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Comparative efficacy of *in vitro* methods to culture rose powdery mildew (*Podosphaera pannosa* (Wallr.:Fr.) de Bary 1870)

NEETHU K. CHANDRAN[1,2] and SUBBARAMAN SRIRAM[1]*

[1]Division of Plant Pathology, ICAR-Indian Institute of Horticultural Research,
Hessaraghatta lake post, Bengaluru - 560 089, Karnataka, India
[2]Deparment of Biotechnology, Center for post graduate studies, Jain University,
Bengaluru - 560 011, Karnataka, India
*Email: sriram@iihr.res.in

ABSTRACT: Powdery mildew of rose caused by *Podosphaera pannosa* (Wallr.:Fr.) de Bary 1870, is one of the most serious fungal diseases of roses grown in poly houses and field. Powdery mildew pathogen is biotrophic and no species of it can be grown in culture apart from its host for any significant duration and none can grow on dead plant material. Three different parameters *viz.* effect of temperature, effect of medium or support and inoculation method were evaluated in the present study for the maintenance of powdery mildew pathogen under laboratory conditions. The disease incidence was observed after 3 to 10 days post inoculation (dpi). The results showed that the temperature range 20°C to 23°C with spore dusting as inoculation method was the best method to maintain powdery mildew *in vitro*. Moist filter paper along with water agar, streptomycin (50 ppm) and 0.03% benzimidazole were found to be suitable matrix or support for rose leaves during incubation period. Using young twigs was found to be the most reliable method compared to detached rose leaves for disease development. The present study results provide a reliable method for *in vitro* maintenance of rose powdery mildew.

Keywords: *In vitro* culture, powdery mildew, *Podosphaera pannosa,* rose

INTRODUCTION

Powdery mildew fungi are biotrophic obligate ubiquitous pathogens of various field and greenhouse crops worldwide. Because of the constant favorable environment in polyhouses, they are extremely aggressive in such situations. Powdery mildews are found on many economically important crops and affect many ornamental crops with the highly conspicuous colonies that cause loss of aesthetic value. The powdery mildew fungi belong to phylum Ascomycota and family *Ersiphaceae*. This pathogen is differing from the vast majority of other fungal pathogens. The mycelium grows superficially over the host tissue. Appresorium, specialized structure formed at the point of infection, help in penetration which is followed by formation of haustoria, the feeding organ that draw nutrients from host cells without killing them.

Powdery mildew pathogen engages in fascinating interactions with its host. The complex organs of infection formed by powdery mildew are among the best studied host pathogen interfaces in plant pathology. The study of biotrophic pathogen such as powdery mildews (ascomycetes) has significantly contributed to our current knowledge about the molecular basis of basal and isolate specific mechanisms.

Worldwide approximately 500 powdery mildew species are able to inhabit about 10000 distinct plant species. Powdery mildew disease of rose caused by *Podosphaera pannosa*, is one of the most-severe diseases of roses grown in polyhouses (Linde and Debener. 2003). Among ornamental plants rose has the greatest economic value worldwide (Hattendorf and Debener. 2007) andpowdery mildew is the economically the most important fungal disease of rose in green house and field production. Powdery mildew infection leads to complete defoliation of roses and subsequent losses of plant material. The disease is characterized by the emergence of white powder like fungal growth, which consists of the mycelium bearing conidiophores and conidia, on the surface of infected leaves. Pathogen is particularly prevalent in low temperature and humid climates where it frequently causes significant yield losses and reduction in the product quality (Micali *et al.* 2008, Holt *et al.* 2000).

The maintenance of powdery mildew culture is important to understand the resistance mechanism, host pathogen interaction that in turn helpsbreeding programs and disease management. *In vitro* maintenance of powdery mildew is important because of its biotrophic nature (Mehta *et al.* 2008; Leus *et al.* 2003). Compared

to other host plant, *in vitro* maintenance of powdery mildew on rose plant is limiting. In the present study different methods were evaluated for maintenance of powdery mildew *in vitro* on rose leaves and disease development.

MATERIALS AND METHODS

Fungal isolate and plant material for inoculation

Mono-conidial isolates of *Podosphaera pannosa* were collected from naturally infected poly house rose plants for experimental inoculation. The rose leaves were collected at early stages of infection to avoid other contaminants that come along with late stages of powdery mildew infection. Powdery mildew conidia from naturally infected leaves were transferred to healthy rose leaves under sterile condition. Susceptible rose genotype (Confetti) that wasgrown in poly house of Indian Institute of horticultural research (IIHR) was selected based on field evaluation of powdery mildew incidence for the inoculation experiment. The leaves collected for inoculations were free from powdery mildew or any other disease.

