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Full Length Research Paper

Diversity analysis of *Rhizoctonia solani* causing sheath blight of rice in India

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Sheath blight (*Rhizoctonia solani*) causes severe loss in the production of rice in India. Twenty five (25) isolates were collected from different parts of India. Morphological and cultural characters were investigated, on the basis of colony colour, growth pattern, hyphal width, colony growth, formation of sclerotia, size of sclerotia, diameter and number of sclerotia. Pathogenic variability was studied on highly susceptible cultivar Pusa Basmati-1 and isolates were classified into two major groups that is, highly virulent and virulent. Genetic variability of *R. solani* was also analyzed using 10 RAPD markers and on the basis of Jaccard's **similarity coefficient, 4 major clusters were formed. The range of genetic similarity varied from 17 - 77%. Maximum similarity (77%) was found between two isolates (RS-14 and RS-15) both from Kerala whereas, lowest similarity (17%) was observed between RS-22 (Delhi) and RS-4 (Punjab). Isolates from same geographical regions showed similarity in DNA profiles except few isolates from Uttar Pradesh and Punjab. Isolates were classified based on morphological, cultural characters and some isolates were identified as highly virulent and virulent.**

Key words: Rice, sheath blight, *Rhizoctonia solani*, cultural, pathogenic, molecular, variability.

INTRODUCTION

Sheath blight is one of the serious diseases caused by *Rhizoctonia solani* Kühn (*Thanatephorus cucumeris*) (Frank) Donk. Sheath blight disease of rice occurs in all rice production areas world over (Savary et al., 2006). Yield losses of 5-10% have been estimated for tropical lowland rice in Asia (Savary et al., 2000). A modest estimation of losses due to sheath blight of rice in India has been reported to be up to 54.3% (Chahal et al., 2003).

In present days, attempts are being made to control rice sheath blight disease using cultural practices, use of resistant cultivars (Premalatha, 1990), fungicides, and biological control methods. In India, breeding for sheath blight resistant varieties has been a priority area of research. However, lack of adequate information on the genetic variability of the fungal population occurring in India, non-availability of resistant donors and the non-availability of appropriate markers are some of the

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limiting factors for developing suitable strategies for control measure (Neeraja et al., 2002). However, there is no resistant cultivar available for practical use and the present intensive rice cultivation practices offer a favourable condition for disease development. Also there is considerable pressure from environmental scientists to decrease emphasis on chemical control. Breeding for disease resistance, though most practical and feasible method, it could not be a final solution because the potential variability of most pathogens will not permit any currently successful variety to remain resistant for an indefinite period.

In *R. solani* variability is being observed due to mutation, and heterokayrosis. These processes affect the morphological, cultural, pathogenic and molecular charac-ters of *R. solani* population. Subsequently, disease epidemiology is also affected. However, understanding of disease epidemiology and host- pathogens interactions is highly dependent on knowledge of the pathogen diversity. Considerable variability is reported on the basis of cultural, morphological and virulence character in *R. solani* (Sunder et al., 2003). Many problems associated with studying different level of diversity in *Rhizoctonia* are best addressed through the use of molecular genetic markers.

At the species level, molecular markers aid in the development of species concept by providing information about the limit of genetically isolated group in relation to pattern of morphological variation and mating behavior; at the population level, molecular markers provide a basis for identifying pattern, dispersal and colonization in spatial and temporal distribution (Vilgalys and Cubeta, 1994). Duncan et al. (1993) concluded that random amplified polymorphic DNA- polymerase chain reaction (RAPD-PCR) analysis is a very useful alternative to anastomosis grouping for identification of isolates of *R. solani*.

Molecular markers are used as important tools for characterization of genetic diversity in pathogens where morphological characteristic are either absent or not able to differentiate isolates properly (Sharma et al., 1999). Moreover morphological characters are also influenced by environmental and cultural conditions. Therefore, problems associated with studying different levels of genetic diversity in *R. solani* have been suggested to be best addressed by use of molecular techniques (Toda et al., 1999).

Molecular techniques have become reliable and are highly suitable tools for identifying pathogen species and for assessing genetic variation within collections and populations (Sundravadana et al., 2011). Recently, Banerjee et al. (2012) reported that the RAPD can still be considered as a reliable, efficient and effective marker technology for determining genetic relationships in *Rhizoctonia* spp. Therefore, present investigations were conducted to study the morphological, cultural, pathogenic and molecular variability of *R. solani* collected from different geographical locations of India.

MATERIALS AND METHODS

Isolation of *Rhizoctonia solani*

In the present study, rice plants infected with sheath blight disease were collected from different agro climatic zones of India (Figure 1). Diseased leaf sheath were cut into 1 to 1.5 cm long bits. The bits were cleaned in sterilized distilled water and surface sterilized with 0.25% sodium hypochlorite solution for 30-60 s, washed thoroughly three times with sterilized distilled water and blot dried. These bits were then placed on potato dextrose agar (PDA) medium in Petri plates and were incubated at 27-30°C for 2-3 days. After three days incubation, fine radiating mycelium growth was observed from the edge of infected bits. A small bit of mycelium was transferred onto the PDA slants and thus pure culture was obtained. Cultures were maintained on sterile potato dextrose agar slants at 4.0°C.

Morphological variability

Hyphal and sclerotial characteristics

Mycelium of 48 h old cultures were stained with 0.5% aniline blue in lacto phenol or only in water without any dyes followed by hyphal widths of each isolate was measured under microscope with 20 observations (each isolates and mean of observation was reported) was, before taking observation with microscope; microscope was calibrated with stage and ocular micrometer. Four days old fungus hyphae were mounted in water on microscopic slide for recordings type of septa, constriction and angle of branching. Mycelial discs of 5 mm diameter from three days old cultures of each isolate was transferred into the center of sterilized PDA plates and incubated for 10 days at 28±2°C to determine sclerotial characteristics and then after observations were recorded visually by method described by Burpee et al. (1980). Sclerotia formation pattern (central, peripheral and scattered), number of sclerotia and time taken for sclerotia formation were recorded. Diameters of the sclerotia were measured in respect of 20 random sclerotia with the help of Digital Vernier Calipers (Mitutoyo Corp, Japan).

Cultural variability

Colony characteristics

The colour of colony was determined with the help of Munsell's soil color chart (Munsell Color Company, Inc., 1954). The culture and key colour card was placed side by side against white background under sun light for comparison.

Growth pattern was recorded by visual observation according to the growth of hyphae: abundant, Aerial mycelium obscures surface mycelium and touches the cover of the Petri dish; moderate, Aerial mycelium obscures surface mycelium, but does not touch the cover of Petri dish, and scarce, aerial mycelium does not obscure surface mycelium. Radial growth rate was measured for each isolate with three replications using meter scale at interval of 24, 48 and 96 h after incubation of the inoculated Petri dish at 28±2°C.

Pathogenic variability

An experiment was conducted in the National Phytotron facility at, IARI, New Delhi during *Kharif* season 2006-2007 and rice cultivar highly susceptible to sheath blight disease Pusa Basmati-1 (Pusa 167 × Karnal Local), was used for the study of pathogenic variability against all the isolates. The seedlings were raised in earthen pots of 10 "diameter. Transplanting was done after 25 days of sowing in

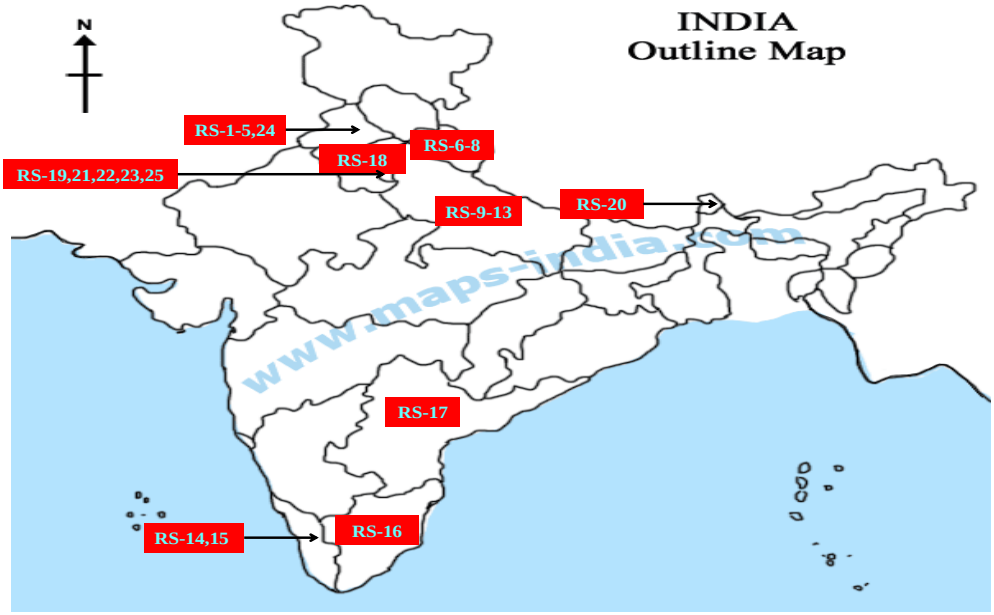

Figure 1. Indian map depicting the geographical location of samples collected for the study: 1-5 and 24 from Punjab; 6-8 from Uttarakhand; 9-13 from Uttra Pardesh; 14-15 from Kerala; 16 from Tamilnadu; 17 from Andhra Pradesh,;18 from Haryana; 19,21,22,23,25 from New Delhi and 20 from Sikkim.

earthen pots of 10 "diameter. Three replicates of five plants per pot were maintained with one control treatment without inoculation.

Mass multiplication of inoculum

The inoculum of each isolate was multiplied following the procedure described by Bhaktavatsalam et al. (1978). Shoots of water sedge (*Typha angustata*) were cut into pieces of 4-5 cm long washed thoroughly and soaked in Typha medium (peptone: 10.0 g, Sucrose: 20 g, K_2HPO_4: 0.1g, $MgSO_4$: 0.1 g, Distilled water: 1 L) for 5 min. The pieces were drained for excess water and later these were filled loosely to one third volume of 250 ml conical flask and sterilize in autoclaved at 1.05 kg/cm^2 for 20 min each for two consecutive days. The sterilized typha flask was inoculated with 5 mm diameter disc of actively growing mycelium of the each isolates and incubated for 15 days at 28±2°C. These colonized typha pieces were used as inoculum.

Method of inoculation

Plants of cv.PB-1 were inoculated at the maximum tillering stage (30-35 days after sowing) with colonized typha pieces. Two pieces of typha were placed between tillers in the central region of rice hills, just above the water level. Water level (5-10 cm) was maintained constantly for ensuring enough humidity to promote disease development and one pot was kept as a control without inoculation. All agronomic practices were followed according to requirement of cultivars.

Observation recorded

Relative lesion height

The relative lesion height (RLH) was recorded at two stages; first observation was taken at 20 days after inoculation and second observation at 35 days after inoculation. The lesion height and plant height were measured. RLH was calculated by the formula given by Sharma et al. (1990). Rice sheath blight grade chart 0-9 (IRRI, 1996) was used for recording reaction and lesion height. Based on disease reaction, isolates were categorized as 0-3.9: avirulent; 4-7.9: moderately virulent, 8-9: virulent according to classification of Neeraja et al. (2002).

Molecular variability

Potato dextrose broth (PDB) was used for mycelial growth of fungus for extraction of DNA. Fifty millilitre of sterilized PDB medium was inoculated with 5.0 mm disc of the fungus from actively growing cultures of different isolates and incubated for five days at 28°C. The mycelial mats were harvested by filtering through sterilized Whatman paper No.1 filter.

DNA extraction

Total DNA extraction was carried out by cetyl-trimethyl ammonium bromide (CTAB) method described by Murray and Thompson (1980) with slight modifications. One gram mycelial mats were ground to fine powder in liquid nitrogen using prechilled mortar and pestle. The powdered mycelium was transferred into sterilized centrifuge tubes containing 10 ml of pre-heated (65°C) 2% CTAB DNA extraction buffer (100 mM Tris Hcl, 50 mM EDTA, 5 M Nacl, 2 g CTAB w/v). The supernatant was discarded and pellet washed with 70% ethanol and recentrifuged at 10,000 rpm for 10 min. The pellets were air dried until smell of ethanol was completely removed from the tubes. The crude DNA pellet was resuspended in TE buffer (10 mM Tris, 0.1 M EDTA buffer, pH; 8.0). Subsequently, RNA from the total nucleic acid was removed by treatment with RNaseA. The quality and quantity of isolated DNA was checked by taking absorbance at 260 and 280 nm, in a spectrophotometer followed by running the dissolved DNA in 0.8% agarose gel alongside

uncut λ DNA of known concentration. The resuspended DNA was then diluted in TE buffer to for use in PCR amplification and stored at -20°C.

Randomly amplified polymorphic DNA (RAPD) analysis

To reveal the genetic diversity among the *R. solani* isolates using RAPD markers, the PCR reaction conditions were standardized. A set of 23 random decamer primers were used for the preliminary screening on *R. solani* out of which only 10 RAPD primers were found better in respect of polymorphic pattern and reproducibility were used for this study. The polymerase chain reaction was carried out in a final volume of 25 µl containing 2.5 µl of 10x assay buffer (100 mM Tris-Cl; pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin), 0.04 mM of each dNTPs (dCTP,dGTP,dATP,dTTP) (MBI Ferment Inc. USA) 0.4 µM of primer (Table 4), 1.0 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 20 ng of template DNA. The amplification reaction was performed in a Thermal Cycler (Eppendorf AG, Germany) programmed for 39 cycles: 1st cycle of 5 min at 94°C followed by 38 cycles each of 1 min at 94ºC, 1 min at 36°C and 2 min at 72°C. The final extension step consisted of one cycle of 10 min at 72°C for complete polymerization. After completion of the PCR, 2.0 µl of 6X loading dye (MBI Ferment Inc. USA) was added to the amplified products and were loaded on 1.2% (m/v) agarose (MBI Ferment Inc. USA) gels and electrophoresis was carried out at 60 V for 3 h in 1x TAE buffer, stained with ethidium bromide and visualized under UV light trans illuminator. Gel photographs were scanned through Gel Doc System (Syngene, Cambridge, U.K). The sizes of the amplification products were estimated by comparing them to standard DNA ladder (1 Kb DNA ladder; MBI Ferment Inc. USA). All the result was repeated twice for confirmation of the polymorphism.

RAPD data scoring

Each amplification product was considered as RAPD marker and was scored across all samples/ isolates. Data were recorded in binary matrix as presence (1) or absence (0) of band products from the examination of gel photographs. Jaccard's similarity coefficient (Jaccard, 1908) was measured and a dendrogram based on similarity coefficients was generated by using the un-weighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) and the SHAN clustering. All the analysis were done by using the computer package NTSYS-PC version 2.02e (Rohlf, 1997).

Statistical analysis

Morpholological cultural and pathogenic study was conducted in Completely Randomized Design and SAS software (SAS Institute, version 9.1, Cary, NC) used for statistical analysis using Duncan Multiple Range Test (DMRT). F-test was used as statistical test and level of LSD was P=0.05.

