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Determination of morphometric, biochemical and genetic variation in *Sclerotium delphinii* isolates

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Variability among four isolates of *Sclerotium delphinii* isolated from infected diseased Khirni (*Manilkara hexandra*); chafa (*Plumeria rubra acutifolia*) and Jackfruit (*Artocapus heterophyllus*) plants of Western Maharashtra region of India were studied. This is the first report on presence of *S. delphinii* species in India. These isolates varied in colony morphology, mycelial growth rate, sclerotia formation period, sclerotia size and color. On the basis of morphological characters, these isolates were identified as *S. delphinii* and were also confirmed in Indian type culture collection (ITCC), New Delhi. In mycelial compatibility study, isolates of *S. delphinii* did not intermingle with *Sclerotium rolfsii* isolates indicating genetic variation among the species. The isolates of *S. delphinii* also showed distinct differences in their oxalic acid content. The classification and variability was further confirmed using molecular studies. It was observed that only morphological features and MCGs differentiated the *Sclerotium* species rather than molecular characterization because they shared some common bands of DNA at genetic level, but showed distinctness in morphological and MCGs study.

Key words: Variation, cultural; biochemical, *Sclerotium delphinii*, RAPD-PCR.

INTRODUCTION

Sclerotium is a devastating soil borne plant pathogenic fungus which causes diseases in over 500 plant species (Punja, 1985; Harlton et al., 1995; Cilliers et al., 2000; Okabe and Matsumoto, 2000; Okabe et al., 1995) and is prevalent in warm, temperate and subtropical regions of the world. Symptoms of the fungus include crown and root rot, stem rot, stem canker and damping off (Aycok, 1966; Punja, 1995).

The Genus sclerotium includes three species *viz*., *Sclerotium rolfsii, Sclerotium delphinii and Sclerotium coffeicola* which are differentiated from each other on the basis of morphological features (Punja and Damiani,

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1996). *S. delphinii* produces larger and irregular shape sclerotia (Harlton et al., 1995; Okabe et al., 2000) lighter brown to orange in colour (Stevens 1931; Harlton et al., 1995; Punja and Damiani,1996). *S. delphinii* is reported to occur on ornamental and bulb plants. It is not clear whether these differences in host occurrence and morphology of sclerotia are sufficient to warrant a separate species of *S. delphinii* from *S. rolfsii* (Harlton et al., 1995; Punja and Damiani,1996)

Cultures of *S. rolfsii* originating from different geographical areas and host frequently displayed variation in morphological characteristics (Punja and Gregon, 1983). However, research work has not been done to study the variation among the *S. delphinii* isolates from different host in India. Therefore, detailed investigation on variation among the *S. delphinii* isolates from different host with regards to morphological, biochemical, genetical and molecular characters were carried out to differentiate them.

MATERIALS AND METHODS

Collection of different isolates of *S. delphinii*

Hard woody plants like khirni (*Manilkara hexandra*), jackfruit (*Artocapus heterophyllus*) and chafa (*Plumeria rubra acutifolia*) showing symptoms of collar rot/root rot in Western Maharashtra region of India were subjected to isolation of sclerotia on potato dextrose agar (PDA) medium.

Four cultures resembling *S. delphinii* were obtained from these isolations. Mycelial growth obtained was purified, sub cultured and maintained on potato dextrose agar in the form of mycelial and sclerotial bodies. These mycelia were used for morphological, pathological, biochemical and molecular studies (Punja and Sun, 2001).

Identification of *S. delphinii* isolates

The isolated fungal cultures were identified as *S. delphinii* on the basis of their morphological and sclerotia characters and were further confirmed from Indian Type Culture collection (ITCC), New Delhi, with allocated ITCC numbers 7168.68/6426 for khirni, 7167.08/6425 for chafa and 7166.08/6424 for jackfruit.

Morphological variation

Growth characteristics of *Sclerotium* isolates of khirni, jackfruit and chafa were studied on PDA medium. Circular discs (5 mm diameter) of an actively growing fungal colony (3-4 days old) of these isolates were taken out with the help of cork borer and was placed in the centre on PDA medium in the plate with the mycelial side facing downwards under aseptic conditions. The plates were incubated at 28°C for seven days.

