure-infiltrate the leaf intracellular spaces. The vascular system of the leaf should be avoided for injection.

Detached leaves were kept in sealed Petri dishes with 1% agar and placed in growth chambers at 25°C and 70% relative humidity. The Petri plates were observed for *A. brassicae* initial symptoms at intervals of 24 h after pathogen inoculation and the infected leaves were examined up to 120 h after pathogen inoculation (Figure 1). The numbers of disease lesions were counted on the detached leaves in all the inoculation methods at 24, 48, 72, 96 and 120 h after inoculation of pathogen.

Statistical analysis

The data were analyzed using statistical software analysis SAS version (9.3). Repetitive measurement anova is a suitable and appropriate approach for this particular data analysis.

RESULTS AND DISCUSSION

In this analysis, four variables were taken: 1) treatment (different types of artificial inoculation methods namely spraying, infiltration, wounding, spore suspension drop and spore suspension drop along with agarose method); 2) time (different time intervals 24, 48, 72, 96 and 120 h after pathogen inoculation); 3) reading (disease lesions) and 4) subject (number of plants). In the repetitive measurement anova, reading was taken as continuous variable and treatment, time and interactions of treatment as a fixed effect and subject under treatment as a random effect. This approach is also known as linear modeling approach. Number of lesions on the detached leaves in all the inoculation methods at 24, 48, 72, 96 and 120 h after inoculation of pathogen were taken as the criteria to find out the best artificial inoculation method

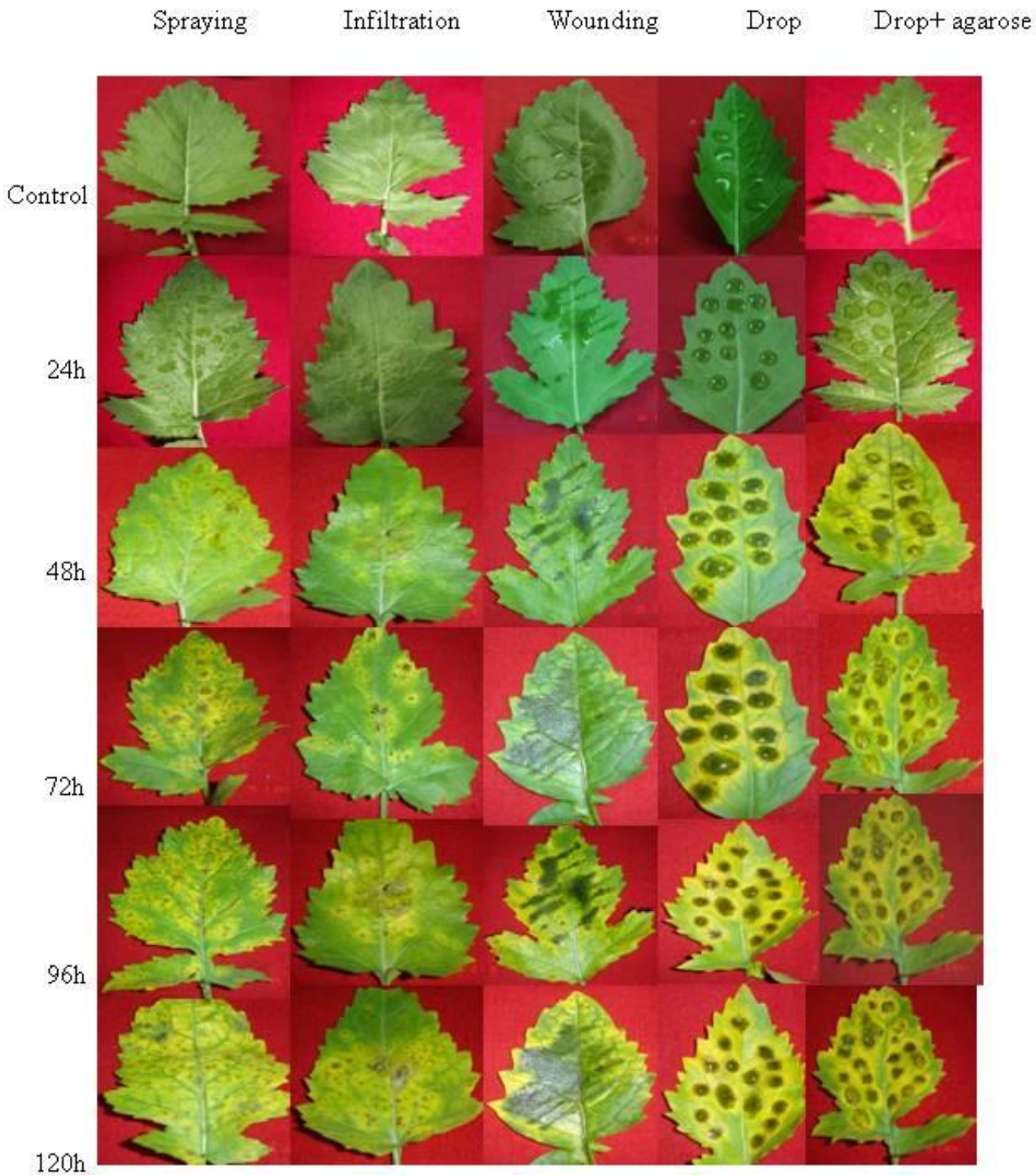

Figure 1. Development of initial black/brown disease lesions on the detached leaves of *Brassica juncea* using different pathogen inoculation methods (spraying, infiltration, wounding, drop and drop plus agarose) at different time intervals (24, 48, 72, 96 and 120 h) after pathogen inoculation.

for the pathogenesis studies.