RESULTS AND DISCUSSION

Isolation of *R. solani*

In the present study, 25 isolates were taken for studying variability. Sheath blight infected rice plants were collected and the pathogen *R. solani* was isolated and purified by single hyphal tip / single sclerotial method. Cultures were maintained on sterile PDA slants in test tube, at 4°C for further study.

Cultural variability

Colony colour

The colour of the colony was observed from the bottom side of Petri dish. Based on the colony pigmentation, all the isolates were assigned into five groups: light brown, yellowish brown, whitish brown, dark brown and very pale brown. Six isolates were found light brown, five isolates were found yellowish brown, four isolates were whitish brown in colour, six isolates were dark brown and four isolates (RS-9, RS-17, RS-21 and RS-23) were very pale brown (Table 1). Sunder et al. (2003) also reported that colony colour ranged from brown, light brown, dark brown, and yellowish brown. The discolorations of the growth media is mainly attributed to the production of pigments by the pathogens. The difference in the intensity of the colour may also correspond to the amount of pigments released by respective isolate in the media.

Growth pattern

On the basis of growth pattern, the isolates were categorized into three groups: abundant, moderate and scarce growth. Eight isolates (RS-12, RS-14, RS-15, RS-19, RS-21, RS-23, RS-24, RS-25) showed abundant growth, four isolates were moderate and 13 isolates only scarce growth pattern (Table 1). Burpee et al. (1980) also reported that three groups of growth pattern that was abundant, moderate, and slight.

Diameter of growth rate

Diameter growth rate was recorded after 24, 48 and 72 h after incubation of the inoculated Petri dsih at 28±2°C (Table 1). On the basis of mean of three readings, isolates were classified into three groups: fast, medium and slow growing. Fast growing (>65 mm) 12 isolates included RS-1, RS-5, RS-6, RS-7, RS-10, RS-14, RS-15, RS-17, RS-18, RS-20, RS-23, RS-24 and were categorized into group 1. Medium growing (60-65 mm) 11 isolates included RS-2, RS-3, RS-4, RS-8, RS-9, RS-11, RS-12, RS-13, RS-16, RS-21, RS-25 and were categorized into group 2 and the remaining two, slow growing isolates (40-59 mm) included RS-19, RS-22 were categorized into group 3.

Morphological variability

Morphological diversity was studied based on the phenotypic (morphological) appearance of the isolates. The observations were recorded on the hyphal

Table 1. Cultural characteristic of different isolates of R. *solani* on PDA medium.

Isolate	Place of collection	Colony colour	Growth pattern	Colony growth dia (mm)			Mean diameter (mm) for categorization of the isolates
				24 h*	48 h*	72 h*	
RS- 1	Jalandhar, Punjab	Light brown	Scarce	16.00	90.00	90.00	65.33
RS-2	Kapurthala, Punjab	Whitish Brown	Scarce	14.00	83.50	90.00	62.50
RS-3	Moga, Punjab	Dark brown	Moderate	14.00	90.00	90.00	64.66
RS-4	Ludhiana, Punjab	Whitish brown	Scarce	12.00	82.00	90.00	61.33
RS-5	Ramgarh, Punjab	Yellowish brown	Scarce	29.50	90.00	90.00	69.83
RS-6	Pant Nagar, Uttarakhand	Whitish brown	Scarce	28.00	90.00	90.00	69.33
RS-7	Haldi, Uttarakhand	Yellowish brown	Scarce	24.50	90.00	90.00	68.16
RS-8	Rudrapur ,Uttarakhand	Yellowish brown	Scarce	16.25	84.75	90.00	63.66
RS-9	Kanpur, Uttar Pradesh	Very pale brown	Moderate	8.75	88.75	90.00	62.50
RS-10	Faizabad, Uttar Pradesh	Light brown	Scarce	23.50	90.00	90.00	67.83
RS-11	Ghaziabad ,Uttar Pradesh	Light brown	Scarce	13.50	81.66	90.00	61.72
RS-12	Merrut, Uttar Pradesh	Light brown	Abundant	19.50	81.83	90.00	63.77
RS-13	Merrut, Uttar Pradesh	Yellowish brown	Moderate	15.75	85.75	90.00	63.83
RS-14	Pattambi, Kerala	Dark brown	Abundant	31.25	76.16	90.00	65.80
RS-15	Moncopu, Kerala	Dark brown	Abundant	24.00	81.66	90.00	65.22
RS-16	Maduari, Tamil Nadu	Whitish brown	Scarce	13.25	89.25	90.00	64.16
RS-17	Rajendranagar,Andhra Pradesh	Very pale brown	Moderate	29.00	90.00	90.00	69.66
RS-18	Hisar, Haryana	Dark brown	Scarce	29.50	83.25	90.00	67.58
RS-19	IARI Farm, New Delhi	Light brown	Abundant	17.00	61.00	90.00	56.00
RS-20	Gayalshing, Sikkim	Light brown	Scarce	31.66	90.00	90.00	70.55
RS-21	IARI Farm, New Delhi	Very pale brown	Abundant	17.50	86.50	90.00	64.66
RS-22	IARI Farm, New Delhi	Dark brown	Scarce	6.0	48.50	77.00	43.83
RS-23	IARI Farm, New Delhi	Very pale brown	Abundant	27.00	90.00	90.00	69.00
RS-24	Hoshiarpur, Punjab	Dark brown	Abundant	20.50	90.00	90.00	66.83
RS-25	IARI Farm, New Delhi	Yellowish brown	Abundant	23.25	67.83	90.00	60.36
CD (P=0.05)				1.65	1.36	1.58	1.69

*****Means of three replications. RS-24 and 25, maize isolates.

characteristics and several sclerotial features of 25 isolates grown on PDA medium after specific incubation period. The basic characteristics of *R. solani* are mycelium branching at right angles, characteristic constriction at the point of branching and formation of septum near the point of origin of the branch. It was an obvious observation for the mycelial branching at right angles as a known feature of *R. solani* (Sneh et al., 1991). Microscopic studies revealed that all the 25 isolates of *R. solani* in the present study characteristically had hyphal branching at right angle, constriction at the point of branching of the mycelium and presence of a septum near the branching junction which is of immense taxonomical importance. Hyphal width ranged from 4.75 to 7.43 µm. Maximum hyphal width (7.43 µm) was observed in isolate RS-22 (New Delhi) while minimum (4.75 µm) was observed in isolate RS 20 (Sikkim). On the basis of hyphal width the isolates were grouped into two categories (Group1: 4.0-6.0 µm and Group 2: 6.1-8.0 µm). Thirteen isolates were categorized into group 1 while 12 isolates (RS- 1, RS- 3, RS-6, RS- 8, RS- 9, RS-13, RS- 15, RS- 21, RS- 22, RS- 23, RS- 24, RS- 25) formed group 2 (Table 2).

Sclerotial characteristics

Observations for the variation in the sclerotial characteristics were taken such as the number, size, formation of sclerotia and time taken for initiation of sclerotial formation. Number of the sclerotia ranged from 0 to >60. No sclerotium was formed in isolate RS-22 and was categorized into group 1 (poor). None of the isolate was categorized in group 2 (fair) and group 3 (moderate). Group 4 (good) included six isolates (RS-2, RS-8, RS-14, RS-15, RS-19, RS-24), Group 5 (very good) included seven isolates (RS-4, RS-7, RS-12, RS-13, RS-20, RS-23, RS-25) and group 6 (excellent) included 11 isolates (Table 2). Mostly isolates were had more number of sclerotia except RS-22 from New Delhi, which was hadno sclerotia at all. According to Meyer, (1965) sclerotia may be absent in some *R. solani* isolates under certain cultural

Table 2. Formation of sclerotia, average diameter, no. of sclerotia per Petridish and hyphal width of different isolates of *R. solani.*

Isolate	Formation of sclerotia	*Average sclerotia diameter (mm)	Number of sclerotia/ Petridish	*Average hyphal width (µm)
RS- 1	Scattered	1.47^{egdfch}	Excellent	7.13^{ba}
RS-2	Peripheral	1.52^{egdfc}	Good	5.64^{edfc}
RS-3	Central	1.73^{bdac}	Excellent	7.13^{ba}
RS-4	Scattered	1.64^{ebdfc}	Very good	5.35^{edf}
RS-5	Central	1.34^{egfh}	Excellent	5.05^{ef}
RS-6	Peripheral	1.29^{gfh}	Excellent	7.13^{ba}
RS-7	Scattered	1.72^{bdac}	Very good	5.95^{ebdfc}
RS-8	Scattered	1.72^{bdac}	Good	6.83^{bac}
RS-9	Scattered	1.13^{h}	Excellent	7.13^{ba}
RS-10	Central	1.59^{egdfc}	Excellent	5.94^{ebdfc}
RS-11	Scattered	1.54^{egdfc}	Excellent	5.94^{ebdfc}
RS-12	Scattered	1.56^{egdfc}	Very good	5.94^{ebdfc}
RS-13	Scattered	1.39^{egdfh}	Very good	6.81^{bac}
RS-14	Peripheral	1.81^{bac}	Good	5.94^{ebdfc}
RS-15	Central	1.82^{bac}	Good	6.54^{bdac}
RS-16	Peripheral	2.00^{ba}	Excellent	5.94^{ebdfc}
RS-17	Central	1.75^{bdac}	Excellent	5.94^{ebdfc}
RS-18	Peripheral	2.03^{a}	Excellent	5.05^{ef}
RS-19	Peripheral	1.68^{ebdac}	Good	5.05^{ef}
RS-20	Peripheral	1.60^{egdfc}	Very good	4.75^{f}
RS-21	Scattered	1.49^{egdfc}	Excellent	6.83^{bac}
RS-22	-	-	Poor	7.43^{a}
RS-23	Central	1.27^{gh}	Very good	7.13^{ba}
RS-24	Central	1.28^{gfh}	Good	6.24^{ebdac}
RS-25	Central	1.40^{egdfh}	Very good	6.24^{ebdac}
CD(P=0.05)		0.27		0.35

Scale for number of sclerotia; 0, Poor; 1-10, fair; 11-20, moderate; 21-40, good; 41-60, very good; >60, excellent. *Avg. of 20 observations; Mean in a column followed by the same letter are not significantly different according to DMRT.

conditions therefore the absence of sclerotia does not mean that it is not a mycelium from *R. solani*. On the basis of diameter of sclerotia, the isolates were categorized into 2 groups. Group 1 had diameter range from 1.13-1.5 mm and Group 2 from 1.5-2.03 mm. Diameter of the sclerotia was observed maximum in isolate RS 18 (2.03 mm) and minimum in RS-9 (1.13 mm). Nine isolates (RS-1, RS-5, RS-6, RS-9, RS-13, RS-21, RS-23, RS-24, RS-25) were categorized into group 1 while 15 isolates were categorized into group 2 (Table 2). Basu et al. (2004) also reported that sclerotial diameter ranged from 0.23 to 1.91 mm and found that the abundance and size of sclerotia determine the virulence of an isolate. Butranu (1988) observed that the number, viability, size, and weight of sclerotia of *R. solani* could not be correlated with rice sheath blight intensity. In the present studies though there was a correlation between the bigger size of sclerotia and high virulence in isolates RS-16, RS-18, similar pattern was not followed by other isolates.

Formation of sclerotia was observed in the Petri dish and classified into three groups. Sclerotia formed in the central region with a ring formed group 1 (RS-3, RS-5, RS-10, RS-15, RS-17, RS-23, RS-24, RS-25; eight isolates). Other 7 isolates (RS-2, RS-6, RS-14, RS-16, RS-18, RS-19, RS-20) were found in peripheral manner and classified into group 2. Those isolates which could not be classified either peripheral or central formed a separate group 3, which is scattered (Figure 2). Singh et al. (2002) reported sclerotial formation in the same manner that is, central/peripheral/scattered. There was also variation in the time taken for initiation of sclerotial formation; it ranged from 3 to 5 days. Four isolates (RS-5, RS- 6, RS- 9, RS- 24) took 3 days for initiation of sclerotial formation, 17 isolates (RS-1, RS-3, RS-4, RS-7, RS-8, RS-10, RS-11, RS-12, RS-13, RS-15, RS-16, RS-17, RS-18, RS-20, RS-21, RS-23, RS-25) took 4 days for initiation of sclerotial formation. Three isolates (RS-2, RS-14, RS-19) took five days for initiation of sclerotial formation. Mostly, isolates take four days for initiation of

Figure 2. Formation of Sclerotia **a.** Central. **b.** Peripheral. **c.** Scattered.

sclerotial formation. Meena et al. (2001) also observed that time taken for sclerotia formation ranged from 3-11 days.

Pathogenic variability

Pathogenic variation of the isolates was studied in Phytotron on cultivar PB-1 in 2006-2007. The data revealed that all the isolates can be classified into two groups on the basis of disease score given by Neeraja et al. (2002). No isolate was observed in avirulent group. Thirteen isolates having disease score of 7.0, belonged to moderately virulent group (RS-1, RS-2, RS-4, RS-7, RS-9, RS-10, RS-12, RS-13, RS-14, RS-19, RS-22, RS-23, RS-25) and 12 isolates having disease score of 9.0, belonged to virulent group (Table 3). All the isolates showed maximum relative lesion height at tillering stage but subsequently decreased at panicle initiation stage except one isolate RS-13 was at par both the stages. Maximum relative lesion height (75.96%) was observed in isolate RS-18 and minimum relative lesion height (55.81%) was observed in isolate RS-25 (Maize- isolate).

Three characters are significant from pathogenicity point of view. Firstly, isolates may cause several types of diseases and symptoms. Secondly the isolates may vary from avirulent to aggressively virulent state, and finally the host range among isolates may vary from limited to extremely wide (Saxena, 1997). Correlation between aerial mycelium growths and virulence pattern was reported by Tu (1967) and Akai et al. (1960). The former found that strain with less aerial mycelium were more virulent, whereas the latter found that the strains with poor mycelium growth were less pathogenic. In the present study also RS-16 and RS-18 having less aerial mycelium were more virulent. But also RS-21 was found more virulent even though it had more aerial mycelium. Diameter growth rate differed according to growth rate of the different isolates. Fast and medium growing isolates were more pathogenic than the slow growing isolates. Basu et al. (2004) found that there was no correlation between the mycelial growth of an isolates and its virulence on the host while Wamishe et al. (2007) reported that the aggressiveness of each isolates could be predicted based on the speed of growth in Petridish.

Molecular variability

Scoring and analysis of RAPD-PCR amplification

Twenty five (25) isolates of *R. solani* isolates collected from different agro-climatic zone of India were analyzed using 23 random decamer primers, out of which 10 primers produced reproducible and scorable bands which were used for study. The total number of 126 bands were amplified from 10 primers, out of which only 2 bands were found to be monomorphic in OPA 13 (one band) and OPF 6 (one band), thereby giving an estimate of profound (>98%) polymorphism (Table 4). The bands with the same mobility were considered as identical fragments, receiving equal values, regardless of the staining intensity. Contrastingly, the polymorphic bands indicate the fragments observed in more than one species with different electrophoretic mobility.