Morphological characteristics of the fungus like growth rate, type of colony, growth pattern, radial colony diameter, development of sclerotia and its number, weight and diameter of sclerotial bodies, days required to form the sclerotia, color, type and location of sclerotial bodies for these isolates were recorded up to seven days at 24 h intervals.

Biochemical variation

Oxalic acid estimation

Richard's broth (KNO_3 10 g; KH_2PO_4 5 g; $MgSO_4.7H_2O$ 2.5 g; sucrose 35 g; distilled water 1000 ml) was used for estimation and quantification of oxalic acid. Fifty milliliter Richard's broth (in 250ml flasks) was inoculated with two 5 mm^2 mycelial disc of actively growing colony of *S. delphinii* isolates and was incubated for 14 and 21 days at 28 ± 1°C. All experiments were performed in triplicates. After desired incubation, cell free cultural filtrate was obtained through Whatman filter paper No.1 and subsequently through sortorious filter paper. The filtrate was centrifuged at 10000 rpm for 10 min. The supernatant of each cultural filtrate (10 ml) was used for the quantitative determination of oxalic acid as per the method suggested by Mahadevan and Sridhar (1986). Biomass of each isolate was also measured.

Mycelial compatibility/incompatibility among the isolates

Four isolates of *S. delphinii* and four isolates of *S. rolfsii* were paired against each other on PDA medium to determine mycelial compatibility among them. For this mycelial bits taken from the edge of an actively growing colony (three to four days old) of each isolate was placed approximately 2.5 to 3.5 cm apart on opposite sides of each other on PDA medium in 100×15 mm Petri plates and incubated at 28 ± 2°C.

Three isolates were usually paired on one dish and pairing test was repeated thrice. The pairing was examined under stereo microscope after seven days and up to 15 days for the presence of an antagonistic (barrage or aversion) zone in the region of mycelial contact (Powell, 1995).

Molecular characterization

Procedure for extraction and purification of DNA of fungus S. rolfsii

Genetic similarities and difference among isolates of *S. delphinii* were assessed through randomly amplified polymorphic DNA (RAPD) analysis as described by Punja and Sun (2001) and was also compared with S. *rolfsii* isolates. DNA from each isolate under observation was extracted from the mycelial mat of each isolate grown on PDA plate for seven days at 28°C.

For this, aerial mycelia were collected by scraping it from the agar surface with sterile scalpel blade and the mycelial mat was suspended in sterile distilled water for washing. These mats were further taken out and placed in aluminum foil for drying at 60°C in hot air oven for half an hour.

After drying, the mycelial mat were collected in another aluminum foil and kept in freeze at 4°C. Dried fungal mat (20 mg) grounded to fine powder under liquid nitrogen was used for isolation of DNA with DNeasy mini kit (Qiagene).

Primer selection and DNA amplification

The decamer primers (OPERON) Set B, D and E were initially screened to detect polymorphism among seventeen isolates of *Sclerotium* spp. The primers which indicated the higher degree of polymorphism were selected for the diversity analysis studies. DNA sequences (5"-3") of all primer used for DNA amplification are mentioned in Table 1.

Table 1. RAPD primers and their sequences.

Primer name	Primer sequences (5'- 3')	No. of polymorphic bands	No. of monomorphic bands	Polymorphism (%)
OPB - 11	GTAGACCCGT	6	01	85.71
OPB - 12	CCTTGACGCA	10	-	100
OPB - 16	TTTGCCCGGA	17	-	100
OPB - 20	ACTTCGCCAC	13	-	100
OPD - 7	TTGGCACGGG	09	-	81.82
OPD - 13	AAGCCTCGTC	11	-	100
OPD - 16	AGGGCGTAAG	14	-	100
OPD - 20	ACCCGGTCAC	14	01	93.33
OPE - 12	TTATGGCCCC	04	01	80.00
OPE - 16	GGTGACTGTG	16	-	100
OPE – 20	AACGGTGACC	09	01	90.00

