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Original Research Article

Isolation and Identification of *Uncinula necator* Associated with Grapevine from Marathwada Region

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Keywords

Uncinula necator, 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 jellys, 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 *Uncinula necator* 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 *Uncinula necator*.

Introduction

According to the Food and Agriculture Organization (FAO), 75,866 square kilometers of the world are dedicated to grapes. Approximately 71% of world grape production is used for wine, 27% as fresh fruit, and 2% as dried fruit. A portion of grape production goes to producing grape juice to be reconstituted for fruits canned "with no added sugar" and "100% natural". The area dedicated to vineyards is increasing by about 2% per year.

Many varieties of grapes are susceptible to fungal diseases such as downy mildew, powdery mildew, anthracnose etc. these diseases have effects on grape production on worldwide. There are many fungal, bacterial, viral etc. diseases affect grape vine. Out of these fungal diseases adversely affect the production of grapes in large quantity. Due to the powdery mildew infection on all green tissue infected clusters drop blossoms before the fruit sets and

reduces fruit quality. Mainly early season control reduces the total number of fungicide applications. Susceptible varieties often require late-season sprays. Optimum temperatures for disease development are 68-77°. If not disease controlled, powdery mildew reduces vine growth, yield, and winter hardiness. 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. Several approaches have been taken i.e. introducing genes from other plants or bacteria encoding enzymes like chitinase or glucanase (Kortecamp, 2006). A genetically controlling fungal infection-protein 1 (VvLTP1) and 1420bp has been introgressed into *V. vinifera* using a pseudo-backross strategy, and genetic markers have previously been identified that are linker o the resistance locus. Laquitaine *et al.*, (2006) enhanced protection against Botrytis cinerea; and introducing (Kittkert, *et al.*, 2000) plant genes to enhance innate plant defence mechanisms (e.g. achieving phytoalexins, proteinase inhibitors, or toxic proteins); invoking the hypersensitive reaction (Cohen, *et al.*, 1999). Plants varieties that are naturally resistant to specific types of fungal diseases are often programmed to have individual cells quickly die at the site of fungal infection. Even with such research having begun many years ago, no commercial cultivars of vine are available today that use these approaches.

The aim of this work was to determine the presence of pathogenic fungi causing trunk diseases in these plants in order to decide the sanitation procedures to apply to the plant material, if needed. This is of great importance because these plants are proposed for the production of certified clones of grapevine. Keeping all these aspects in view, the present study were undertaken to develop isolation method for *Uncinula necator* from grape leaves and to identify the isolated *Uncinula necator* by microscopy and molecular studies examination.

The experiments described in this chapter were conducted during 2016-17. The materials and methods used in the present investigations are broadly described the following heads.

Materials and Methods

On the basis of symptoms and signs the *Uncinula necator* resistance and susceptible leaves were collected form "Ausa and Osmanabad Road Grape Garden", Dist-Latur (Plate 1).

Material collection

Uncinula necator 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 *Uncinula necator*.

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.

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.

Media preparation

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.

Streaking for isolation by the quadrant method

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).

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.

Staining of fungus from culture

Take a grease free slide.

Add a lactophenol cotton blue solution on slide.

Sterilize the inoculation loop or needle and cool it then transfer mycelial growth onto the LCB strain and press it gently so that it can easily mix with the stain.

Take a clean cover slip and with the help of a forceps place the cover slip on mycelial growth + LCB

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

Observe the preparation under low and high power objectives of microscope.

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).

Fungal genomic DNA extraction

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

DNA isolation protocol

DNA was isolated by using Maniatis *et al.*, (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:

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

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 *Uncinula necator* of grapevine leaves. RAPD primers were used to screen the genomic DNA of *Uncinula necator* of grape leaves.

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 *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.

To check out for potential co-segregation of DNA fragments and *Uncinula necator* resistant phenotypes.

Results and Discussion

Development of isolation method for *Uncinula necator* from grape leaves

The diseased samples were washed thoroughly under tap water and allowed to dry in shade under laboratory conditions. The infected portion along with some healthy part was cut into small pieces and they were surface sterilized with 1:1000 mercuric chloride solution for one minute.

The excess traces of mercuric chloride on the surface of the leaf and shoot bits were removed by washing 2-3 times in sterile distilled water and such bits were then transferred aseptically to Petri dishes containing Potato Dextrose Agar (PDA) medium. The inoculated Petri dishes were then incubated at 28 + 10C and growth of fungus was observed periodically. The pure colonies that developed from these infected leaf/stem bits were then transferred onto the PDA slants aseptically.

The same day as the lab presentations on downy mildew of grapevine, we were set up moist chambers from which we were isolating downy mildew (Stanley, 1992).