Maximum number of the bands were in the size range of > 250-2500 bp whereas minimum numbers were in the range of 250-1500 bp. Out of 10 primers used, the primers such as OPC 18, and GCC 1 generated maximum of total 18 bands which were polymorphic in nature. Out of 10 primers some primers namely OPA-10, OPF-06 and OPZ-20 were found to be good for the isolates of *R. solani* Kühn. Primer OPZ-20 could distinguish the isolates RS-16 (TN), RS-18 (Haryana) from each other and about 1300 bp size of the band was common in all the isolates except RS-25. About 750 bp band was common in all isolates except four isolates that is, RS-4, RS-17, RS-20, RS-22 (Figure 3a). A band about850 bp obtained with primer OPA-10 was common in RS-2, RS-6, RS-8, RS-11, RS-12, RS-13 and it could

Table 3. Virulence pattern of different isolates *R. solani* in Phytotron (Temp. 28°C, RH 100%) on Pusa Basmati -1.

Isolate	Relative lesion height (%)			Disease score	Category	Grade
	*Maximum tillering stage	*Panicle initiation stage	Mean			
RS-1	65.84	56.94	61.39^{ijfhkg}	7	MV	S
RS-2	71.61	55.66	63.63^{eidjfhkg}	7	MV	S
RS-3	81.29	59.62	70.45^{ebdac}	9	V	HS
RS-4	69.47	54.10	61.79^{ijfhkg}	7	MV	S
RS-5	78.43	53.19	65.81^{ebdfhcg}	9	V	HS
RS-6	75.61	58.58	67.09^{ebdfcg}	9	V	HS
RS-7	66.37	61.64	64.01^{eidjfhcg}	7	MV	S
RS-8	76.82	59.15	67.98^{ebdfc}	9	V	HS
RS-9	61.51	51.33	56.42^{ijk}	7	MV	S
RS-10	60.71	58.29	59.50^{ijhkg}	7	MV	S
RS-11	74.63	60.31	67.47^{ebdfcg}	9	V	HS
RS-12	75.18	54.80	64.99^{eidfhcg}	7	MV	S
RS-13	63.96	64.00	63.98^{eidjfhcg}	7	MV	S
RS-14	71.03	53.09	62.06^{ijfhkg}	7	MV	S
RS-15	75.21	56.89	66.05^{ebdfhcg}	9	V	HS
RS-16	82.81	64.47	73.64^{ba}	9	V	HS
RS-17	79.90	63.04	71.47^{bdac}	9	V	HS
RS-18	78.65	73.27	75.96^{a}	9	V	HS
RS-19	62.80	51.78	57.29^{ijk}	7	MV	S
RS-20	79.75	64.54	72.15^{bac}	9	V	HS
RS-21	73.12	59.11	66.11^{ebdfhcg}	9	V	HS
RS-22	61.69	55.89	58.79^{ijhk}	7	MV	S
RS-23	73.52	50.97	62.24^{eijfhkg}	7	MV	S
RS-24	77.16	59.32	68.24^{ebdfc}	9	V	HS
RS-25	61.39	50.23	55.81^{jk}	7	MV	S
CD (P=0.05)			6.78			

*****Means of three replications. Mean in a column followed by the same letter are not significantly different according to DMRT; M V, moderately virulent; V, virulent; S, susceptible; HS, highly susceptible.

Table 4. Primers sequence, number of polymorphic and monomorphic bands, percent polymorphism and size range of amplicons.

Primer Name	Primer Sequences (5'to3')	Total number of bands amplified	Polymorphic bands	Monomorphic bands	Percent polymorphism	Size range of amplicon (bp)
OPA-10	GTGATCGCAG	11	11	0	100	250-2000
OPA-13	CAGCACCCAC	8	7	1	90	250-2000
OPC-18	TGAGTGGGTG	18	18	0	100	250-2000
OPC-19	GTTGCCAGCC	11	11	0	100	250-2000
OPF-06	GGGAATTCGG	9	8	1	90	250- ~1750
OPH-18	GAATCGGCCA	14	14	0	100	250-2000
OPQ-1	GGGACGATGG	12	12	0	100	250-2000
OPR-1	TGCGGGTCCT	12	12	0	100	250-1500
OPZ-20	ACTTTGGCGG	13	13	0	100	250-2000
GCC-1	ATGGATCCGC	18	18	0	100	250-2500
Total		126	124	2		

be distinguished as RS-23 and RS-24, isolates RS-16 (TN) with RS-18 (Haryana) and RS-20 with RS-21. RS-16 and RS-18 could be distinguished by primer OPC-19 and 500 bp size of the band was common most of the isolates.

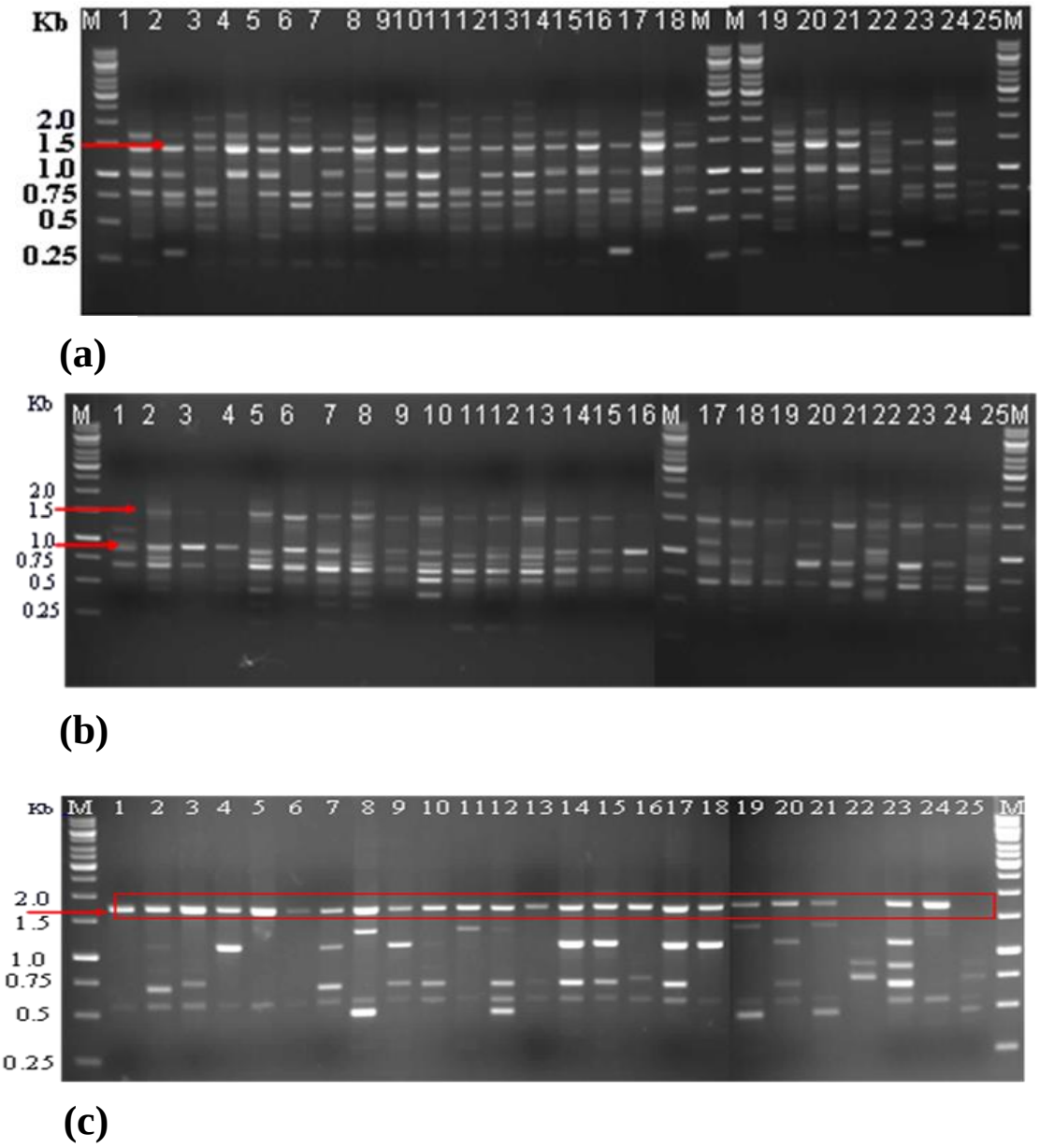

Figure 3. a. RAPD Profile of 25 isolates of *R. solani* (Lane1-25) with primer OPZ- 20. **b.** RAPD Profile of 25 isolates of *R. solani* (lane 1-25) with primer OPA-13. **c.** RAPD profile of 25 isolates of *R. solani* (Lane1-25) with primer OPF-6 and M=1 Kb molecular marker. *Arrows indicate common band in most of the isolates.

RS-24 and RS-25 could be distinguished by primer OPA-13 and 1.5 kb band was monomorphic in all the isolates (Figure 3b).

OPF-06 gave about 1750 bp bands in all the isolates except RS-22 and RS-25 (Figure 3c). A total of 10 RAPD primers data was used to construct an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogam based on Jaccard's similarity coefficient. The relationships between isolates can be more clearly represented by similarity matrix. The degree of genetic similarity/relatedness among the isolates was measured by Jaccard's similarity coefficient and varied considerably from 17 to 77%. Maximum similarity (77%) was found between two isolates, RS-14 and RS-15; both were collected from Kerala whereas, the lowest similarity (17%) was found between RS-22 (New Delhi) and RS-4 (Punjab) isolates. Cluster analysis based on UPGMA grouped the 25 isolates into four major clusters.

First four clusters were classified into sub-clusters. The first clusters consisted of four isolates which further contained two sub clusters. Sub clusters Ia contained only one isolate RS-1(Punjab) and sub clusters Ib con-

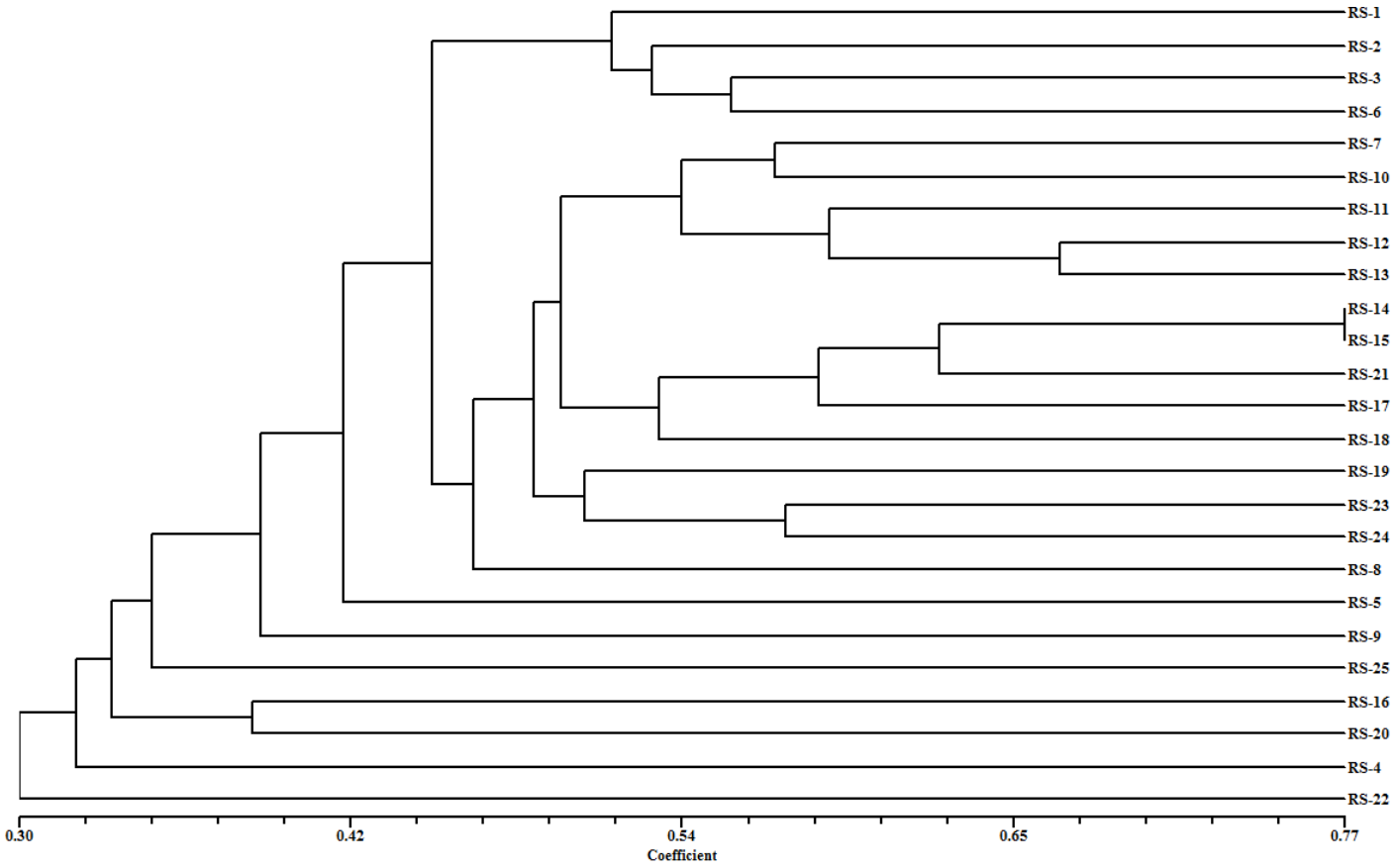

Figure 4. RAPD based dendrogram of *Rhizoctonia solani* isolates constructed using unpaired group arithmetic average (UPGMA).

tained 3 isolates that is, RS-2, RS-3 and RS-6. In this cluster isolates RS-3 and RS-6 had 55% genetic similarity followed by RS-2 and RS-1 with 48% similarity (Figure 4). The second clusters contained five isolates. The second clusters also further divided into two sub clusters viz IIa and IIb. Sub cluster IIa included two isolates, RS-7 and RS-10 with 57% genetic similarity. Sub cluster IIb contained three isolates, RS-11, RS-12 and RS-13. These isolates belonged to Uttar Pradesh. Similarity between RS-13 and RS-12 was 67% however these isolates belong to Meerut in UP. The third clusters consisted of five isolates. This cluster further divided into sub clusters. Four isolates (RS-14, RS-15, RS-21, and RS-17) in a cluster and RS-18 (Haryana) in another sub cluster. Isolates RS-14 and RS-15 showed 77% and both were from Kerala. Fourth cluster contained three isolates viz. RS-19, RS-23 and RS-24 (Punjab- Maize). It was an interesting observation that both RS-23 and RS-24 were from different host and it comes under in same cluster with 57% similarity. The 3 D plot analysis also revealed wider variation among the collected isolates used for this study (Figure 5).