DNA amplification

The RAPD-PCR reaction was carried out in 20 µL volume. The PCR reaction mixture contained 10X Taq DNA polymerase Buffer (GeNei, Banglore), 2 µL dNTP mix (10mM dNTP GeNei, Banglore), 1 µL of respective primers, Taq DNA Polymerase 0.5 µL (5 units μL^{-1} GeNei, Banglore) and 1 µL template DNA (100 ng μL^{-1}). 13.5 µL deionized water was used to make the total reaction volume up to 20 µL. PCR tubes were placed in Thermocycler (Eppendrorp, Germany) for DNA amplification using the following PCR programme: (Cycle, 1 Initial denaturation at 94°C for 5 min, Cycle-2, 45 denaturation at 94°C for 1 min; Primer annealing at 35°C for 1 min, Primer extension at 72°C for 2 min and final extension at 72°C for 10 min).

On completion of PCR amplification, the tubes were removed from the Thermocycler. 1 µg of 100 bp gene ruler DNA (Fermentas) ladder was used as a marker. After adding 4 µL of loading buffer dye (0.1% bromophenol blue, 0.5% xylene cyanol FF 30% Glycerol), the 20 µL of RAPD Product was loaded on 1.2% agarrose gel using Tris acetate EDTA (TAE) buffer (pH -8). Electrophoresis was performed for 3 h at 80 V in an submarine electrophoresis apparatus (Bio Rad No.96). The gel was stained with ethidium bromide (0.1%) and photographed under UV illumination. The fungal isolates were compared with each other by using their RAPD-PCR profiles and bands of DNA fragments were scored as (1) for present and (0) for absent for each of the primer used. The binary data were analyzed under the SIMQUAL module using DICE coefficient (Nei and Lei, 1979). A dendrogram based on the UPGMA clustering method (SAHN) (Sneith and Sokal, 1973) was constructed using NT SYS- PC software (Rohlf, 1998). Principal coordinate analysis (PCO) was performed to estimate the genetic distance between each group of the isolates using NT-SYS software. It involved first transforming similarity value to a scaler product by DCENTER, then analyzing the product matrix by EIGEN to get Eigenvector (which is PCO) and Eigen values and finally getting its 2D/3D scatter plot graph.

RESULTS

Four isolates of *S. delphinii viz*., Jackfruit, chafa, khirni-1 and khirni-2 varied greatly in their morphological characters (Table 2). The isolate of chafa and jackfruit produced filamentous mycelial growth whereas khirni-1 and khirni-2 isolate produced fluffy mycelial growth. Growth rate of khirni-2 and chafa isolate was much faster (30 and 29 mm/day respectively) than khirni-1 (28mm/day) and isolate of jackfruit was slowest in rate (23 mm/day). All isolates produced globose to irregular shape light brown color sclerotia of medium to large size (1.9 to 3 mm) and this is the typical character of *S. delphinii.* However, the size of the sclerotial bodies varied among these isolates (Figure 1). The chafa isolate produced the sclerotia having highest diameter of 3 mm, whereas the rest of the isolates were in the range of 1.9 to 2.1 mm. In general, 9 to 11 days were required for formation of sclerotial bodies. The number of sclerotial bodies per plate was in the range of 60 to 104. Presence of basidial stage was detected in khirni isolate but not in jackfruit and chafa isolate.

Production of oxalic acid and fungal biomass (mycelial mat) varied significantly among four different isolates of *S. delphinii* (Table 3). Maximum oxalic acid (8.86 mg/g of mycelial mat) production was recorded in chafa isolate followed by khirni-1 (6.16 mg/g) and khirni-2 (5.54 mg/g) at 14 days after incubation, whereas isolate of jackfruit produced low oxalic acid (4.94 mg/g). However, in the mycelial compatibility reaction studies of four isolates of *S. delphinii* and *S. rolfsii* making 36 pairings for comparison on PDA medium (Table 4) revealed that all the four isolates of *S. delphinii* were different from *S. rolfsii* and this did not intermingle with *S. rolfsii* isolates. The *S. delphinii* isolates were only compatible with each other except Khirni- 1 isolate, which formed the separate mycelial compatibility group (Figure 2). Among four isolates, in two MCGs were detected three isolates *viz.,* Jackfruit, Chafa and Khirni-2 under the same MCG and Khirni-1 isolate formed the separate MCG. This indicates the extent of diversity amongst *S. delphinii* and *S. rolfsii* isolates.

