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Original Research Article

Isolation and Identification of *Plasmopara viticola* Associated with Grapevine from Marathwada Region

M. A. Mane[1*], S. S. Bodke[1] and R. N. Dhawale[2]

[1]Department of Botany and Horticulture, Yeshwant Mahavidyalaya, Nanded, Swami Ramanand Teerth Marathwada University, Nanded-431 606, Maharashtra, India
[2]Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Vasantrao Naik Marathwada Agricultural University, Parbhani-431 402 (MS), India
**Corresponding author*

Keywords

Plasmopara viticola, Marathwada, Grape, RAPD, Microscopy, OPF-18

A B S T R A C T

Grape (*Vitis vinifera*) is species of vitis, belongs to the family Vitaceae. It is native to Mediterranean region, central Europe and south western Asia from Morocco and Portugal north to southern Germany and east to northern Iran. A grape is a fruit, botanically a berry, of the deciduous woody vines of the flowering plant genus *Vitis*. Grapes can be eaten fresh as table grapes or they can be used for production of wine, jam jellies, grape seed extract, raisin, vinegar etc. Presently, in India it is most commonly known as 'Draksha' in Marathi and 'Angur' in Hindi. Powdery mildew gives an undesired, off-flavor to wine but it is not a concern for grape juice. Mainly fungal plant diseases are usually managed with applications of chemical fungicides or heavy metals. In some cases, conventional breeding has provided fungus resistant cultivars. Genetic engineering enables new ways of managing infections. In this study 10bp oligonucleotide primers (Operon kit) were tested. DNA samples isolated from *Uncinula necator* of grape sample and amplification were repeated at least thrice on 1.5% agarose gel and only bands reproducible on several runs were considered for analysis. For confirmation the specific RAPD product originated from the *Plasmopara viticola* resistant region was identified to this region. Isolated DNA was used in PCR reaction for amplification with primer OPF-18 and OPF-19. The specific band of both primers was also found in *Plasmopara viticola*.

Introduction

Downey mildew/*Plasmopara viticola* is endemic on wild *Vitis* species of North America. It was first observed in Europe in 1878. It was probably introduced into Europe with American grape cuttings used to replant the French vineyards destroyed by phylloxera. Its appearance in Europe was not a real surprise, as several voices had expressed concern (Cornu, 1872 cited in Muller and Sleumer, 1934) about the danger of the accidental introduction of "*Peronospora viticola*". *Plasmopara viticola* (Order: Peronosporales, Family: Peronosporaceae) is a heterothallic (Wong, *et al.*, 2000) diploid obligate *Biotrophico omycete*. The biology was studied in detail beginning in 1900 and it was immediately clear that the pathogen overwintered as oospore in the leaf or berries residues on the surface layer (Gregory, 1915). The incidence of downy mildew has increased in recent years in Europe, requiring costly crop

protection treatments that have negative effects on the environment and that may adversely affect the quality of any wine produced pevines (Clark and Spencer-Phillips, 2000). This has led to the formation of groups of viticulturalists, researchers and winery owners devoted to the establishment of a more sustainable and environmentally-friendly type of viticulture that reduces the use of such treatments to a minimum. Such a goal requires the use of strategies based on an in-depth knowledge of the biology of *P. viticola* and the environmental conditions that favor its growth. Additionally, knowledge of the susceptibility of different grapevine varieties to downy mildew is essential to select those more tolerant or resistant to the disease in a growing area.

The conditions under which *P. viticola* can sporulate in susceptible tissue under controlled conditions were analyzed in detail by Blaeser (Blaeser, 1978; Blaeser and Weltzien, 1978; 1979). Most simulation models still rely on Blaeser's parameters, including a minimum of 98% relative humidity and 4 h of darkness, a minimal temperature of 13°C and an optimal temperature of 19°C.

Sporulation proceeds in darkness, but not in the light, and is completed within 7 h. It is inhibited by irradiation with white light (Rumbolz *et al.,* 2002), near-UV light of 310–400 nm or green light (500–560 nm) at intensities >3–3.5 Wm-2 (Brook, 1979). The lifespan of the zoosporangia decreases as the water saturation deficit increases. The zoosporangia are thought to be dispersed by rain splash, as they are detached by water. So there is a need of rapid growth in agriculture sector not only for self-reliance, but also to bring about equitable distribution of income and wealth in rural areas as well as to reduce poverty and improve the quality of life (Davies, 2009).