Remaining isolates did not share any of the group except two isolates, RS-20 (Sikkim) and RS-16 (Tamil Nadu) which showed a distinct group and its similarity was 38%. And other isolates for example RS-8, RS-5, RS-9 and RS-4 did not come in a cluster. RS-22 from New Delhi showed a distinct group; it might be the reason why it was morphologically different from the other remaining isolates. It had no sclerotia formation and highest hyphal width. Isolates RS-24 and RS-25 from maize isolates revealed only 38% similarity. In this study some specific bands sizes (~500 bp, 1500 bp, 1550 bp ~1750 bp, ~750 bp, and ~ 1300 bp) were observed with different primers and was specific to *R. solani*, that regions could be used for development of specific markers for detection of *R. solani* isolates. More recently the use of molecular markers has given a boost to analysis of accurate variations among various isolates of the pathogen. Random Amplified Polymorphic DNA technique has been used consistently to determine the genetic variation and subsequently correlating it with the variation in the virulence pattern of the pathogen. The genetic differences among isolates were determined by a means of RAPD analysis with different random primers. Any decamer oligonucleotide can be used as a primer, and the same primers have been used with animals, plants, fungi, and bacteria. RAPD-PCR has been success

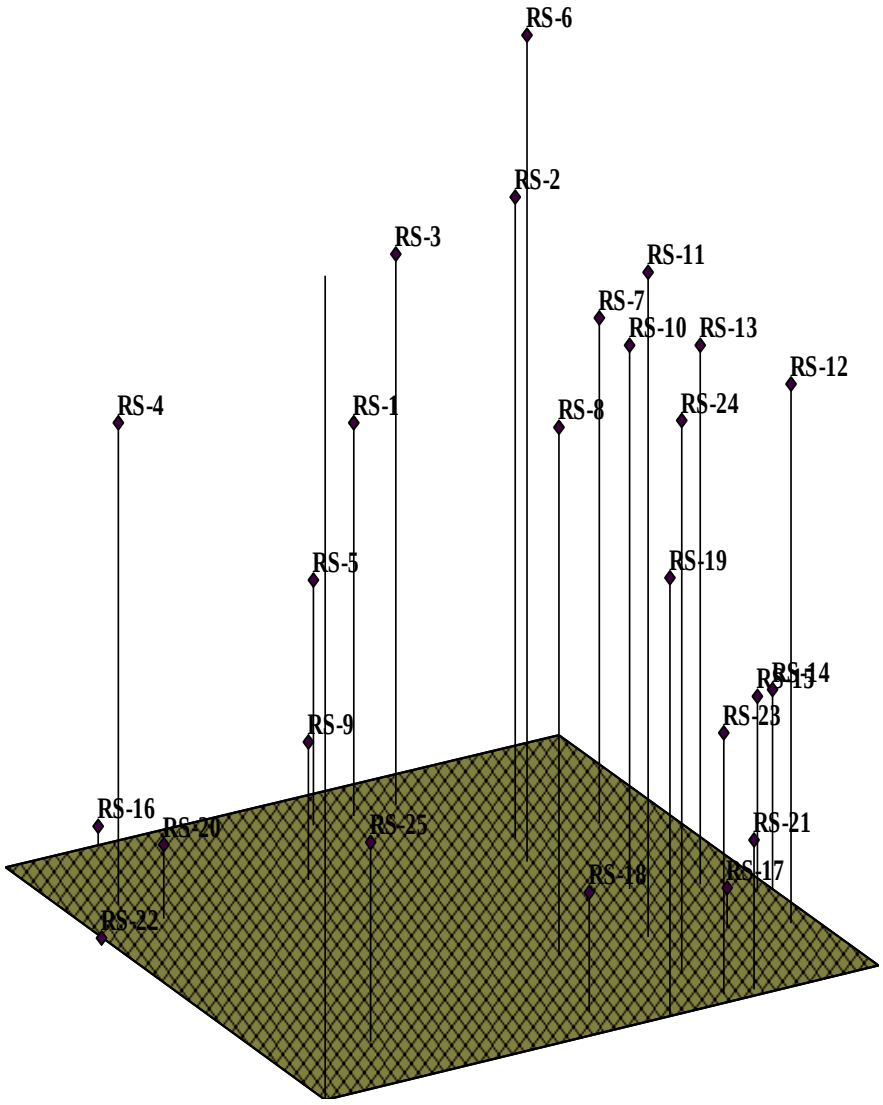

Figure 5. Three dimensional distribution of *R. solani* isolates based on RAPD markers.

fully used to differentiate strains with species of plants, bacteria, animals, and fungi (Williams et al., 1990).

Sharma et al. (2005) reported that French bean isolates of *R. solani* collected from different geographical areas were grouped in two separate clusters on the basis of same host and same geographical regions and 29.17% of the isolates did not share any of the clusters indicating the high variability within pathogen population. Guleria et al. (2007) reported that 19 isolates of *R. solani* from Punjab formed 5 clusters on the basis of variety specific grouping, which can be explained on the basis of similar virulence nature of these isolates towards the rice variety. Variation among isolates in *R. soalni* from upland crop seemed to be partially correlated with geographical origin and virulence.

Using RAPD-PCR, Duncan et al. (1993) were also able to identify heterogeneities within groups of isolates which originate in the same location and also reported that difference in patterns between isolates from the same geographic region tended to be fewer than difference between isolates from different regions. Pascual et al., (2000*)* reported that 30 isolates of *R. solani* AG1-IA isolates from maize in the Philippines and Japan. These were resolved into seven groups of AG1-IA through RAPD fingerprints at 75% similarity level. Yang et al. (1996) observed that genetic variation of 12 isolates from Alberta and 3 from Alaska were analyzed by random amplified polymorphism DNA (RAPD) assay using different oligonucleotide primers.

There was considerable variation within the *R. solani* AG-9 group; this suggests the AG-9, considered indigenous to Alaska, is present in a variety of environment and different geographic areas. Singh et al. (2002*)* studied variability among 46 isolates from hill areas (Uttrakhand)

and plain areas of U.P. in India. They analyzed intra-field variability in *R. solani* through RAPD fingerprinting and found high variability among them.

Conclusion

R. solani has sterile mycelia and it produced sclerotia. It showed greater variation in cultural/morphological characteristics, for example size of sclerotia and formation of sclerotia. Wide range of pathogenic variability was observed in *R. solani*, because within district of a state isolates revealed moderately virulent and virulent categories. It might be a reason that the isolates produced greater number of clones in this district. Primer OPF-06 could be used to develop specific marker for detection of *R. solani*. From this study it could be concluded that virulent isolates (RS-16, RS-18, RS-20 and RS 21) should not have similar genetic makeup. Even though, *R. solani* isolates from maize also did not have similar banding pattern.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Characterization of pathogen responsible for infection in bhindi plant by using phytoplasma specific universal primers

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Infection in bhindi plant (*Abelmoschus esculentus*, Family-*Malvaceae*) is very common in Gorakhpur district of Eastern U.P. region of India. To detect the pathogen, genomic DNA was isolated from the infected bhindi plant sample and amplified through polymerase chain reaction (PCR) by using P1 forward primer and Tint reverse primer, which were phytoplasma specific universal primers. Obtained PCR amplicons were cloned and sequenced. BLAST analysis discovered their 91% identity with the members of 'Uncultured bacterium'. Phylogenetic tree also make their relationship with uncultured bacterium. Obtained nucleotide sequence was submitted to NCBI through accession number KF663567. To the best of our knowledge, this is the first report of "uncultured bacterium" (473 bp), from Gorakhpur district of Eastern U.P. region of India, associated with infection in bhindi plant.

Key words: *Abelmoschus esculentus*, Gorakhpur district, Uncultured bacterium, Acc. No. KF663567.

INTRODUCTION

Abelmoschus esculentus (L.) (Moench) belonging to the family *Malvaceae*, usually known as bhindi, is one of the important vegetable crops grown in tropical, subtropical and warm regions (Charier, 1984). This is a rich source of iodine. The states, Uttar Pradesh, Assam, Bihar, Orissa, Maharashtra, West Bengal and Karnataka are the major producers of this vegetable (Prakasha et al., 2010). bhindi, exported from India as a fresh vegetable, comprises 70% of the total fresh vegetable earnings, apart from onion (Anonymous, 2000). There are several pathogens, which causes 20 to 30% total loss of this vegetable (Hamer and Thompson, 1957).

Plants infected by phytoplasma shows a variety of symptoms (Bertaccini, 2007; Bertaccini and Duduk, 2009). During survey period, we observed symptoms of leaf distortion, leaf curling and overall stunting of infected bhindi plant which make suspicion for phytoplasmal infection. This infection adversely harms the bhindi pods production and their quality. So, in the present study, we tried to identify pathogen causing leaf distortion, leaf

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Table 1. PCR Components used in present study.

PCR components (concentration)	Volume (µl)
Template DNA	5.0
P1 forward primer (10 µM)	1.0
Tint reverse primer (10 µM)	1.0
10X PCR buffer	2.5
50 mM $MgCl_2$	0.75
10 mM dNTP mixture	0.5
Taq DNA Polymerase (5 U/µl)	0.2
Sterile distilled water	14.05

Table 2. PCR conditions during the present study.

Temperature (°C)	Time (min)	Cycles
95	4	1
94	1	35
56	1	
72	1	
72	10	1
4	∞	

curling and overall stunting of bhindi plants of Gorakhpur district of U.P., India.

To our knowledge, there is little work done on molecular characterization of pathogen responsible for infection in bhindi plant of Gorakhpur district of U.P., India.

MATERIALS AND METHODS

Plant samples

Infected bhindi (*A. esculentus*) plant specimens, showing possible symptoms of phytoplasamal infection, that is, leaf distortion, leaf curling and overall stunting were collected from Gorakhpur district.

DNA extraction

There are series of different extraction procedures performed for phytoplasmal DNA. Each procedure of extractions involved the collection of sufficient plant material to perform the DNA extraction. Here, we followed procedure published by Ahrens and Seemüller (1992) and included a phytoplasma enrichment step. An amount of 1.5 g of infected plant material was incubated for 10 min in 8 ml of phytoplasma grinding buffer in a mortar maintained on ice, and then finely crushed with a pestle, adding 5 ml of PGB. The homogenate was then centrifuged for 5 min at 2,500 *g*. The supernatant of each sample was transferred to clean tubes and centrifuged for 25 min at 18,000 *g*. The pellet was dissolved in 1 ml CTAB buffer. After 1 h incubation at 60°C, the nucleic acids were purified by chloroform-isoamyl alcohol (24:1), and centrifuged at 12,000 *g* for 10 min. An equal volume of cold isopropanol was added to the drawn aqueous phase, and then incubated in ice for 1 h and then centrifuged at 12,000 *g* for 10 min. After centrifugation, 1 ml 70% ethanol was added and centrifuged at 12,000 *g* for 10 min. Supernatant was decanted and the pellet dried at 37°C for 30 min. DNA was dissolved in 30 µl of sterile water.

Phytoplasma grinding buffer (PGB)

The phytoplasma grinding buffer contained 100 mM K_2HPO_4, 30 mM KH_2PO_4, 10% sucrose, 0.15% bovine serum albumin fraction, 2% polyvinylpyrrolidone-10 and 25 mM ascorbic acid.

CTAB buffer

The CTAB buffer contained 2% CTAB, 100 mM Tris pH 8, 1.4 M NaCl and 20 mM EDTA.

Target gene

Target gene was 16s-23s rRNA spacer regions.

Primers used in the study

Primers used in the study were universal phytoplasma specific primer pair, which were P1 forward primer: 5'AAGAGTTTGATCCTGGCTCAGGATT3' and Tint reverse primer: 5'TCAGGCGTGTGCTCTAACCAGC3'.

PCR setup

Genomic DNA from the test samples was PCR amplified using the PCR components as mentioned in Table 1. The reactions were cycled using a 2720 thermal cycler (Applied Biosystems) according to the PCR conditions mentioned in Table 2.

Agarose gel electrophoresis of PCR products for confirmation of PCR amplification

After PCR is completed, the PCR products were checked on 1% Agarose by Agarose Gel Electrophoresis and amplicon size was compared using reference Ladder. 1% agarose gel spiked with ethidium bromide at a final concentration of 0.5 µg/ml was prepared using agarose (LE, Analytical Grade, Promega Corp., Madison, WI 53711 USA) in 0.5X TBE buffer. 5.0 µl of PCR product was mixed with 1 µl of 6X Gel tracking dye. 5 µl of g Scale 1000 bp size standard (geneOmbio technologies, India) was loaded in one lane for confirmation of size of the amplicon using reference ladder. The DNA molecules were resolved at 5 V/cm until the tracking dye was 2/3 distance away from the lane within the gel. Bands were detected under a UV trans illuminator. Gel images were recorded using BIO-RAD GelDocXR gel documentation system. The PCR product of size 1500 bp was generated through this reaction.

Cloning of PCR products

PCR product obtained from PCR analysis of bhindi plant was gel eluted using Invitrogen Gel DNA purification kit as per the manufacturer's instructions. This product was then ligated to TOPO vector and cloned in TOP 10 *Escherichia coli* ultra competent cells. The transformants were selected by following a blue white screening procedure. The putative recombinant clones were confirmed by colony PCR using M13 PCR primers. The recombinant clone confirmed by having the insert from the phytoplasma PCR was subjected to sequencing. The sequencing was performed using Tint Primer.

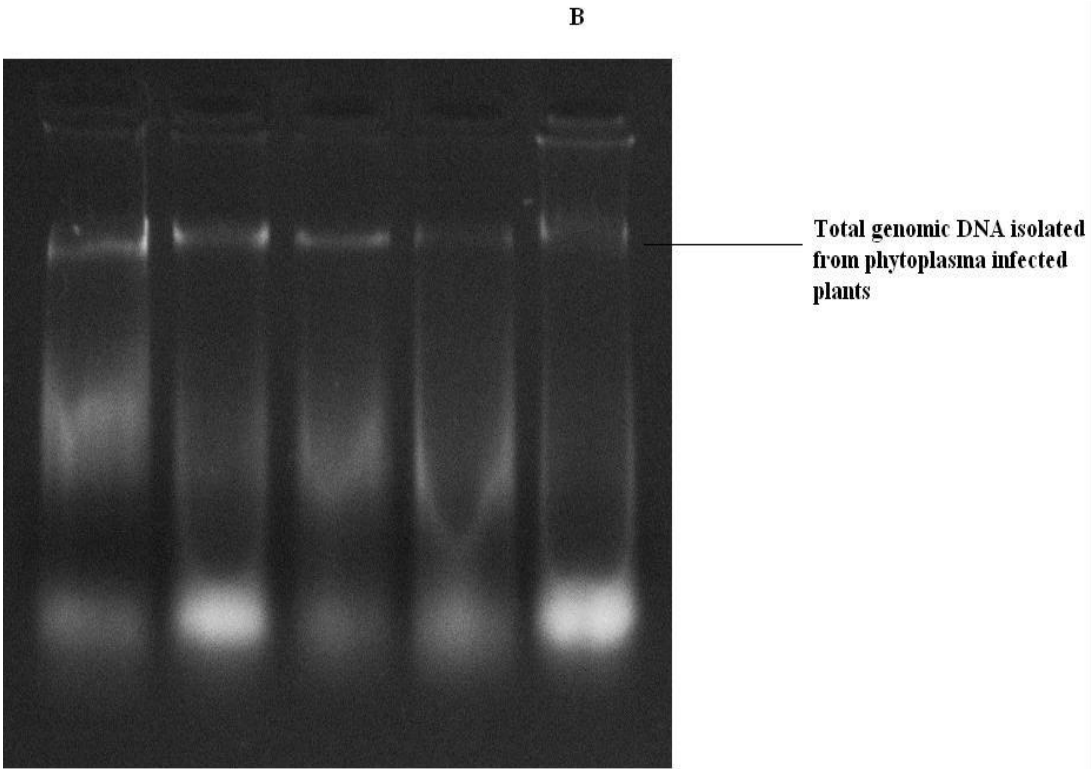

Figure 1. Genomic DNA QC image (1% agarose (w/v) gel electrophoresis). Well No. B, bhindi infected.