Table 2. Morphological variation among the isolates of *S. delphinii*.

Sclerotium delphinii isolate of	Colony character	Mycelial growth (mm/day)	Sclerotial formation pattern	Sclerotial Diameter (mm)	Color of sclerotia	No. of days for sclerotia formation	Weight of 100 sclerotia	No. of sclerotia per plate	Basidial stage present/absent
Jackfruit	Filamentous	23	Pattern5	2.1	Light brown	9	0.07	90	_
Chafa	Filamentous	29	Pattern 5	3.0	Light brown	10	0.23	60	_
Khirni-1	Fluffy	28	Pattern 5	2.0	Light brown	11	0.09	96	+
Khirni-2	Fluffy	30	Pattern 4	1.9	Light brown	9	0.08	104	+

Pattern no. 1, Small size (1 to 1.5 mm), round sclerotia, spread all over the plate but more in number preferably at margin side; Pattern no. 2, Medium size (1.5 to 2 mm), globose sclerotia spread all over the plate. Pattern no. 3, medium size (1.5 to 2.5 mm), round sclerotia spread all over the plate. Pattern no. 4, Medium size (1.5 to2.0 mm), globose to irregular sclerotia spread all over the plate. Pattern no. 5, Large size (2 to 3 mm), globose to irregular sclerotia spread all over the plate.

Figure 1. *Sclerotium delphinii* isolates of: a) Chafa b) Jackfruit c) Khirni-1 d) Khirni-2.

To assess the genetic variability within the species, 13 isolates of *S. rolfsii* and four isolates of *S. delphinii* collected from different crop plants were analyzed. RAPD-PCR generated very distinct amplification products with considerable variability between the isolates of *Sclerotium* spp. In total, 129 fragments were generated from 11 RAPD primers (Table 1). The number of RAPD fragments produced per primer varied between 5 and 17 and ranged in size from 250 to 1358 bp. However, OPB 16 was found to be the most useful primer yielding polymorphic bands within and between fungal species (Figure 3). UPGMA cluster analysis of RAPD data separated the isolates of each particular species of *Sclerotium* into unique group based on genetic similarity coefficient. Though all isolates of *Sclerotium* spp. were genetically variable, they share some common bands indicating phylogenetic relationships among themselves. A dendrogram representing the genetic relationships among the isolates was developed and presented in Figures 4 and 5). On this basis the isolates of *Sclerotium* spp. that is, *S. rolfsii and S. delphinii* can be classified into six broad groups. Among These groups, group III consisted of *S. delphinii* isolates consisted again of two subgroup *viz.,* III_a (Khirni- 1 and Khirni- 2 isolates) and III_b (Jackfruit and Chafa isolates) subgroup consisted of four isolates each with 0.75 to 1.0 genetic similarity coefficient.

DISCUSSION

Variability among four Indian isolates of *S. delphinii* isolated from infected diseased Khirni (*Manilkara hexandra*), chafa (*Plumeria rubra*

Table 3. Production of oxalic acid by different isolates of *S. delphinii* at 14 and 21 DAI.

S. rolfsii isolate of	Mycelial mat (gm) produced at days		Amount ((mg/ml)of oxalic acid produced at days	
	14	21	14	21
Jackfruit	0.59	1.22	2.92 (4.94)	3.03 (2.48)
Chafa	0.23	0.25	2.04 (8.86)	2.19 (8.76)
Khirni-1	0.36	0.93	2.22 (6.16)	2.56 (2.75)
Khirni-2	0.37	0.35	2.05 (5.54)	2.24 (6.05)

Figures in parenthesis indicate mg of oxalic acid/g of mycelial mat of respective isolates

Table 4. Mycelial compatibility/incompatibility reaction among different isolates of *S. rolfsii* and *S. delphinii*.