With the development of tools allowing the genetic characterization of single genotypes and identification of the genotype of a single lesion, it became possible to determine whether an epidemic was indeed caused by one or a few clones deriving from a few primary infections, as had been assumed. RAPDs were the first type of markers to be used for this work. Although the power of this type of marker is limited, RAPDs can be used to detect genetic differences between genotypes in a single comparative experiment. However, since *P. viticola* is an obligate biotroph, doubts remain as to whether other genetic material collected with the sporangia may have been the source of the observed differences. In 1998, Kump reported a high level of variability between batches of sporangia collected from individual leaves with single oil spots in a vineyard in northern Switzerland.

Attributing a specific genotype and, therefore, a unique oosporic origin to each specific RAPD banding pattern, they concluded that the ratio between primary and secondary lesions was higher than expected and recommended that the quantitative role of oospores in epidemics of grape downy mildew be reconsidered (Kump *et al.,* 1998).

In the view of above constraint, the present study being proposed to take initiatives for to develop isolation method for *Plasmopara viticola* from grape leaves and to identify the isolated *Plasmopara viticola* by morphology and molecular marker.

Materials and Methods

On the basis of symptoms and signs the *Plasmopara viticola* resistance and susceptible leaves were collected from "Aurangabad, Osmanabad, Latur districts of Marathwada region (Plate 1).

Material collection

Plasmopara viticola infected leaves were collected from different locations of Marathwada region. The same day as the lab presentations on downy mildew of grapevine, we were set up moist chambers from which we were isolating *Plasmopara viticola*.

Preparation and procedure of petri plate moist chamber

Leaves or stem were made into small pieces and tape them onto a sterile slide. It is good to leave a few pieces untreated because some pathogens are very sensitive to sterilization. Heavily damaged tissue was not selected to avoid saprophytic organisms.

Filter or blotting paper disk was placed on the bottom and in the lid of petri dish and moisten it thoroughly with sterile water. The slide was placed on the paper disk. The plate was closed and sealed with parafilm to hold in moisture.

Incubated at 18-22^{0}C under alternating cycles of light and darkness (10h light/14h darkness) (many fungus will not develop to the reproductive stage without this alternating light/darkness regimen).

Direct plating

Often it is most convenient to place fungal materials that are of interest directly on a nutrient agar medium, because it is widely used. It is a simple technique, requiring the placing of small bits of the leaf samples on the surface of the agar or the pouring of melted but cooled agar over the fragments.

After a few days' incubation fungal growth appear on the surface, and can be transferred into pure culture.

Aseptic technique

In most microbiological procedures, it is necessary to protect instruments, containers, media, and stock cultures from contamination by microorganisms constantly present in the environment.

Aseptic technique involves the sterilization of tools, glassware, and media before use as well as measures to prevent subsequent contamination by contact with non-sterile objects.

Media preparation

The media most commonly used are nutrient agar (bacteria), potato dextrose agar (fungi), and Sabouraud dextrose agar (fungi).

To prepare potato infusion, boil 200g sliced, unpeeled potatoes in 1 liter distilled water for 30 min.

The medium was filtered through cheese cloth, saving effluent, which is potato infusion.

Filtrate was mixed with dextrose, agar and water and boil to dissolve.

Final pH, 5.6 ± 0.2.

Autoclaved for 15 min at 121°C.

20-25 ml of media dispensed into sterile 15 × 100 mm petri dishes.

Isolation and pure culture development

To obtain the pure culture of infecting microorganisms they should be cultured on suitable medium containing appropriate amount of nutrients. For the development fungal culture strains PDA i.e. Potato Dextrose Agar Medium is used.

The infected leaf samples were cut into 3mm pieces with sterile razor blade, surface-sterilized in 1% hypochlorite solution for 2 minutes, then placed on Potato Dextrose Agar (PDA) and incubated at room temperature for 5 days. After incubation, colonies of different shape and colors were observed on the plates. A pure culture of each colony type on each plate was obtained and maintained (As per contamination subculture was carried out). The maintenance was done by sub-culturing each of the different colonies onto the SDA plates and incubated at room temperature again for 5 days (Jha, 1995).

Procedure for preparation of pure culture for *Plasmopara viticola*

Streaking for isolation by the quadrant method

Potato Dextrose Agar (PDA) plates were obtained. These culture media dishes were turned bottom side up and labeled the perimeter of the dishes with initials, date, section number and table number, temperature of incubation, type of medium and specimen. The plates were inverted and incubated plate at 30°C - 37°C.

The reason the plate is inverted is the fact that the air space between the dish lid and the agar surface is saturated with moisture; during incubation the moisture condenses on the upper lid as droplets. As these droplets collect into a large drop, the water drips onto the agar surface causing the spread and mixing of colonies. Inversion of the plate eliminates this problem.