DNA sequencing

Using the gene specific sequencing primers and ABI BigDye® Terminator v3.1 Cycle Sequencing reaction kit (Applied Biosystems, USA), the insert DNA was sequenced.

BLAST analysis

BLAST analysis was conducted on the finally obtained sequence at http://blast.ncbi.nlm.nih.gov/Blast.cgi by using BLASTN 2.2.28+ program (Stephen et al., 1997). Sequence alignment was performed by sing clustalW sequence alignment tool available at http://www.genome.jp/tools/clustalw/.

Phylogenetic analysis

Genetic distance in the tree was calculated by default using Blast tree viewer. Finally, the obtained sequence was submitted to GenBank. The sequence generated from the present study and reference strains sequence retrieved from GenBank were used for phylogenetic analysis.

RESULTS AND DISCUSSION

During the course of survey for suspected phytoplasmal diseases in vegetable plants of different parts of Gorakhpur District, we were observed several plants. Among them, maximum suspected plant samples were collected for further characterization of their causative pathogen. PCR reactions with P1 and tint primer pairs resulted in the production of the PCR product of size approximately 1500 bp (Figures 1 and 2), which were further cloned and sequenced: Finally obtained nucleotide sequence (473bp) was deposited into NCBI (Acc. No. KF663567).

Total 473 bp: 1 gtcgtcagct cgtcccgtga ggtgttaggt taagtcctat aacgagggca acccctgttt; 61 ttagttgcca gcacgtaatg ttgggaactt taacaagact tccggtgaaa actgtgagga; 121 agggggggat gaggtcaaat tatcacggcc cttacgtcct gggccacaca cgtggtacaa; 181 taggcggtcc agagagcagc tacctagtga taggatggga atctataaaa ccgttctcag; 241 tttggatcgg agtttgcaac ttgactccgt gaagctggaa ttgctagtaa taggatatca; 301 cccatgatcc ggggaatggg ttcccgggcc ttgtaaaccc cgcccgtcaa accgtggaag; 361 ctggggttac cagaagtcgg tgaccgcaag gagtttccta gggtaaaact ggtaactagg; 421 gataagttta aacaagtaac tagtaccgga aggagcgcct ggaacacctc ctt.

Detected organism having 473 bp from total amplified PCR product (1500bp) indicates that amplifiable gentic materials were presented into them, but in smaller fragments.

The 16S rDNA sequences obtained from amplified and cloned products were analyzed by multiple sequence alignment with nucleotide sequences of other 16S rDNA

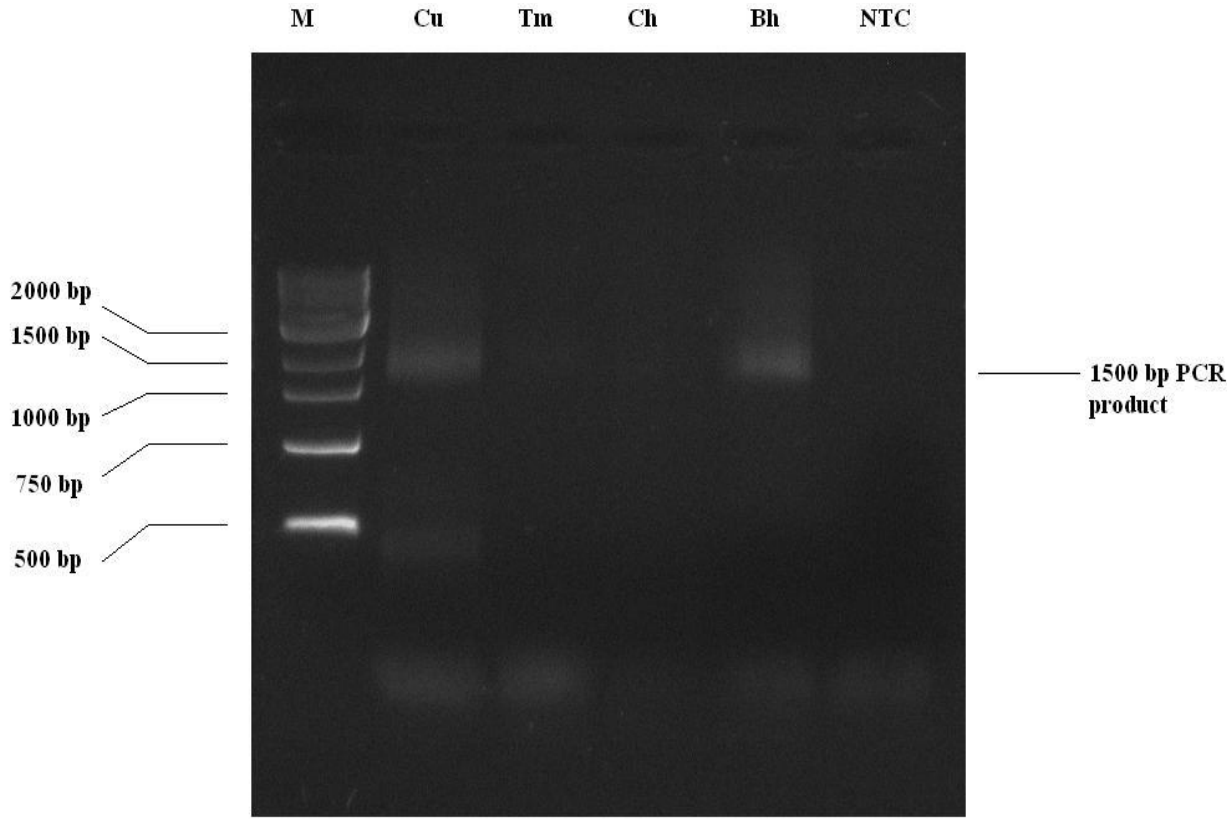

Figure 2. Representative data for PCR amplification: 1% Agarose (w/v) gel electrophoresis of 16S rRNA gene PCR products. Lane M, 1000 bp ladder; Lane Bh, bhindi plant; Lane NTC, negative control.

sequences from microorganism available at GenBank database of NCBI using BLASTN 2.2.28+ program (Stephen et al., 1997).

Blast analysis of obtained gene sequence (Acc. No. KF663567) showed 91% identity with uncultured bacterium sp. and uncultured flavobacterium sp., for example uncultured bacterium partial 16S rRNA gene, clone E130 (Acc. No. AM500800.1), Uncultured bacterium partial 16S rRNA gene, clone SMA4 (Acc. No. AM183001.1), etc. Their identities were furthered confirmed by phylogenetic analysis (Figure 3).

Bacteria are single-celled microorganisms which may be plant pathogens (causing disease), plant asymptomatic bacteria (have no evident effects), and plant growth-promoting bacteria (PGPB) (push up plant growth) (Vidaver and Lambrecht, 2004).

PGPB may be advantageous to plants by several ways such as auxin production, nitrogen fixation (Compant et al., 2005; Watanabe et al., 1979). They may be rhizospheric bacteria (live at root surface), symbiotic bacteria and endophytic bacteria (live inside the plant) (Bacon and White, 2000; Bacon and Hinton, 2006). Bacterial endophytes were first discovered in Germany in 1903 (Freeman, 1903; Tan and Zou, 2001) and defined as “microorganisms that colonize healthy plant tissue without causing obvious symptoms or producing obvious injuries to the host” (Bacon and White, 2000; Bacon and Hinton, 2006). So, that bacterium is not considered as endophyte which causes infections and produces symptoms for disease. By phylogenetic view, endophytic bacteria placed between saprophytic bacteria and plant pathogens (Hallmann et al., 1997).

In infected plants, phytoplasmas colonize sieve cells of phloem tissue and characteristically induce disease symptoms by disturbing their normal metabolic pathways (Chang, 1998; Curkovic´-Perica et al., 2007). They are unique bacteria, as they inhibit insects and plants (Xiaodong et al., 2006). Phytoplasma falls into class Mollicutes (soft-skinned bacteria), due to absence of an outer cell wall and generally have small genomes, low G-C content and essential metabolic activities (Bove, 1997). Mollicutes are directly associated with low G-C, Gram-positive bacteria for example Bacillus, Clostridium and Streptococcus species (Weisburg et al., 1989; Woese, 1987).

Phylogenetic investigation shows that phytoplasma come down from gram-positive, walled bacteria but way by which first phytoplasma originate still unknown (Wei et al., 2008). They are pleomorphic bacteria which fall from an acholeplasma-like ancestor and have small, AT-rich

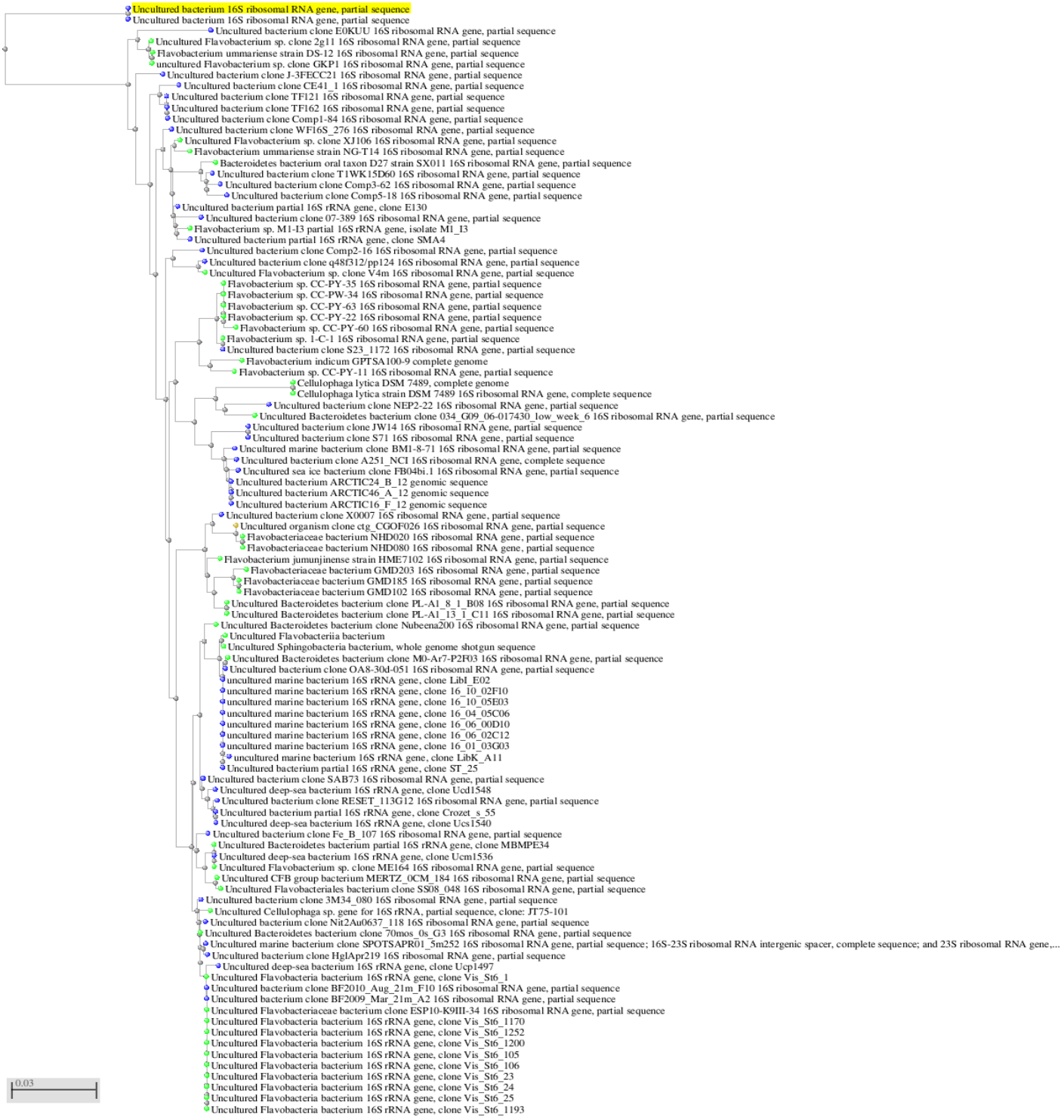

Figure 3. Phylogenetic tree (neighbor joining).

genomes through which they can live in two hosts and act as pathogen (Gundersen et al., 1994; Lee et al., 2000).

Phytoplasmal genomes made up by repeated genes, structured in units of nearly 20 kb, called PMUs (Potential Mobile Units) which involved in phytoplasma genome instability and recombination (Dickinson, 2010). Although PMU is a mobile unit, but it may engage in phase-variation mechanism by which phytoplasma can live in plant and vector (Dickinson, 2010). Phytoplasmal genomes

are special due to their unique structural design, having genes repetitively clustered in non-randomly distributed segments called “Sequence Variable Mosaics” (SVMs) that were formed through repeated, targeted attacks by mobile elements (Jomantiene and Davis, 2006; Jomantiene et al., 2007, Wei et al., 2008).

Wei et al. (2008) discovered that ‘cryptic prophases’ or prophage genome remnants form important structural constituent of phytoplasmal genomes and phage-mediated gene exchange which allow them to live into plant and insect host, for infection and to start events that initiate evolution of phytoplasma clade.

Phytoplasma posses extremely reduced genomes in comparison to other mollicutes, which is responsible for their unique metabolism by which they cannot artificially cultured (Xiaodong et al., 2006).

Phytoplasma produces several symptoms such as witches' broom, phyllody, generalized yellowing, decline and stunting of plants which indicates that they inhibit normal plant development (Hogenhout et al., 2008).

In present study, we also observed symptoms of leaf distortion, leaf curling and overall stunting of plant which makes suspicion that causative pathogen may have some relation with phytoplasma.

Conclusion

Here, we identified an “uncultured bacterium sp.” responsible for infection in bhindi plant (*A. esculentus*) of Gorakhpur district of Eastern U.P. region of India. Their nucleotide sequence deposited in GenBank has accession number KF663567. On the basis of visible symptoms of infection and positive PCR amplification with universal phytoplasma specific primers (P1/Tint), we can believe that the identified organism has some phytoplasmal nature.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

Effect of elicitation on picrotin and picrotoxinin production from *in vitro* products of *Picrorhiza kurrooa*

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Picrorhiza kurrooa **Royel ex. Benth. is an important medicinal plant of Himalayan region and a good source of iridoid glycosides. Picrotin and picrotoxinin are compounds produced by *P. kurrooa* which are widely used in treatment of hepatic diseases. Elicitation is one of the best effective methods which enhance secondary metabolite production in plants. In the present study effect of elicitors for example, Methyl jasmonate (100 µM) and yeast extract (0.5 mg/ml) on the production of picrotin and picrotoxinin from *in-vitro* products of *P. kurrooa* were studied. Yeast extract (0.5 mg/ml) was found more efficient than the methyl jasmonate for enhancing the production of picrotin and picrotoxinin in roots of *P. kurrooa*. Higher amount of picrotin (2.47 µg/g dry wt.) and picrotoxinin (45.2 µg/g dry wt.) were recorded in *in-vitro* products in comparison to control plant. Genetically stable *in vitro* plants were used to assess the effect of elicitation. Genetic stability was detected with Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers. The percentage of polymorphic bands in the RAPD and ISSR analysis were 2.5 and 7.02%, respectively. The similarity coefficient revealed that differences between tissue culture raised plants and mother plant was not remarkable by both RAPD and ISSR analysis.**

Key words: Genetic fidelity, plant tissue culture, inter simple sequence repeats, West Himalaya.