Sclerotium isolate of	1	2	3	4	5	6	7	8
Chickpea	NI	A	NI	NI	A	A	A	A
Soybean		NI	A	A	A	A	A	A
Groundnut			NI	NI	A	A	A	A
Sugarbeet				NI	A	A	A	A
Khirni-1*					NI	A	A	A
Khirni-2*						NI	NI	NI
Jackfruit*							NI	NI
Chafa*								NI

NI = Normal intermingling, A = antagonistic reaction; *indicates *Sclerotium delphinii*.

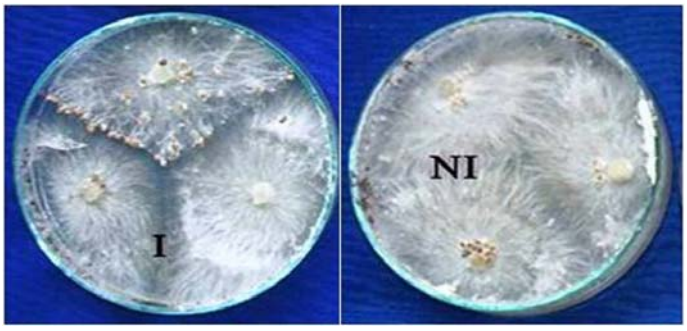

Figure 2. Mycelial compatibility/incompatibility among the isolates (Where I = Inhibition, NI = Normal intermingling).

acutifolia) and Jackfruit (*Artocapus heterophyllus*) plants of Western Maharashtra region of India were studied. These isolates varied in colony morphology, mycelial growth rate, sclerotia formation period, sclerotia size and color. On the basis of morphological characters, these isolates were identified as *S. delphinii.* Punja and Damiani (1996) reported that the sclerotia of *S. delphinii* isolates were lighter brown to orange in colour. Stevens (1931) reported 2.18 mm diameter of sclerotial bodies in *S. delphinii.* Adandonen (2000) reported variation in

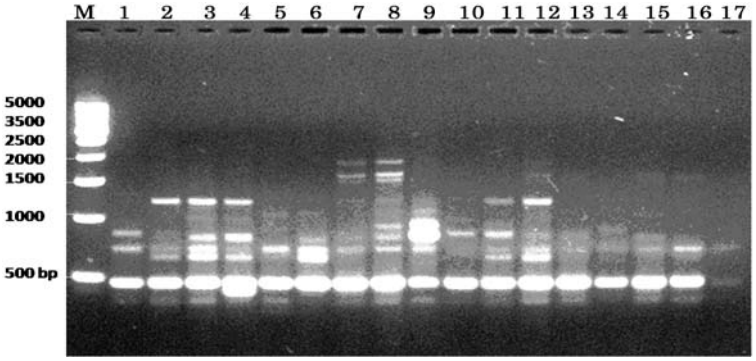

Figure 3. 1.2% agarose gel electrophorosis and amplification profiles of seventeen different isolates of *Sclerotium* species by OPB-16
(Where, M represent DNA (5000 bp) Ladder Lane 1: *Sclerotium* species isolates of Cotton, Lane 2: Sugar beet, Lane 3 :Groundnut, Lane 4: Chickpea, Lane 5: Isabgol, Lane 6: Tomato, Lane 7: Khirni -1*, Lane 8: Khirni-2*, Lane 9: Wheat-2, Lane 10: Wheat-1, Lane 11: Bottle gourd, Lane 12: Mango, Lane 13: Lily, Lane 14: Tuberose, Lane 15: Chafa*, Lane 16: Jackfruit*, Lane 17: Dahalia) *indicates isolate of *Sclerotium delphinii*.