Identification of isolated fungi

The technique of James and Natalie (2001), was adopted for identification of the unknown isolated fungi using cotton blue in lactophenol stain.

Lactophenol cotton blue technique for fungus isolation

Fungus is eukaryotic organisms and they are mainly classified into two main groups yeast and molds. Fungal structure includes sporangiospores, mycelium, spores etc. The lactophenol cotton blue wet mount is simply and widely used method for staining of fungus.

The main components of LCB staining

Phenol - fungicidal in nature
Lactic acid - preserves fungal structures
Cotton blue - stains the chitin in the fungal cell wall and cytoplasm

Staining of fungus from culture

A grease free slide was taken.

A lactophenol cotton blue solution was added on slide.

The inoculation loop or needle was sterilized and cooled it then transferred mycelial growth onto the LCB strain and pressed it gently so that it can easily mix with the stain.

A clean cover slip was taken and with the help of a forceps the cover slip was placed on mycelial growth + LCB

With the help of blotting paper, the excess strain was wipe.

The preparation was observed under low and high power objectives of microscope.

Morphological characterization of isolated sample of downy mildew from grapevine leaf samples

The technique of James and Natalie (2001) was adopted for identification of the

unknown isolated fungi using cotton blue in lactophenol stain. The identification was achieved by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the mycelium from the fungal cultures was removed and placed in a drop of lactophenol. The mycelium was spread very well on the slide with the aid of the needle. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with x10 and x40 objective lenses respectively. The species encountered were identified in accordance with Cheesbrough (2000).

Genomic DNA extraction from *Plasmopara viticola*

Preparation of stock solutions for DNA extraction by using Dr. Shunxue, JK lab and electrophoresis.

DNA isolation protocol

This standard protocol is based on the following reference:

[Mania and T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, 10423 MV.]

Determination of quantity and quality of isolated DNA

Determination of quantity and quality of isolated DNA was done by spectrophotometer (Hitachi-U2900®). The instrument was set to a blank with 50 µl of distilled water. After that 49 µl distilled water and 1 µl of sample were added in Eppendorf® cuvette and the quantity and quality in nanogram at A260/ A280 nm was determined. The ratio higher than 2.0 indicated the impurity of protein and less than 1.8 indicated RNA impurity in sample. The amount of DNA was calculated by using the formula:

$$\text{DNA } (\mu g/\mu l) = \frac{A260 \times 50 \times \text{dilution factor}}{1000}$$

Dilution of DNA sample

Part of DNA samples were diluted with appropriate quantity of sterilized distilled water to yield a working concentration of 25ng/µl and stored at 4^0C until PCR amplification.

Optimization of PCR condition for RAPD

Genomic DNA was prepared from equal volumes of standard DNA (20ng/µl) from downy mildew of grapevine leaves. RAPD primers were used to screen the genomic DNA of downy mildew of grape leaves for determining the presence or absence of RAPD marker linked to RPv-1 gene (Resistant to *Plasmopara viticola*) of resistance of downy mildew of grape (Luo Su-Lan, 2001). The reaction was carried out in a volume of 25µl and was prepared as follows: 20ng of genomic DNA, 1U *Taq polymerase*, 1.5mmol/l $MgCl_2$, 2.5µl 10x reaction buffer, 150µmol/l dNTPs and 4pmol/l primer. Each reaction solution was overlaid with one drop of mineral oil to prevent evaporation. Amplification reactions was performed in a 96-well thermocycler (Eppendorf Authorised Thermal Cycler PCR) programmed as follows: (94^0C for 1min; 36^0C for 1min; 72^0C for 10min.

Agarose gel electrophoresis

Agarose gel electrophoresis unit was cleaned properly before use. Agarose gel (1.5%) was prepared by dissolving 1.5g agarose powder in 100 ml 1XTAE buffer

and heated in a microwave oven. Then 10mg/ml ethidium bromide was added to it after cooling down to 50°C. The gel was poured in gel casting tray in which comb was inserted and kept for 1 hr.

After solidification the comb was removed. 5µl DNA was mixed with 1µl to 6X gel loading dye and loaded on the gel. The electrophoresis was carried out at 100 volts for 1.5 hr using 1X TAE buffer.

Identification of RAPD marker by using molecular weight of amplified band of RAPD primer

10bp oligonucleotide primers (Operon kit) were tested. DNA samples isolated from downy mildew of grape sample and amplification were repeated at least thrice on 1.5% agarose gel and only bands reproducible on several runs were considered for analysis. To check out for potential co-segregation of DNA fragments and downy mildew resistant phenotypes.

