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Original Research Article

Isolation and Identification of *Elsinoe ampelina* Associated with Grapevine from Marathwada Region

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A B S T R A C T

The anthracnose of grapes is caused by an ascomycete, *Elsinoe ampelina* (de Bary) Shear. De Bary (1874) described the pathogen as *Sphaceloma ampelinum*, the imperfect state and this anamorphic name was used for long time. Recent reports throw light on the fact that *Colletotrichum gloeosporioides* (Stonem) Spauld and Schrenk (*Colletotrichum* state of *Glomerella cingulata)* is also involved in causing anthracnose of grapes during rainy season/ that occurs only late in the season. Hence, there is a need to establish this fact. The present investigation on standardization of method for isolation of *Elsinoe ampelina* from grape leaves and its identification on the basis of morphological and molecular marker revealed the use established methods are more time consuming and laborious so this method provides easy and rapid isolation of *Elsinoe ampelina* from grape fruit. These isolated *Elsinoe ampelina* and the OPF-13 and OPF-09 and more different primers will be utilized for development of SCAR markers through PCR techniques. This study will be more reliable, easy, and specific for SCAR marker of *Elsinoe ampelina* resistant genes from grape leaves will more useful in early screening than the other pathogens resistance with different physiological forms.

Keywords

Vitis vinifera, *Elsinoe ampelina*, OPF-09, OPF-13, Marathwada

Introduction

Grape (*Vitis vinifera* L.) is among the oldest plants (90-95 million years) on earth, existing almost at the time when dinosaurs flourished as evidenced by the recent discoveries from western Kazakhaton (Shanmugavelu, 2003). Grape is the most important temperate fruit crop that has acclimatized to the subtropical and tropical agro-climatic conditions. It is a fairly good source of minerals like calcium, phosphorus, iron and vitamins such as B1 and B2. Grape is known for its cultural dualism between subsistence-oriented growers and export oriented large corporate growers in India. It has become the most remunerative commercial farming enterprise and as such, India exports a large quantity of fresh grapes valued at Rs.110 crores annually to different countries around the world (Anon., 2004b).

At present the total area under grapes in India is estimated to be 57,800 ha with an annual production of 1.47 million tonnes (Chandha, 2006). Among the different

states, Maharashtra, Karnataka, Andhra Pradesh, Tamil Nadu, Uttar Pradesh, Punjab and Haryana are the major producers of grapes of different varieties. Maharashtra stands first occupying the largest area of 41,400 ha under grape cultivation accounting for about 71.5 percent of total area and 80 per cent of the production of grapes in the country (Anon., 2006). The varieties such as, Thompson Seedless and its mutants like Sonaka, Manik Chaman, Tas-E-Ganesh and Sharad Seedless are predominantly grown in the northern parts of Maharashtra contributing 60 per cent of the states grape production. The losses due to insect pests and diseases are high and for their management many sprays of several pesticides are required which accounts to 30 percent of the total cost of production. Among the diseases occurring on grapes, fungal diseases are the most destructive followed by a few bacterial, viral and nematode infections of minor importance. The incidence of diseases depend not only on the presence of the pathogen but also on the vineyard management practices and environmental factors like temperature, rainfall, and humidity which has an important bearing on the epidemics of any disease.

Anthracnose of grapes popularly referred to as "Bird's eye spot" is one of the most important diseases of grapes after downy mildew and powdery mildew. The disease was first referred by Pliny in Italy in the 1st century of Christian era and later reported by Burrill during 1886 from Illinois, U.S.A. It was observed that the disease was present in Europe for centuries and was introduced into the United States through cuttings/young vines from European grapes. In India, the disease was first recorded in 1903 near Pune (Butler, 1905). The disease is widely prevalent in Maharashtra, Karnataka, Punjab, Haryana, Andhra Pradesh, Uttar Pradesh and Tamil Nadu. Extremely high losses due to anthracnose of grapes have been reported in South Africa and South America. In Chile during 1950-51, the crop was lost on several hundred acres of susceptible varieties covering 90 percent area and causing 83 to 100 per cent loss (Anderson, 1956). In India, anthracnose has become a potential threat to grape cultivation. In north India, it appears every year and reduces the quality and quantity of the crop apart from making vines weak (Thind *et al.*, 1992), where the disease is most damaging being widely prevalent from July to October. In south India (mainly Maharashtra and Karnataka), the disease prevails from June to October with peak damage during May-July. The ruling variety in Karnataka, Thompson Seedless suffers huge losses due to this disease resulting in drastic reduction in productivity if left unchecked or proper control measures are not adopted. The annual loss due to anthracnose of grapes is estimated to be 15-30 per cent (Anon., 2006). Kore and Gurme (1978) studied the growth and sporulation of *S. ampelinum* on various solid and liquid media and found out that superior growth and sporulation was observed on potato dextrose agar in solid media and Richards' medium supported the maximum mycelial growth in liquid media. Hence, it is needed to know the behavior of different isolates collected from different regions during survey on solid and liquid media of synthetic, semi- and non-synthetic origin.