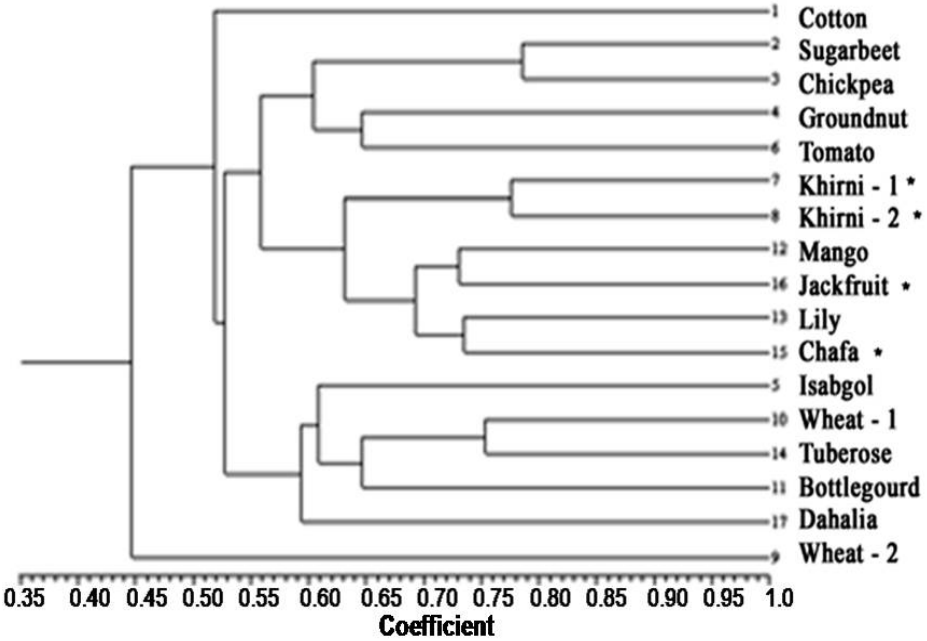

Figure 4. Dendrogram representing the genetic relationship among seventeen different isolates of *Sclerotium* sp.

growth rate, sclerotial numbers and time required for first appearance of sclerotia in isolates of *S. rolfsii* and *S. delphinii* collected from different villages in the Oueme valley. The present findings are in line with the morphological characters of *S. delphinii* species described by above workers and are also confirmed from Indian Type Culture Collection, (ITCC) New Delhi. This is the first report of presence of *S. delphinii* species in India.

Production of oxalic acid and fungal biomass (mycelial mat) varied significantly among four different isolates of *S. delphinii.* Ansari and Agnihotri (2000) characterized 44 isolates of *S. rolfsii* from soybean and classified them into four groups on the basis of quantity of oxalic acid produced. Sharma et al. (2002) recorded maximum production of oxalic acid after 14 days of incubation. Shukla and Pandey (2006) also observed maximum

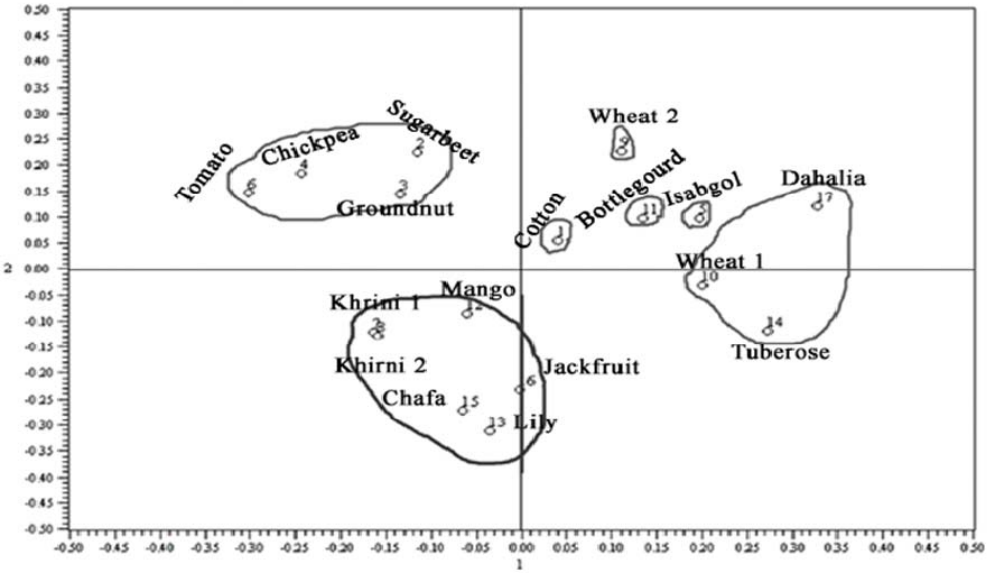

Figure 5. RAPD 2D PCO scatter plot representing the genetic relationship among seventeen different isolates of *Sclerotium* spp.