Results and Discussion

Development of isolation method for *Plasmopara viticola* from grape leaves

Direct isolation of fungi is often more effective if the natural substrate has been kept moist for one to several weeks to allow fungus to grow and sporulate. The easiest method involves a container called a moist chamber. Moist chambers can take any number of forms, but are basically containers holding a material such as cotton, blotting paper, cloth, sterile sand or soil, or peat moss that can be kept moist for several weeks. The specimen is placed on top of the moist material and left until fungus begins to grow on it. Incubate plate at 18-22^{0}C under alternating cycles of light and darkness (10h light/14h darkness) (many fungus will not develop to the reproductive stage without this alternating light/darkness regimen).

Direct plating

Often it is most convenient to place fungal materials that are of interest directly on a nutrient agar medium, because it is widely used. It is a simple technique, requiring the placing of small bits of the leaf samples on the surface of the agar or the pouring of melted but cooled agar over the fragments. After a few days' incubation fungal growth appear on the surface, and can be transferred into pure culture (Plate 2).

Sterilization of glassware's and preparation of media

The glassware's viz. culture tubes, bottles, Petri dishes, pipettes, beakers, measuring cylinder, conical flask etc. for sterilization of culture tubes and bottles they were closed with non-absorbent cotton and caps. Petri dishes, pipettes, beakers, measuring cylinder, conical flask etc. were sterilized by wrapping by wrapped in an aluminium foil and kept in wire mesh basket prior to autoclaving. The wire mesh basket containing glassware's were autoclaved at 15lbs. at 121^{0}C for a 30 min followed by drying in hot air oven at 80-100^{0}C for 1hr. for removal of excess moisture. Forceps and scalpel like instruments were sterilized by flame sterilization technique. The culture showing unwanted microbial growth (contamination) was discarded after autoclaving in order to destroy the source of contaminants.

Isolation and pure culture development

To obtain the pure culture of infecting microorganisms they should be cultured on suitable medium containing appropriate amount of nutrients. For the development

fungal culture strains PDA i.e. Potato Dextrose Agar Medium is used.

The infected leaf samples were cut into 3mm pieces with sterile razor blade, surface-sterilized in 1% hypochlorite solution for 2 minutes, then placed on Potato Dextrose Agar (PDA) and incubated at room temperature for 5 days (Jha, 1995). After incubation, colonies of different shape and colors were observed on the plates.

A pure culture of each colony type on each plate was obtained and maintained (As per contamination subculture was carried out). The maintenance was done by sub-culturing each of the different colonies onto the SDA plates and incubated at room temperature again for 5 days (Figure 1).

Pure culture development by streaking for isolation by the quadrant Method

Obtain one Potato Dextrose Agar (PDA) plates. Turn these culture media dishes bottom side up and label the perimeter of the dishes with initials, date, section number and table number, temperature of incubation, type of medium and specimen.

Draw two perpendicular lines with a marker on bottom of the plate to divide the circle into 4 quadrants.

Note: After you become proficient in streaking, you could visualize each petri dish divided into quarters instead of actually drawing lines.

Holding an inoculating loop between your thumb and index finger, insert the wire portion into the Bunsen burner flame, heating the entire length of the wire until it is red and glowing. Allow the wire to cool before doing the next step. Do not wave the loop in the air.

Note: The wires of your loops are made of special alloy that makes them heat fast and cool fast. Still, the loop takes about a minute to get down to room temperature after being in the flame. If your loop is not sufficiently cooled down, it may kill the organisms that it comes in contact with and you may observe no growth on your plates.

Using free hand, pick up the tube containing the mixed culture and gently shake it to disperse the culture. Remove the tube cap or plug with free fingers of the hand holding the sterile inoculating loop and carefully flame the lip of the tube in the Bunsen burner flame.

Tilt the tube to bring the broth culture within 1 inch from the lip of the tube. Insert the sterile loop and remove a small amount of growth; a loopful is usually sufficient. Try not to touch the sides of the tube with the loop.

Flame the tube lip again, carefully replace the tube cap or plug, and return the culture tube to the test tube rack.

Expose the agar surface of each plate for inoculation by raising the lid at an angle over the agar, thus keeping the plate surface protected from aerial contamination.

Apply the mixed culture on the loop onto the first quadrant by sweeping the area of this quadrant. Spread the specimen out well.

The loop was flamed and allowed to cool. May cool the loop in an uninoculated area of the medium. NOT to wave it in the air to cool.