INTRODUCTION

Picrorhiza kurrooa Royle ex Benth. (family: Scrophulariaceae; local/trade name: Kutki), an important medicinal herb endemic to alpine Himalaya (Thakur et al., 1989), is distributed between 2800-4800 m altitude. A group of active compounds of great pharmaceutical interest like picrotin, picrotoxinin, pikroside I and II are produced mainly in the roots and runners of this species. The extract of runners and roots of this plant has been used since long in several Ayurvedic preparations. Picrotin and picrotoxinin are used as Picrotoxin, an equimolar mixture of picrotoxinin ($C_{15}H_{16}O_6$) and picrotin ($C_{15}H_{18}O_7$). It acts as a non-competitive channel blocker

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Abbreviations: MS, Murashige and Skoog's medium; **RAPD,** Random Amplified Polymorphic DNA; **ISSR,** inter simple sequence repeats; **MeJA,** Methyl Jasmonate.

for the GABAA receptor chloride chan-nels (Rho et al., 1996). In modern medicine it is used in hepatic disorders, gastric troubles, pregnancy, anemia and asthma (Hussain, 1984; Kirtikarand Basu, 1984). Due to indiscriminate collection from wilds and lack of organized cultivation this plant is listed in 'endangered' category (Samant et al., 1998; Ved et al., 2003). Therefore, a method for rapid and efficient multiplication of this medicinal plant is highly desired.

Plant tissue culture is a prospective technique not only in the protection of natural plant resources, but also has potential commercial interest. The crucial part of any *in vitro* propagation system is mass and rapid production of plantlets which are phenotypically uniform and genetically similar to the mother plant, otherwise the advantage of desirable characters of elite/supreme clones cannot be achieved. Molecular markers such as RAPD and ISSR can be used advantageously for this purpose (Isabel et al., 1993; Rani et al., 1995; Rani and Raina, 2000; Agnihotri et al., 2009, Mishra et al., 2011a).

Plant-based molecules are continuously gaining wide spread acceptance due to their effective therapeutic properties. Production of picrotin and picrotoxinin from plant tissue cultures is important because intact plants produce this compound only in small amounts. The reported yield of active ingredients (picrotin and picrotoxinin) from field-grown plant of *P. kurrooa* is 0.64 and 7.49 µg/g dry weight (DW) (Mishra et al., 2011b). Several papers have been published on micro-propagation (Lal et al., 1988; Chandra et al., 2004; Chandra et al., 2006, Sood and Chauhan, 2011), synthetic seed formation (Mishra et al., 2011a), hairy root culture and regeneration from hairy roots of *P. kurrooa* (Mishra et al., 2011b; Rawat et al. 2013), however, assessment of secondary metabolites, that is, picrotin and picrotoxinin in tissue culture raised plants of *P. kurrooa* has not reported so far.

Elicitation enhances the accumulation of secondary metabolites and improves the productivity of plant tissue cultures (Zhao et al., 2005; Putalun et al., 2007; Kamonwannasit et al., 2008). It is also reported that, no specific elicitor has a general effect on different plant species and optimum concentration and type of elicitor is likely to vary from species to species (Putalun et al., 2010). A number of elicitors like jasmonates, salicylates, chitosan, yeast extract, ascorbic acid, and fungal culture filtrate (Komaraiah et al., 2002; Walker et al., 2002; Orlita et al., 2008; Karwasara et al., 2011a, b) have been investigated to enhance yield of plant-based secondary metabolites. MeJA has been used as an elicitor for enhancing the production of secondary compounds in plant cell cultures such as anthocyanin in *Melastoma malabathricum* (See et al., 2011) and in hairy roots cultures such as glycyrrhizin in *Glycyrrhiza inflata* (Wongwicha et al., 2011). Putalun et al. (2010) found the yeast extract enhance the production of plumbagin in the roots of *Drosera burmanii* by 3.5-fold when compared to the control plants. Similarly, Chandra and Chandra (2011) reported that elicitation with yeast extract increased decursin accumulation by 3-fold in the roots of *Angelica gigas*. Moreover, yeast extract significantly increased the intracellular content of both scopolamine and hyoscyamine in roots of *Brugmansia candida* (Pitta-Alvarez et al., 2000).

In view of this, *in vitro P. kurrooa* cultures looked favourable for the production of secondary metabolites. The present finding is the first report on the effect of elicitors for the enhancement of picrotin and picrotoxinin production from genetically stable tissue culture raised plants of *P. kurrooa*.

MATERIALS AND METHODS

Plant material

Runners of *P. kurrooa* Royle ex Benth. (common name: Kutki) were collected from Pindari area (30° 6' to 39° 15' N and 70° 55' to 80° 5' E, 3400 m amsl, District Bageshwar, Uttarakhand) in Kumaun Himalaya, India. These were brought to the laboratory and used to develop *in vitro* cultures of *P. kurrooa* as per published method (Chandra et al., 2004, 2006). *In vitro* proliferating shoot cultures of *P. kurrooa* were used for genetic fidelity analysis and elicitation.

Chemicals

All the chemicals used in tissue culture were purchased from Hi-media. All HPLC grade chemicals, MeJA and yeast extract were purchased from Sigma (Sigma Chemical Company, St. Louis, USA). Chemicals used in genetic fidelity analysis were purchased from Genei (M/s Bangalore Genei, India), whereas RAPD and ISSR primers were purchased from Operon Technologies Inc. (Alameda, California, USA) and University of British Columbia, Biotechnology Laboratory, Vancouver, Canada, respectively.

Genetic stability analysis

DNA was isolated from field grown mother plant (MP) and tissue culture raised plants (T1, T2 and T3) randomly taken from three batches.

DNA isolation, RAPD and ISSR fingerprinting

DNA was extracted using N-cetyl-N,N,N-trimethylammonium bromide (CTAB) as described by Doyle and Doyle (1987) with modifications. In brief, fresh leaf material (200 mg) was washed and ground in liquid nitrogen. Then 10 ml of preheated extraction buffer [2% CTAB (w/v), 0.2% β-mercaptoethanol (v/v), 100 mM Tris-HCl (pH 8.0), 20 mM ethylene diamine tetraacetic acid (EDTA) and 1.4 mM NaCl] were added to the powdered material. After incubating the homogenate for 1 h (at 65°C) an equal volume of chloroform : isoamyl alcohol (24:1) was added and centrifuged at 10000 rpm for 20 min. DNA was precipitated with 1/10 volume of 3 M sodium acetate and an equal volume of isopropanol followed by centrifugation at 10000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, air dried and then resuspended in 200-300 µL Tris EDTA (TE; 1 mM). Quantification was performed by visualizing

under UV light, after electrophoresis on 0.8% agarose gel stained with ethidium bromide. This resuspended DNA was then diluted in sterile distilled water to 5 ng μl^{-1} concentration for use in amplification reactions.

A total of 31 random decamer oligonucleotides purchased from was used as single primers for the amplification of RAPD fragments. Polymerase chain reactions (PCRs) were carried out in a final volume of 25 µL containing 20 ng template DNA, 200 µM each deoxynucleotide triphosphate, 20 ng of decanucleotide primers, 1.5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100 and 0.5 U Taq DNA polymerase. Amplification was achieved in a Thermocycler (Biometra; Germany) programmed for a preliminary 5 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 36°C for 1 min and extension at 72°C for 1 min, finally at 72°C for 10 min.

Thirty-three ISSR primers (16-17 mer) randomly selected from primer set 9 were used for PCR amplification. All the amplification conditions were same as described above in RAPD section except annealing temperature which was 52°C for ISSR analysis.

Amplification products were separated by electrophoresis on 1.5% agarose gels run in 1X TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Alpha ImagerTM IS-2200, San Leandro, CA, USA). PCRs were repeated at least twice to establish reproducibility of results.

Data analysis

Each polymorphic band was considered as a binary character and was scored 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. Only intensely stained, unambiguous, and reproducible bands were scored for analysis. Similarity index was estimated using Dice coefficient of similarity (Nei and Li, 1979). Cluster analyses was carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) using Gene Profiler 1-D Phylogenetic analysis and Data basing Software.

Elicitor treatment: Stock preparation and addition of elicitors

Methyl Jasmonate

Methyl Jasmonate (MeJA), a signaling intermediate, was dissolved in 95% ethanol, filter sterilized and tested at a final concentrations of 50, 100, 150, 200 µM.

Yeast extract

Yeast extract was prepared as an aqueous stock (10 mg/mL) having pH 5.7, filter sterilized and tested at 0.5, 1.0, 1.5, 2.0 mg/l concentrations.

Four weeks old *in vitro* established plants were used to check the effect of elicitation on secondary metabolite production. Plantlets were maintained in MS (Murashige and Skoog, 1962) liquid medium at 25 ± 1°C under 16 h light/day with agitation (80 rpm). Elicitors were added to 28-day-old well rooted plant cultures, which were harvested after elicitor treatment at 5 and 10 days. Each experiment was done in triplicate. Untreated tissue culture raised plants of the same age were used as control.

Extraction of active ingredients

In-vitro raised plants were used to extract picrotin and picrotoxinin. The fresh weight and dry weight after lyophilization were determined. The powdered samples (0.5 g DW) were extracted with 100 ml of 70% ethanol in a soxhlet apparatus, and then dried crude extract was dissolved in 10 ml of water:methanol:isopropanol: acetonitrile (60:30:5:5) and quantification by HPLC was done using standards (Purohit et al., 2008; Mishra et al., 2011b). A standard curve was prepared by 10, 20, 30, 40 and 50 mg/L solutions of picrotin and picrotoxin standard (Sigma Chemical Company, St. Louis, USA).

HPLC analysis

Purified samples (20 µL) were analyzed in a HPLC system (LaChrom, L-7100, Merck Hitachi, Japan; Column RP-18e; 5 µm; Purosphare Star, Merck). Samples were eluted in isocratic mode with water:methanol:isopropanol:acetonitrile (60:30:5:5) at a flow rate of 0.5 ml/min. Detection was done at 220 nm using UV detector L 7400. The calculations of picrotin and picrotoxinin were carried out on peak area basis using standard curves made with reference standard compound (Mishra et al. 2011b).

Statistical analysis

Results were reported as the mean ± standard deviation (SD) and analyzed by the least significant difference (LSD) test. Differences with $P<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Elicitor treatment

In the present work attempts were made for the improvement in picrotin and picrotoxinin production by means of elicitor treatment to the genetically stable *in vitro* culture of *P. kurrooa* (Figures 2 and 3). MeJA and yeast extract were tested at varying concentrations.

Figure 2 shows effect of various concentration of MeJA (50, 100, 150 and 200 µM) on picrotin (Figure 2A) and picrotoxinin (Figure 2B) production. A direct correlation between incubation time (5 or 10 days) and the yield of picrotin was observed. Significant increase was observed in picrotin production after 10 days treatment of MeJA. MeJA at 50 µM gave the highest concentration of picrotin (1.56 ± 0.2 µg/g dry wt) which was 2.26-fold higher than the control plants (0.69 µg/g dry wt). In case of picrotoxinin, no significant difference was observed in cultures treated with MeJA at day 5. Maximum production (38.4 ± 2.2 µg/g dry wt) was recorded with 50 µM MeJA whereas; minimum production (30.4 ± 2.4 µg/g dry wt) was recorded with 200 µM concentration of MeJA at day 10 (Figure 2B). The results showed that various concentrations of MeJA (50-200 µM) have different eliciting influences. Similar results have been reported by Loc et al. (2014).

It has already been reported that exogenous application of MeJA to the plant cell culture or intact plant is known to stimulate biosynthesis of secondary metabolites (Mueller et al., 1993; Fang et al., 1999). Jasmonates are known as signalling intermediates in the

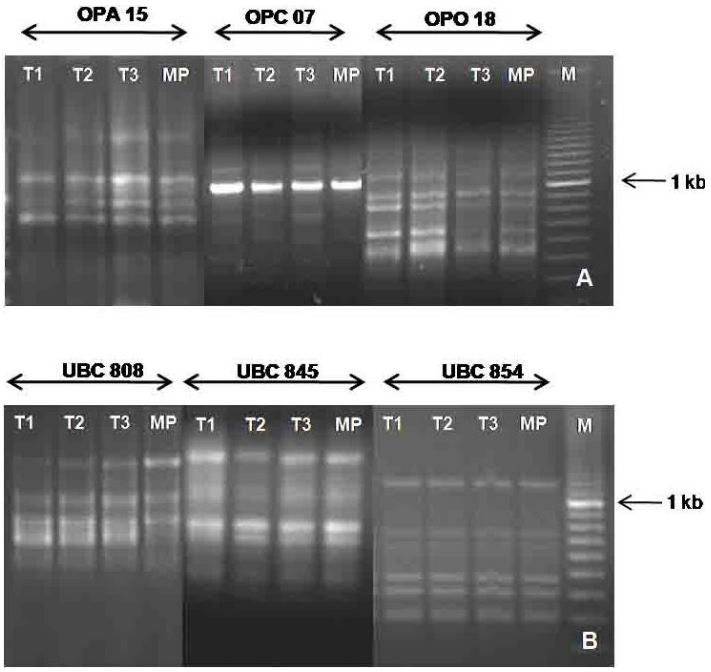

Figure 1. Assessment of genetic fidelity of tissue culture products with their parent *Picrorhiza kurrooa* A. RAPD amplification profiles with primer OPA 15 OPC 07 and OPO 18; *M* is molecular weight marker (3kb); T1-T3 tissue culture raised plants; MP is mother plant B. ISSR amplification profiles with primer UBC-808, UBC 845 and UBC-854; *M* is molecular weight marker (3kb); T1-T3 tissue culture raised plants; MP is mother plant.

wound and/or elicitor-activated expression of plant defence genes in parsley (*Petroselinum crispum*) cell cultures and transgenic tobacco (*Nicotiana tabacum*) plants (Ellard-Ivey and Douglas, 1996; Robert and John, 1997). It has also reported that MeJA stimulated a multi-component defence response in leaves and suspension-cultured cells of *Vitis vinifera* and enhanced production of artemisinin in *Artemisia annua* cell culture (Repka et al., 2001; Baldi and Dixit, 2008). Shohael et al. (2007) reported MeJA induced over-production of eleutherosides in somatic embryos of *Eleutherococcus senticosus* cultures in bioreactors.