The present investigation aims to find out such a phenomenon among the isolates isolated form Marathwada areas. There is plenty information on source of survival of the anthracnose of grape pathogen. Keeping all these aspects in view, the present studies were undertaken with identification and development of isolation method for *Elsinoe ampelina* from grape leaves.

Materials and Methods

Materials

On the basis of symptoms and signs the *Elsinoe ampelina* resistance and susceptible leaves were collected from different locations of Marathwada region.

Material collection

Elsinoe ampelina infected leaves were collected from different locations of Marathwada region by using moist chambers. The same day as the lab presentations on infected leaves of grapevine, were set up moist chambers from which *Elsinoe ampelina* was isolated. 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).

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

The glassware's were sterilized by wrapping by wrapped in an aluminum 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, Autoclave it for 15 min at 121°C, 20-25 ml of media dispensed into sterile 15 × 100 mm petri dishes. For the development fungal culture strains PDA i.e. Potato Dextrose Agar Medium is used.

Isolation and pure culture development

The infected leaf samples were cut into 3mm pieces with sterile scalpel 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 or need 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).

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. After attending all the precautions pick up the culture on media.

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. Flame the loop and allow it to in an uninoculated area of the medium. NOT to wave it in the air to cool. Now streak the inoculum from quadrant 1 into quadrant 2. Use smooth, nonoverlapping strokes. Utilize the entire quadrant 2 as shown in the figure below. Flame the loop when done. Let the loop cool.

Now streak the inoculum from quadrant 2 into quadrant 3 by smooth, non-overlooking strokes again. Flame your loop one more time and let it cool. Now bring some inoculum from quadrant 3 into quadrant 4 in the same manner as for other previous quadrants. Flame your loop and cool. Invert the plates and incubate plate at 30°C-37°C.

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.

Lactophenol cotton blue technique for *Elsinoe ampelina* staining by Natalie (2001) method

Fungal structure includes sporangiospores, mycelium, spores etc. The lactophenol cotton blue wet mount is simply and widely used method for staining of fungus. 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 *Elsinoe ampelina* 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.

Molecular identification of isolated *Elsinoe ampelina* from grapevine leaf samples

Elsinoe ampelina genomic DNA extraction

Preparation of stock solutions for DNA extraction by using Dr. Shunxue, J. K. lab and electrophoresis. According to Mania *et al.*, (1982).

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.

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 and agarose gel electrophoresis

DNA samples of working concentration of 25ng/µl and stored at 4^0C until PCR amplification. 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 *Elsinoe ampelina* 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 unit was cleaned properly before use. Agarose gel (1.5%) was prepared by dissolving 1.5% agarose gel.

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 against *Elsinoe ampelina*.

Results and Discussion

In this experiment the development of isolation method for *Elsinoe ampelina* from grape leaves and identified the isolated *Elsinoe ampelina* by morphology and molecular marker. The results were presented as follows:

Development of isolation method for *Elsinoe ampelina* from grape leaves

On the basis of symptoms and signs the *Elsinoe ampelina* infected leaves were collected from different locations of Marathwada region. (Plate 1).

Methodology

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.

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.

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 appears on the surface, and can be transferred into pure culture (Plate 2a).

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 (Plate 2b).