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.

Procedure of petri dish 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 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).

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).

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).

Streaking for isolation by the quadrant method

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).

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.

Two perpendicular lines with a marker on bottom of the plate were made to divide the circle into 4 quadrants.

Holding an inoculating loop between thumb and index finger, the wire portion was inserted into the Bunsen burner flame, heating the entire length of the wire until it is red and glowing and allowed to cool before doing the next step.

Using free hand, the tube containing the mixed culture was picked up and gently shacked it to disperse the culture.

The tube cap or plug was removed with free fingers of the hand holding the sterile inoculating loop and carefully flamed the lip of the tube in the Bunsen burner flame.

The tube was tilted to bring the broth culture within 1 inch from the lip of the tube. The sterile loop was inserted and a small amount of growth was removed; a loopful is usually sufficient. Try not to touch the sides of the tube with the loop.

The tube lip was flamed again, carefully the tube cap or plug was replaced, and the culture tube was returned to the test tube rack.

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

The mixed culture was applied on the loop onto the first quadrant by sweeping the area of this quadrant. The specimen was spread 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.

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.

DNA isolation protocol

DNA was isolated by using 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) 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 hr 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.

Gel electrophoresis by using RAPD primers

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. To check out for potential co-segregation of DNA fragments and *Uncinula necator* resistant phenotypes.

To confirm that the specific RAPD product originated from the *Uncinula necator* Resistant region and select a molecular marker to this region. DNA three four

grapevine leaf samples was used for amplification with primer OPF-18 and OPF-19. The specific band of both primers was also found in *Uncinula necator* (Figure 3). Therefore, DNA samples of lane number 1, 2 and 3 considered as *Uncinula necator* isolated from grape.

The present investigation on standardization of method for isolation of *Uncinula necator* from grape leaves and its identification on the basis of morphological and molecular marker revealed some conclusions viz., the use established methods are more time consuming and laborious so this method provides easy and rapid isolation of *Uncinula necator* from grape fruit. These isolated *Uncinula necator* and the OPF-18 and OPF-19 will be utilized for development of SCAR markers through PCR techniques. This study will be more reliable, easy, and specific for SCAR marker of *Uncinula necator* resistant genes from grape leaves will more useful in early screening than the other pathogens resistance with different physiological forms. It also provides a good platform for finding out control measure on this disease.

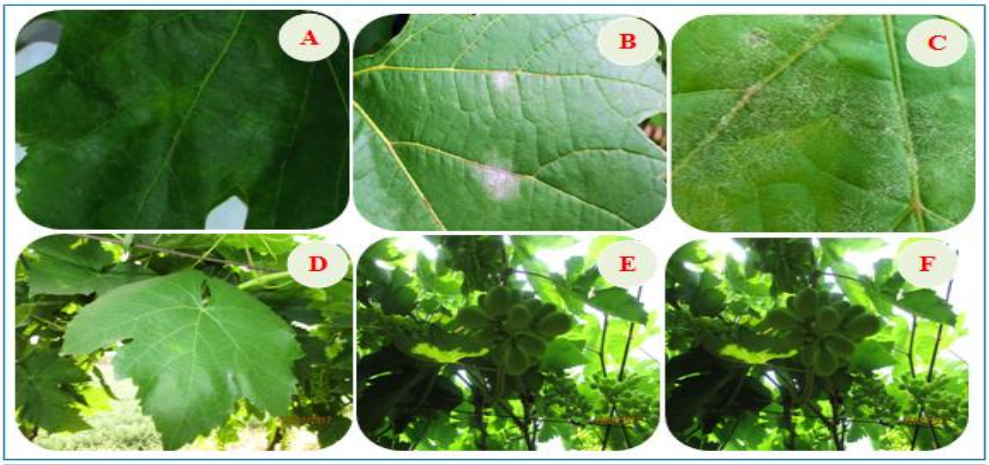

Plate 1 Powdery mildew / *Uncinula necator* sample collection from grape gardens of Marathwada region
Sample No. A to C PM Susceptible grape plants
Sample No. D to F PM Resistant grape plants