Effect of yeast extract (0.5, 1.0, 1.5 and 2.0 mg/ml) was examined on picrotin and picrotoxinin production in *P. Kurrooa* (Figure 3). Significant increase was observed in picrotin production after 10 days treatment of yeast extract (Figure 3A). Maximum increment in picrotin (2.47 ± 0.2 µg/g dry wt, 3.8 fold) was recorded with 0.5 mg/ml yeast extract. Results showed that picrotin accumulation decreased at higher concentrations of yeast extract (1.0-2.0 mg/ml). Effect of yeast extract on picrotoxinin production is presented in Figure 3B. A clear response of plant was observed by yeast extract after 10 days (Figure 3B) for picrotoxinin production. Yeast extract at 0.5 mg/ml showed the highest influence on picrotoxinin production (45.2 ± 5.2 µg/g dry wt) which was 1.88 fold higher than the control plants. Minimum production of picrotoxinin was recorded with 2.0 mg/ml (32.0 ± 3.1 µg/g dry wt).

Results of the study are similar to those of Putalun et al. (2010), Goyal and Ramawat (2008), Turgut-Kara and Ari (2011) and Cai et al. (2012), who reported elicitation efficiency of yeast extract on the biosynthesis of isoflavonoids, cytochrome P450 and anthocyanin, and phenolic acid respectively. Wilczańska-Barska (2012) reported that yeast extract (50 mg/mL) increased acteoside production by 1.4-fold and flavone production by 1.7-fold after 7 and 14 days of elicitation. Among various carbon and nitrogen sources, glucose, peptone and yeast extract were found to be the most favourable for palmarumycin C^{13} production (Zhao et al., 2013). The probable reason for the effects of yeast extract on the enhancement of plant based secondary metabolites could be attributed to its complex composition and the presence of cations like Zn, Ca, and Co (Suzuki et al., 1985).

Analysis of genetic fidelity by RAPD and ISSR

Initial screening of 31 decamer RAPD primers resulted in selection of 16 oligonucleotides, which produced clear amplification products. Each primer produced a unique set of amplification products ranging in size from 0.3 to

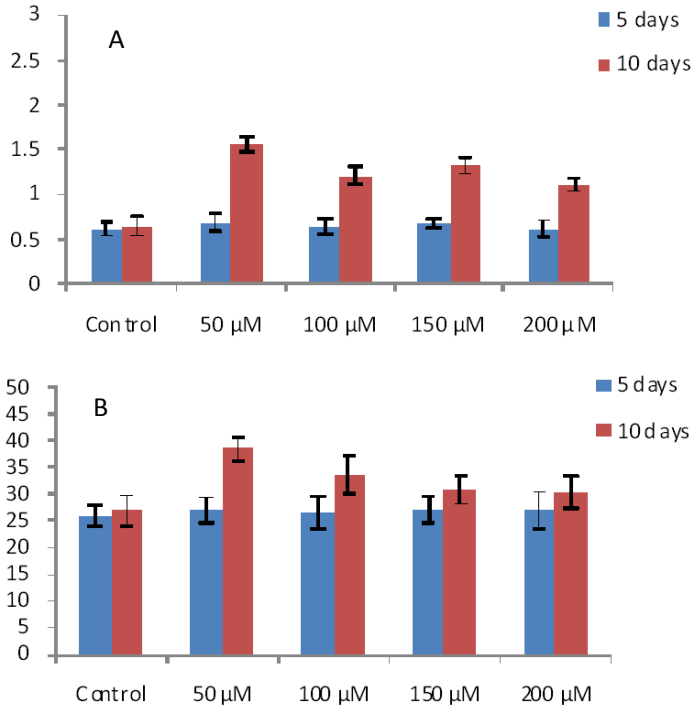

Figure 2. Effect of methyljasmonate on concentration of picrotin (A) and picrotoxinin (B) content of *P. kurrooa.*

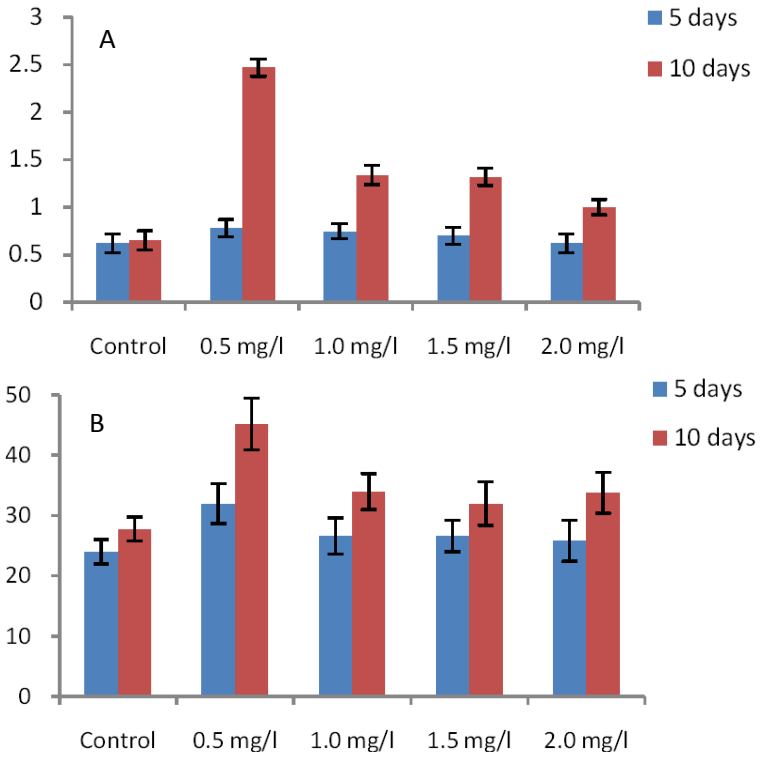

Figure 3. Effect of yeast extract on concentration of picrotin (A) and picrotoxinin (B) content of *P. kurrooa.*

Table 1. Total number and size range of amplified fragments and number of polymorphic fragments generated by 16 random primers by RAPD analysis.

Primer	Primer sequence	Total No. of amplified products	No. of polymorphic bands	Size range (kb)	% polymorphic bands
OPA-2	5' TGCCGAGCTG 3'	4	0	0.3-2.4	0
OPA-3	5'AGTCAGCCAC 3'	5	0	0.6-2.9	0
OPA-7	5'GAAACGGGTG 3'	4	0	0.5-1.6	0
OPA-10	5'GTGATCGCAG 3'	4	0	1.0-1.3	0
OPA-11	5'CAATCGCCGT 3'	7	0	0.5-2.4	0
OPA-15	5'TTCCGAACCC 3'	5	1	0.6-2.8	20
OPA-16	5'AGCCAGCGAA 3'	5	0	0.5-1.6	0
OPA-19	5'CAAACGTCGG 3'	8	0	0.5-1.8	0
OPC-2	5'GTGAGGCGTC 3'	8	0	1.0-1.3	0
OPC-7	5'GTCCCGACGA 3'	1	0	0.5-2.8	0
OPC-13	5'AAGCCTCGTC 3'	8	0	0.4-1.2	0
OPC-16	5'CACACTCCAG 3'	5	1	0.5-1.8	20
OPC-18	5'TGAGTGGGTG 3'	8	0	0.4-1.9	0
OPC-19	5'GTTGCCAGCC 3'	2	0	0.3-2.4	0
OPO-15	5'TGGCGTCCTT 3'	3	0	0.5-1.8	0
OPO-18	5'CTCGCTATCC 3'	5	0	0.3-2.4	0
Total		82	2	0.3-2.9	2.5

Table 2. Total number and size range of amplified fragments and number of polymorphic fragments generated by 12 primers by ISSR analysis.

Primer	Primer sequence	Total No. of amplified products	No. of polymorphic bands	Size range (bp)	% polymorphic bands
UBC-803	(AT)8 C	2	0	0.5-2.7	0
UBC-808	(AG)8 C	4	1	0.5-2.7	25
UBC-811	(GA)8 C	4	0	0.6-2.9	0
UBC-820	(GT)8 C	5	2	0.3-2.9	40
UBC-822	(TC)8 A	8	0	0.6-2.9	0
UBC-823	(TC)8 C	4	0	0.6-1.5	0
UBC-827	(AC)8 G	6	0	0.2-2.4	0
UBC-830	(TG)8 G	5	1	0.6-2.9	20
UBC-841	(GA)8 YC	4	0	0.5-2.7	0
UBC-845	(CT)8 RG	5	0	0.6-2.9	0
UBC-849	(GT)8 YA	6	0	0.2-1.4	0
UBC-855	(AC)8 YT	4	0	0.6-2.9	0
Total		57	4	0.2-2.9	7.02

Y= C; T and R= A,G

2.9 kb. The number of bands for each primer varied from 1 (OPC 07) to 8 (OPA-19, OPC-2, OPC-13 and OPC-18; Table 1; Figure 1A). These 16 primers used in this analysis yielded 82 scorable bands with an average of 5.12 bands per primer. Of the 82 fragments scored from these primers 80 were monomorphic and 2 were polymorphic (2.5%) producing similarity values that ranged from 0.97 to 1 with a mean of 0.985.

A total of 12 out of 33 ISSR primers were selected as suitable in terms of reproducibility of bands for the present study. The number of bands for each primer varied from 2 (UBC-803) to 8 (UBC-822) (Table 2; Figure 1B). Twelve primers yielded 57 scorable bands with an average of 4.75 bands per primer. Of the 57 fragments scored from these primers 53 were monomorphic and 4 were polymorphic (7.02%). Similarity values of the samples ranged from 0.96 to 1 with a mean of 0.98. A comparative account of RAPD and ISSR are presented in

Table 3. Summary of RAPD and ISSR amplified products from samples of *P. kurrooa*.

Description	RAPD	ISSR
Total bands scored	82	57
Number of monomorphic bands	80	53
Number of polymorphic bands	2	4
Percentage of polymorphism	2.5	7.02
Number of primers used	16	12
Average polymorphism per primer	0.12	0.33
Average number of fragments per primer	5.12	4.75
Size range of amplified fragments (kb)	0.3-2.9	0.2-2.9

Table 3. Results indicating genetic differences between them were not remarkable. Therefore, in the screening of tissue culture raised plants only chemical components and growth properties of the plants needed consideration. The use of two types of markers, which amplify different regions of the genome, allows better analysis of genetic stability or variability in the tissue culture raised plants.

In conclusion, this study represents the successful *in vitro* culture-based approach for the production of picrotin and picrotoxinin from *P. kurrooa*. Yeast extract at 0.5 mg/mL was the most effective treatment for the enhancement of picrotin and picrotoxinin. Our findings indicate that the application of elicitors can enhance the capacity of *P. kurrooa* cultures to produce high amount of secondary metabolites for pharmaceutical purposes. The results of the present study can be pooled with other yield enhancement strategies like precursor feeding, chemical and hormonal treatment for further enhancement in secondary metabolite production.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Genetic characterisation of avocado (*Persea americana* Mill.) in two regions of Ghana

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Avocado (*Persea americana* Mill.) is a nutritious and economic tree crop with cultivations scattered all over Ghana. Although, there have been some genetic diversity research on this crop in some parts of the world, rather limited research has been done on avocado in Ghana. A study was therefore conducted to assess its genetic diversity in the Ashanti and Central Regions of Ghana. Microsatellites analyses revealed 115 different amplification fragments ranging from 5 to 22 alleles per locus, with an average of 11.5 alleles per locus. All the microsatellites were highly informative with both genetic diversity and polymorphic informative content (PIC) higher than 0.5. Using the unweighted pair group method with arithmetic averages (UPGMA), the genotypes were clustered into groups. The wide genetic diversity among the accessions indicates that there is a wide genetic base for improvement of the crop through breeding and selection in Ghana.

Key words: Amplification fragments, polymorphic information content, accessions microsatellites, alleles.

INTRODUCTION

The conservation of crops in the world crucially depends on the knowledge of their genetic diversity. Molecular markers, such as simple sequence repeats (SSRs), reveal diversity at the DNA level; and thus provide a fundamental tool for germplasm conservation and genotyping. Avocado is one of the highly marginised crops in Ghana though it has a very high nutritive value and a good potential for commercial production (Campbell and Malo, 1976; Morton, 1987; Verheij and Coronel, 1991; FAOSTAT Database, 2001). There have been very few studies that investigated alternative ways of improving the production and conservation of Avocado in Ghana, in contrast to the increasing attention given to the crop elsewhere (Knight, 2002). Consequently, we are witnessing indiscriminate cutting of this important resource in Ghana due to the low profile given to it.

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Traditionally, morphological characters are used to identify the crop for selection and breeding. However, the efficiency of morphological traits has been mixed. Whilst morphological traits have proved useful in distinguishing between species in many cases (Rhodes et al., 1971; Morton, 1987), recent studies reveal that morphological characterisation is not always very informative because the traits can be altered by environmental conditions and thus mislead species identification (Bergh et al., 1973; Furnier et al., 1990; Gutiérrez-Diez et al., 2009). The use of model genetic systems in plant and animal studies nowadays represent a more reliable alternative way that greatly increases our understanding of how genomes regulate phenotype.

The avocado plant, *Persea americana* Mill. (Lauraceae), is indigenous to Central and South America notably Mexico and has developed varieties, which have adapted to a wide range of climatic conditions including those of Asia and Africa (Bergh, 1969; Rhodes et al., 1971). Three botanical varieties of avocado have traditionally been recognised; namely, Mexican, Guatemalan and West Indian. These varieties are distinguishable on the basis of morphological, physiological, and horticultural traits; and they are adapted to different climates and ecological conditions (Bergh, 1995; Bergh and Lahav, 1996). They also correspond to the varieties *P. americana* var. *drymifolia* (Schlecht. *et* Cham.) Blake, *P. americana* var. *guatemalensis* Williams. and *P. americana* var. *americana* Mill., respectively, (Bergh et al., 1973; Bergh, 1995). Several attempts have been made to refine the classification of avocado such as numerical taxonomical methods based on morphological characters (Rhodes et al., 1971). Such methods revealed that cultivars tended to cluster into three groups, representing three varieties (Rhodes et al., 1971).

Further studies applied genetic-based approach such as restriction fragment length polymorphism (RFLP) of chloroplast DNA, ribosomal DNA, and the genes coding for the enzyme cellulase to infer a phylogeny of avocado (Furnier et al., 1990). In that instance, the results lent some support to the current classification which placed the varieties *drymifolia*, *guatemalensis* and *americana* in a single species *P. americana* (Bergh et al., 1973). More recent studies have used the markers of minisatellite and microsatellite DNA to characterise and differentiate botanical varieties of avocado (Lavi et al., 1991; Mhameed et al., 1997; Acheampong et al., 2008).

Microsatellites are said to be highly polymorphic and useful as genetic markers that have been used in defining genetic similarities in crops such as maize, sorghum and wheat (Röder et al., 1995; Smith et al., 1997; Taramino et al., 1997; Uptmoor et al., 2003; Menz et al., 2004). While both DNA fingerprints (DFP) and SSR markers were used to estimate the heterozygosity level in the avocado genome and to define genetic relationships in the *Persea* genus (Mhameed et al., 1996; 1997), none of these markers has so far been used to construct a genetic linkage map of the avocado genome (Sharon et al., 1997) and this does not give a pictorial view of the relationships within avocados. Moreover, most of the research done on avocados are concentrated in the developed world like the United States of America and Israel (Morton, 1987; Furnier et al., 1990; Schnell et al., 2003), whilst we know very little about their genetic diversity in the developing and third world countries like Ghana. Our study aimed at defining the genetic variations between avocado accessions in Ghana using microsatellite. We therefore used the potential of SSRs to track pollen movement (Queller et al., 1993; Jarne and Lagoda, 1996; Goldstein and Pollock, 1997; Sunnucks, 2000) and their suitability for studying genealogical relationships (Goldstein et al., 1995) to characterise the avocados in Ashanti and Central Regions of Ghana for the first time.