Morphological characterization of isolated sample of *Elsinoe ampelina* 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. 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. 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 (Plate 3 & 4).

Plate 1 a Infected grape leaf with *Elsinoe ampelina*

Plate 2a. Direct plating of fungal leaves on nutrient media

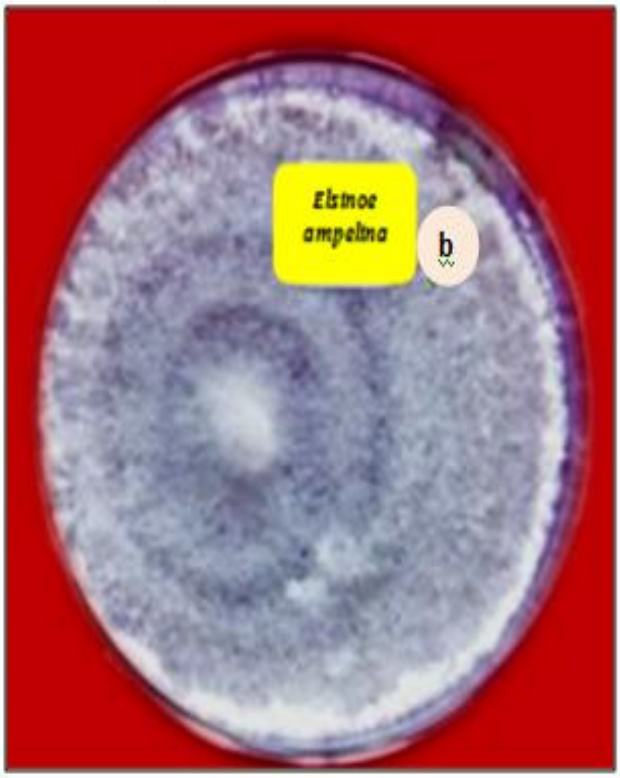


Plate 2b. Pure Culture of *Elsinoe ampelina* Isolated from infected grape leaves

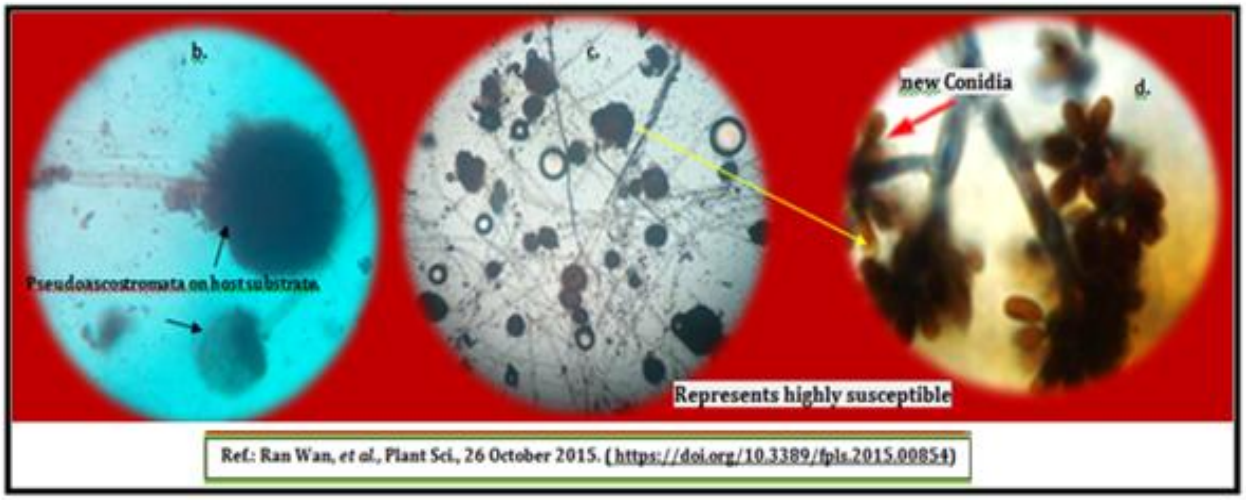
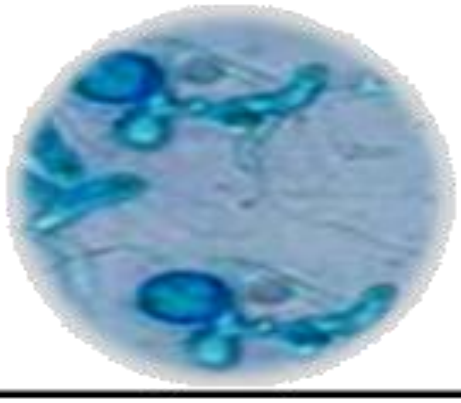