Now the inoculum was streaked from quadrant 1 into quadrant 2. Use smooth, non-overlapping strokes. The entire quadrant 2 was utilized. The loop was

flamed when done and the loop let to cool. Now the inoculum was streaked from quadrant 2 into quadrant 3 by smooth, non-overlooking strokes again. The loop was flamed one more time and let it cool. Now some inoculum was brought from quadrant 3 into quadrant 4 in the same manner as for other previous quadrants.

Note: In this procedure, the number of times you enter back into the preceding quadrant depends on how heavy the initial inoculum is. If the initial inoculum comes from a plate, slant or a heavy broth culture, enter the preceding quadrant only once. However, if the inoculum is obtained from food material, very light broths or any other source where you expect to have few bacteria, you may need to bring the inoculum from the previous quadrant to a new quadrant a few times.

The loop was flamed and cooled.

The plates were inverted and incubated at 30°C - 37°C. The reason the plate is inverted is the fact that the air space between the dish lid and the agar surface is saturated with moisture; during incubation the moisture condenses on the upper lid as droplets. As these droplets collect into a large drop, the water drips onto the agar surface causing the spread and mixing of colonies. Inversion of the plate eliminates this problem.

Note: Plates are always incubated inverted, even (especially) in the refrigerator.

Lactophenol cotton blue technique for identification of DM fungi

The technique of James and Natalie (2001) was adopted for identification of the unknown isolated fungi using cotton blue in lactophenol stain. Fungus is eukaryotic organisms and they are mainly classified into two main groups yeast and molds. Fungal structure includes sporangiospores, mycelium, spores etc. The lactophenol cotton blue wet mount is simply and widely used method for staining of fungus. After lactophenol cotton blue solution treatment observed the prepared slide under low and high power objectives of microscope (Fig. 3).

Morphological characterization of isolated sample of *Uncinula necator* from grapevine leaf samples

The technique of James and Natalie (2001) was adopted for identification of the unknown isolated fungi using cotton blue in lactophenol stain. The identification was achieved by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the mycelium from the fungal cultures was removed and placed in a drop of lactophenol.

The mycelium was spread very well on the slide with the aid of the needle. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with x10 and x40 objective lenses respectively. The species encountered were identified in accordance with Cheesbrough (2000) and shown in Figure 2.

Genomic DNA extraction from *Plasmopara viticola*

Preparation of stock solutions for DNA extraction by using Dr. Shunxue, JK lab and electrophoresis. This standard protocol is based on the following reference:

[Mania and T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning, A Laboratory Manual, Cold Spring Harbor

Laboratory, Cold Spring Harbor, 10423 MV.]

Fungal mat (3g) grown on potato dextrose broth (PDB) was homogenized using pestle and mortar in 4ml of 2 per cent sodium dodecyl sulfate (SDS) for 5 minutes.

To the above solution, 6ml of lysis buffer (2.5mM EDTA, 1% TritonX100 and 50 mM Tris-HCl, pH 8.0) (Appendix III) was added.

The suspension was extracted with equal volume of phenol: chloroform: isoamyl alcohol (5:4:1) and centrifuged at 10,000 rpm for 10 min.

The supernatant was taken into a fresh tube and one tenth volume of 3M sodium acetate and 0.54 volume of isopropanol were added at room temperature, mixed by gentle inversion and kept for 30 min at 2ºC.

The DNA was recovered by centrifugation at 10,000 rpm for 10 min at 4ºC.

The DNA pellet was washed with 70 percent ethanol, air dried and resuspended in 300 µl of T10E1 (10mM Tris-Cl and 1 mM EDTA, pH 8.00).

The genomic DNA isolated was purified according to the protocol described by (Mania, 1982). To the above DNA solution, RNase @100 µg/ml was added and this solution was incubated for two hours at 37ºC on water bath.

The solution was centrifuged at 10,000 rpm for 10 min and the suspension was treated with equal volume of buffered phenol (pH 8.0) and centrifuged.

The upper aqueous layer was taken in a fresh tube and treated with equal volume of phenol: chloroform (1:1 v/v).

This suspension was centrifuged and upper aqueous layer was taken into fresh tube and to this one tenth volume of 3M sodium acetate and 2 volumes of absolute ethanol were added and incubated at 4^0C for 2 hr.

The DNA was pelleted by centrifugation at 10,000 rpm for 10 min. The pellet was washed with 70 per cent ethanol, air dried and dissolved in 100µl of $T_{10}E_1$ buffer and stored at 4ºC until further use.

The concentration of DNA was estimated by use of Nanodrop spectrophotometer.