At 24 h after inoculation of pathogen, drop plus agarose method showed the highest number of initial lesions that is 380 lesions, followed by drop (180), spraying (116), wounding (80) and infiltration (10). At 72, 96 and 120 h after inoculation of pathogen, again the drop plus

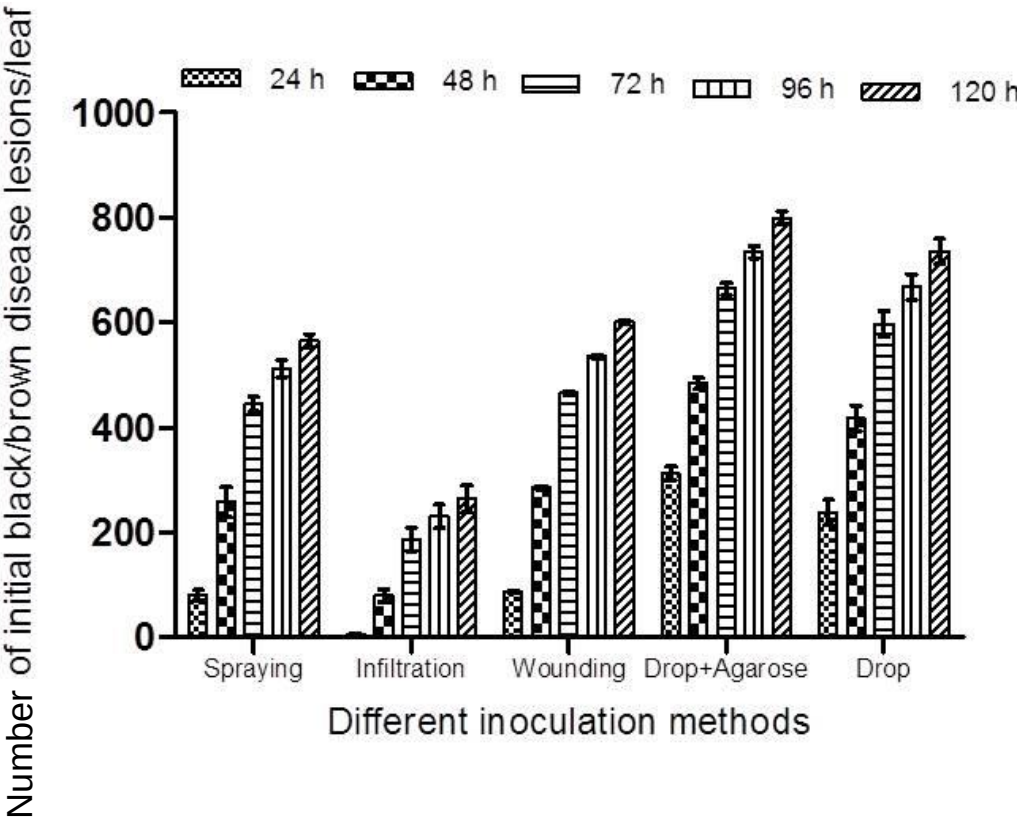

Figure 2. Number of initial black/brown disease lesions on the detached leaf of *B. juncea* using different pathogen inoculation methods (spraying, infiltration, wounding, drop plus agarose and drop) at different time intervals (24, 48, 72, 96 and 120 h) after pathogen inoculation.

Table 1. Repetitive measurement ANOVA showing significance among different pathogen inoculation methods (spraying, infiltration, wounding, drop plus agarose and drop) at different time intervals (24, 48, 72, 96 and 120 h) after pathogen inoculation.

Effect	Num DF	Den DF	F value	Pr>F
Treatment (Method)	4	45	121.80	<0.0001
Time	4	180	4536.65	<0.0001
Treatment* Time	16	180	49.08	<0.0001

DF, Degree of freedom.

agarose method showed the highest number of initial lesions followed by drop, spraying, wounding and infiltration except in 48 h after inoculation of pathogen where drop plus agarose showed the highest number of initial lesions followed by spraying, drop, wounding and infiltration (Figure 2). Linear modeling approach showed that the comparison among all the artificial inoculation methods at all the time intervals was significant ($P< 0.0001$) (Table 1). The mean value of number of disease lesions was highest in drop plus agarose method at all time intervals namely 312.2, 484.2, 664.2, 734.2 and 799.2 at 24, 48, 72, 96 and 120 h after pathogen inoculation respectively.

Several studies have been conducted for comparing different inoculation methods against different pathogens for screening different varieties like Co et al. (2008) evaluated three different smut inoculation techniques: soaking, wounding along with paste and pastes without wounding in sugarcane seedlings and found wound along with paste method was the best method. Buckley et al. (2009) compared side needle and spraying method for evaluating corn resistance to aflatoxin contamination and reported side needle inoculation method better as it produces significantly higher level of aflatoxin contamination. Baayen and Schrama (1990) compared five stem inoculation methods with respect to phytoalexin

accumulation and Fusarium wilt development in carnation and found injection method to be the more effective method.

The findings of the present study suggest that out of five inoculation methods used, spore suspension drop along with agarose inoculation method was most ideal as this fixed the inoculum on the target site. With this technique, a single conidiophore of *A. brassicae* were able to cause local lesions on leaves. This was also reported by Xu and Ko (1998), that even a single conidium containing drop were able to induce the development of local lesion in black mustard leaves. It also produces significantly higher number of disease lesions than the other inoculation methods and has the advantage of being accurate and precise. It is also easy to handle the inoculated plants. This method can also be used for green house screening of different varieties of *B. juncea* against *A. brassicae* and can be considered a good screening method for resistance against *Alternaria* blight in rapeseed mustard.

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