Plate 3. Morphological characterization of isolated sample of *Elsinoe ampelina* from grapevine leaf samples

Plate 4 ***Elsinoe*** **spores observed under x100**

Plate 5 ***Elsinoe*** **spore germination observed under x100**



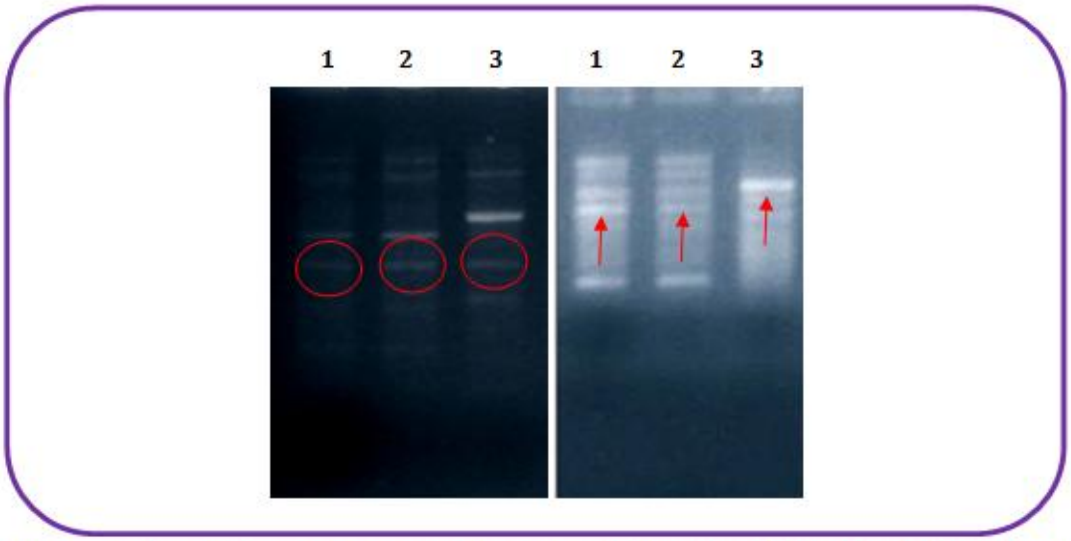


Figure 1 **RAPD marker OPF-13 and OPF-09 produced reproducible banding pattern for** ***Elsinoe ampelina*** **isolated from grape.**

Fungal genomic DNA extraction

Preparation of stock solutions for DNA extraction from *Elsinoe ampelina* was done 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

Gel electrophoresis by using RAPD primers

10bp oligonucleotide primers (Operon kit) were tested. DNA samples isolated from *Elsinoe ampelina* 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 *Elsinoe ampelina* resistant phenotypes.

To confirm that the specific RAPD product originated from the *Elsinoe ampelina* resistant region and select a molecular marker to this region. DNA three four grapevine leaf samples was used for amplification with primer OPF-13 and OPF-09. The specific band of both primers was also found in *Elsinoe ampelina* (Figure 1). Therefore, DNA samples of lane number 1, 2 and 3 considered as *Elsinoe ampelina* isolated from grape.

In this present study, developed the method for isolation of *Elsinoe ampelina* from grape leaves, identified the isolated *Elsinoe ampelina* by morphological level, and in case of molecular marker, To confirm that the specific RAPD product originated from

the *Elsinoe ampelina* Resistant region and select a molecular marker to this region. DNA three four grapevine leaf samples was used for amplification with primer OPF-13 and OPF-09. The specific band of both primers was also found in *Elsinoe ampelina*. Therefore, DNA samples of lane number 1, 2 and 3 considered as *Elsinoe ampelina* isolated from grape.

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