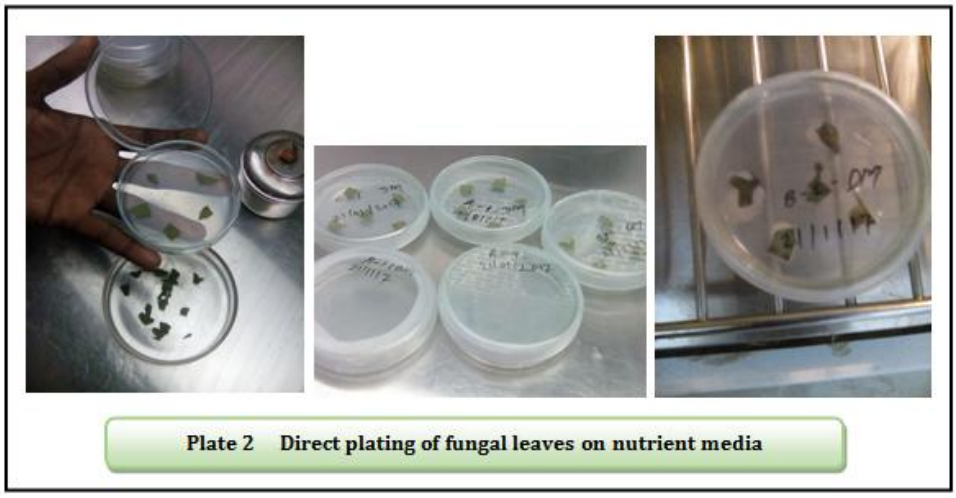

Plate 2 Direct plating of fungal leaves on nutrient media

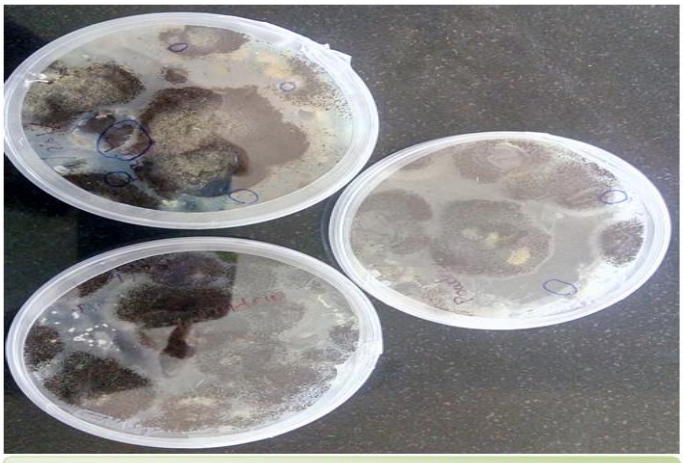

Figure 1 Pure culture development by direct method in a petri plate

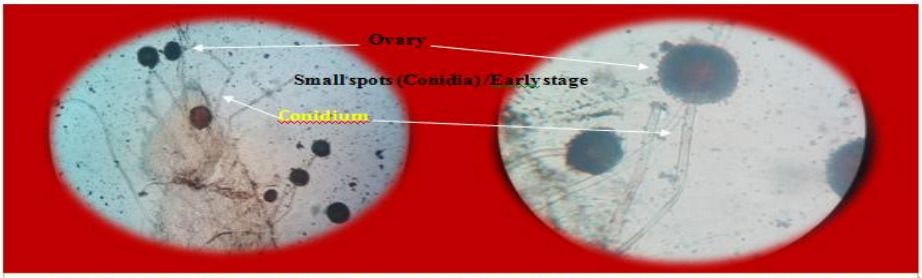

Figure 2 Microscopic identification of early stage of *Uncinula necator* collected from Marathwada grape gardens

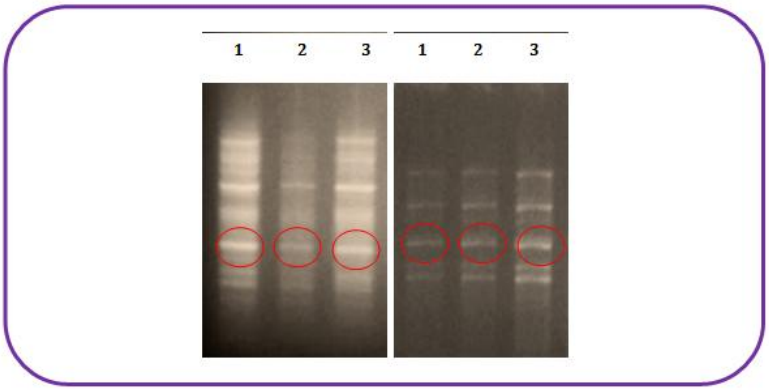

Figure 3 The RAPD marker OPF-18 and OPF-19 produced reproducible banding pattern for *Uncinula necator* isolated from grape.

Table.1 Media composition for isolation of *Uncinula necator*

Chemicals	Weight
Potato infusion	200gm
Dextrose	20gm
Agar	20gm
Distilled water	1000ml

Table.2 Lactophenol cotton blue stain composition

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

Table.3 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 disodium 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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