Experimental design

Different methods were evaluated for the maintenance of powdery mildew pathogen under laboratory conditions. Healthy and infected rose leaves were collected from field and polyhouse of ICAR-IIHR. Different methods evaluated for maintenance of powdery mildew culture are described below. The each experimental set up was done with three replications along with control.

Detached leaf assay

Coppery red detached rose leaves with 3-6 leaflets were used for the assay. Preliminary washing of the leaf was done with sterile water. The experiment setup performed in 90 mm and 200 mm Petri plates with different media or support matrix.

Leaf twig assay

The experiment performed in 250 ml beakers with sterile water. The young rose twigs were collected from the polyhouse and the bottom of the twig wrapped with tissue paper (5-6 layers) in such a manner to stand the twigs in the beaker upright. The twigs were directly used for experimental inoculation without any surface sterilization.

Effect of different media

Different media or support used for keeping the rose leaves were moist filter paper, water agar, water agar with streptomycin and 0.03% carbendazim (Linde and Debener. 2003; Schulz *et al.,* 2009). Sterile water was used as the medium for the leaf twig assay. Fifteen ml of water agar was poured into the 90 mm Petri plates with and without antibiotics and fungicides. Rose leaves were collected from polyhouses were washed with sterile water and dried in laminar airflow chamber. Healthy rose leaves were placed in glass 90 mm Petri plates with water agar alone and water agar with streptomycin and 0.03% carbendazim. The exact size filter papers were placed in both bottom and lid of 200 mm Petri plates. Proper moisture was given to the filter paper with sterile water. The petiole of the rose leaves was covered with bit of moist absorbent cotton. The detached leaf assay plates and leaf twig assay was kept ready for powdery mildew inoculation.The whole experimental set up was performed under sterile condition.

Effect of different inoculation methods

Three methods evaluated for inoculation were spore dusting, spore suspension and spore transfer with needle / brush. Heavily infected rose leaves were collected from polyhouse and carefully taken to the inoculation chamber and confirmed the absence of other contaminants along with powdery mildew conidia. Powdery mildew conidia were inoculated on detached leaf assay plates and leaf twig assay. The maximum possible spore load was used for inoculation in all three methods.

Spore dusting: The spores were dusted on the healthy leaves carefully and slowly to prevent the escape of spores. The dusting pattern was done evenly to cover the surface of each leaflet.

Spore suspension: Powdery mildew spores were collected from infected material and suspension was made by mixing the spores with 1 ml sterile water by proper vortexing. Spore suspension was inoculated on healthy leaves with the help of a pipette.

Spore transfer with brush: Spores were transferred from infected leaf to healthy leaves with the help of a thin inoculation brush.

Effect of temperature on maintenance of powdery mildew

Different incubation temperatures were evaluated to find out the optimum temperature required for artificial

Fig. 1. Maintenance of powdery mildew on leaf twig A- experimental setup B C D & E- Initial stages of powdery mildew development F G H &I – Complete growth of powdery mildew (100% leaf area covered)

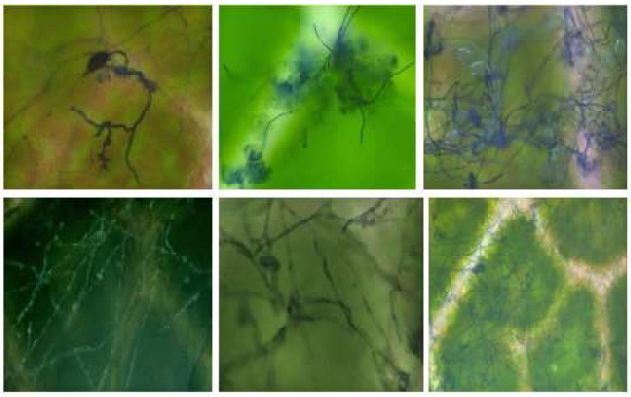

Fig. 2. Microscopic observation (at 10X and 20X) of powdery mildew on rose leaves (leaf twig assay) at 24 - 48hai