Agarose gel electrophoresis

Agarose gel electrophoresis unit was cleaned properly before use. Agarose gel (1.5%) was prepared by dissolving 1.5g agarose powder in 100 ml 1XTAE buffer and heated in a microwave oven. Then 10mg/ml ethidium bromide was added to it after cooling down to 50°C. The gel was poured in gel casting tray in which comb was inserted and kept for 1 hr. The electrophoresis was carried out at 100 volts for 1.5 hr using 1X TAE buffer.

Identification of RAPD marker by using molecular weight of amplified band of RAPD primer

10bp oligonucleotide primers (Operon kit) were tested. DNA samples isolated from downy mildew of grape sample and amplification were repeated at least thrice on 1.5% agarose gel and only bands reproducible on several runs were considered for analysis. To check out for potential co-segregation of DNA fragments and downy mildew resistant phenotypes. A recombination was obtained in between OPA18-1500 and RPv-1. To confirm that the specific RAPD product originated from the *Plasmopara viticola* Resistant region

and select a molecular marker to this region. DNA from four grapevine leaf samples was used for amplification with primer OPA18. The specific band of OPA18 primer with 1500bp was also found in *Plasmopara viticola* Resistant grapevine leaf samples (Figure 4). Therefore, DNA samples of lane number 1, 2 and 4 considered as *Plasmopara viticola* Resistant and DNA samples of lane number 3 is susceptible to *Plasmopara viticola*.

In this present study,

Developed the method for isolation of *Plasmopara viticola* from grape leaves.

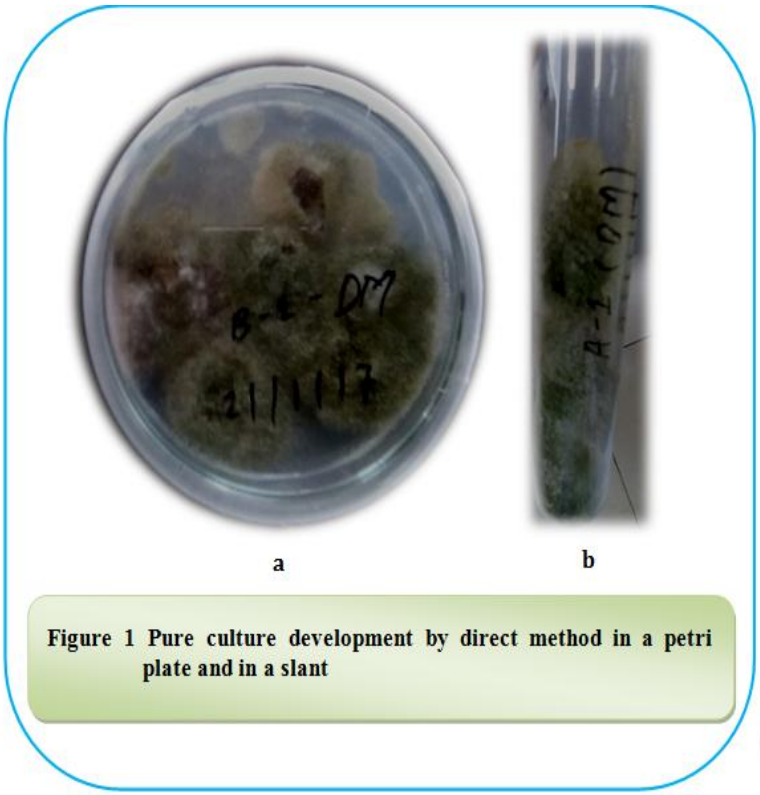
In second objective, here identified the isolated *Plasmopara viticola* by morphological level on the following observations levels:

Substomatal vesicles with short hyphe developing from haustorium (arrow) has been initiated 3.5 hrs infection. Intercellular mycelium showing the irregular shape. Haustoria are not visible in this photograph. 40hrs. infection.

Extensive colonization of the hyphe – 72 hrs infection.

Intercellular mycelium developing from the substomatal cavity showing haustoria (40hrs.)