oxalic acid after incubation for fourteen days. Punja and Jenkins (1984) reported that isolates of *S. rolfsii* varied in oxalic acid production in the culture. Present investigations on oxalic acid production of S. *delphinii* are in agreement with variation in oxalic acid content of *S. rolfsii.*

In mycelial compatibility study, isolates of *S. delphinii* did not intermingle with *S. rolfsii* isolates inflicting genetic variation among *S. delphinii* and *S. rolfsii* species. In the present study, two MCGs were detected among four isolates of *S. delphinii.* The three isolates *viz.,* Jackfruit, Chafa and Khirni-2 is under the same MCG and Khirni-1 isolate formed the separate MCG and in corroboration with the study undertaken by Harlton et al. (1995) where paired 119 isolates of *S. rolfsii*, 11 of *S. delphinii* and 2 of *S. coffeicola* and recorded 49, 3 and 2 MCGs, respectively. Remesal et al. (2013), Twelve MCGs (i–xii) were identified among 459 *S. rolfsii* isolates. MCG iii was the most prevalent group in all countries except Italy. MCG i, the most abundant group (64Æ7% of isolates) was identified in Portugal and Spain. The remaining MCGs were restricted to various regions within one country (ii, vi and ix) or different countries (v), or to specific localities (iv, vii, viii, x, xi, xii).MCGs iv, vii and x each comprised one isolate.

The RAPD analysis was reported to be efficient for studying the phylogenetic relationships among the *Sclerotium* spp. RAPD-PCR analysis had also been used by other workers to study the genetic variability among isolates of *S. rolfsii* collected from different geographical regions and hosts. Punja and Sun (2001) as compared to 128 isolates of *S. rolfsii* from 36 host species and 283 geographic regions by means of RAPD-PCR which confirmed that many isolates from the same host belong to the same MCG. Almeida et al. (2001) studied the variation among 30 isolates of *S. rolfsii* from different hosts and region of Brazil by undertaking analysis of genomic DNA through RAPD-PCR technique (Le et al., 2012). Based on internal transcribed spacer (ITS) ribosomal DNA sequence analyses, three distinct groups were identified among a total of 103 randomly selected *S. rolfsii* field isolates, with the majority of the isolates (*n* = 90) in one ITS group. *S. rolfsii* isolates originating from groundnut, tomato and taro were all pathogenic on groundnut and relatively sensitive to the fungicide tebuconazole but displayed substantial diversity of various genetic and phenotypic traits, including mycelial compatibility, growth rate and sclerotial characteristics. This technique confirmed that the variability among the isolates of *Sclerotium* spp. in relation to the number, size, colour and location of sclerotia on the surface of the medium. It was observed that only morphological features and MCG's differentiated *Sclerotium* species rather than molecular characterization because they shared some common bands of DNA at genetic level, but showed distinctness in morphological and MCG's study. Thus, the data obtained under this experiment confirmed the efficiency and necessity of morphological, genetical and molecular (RAPD-PCR and ISSR) techniques for determination and estimation of genetic similarities and differences among the isolates of *S. rolfsii* and *S. delphinii* collected for the present study. The data obtained under this investigation confirmed the potential of RAPD-PCR technique for determination of genetic

similarity and differences among *Sclerotium* isolates collected for the present study. Therefore, RAPD analysis was found an efficient and informative DNA marker system to assess genetic relatedness and diversity among different isolates of *Sclerotium* spp. It was observed that only morphological features and MCG's differentiated the *Sclerotium* species rather than molecular characterization because they shared some common bands of DNA at genetic level, but showed distinctness in morphological and MCG's study. Thus, the data obtained under this experiment confirmed the efficiency and necessity of morphological, genetical and molecular (RAPD-PCR) techniques for determination and evaluation of genetic similarities and differences among the isolates of *S. rolfsii* and *S. delphinii*.

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