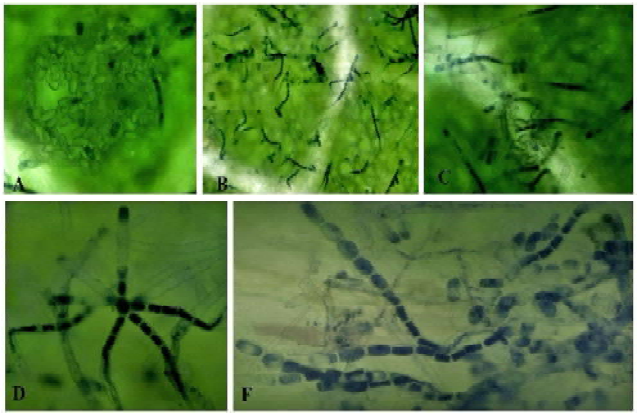

Fig. 3. Microscopic observation of powdery mildew on rose leaves (leaf twig assay) at 108 hai. A-F: Powdery Mildewconidia and conidiophores development.

maintenance of powdery mildew on rose leaves. Three different aspects were planned for the maintenance of the temperature *viz*. incubation at ambient temperature, an artificially made chamber for incubation and a commercially available laboratory purpose incubator.

Ambient temperature: During the experimental setup the ambient temperature range was 18°C to 22°C during night, 20°C to 28°C during day. The powdery mildew inoculated Petri dishes were incubated at room temperature and observed for the emergence of powdery mildew growth.

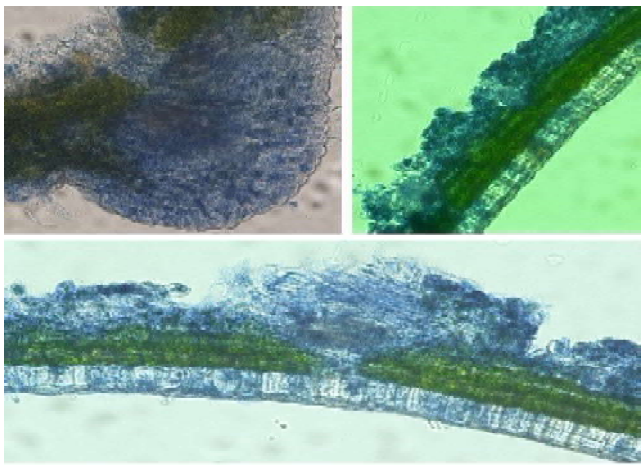

Fig. 4. Cross section of powdery mildew infected leaves at 108 hai. Leaf sections were stained with lacto phenol cotton blue

Table 1. Different parameters evaluated for the study

Media /Support	Inoculation method	Temperature
Filter paper	Spore dusting	22°C
Water agar	Spore suspension	Room temperature (18°C - 22°C during night, 20°C to 28°C during day)
Water agar with streptomycin and 0.03% benzimidazole	Spore transfer with brush	23°C - 25°C with more than 80% relative humidity
Leaf twig assay		

Artificial chamber: The artificial Incubation chamber was made by storage crates. The crates were covered by papers on all sides, spreading blotting sheets inside and polypropylene sheets on top with proper aeration. The temperature was adjusted with wet blotting sheets and cotton. Temperature range 22°C - 24°C was maintained in artificially made incubation chamber and monitored with the help of thermometer and proper humidity range was attained by covering the crates with polypropylene sheets. The powdery mildew inoculated detached leaf assay plates and leaf twig assay plates were kept in this chamberat 23°C - 25°C with more than 80% relative humidity.

Precision lab incubator : Inoculated plates and beakers were kept in precision lab incubator with temperature 22°C and light.

Microscopic observation and disease assessment

Each experimental setup wasobserved for the powdery mildew development and microscopic evaluations were made for the same. The microscopic observations were made at 32 hours after inoculation (hai), 72hai and 108hai to confirm the powdery mildew development and conidia germination on leaf surface. The inoculated rose leaves were stained with lacto phenol cotton blue for microscopic observations. Thin cross sections of rose leaves made with the help of dissection blade and stained for microscopic observation to confirm the powdery mildew penetration.

RESULTS AND DISCUSSION

Effect of different media

Detached leaf assay (DLA) with moist filter paper : The emergence of powdery mildew growth observed after 3 dai. Rate of contamination was more due tothe moisture in the filter paper that was favourable for other fungal contaminants. Freshness of leaves wasthere for shorter period as leaflets showed yellowing and defoliated after 7 dai. The visible growth of powdery mildew was observed after 3-4 dai.