In case of molecular marker, confirmed that the specific RAPD product originated from the *Plasmopara viticola* Resistant region and select a molecular marker to this region. DNA from four grapevine leaf samples was used for amplification with primer OPA18. The specific band of OPA18 primer with 1500bp was also found in *Plasmopara viticola* Resistant grapevine leaf samples (Figure 4). Therefore, DNA samples of lane number 1, 2 and 4 considered as *Plasmopara viticola* Resistant and DNA samples of lane number 3 is susceptible to *Plasmopara viticola*.



a b

Figure 1 Pure culture development by direct method in a petri plate and in a slant

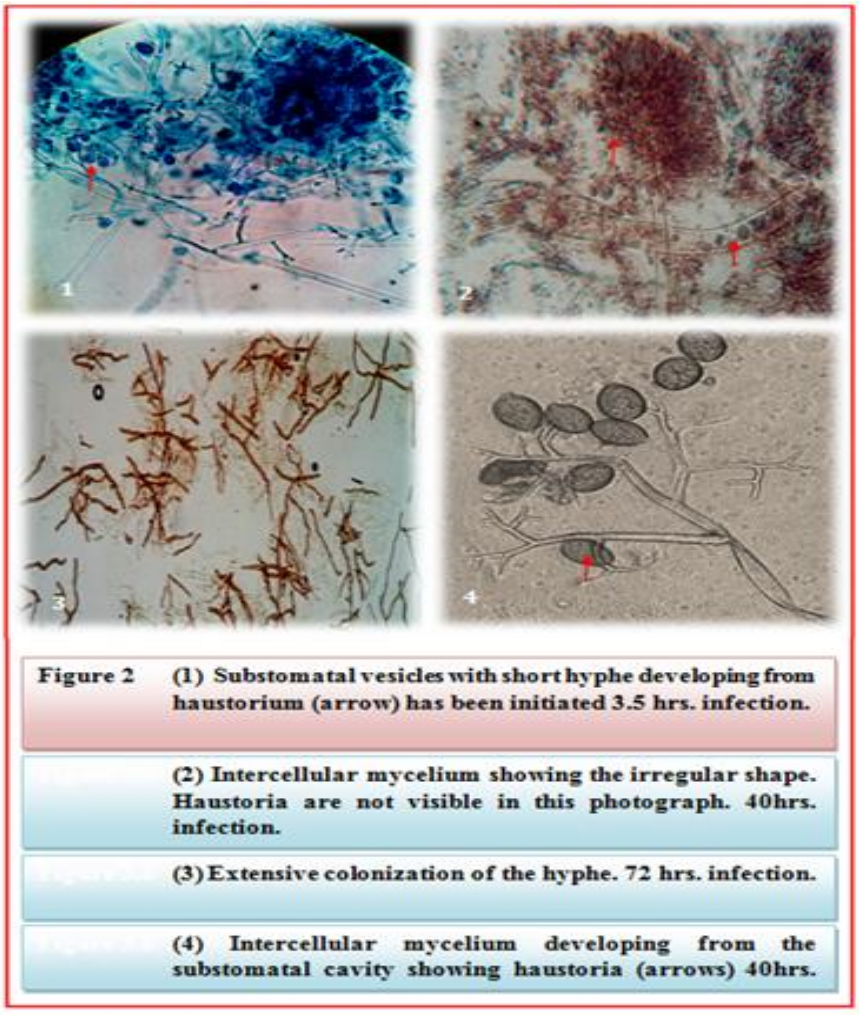


Figure 2 (1) Substomatal vesicles with short hyphe developing from haustorium (arrow) has been initiated 3.5 hrs. infection.

(2) Intercellular mycelium showing the irregular shape. Haustoria are not visible in this photograph. 40hrs. infection.

(3) Extensive colonization of the hyphe. 72 hrs. infection.

(4) Intercellular mycelium developing from the substomatal cavity showing haustoria (arrows) 40hrs.