Detached leaf assay (DLA) with water agar: The experimental set up was performed in 90mm plates with single leaf per plate. Contamination was more in water agar alone plates.The secondary contaminations developed from water agar and started growing on rose leaves. Water agar favours the growth of other

Table 2. Disease assessment

Temperature	Spore dusting				Spore suspension			Inoculation with brush		
	FP	WA	WA+S+C	Leaf twig	FP	WA	WA+S+C	FP	WA	WA+S+C
22°C	+++	+	+++	++++	-	-	-	++	+	++
Ambient temperature	-	-	-	+	-	-	-	-	-	-
23°C - 25°C	+	-	++	+++	-	-	-	+	-	++

Note: Percentage leaf area covered by powdery mildew: (-) 0%,(+) 25%, (++) 50%, (+++) 75%, (++++) 100%,
FP: Filter paper, WA: Water agar; WA+S+C: water agar with streptomycin and carbendazim

contaminants more than filter paper with moisture. There was no proper development of powdery mildew due to the spreading of contamination into the inoculated leaves from agar matrix.

Detached leaf assay with water agar with streptomycin and 0.03% benzimidazole: Leaflets placed onwater agar with streptomycin and benzimidazole (Carbendazim), stayed in good condition during incubation period was after 10days. There was no contamination observed during the incubation period. Powdery mildew emergence was observed and disease scoring are given in Table 2.

Leaf twig assay: Powdery mildew development was more in leaf twig assay compared to other methods. Single inoculation method was (spore dusting) used for this assay. Powdery mildew spores were inoculated by dusting only. Spore suspension and inoculation were not tried for leaf twig assay. Powdery mildew development and spreading was more (++++) on inoculated leaves of twigs than detached leaf assay. It was found to be the most reliable compared to other method because rose twigs were able to survive longer up to 17 days and no contamination observed during incubation period.

Effect of temperature

The optimum temperature observed for spore germination and infection was at 18°C to 23°C. Powdery mildew development initiated first in inoculated leaf twigs kept in 18°C to 23°C and the further disease spread was also more in the same temperature. The pathogen growth was observed in plates incubated in incubation chamber made with crate. Powdery mildew development was not visible in room temperature and more contamination was observed. Leaves got defoliated very soon in detached leaf assay kept in room temperature.

Effect of inoculation methods

Spore dusting was found to be the best method for inoculation as the powdery mildew disease development was more in which spore dusting used as inoculation method. Secondary contamination was observed in leaves inoculated with brush. No powdery mildew growth was observed were spore suspension used as inoculation method.

Powdery mildew development and penetration by hyphal growth was confirmed by microscopic observations at 24-48 hai, 72 hai and 108 hai. The results of present study confirmed the importance of temperature range required for powdery mildew infection process. Extending contamination free setup during inoculation and throughout the incubation period is essential for disease development. Homogenous inoculation method of the pathogen is required for screening of genotypes, breeding and other research programmes and homogenous spore distribution is essential for the disease assessment. Inoculating with spore suspension is easy for spore quantification (Yan *et al.,* 2006) but in the present study the results indicated that there was poor disease development compared tospore dusting method. The assay with detached leaves placed on water agar amended with streptomycin and benzimidazole (0.03%) (Linde and Debener, 2003) resulted in better colonization by the fungus. Use of vacuum setting tower for spore inoculation (Linde and Debener, 2003; Moghaddam *et al.,* 2013) can be applied for large scale screening purposes and needed more inoculum for the experiment. Spore dusting method was found to be the best for small scale screening experiments because the experiment can be performed with less amount of inoculum. Leaf twig assay with spore dusting as inoculation methodis suitable for powdery mildew maintenance under laboratory conditions and varietal screening. Moist filter paper and water agar with streptomycin and 0.03% carbendazim also suitable for powdery mildew maintenance under laboratory conditions but longevity of leaf was shorter. Leaf twig can stay for long (17-20 days). There are different contaminants present in powdery mildew infected leaves so the selection of inoculum is very much critical for the *in vitro* maintenance of the pathogen. Leaves with final stage of powdery mildew colonization have more insects and other contaminants and hence these contaminants also fall on the leaves while used for inoculation. The results of the present study can be used for the maintenance of the powdery mildew for further research work.

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