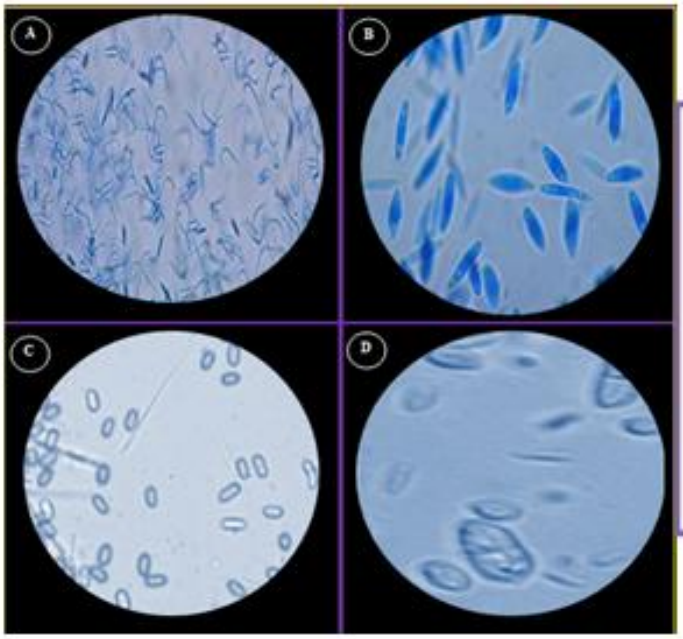


Figure 3

A. Microscopy of *P. viticola* (10x) isolated from grape stem.

B. Hyaline, ellipsoidal and biguttulate alpha -conidia. Scale bar = 10 µm

C. Conidia of *P. viticola* 7.5 µm.

D. Conidia of *P. viticola* 6µm.

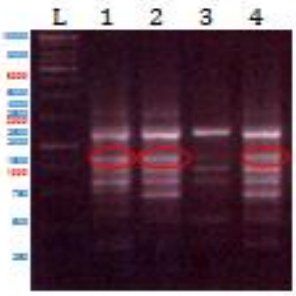


Figure 4 The RAPD marker OPA-18 produced reproducible band of 1500bp (Reference: Luo Su-Lan, *et al.*, 2001)
L= Ladder, Lane 1, 2 & 4 shows [repeated samples] RPv-1 (Resistance to *Plasmopara viticola* of grapevine; Lane 3 shows RPv-1 (Suceptible to *Plasmopara viticola* of grapevine)

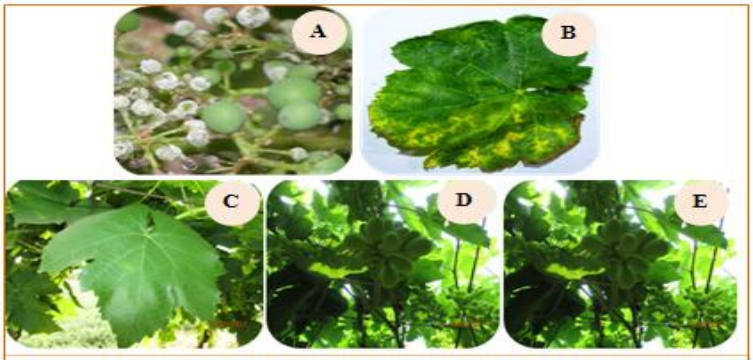


Plate 1 Downey mildew / *Plasmopara viticola* sample collection from grape gardens of Marathwada region
Sample No. A to B DM Susceptible grape plants/samples
Sample No. D to F DM Resistant grape plants

Plate 2 Direct plating of fungal leaves on nutrient media

Table.1 Lactophenol cotton blue stain composition

Cotton blue	0.05gm
Phenol crystals	20gm
Glycerol	40ml
Lactic acid	20ml
Distilled water	20ml

Table.2 Preparation of stock solutions for DNA extraction

Solution	Method of preparation
50mM Tris HCl (pH 8.0) 100 ml	788mg Tris HCl was dissolved in 80ml distilled water. The pH was adjusted to 8.0 by adding concentrated HCl. A total volume was adjusted to 100ml and sterilized by autoclaving.
2% SDS	2gm SDS dissolved in 100ml distilled water
2.5M EDTA (pH 8.0) 100 ml	73.06g EDTA di Sodium salt was dissolved in 80 ml distilled water. The pH was adjusted to 8.0 by adding NaOH pellets. A total volume was adjusted to 100ml. It was dispensed to reagent bottle and sterilized by autoclaving.
5M NaCl 100 ml	29.22g NaCl was taken in to beaker; 50ml of distilled water was added and mixed well. When the salts get completely dissolved, the final volume was adjusted to 100ml. It was dispensed to reagent bottle and sterilized by autoclaving.
70% Ethanol 100 ml	70ml of ethanol was taken and 30ml of distilled water was added, mixed well and dispensed to reagent bottle and stored at 4^0C.
Phenol: Chloroform: Isoamyl alcohol (5:4:1),100ml	Phenol50ml, isoamyl 40ml of chloroform and 10ml of isoamyl alcohol were measured, mixed well and stored in reagent bottle at room temperature.
EtBr (10mg/ml) 1.0ml	10mg Ethidium Bromide was added to 1.0ml of distilled water and it was kept on magnetic stirrer to ensure that the dye has dissolved completely. It was dispensed into amber colored Eppendorf tube and stored at 4^0C.
1X TE buffer (pH 8.0)	1.0ml of Tris HCl (1M), 200µl of EDTA (0.5M) were taken and distilled water was added to adjust the final volume of 100ml, mixed thoroughly, autoclaved and stored at room temperature.
TAE buffer 50X (1 liter) pH 8.0	242g of Tris base, 100ml 0.5M EDTA (pH 8.0) and 57.1ml Glacial acetic acid were taken, and the final volume of 1 liter was adjusted by adding distilled water and the pH was adjusted to 8.0.

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