MATERIALS AND METHODS

We sampled materials from healthy leaves of 71 avocado plants in the Ashanti and Central Regions of Ghana, as well in the vicinity of the Cocoa Research Institute of Ghana (CRIG) in New Tafo in the Eastern region of Ghana. Thirteen avocado plants out of the total of 71 used were from the avocado farm of CRIG. The 13 plants from the CRIG avocado farm were mostly of West Indian *(P. americana var. americana.)* and the Guatemalan (*P. americana* var. *guatemalensis* Williams) varieties. These were used as controls in this study.

Genomic DNA extraction

DNA was extracted following a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Aldrich and Cullis, 1993). Total DNA was washed in 1 ml washing buffer made of 76% ethanol and 10 mM ammonium acetate, and again in 1 ml ethanol (80%). It was then stored at -20˚C. Two microliters of each of the genomic DNA samples was dissolved in 3 µl of TE [1 mM Tris HCl (pH 8), 0.1 m M EDTA (pH 8)] buffer and 1 µl of 6X sample buffer (Bromophenol blue and xyelne cyanol) was added. We used 25 ng/µl of DNA for polymerase chain reaction (PCR).

SSR markers

Ten (10) pairs of microsatellite primers designed by Sharon et al. (1997), and later applied by Schnell et al. (2003) (Table 1) were used for the PCR.

PCR amplification and electrophoresis

We performed amplifications using 16 to 50 ng of genomic DNA as template, 0.5 µM of each of the forward and reverse primers, in a 10 µl reaction volume using the AccuPower^{TM} PCR PreMix (USA Bioneer Inc., Alameda, USA) (DNA polymerase, DNTPs, a tracking dye, and reaction buffer in a premixed format). The PCR reaction was carried out on 2720 Thermal Cycler (Applied Biosystems, Singapore) and consisted of the following cycle: 3 min denaturation at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, 1 min at appropriate primer annealing temperature (Table 1), 1 min extension at 72°C. The amplification finished with an extension at

Table 1. Repeat motifs and primer sequences of the microsatellite loci.

Locus	Repeat Motif	Primer Sequence (5' - 3')	Anneal Temp (°C)
AVAC01	$(TG)_{15}$	F:CTGGTTGCTCTCTTGTCTACATAATA R: CGGTTTTGTAAGTTGATAG	40
AVAG03	$(TC)_{17}$	F: GCACTTCCTAAACTTGCAGGT R: CTGAACATCCAATGACAAACATCC	45
AVMIX04	$(AG)_{12},(CAA)_5,(ACAG)_{10}$	F: CCGTTTGCTTCCTGTATC R: GTTATCCCTTCCACTTTC	50
AVAG05	$(AG)_{10}$	F: GGATCTGATGTGTGGGGGAG R:CCTGTCGGAAAAGACTATGCG	45
AVAG06	$(CT)_{18}$	F: CGACCTCTTCTTATACTC R: GTACCTCTGATAATGAGCAT	40
AVAG10	$(CT)_{22}$	F: GAATTACAAAGCACTAGAG R: GTAGAAAGTGGGCACACAT	45
AVAG13	$(CT)_{18}$	F: CTGCGATAACAACTGGAC R: AACTAGGACCTGAAACCG	50
AVAG 21	$(CT)_{22}$	F: TGTAAGTTTTAACCCCACAA R: AATCACTATTAGAGTTTTCAGTCG	50
AVAG22	$(GA)_{15}$	F: GATCATCAAGTCCTCCTTGG R: GATCTCATAGTCCAAATAATGC	55
AVAG25	$(TC)_{14}$	F: ATGGTTTTTTCCTGCCCTTT R: AACAAGCCCCCTAAAAGAA	50

Source: Adapted from Sharon et al. (1997).

72°C for 10 min, followed by maintenance of the reaction mixture at 4°C at infinity until removed for storage at -20°C.

Electrophoresis

Electrophoresis was done using 3 µl of denaturing buffer (95% formamide, 0.02 M EDTA pH 8, 1% bromophenol blue, 1% xylene cyanol, 10 mM NaOH) added to 3 µl of the PCR products. Equal volume of the denaturing buffer was added to 3 µl DNA ladder (10 bp ladder, diluted to 0.1 µg/µl in doubled distilled water). The mixture was denatured at 95°C for 5 min and then immediately chilled on ice. Each was loaded into a well of a 49-well plate (4 mm thick) for electrophoresis on a DNA sequencing gel containing 6% polyacrylamide, 8 M urea and 1 X TBE (Tris-Boric acid-EDTA buffer). Gels were run at 100 W or V constant power and 2 kV for 2 to 2.5 h, using a Bio-Rad Sequi-Gen® GT Nucleic Acid Electrophoresis Cell (Bio-Rad, Consult EG 261, Belgium) and power pack (Bio-Rad Power Pac 300) (Bio-Rad, Consult EG 261, Belgium) and 1 X TBE as running buffer. The products were visualised by silver staining using the method described by Bassam et al. (1991).

Data analysis

For each gel, the distance migrated by each marker size of the DNA ladder was measured using a ruler. A line graph of the distance travelled by the marker size was plotted for each gel; the equation of the relationship between them was then used to estimate the size of the unknown SSR bands of the PCR products. Allele size and the total numbers of alleles were determined for each SSR locus. Bands for same SSR locus with different molecular weight were scored as alleles. The scored alleles were coded using FlexiBin (Bill Amos, Cambridge, UK; http://www.zoo.cam.ac.uk/departments/molecularecology/FlexiBin.pdf) analysis. Gene diversity values for each locus and the average across all loci for all populations were calculated using Nei's unbiased estimate (Nei, 1973). The numbers of alleles and the allelic frequencies for each SSR, and across all populations as well as the unbiased gene diversity (H_{nb}), and the observed heterozygosity (H_{obs}), for all populations were estimated using PowerMarker version 3.25 (Liu and Muse, 2004). A phylogenetic tree was constructed for all 71 individuals genotyped following the Unweighted Paired Group Method (UPGMA) (Sneath and Sokal, 1973). Following this, dendrograms of genetic relationships among the genotype studied were produced using PHYLIP version 3.5 and TreeView version 1.6.6 (Saitou and Nei, 1987; Felsenstein, 1989).

RESULTS

DNA analysis and microsatellite typing

The 10 microsatellites loci had varying degrees of polymorphism, generating 115 alleles across the population sampled (Table 2). The number of alleles varied from five in AVAG06 to 22 in AVAG21; with an average of 11.5 alleles per locus. All SSR loci used were polymorphic. The average gene diversity was 0.7529 (range = 0.5636 to 0.8907) (Tables 3 and 4). The number, size and frequencies of alleles, observed heterozygosity (Hobs) number of homozygotes, heterozygotes and null alleles across all loci are summarised (Table 3). Allele size ranged from 71 (AVAG06) to 225 bp (AVAG21), with allele frequencies varying between 0.70 (AVOC01) and 64.29% (AVGA13) (Table 2). The allelic

Table 2. Allele size (bp) and percentage frequencies (in brackets) of loci for the 71 avocado samples.

Allele No.	AVAC01	AVAG03	AVMIX04	AVGA05	AVAG06	AVAG10	AVAG13	AVAG21	AVAG22	AVAG25
1	102 (0.72)	98 (0.79)	107 (0.88)	93 (4.62)	71 (18.25)	122 (4.76)	96 (0.79)	157 (0.70)	104 (1.41)	80 (0.79)
2	110 (2.90)	99 (3.97)	162 (6.14)	95 (3.85)	73 (52.38)	128 (3.17)	97 (10.32)	163 (0.70)	107 (4.23)	100 (0.79)
3	111 (7.25)	103 (1.59)	164. (14.91)	97 (34.62)	75 (3.17)	161 (2.38)	99 (1.59)	168 (1.41)	108 (23.94)	102 (05.56)
4	113 (35.51)	107 (41.27)	166 (5.26)	99 (36.15)	77 (25.40)	162 (1.59)	101 (7.94)	169 (2.11)	110 (21.82)	103 (23.02)
5	115 (28.26)	108 (43.65)	168 (1.75)	101 (2.31)	79 (0.79)	173 (0.79)	103 (64.29)	173 (0.70)	116 (2.11)	114 (0.79)
6	121 (3.62)	110 (0.79)	170 (1.75)	107 (0.77)		175 (25.40)	112 (1.59)	175 (1.41)	118 (5.63)	116 (7.14)
7	123 (15.94)	113 (1.59)	172 (2.63)	117 (0.77)		176 (30.16)	120 (3.14)	177 (9.86)	120 (33.10)	118 (1.59)
8	125 (2.90)	114 (3.97)	173 (14.04)	121 (16.92)		178 (4.76)	122 (4.76)	179 (18.31)	121 (1.41)	120 (15.87)
9	127 (1.45)	116 (0.79)	174 (17.54)			181 (0.79)	126 (4.76)	181 (8.45)	123 (3.52)	122 (7.14)
10	129 (1.45)	122 (1.59)	177 (8.77)			190 (3.97)	132 (0.79)	182 (1.41)	124 (2.82)	131 (3.97)
11			178 (10.53)			194 (15.08)		191 (4.93)		134 (1.59)
12			180 (12.28)			195 (5.56)		195 (2.11)		138 (18.25)
13			186 (3.51)			197 (1.59)		203 (1.41)		139 (11.11)
14								207 (4.23)		144 (2.38)
15								209 (2.11)		
16								211 (5.63)		
17								213 (7.04)		
18								214 (0.70)		
19								216 (4.93)		
20								218 (20.42)		
21								220 (0.70)		
22								225 (0.70)		

Table 3. Characteristics of the SSR loci among avocado populations in Ashanti and Central Regions of Ghana.

SSR (Locus)	No. of alleles	Range of allele size (bp)	Range of allele frequencies (%)	$^1H_{obs}$	No. of homozygotes	No. of heterozygotes	No. of null amplifications
AVAC01	10	102-129	0.72 - 35.51	0.4058	40	29	2
AVAG03	10	98-122	0.79 - 43.65	0.4603	34	29	8
AVMIX04	13	107-186	0.88 - 17.54	0.5614	25	32	14
AVAG05	8	94-122	0.77 - 36.15	0.3538	40	24	6
AVAG06	5	71-80	0.79 - 52.38	0.4603	34	30	8
AVAG10	13	122-197	0.79 - 30.16	0.3333	42	21	8
AVAG13	10	96-132	0.79 - 64.29	0.3968	38	25	8
AVAG21	22	157-225	0.70 - 20.42	0.6761	23	48	0
AVAG22	10	104-124	1.41 - 33.10	0.4507	39	32	0
AVAG25	14	80-144	0.79 - 23.02	0.6667	21	42	8
Mean	11.5			0.4765	33.6	31.2	6.2

[1]Observed heterozygosity

frequencies at the different SSR loci varied significantly (Table 3). Across all the accessions screened, 336 (47.3%) loci were homozygous, while 312 (43.9%) loci were heterozygous. The highest H_{obs} (H_{obs} = 0.6761) was recorded at the AVAG21 locus, whereas the lowest (H_{obs} = 0.3333) occurred at the locus AVAG10. The overall mean H_{obs} of all populations, calculated across all loci was 0.4765. A total of 180 genotypes and 115 alleles were detected; with a mean of 11.5 per locus (Table 4). Unbiased gene diversity (H_{nb}) ranged from 0.5636 to 0.8907 with observed heterozygosity (H_{obs}) ranging from 0.3333 to 0.6761 (Table 4).

The 10 SSRs used were all highly significant (AVAC 01: χ^2 = 191.0, *d.f.* = 45, $P < 0.001$; AVAG 03: χ^2 = 166.9, *d.f.* = 45, $P < 0.001$; AVMIX 04: χ^2 = 196.9, *d.f.* = 78, $P < 0.001$; AVAG 05: χ^2 = 88.7, *d.f.* = 28, $P < 0.001$; AVAG 06: χ^2 = 81.8, *d.f.* = 10, $P < 0.001$; AVAG 10: χ^2 = 339.3, *d.f.* = 78, $P < 0.001$; AVAG 13: χ^2 262.8, *d.f.* = 45, $P <$

Table 4. The major allele frequency, genotype number, gene diversity, heterozygosity and polymorphic information content (PIC) of SSR markers used for genetic diversity analysis.

Marker	Major allele frequency	Genotype number	Number observed	No. of alleles	Availability	Gene diversity	Hetero-zygosity	PIC in this study	SMM Index	F
AVAC 01	0.3551	14	69	10	0.9718	0.7599	0.4058	0.7252	< 0.0001	0.4717
AVAG 03	0.4365	13	63	10	0.8873	0.6350	0.4603	0.5672	< 0.0001	0.2825
AVMIX 04	0.1754	23	57	13	0.8028	0.8843	0.5614	0.8731	< 0.0001	0.3728
AVAG 05	0.3615	11	65	8	0.9155	0.7166	0.3538	0.6685	< 0.0001	0.5119
AVAG 06	0.5238	8	63	5	0.8873	0.6267	0.4603	0.5680	< 0.0001	0.2729
AVAG 10	0.3016	20	63	13	0.8873	0.8104	0.3333	0.7874	< 0.0001	0.5939
AVAG 13	0.6429	12	63	10	0.8873	0.5636	0.3968	0.5441	< 0.0001	0.3032
AVAG 21	0.2042	35	71	22	1.0000	0.8907	0.6761	0.8818	< 0.0001	0.2476
AVAG 22	0.3310	18	71	10	1.0000	0.7822	0.4507	0.7513	< 0.0001	0.4296
AVAG 25	0.2302	26	63	14	0.8873	0.8600	0.6667	0.8452	< 0.0001	0.2324
Mean	0.3555	18	64.8	11.5	0.9127	0.7529	0.4765	0.7212	< 0.0001	0.3739

0.001; AVAG 21: χ^2 = 468.6, *d.f.* = 231, *P* < 0.001; AVAG 22: χ^2 = 198.3, *d.f.* = 55, *P* < 0.001; AVAG 25: χ^2 = 156.9, *d.f.* = 91, *P* < 0.001). The polymorphism information content (PIC) value ranged from 0.5441 to 0.8818 with a mean of 0.7212. The proportion of loci with PIC value from 0.5 and above was 100%. We found that 70% of the accessions had PIC values of 0.6 and beyond. The Stepwise Mutation Model Index (SMMIndex) was highly significant, with mean f value of 0.3739.

Phylogenetic analyses

The set of markers used uniquely classified 71 individual plants in this study; it also illustrated the considerable genetic diversity that was present. The resultant dendrogram (Figure 1) defined seven distinct groups. The most genetically distinct genotype was As 37; this did not cluster with any other accession. The largest group consisted of 27 genotypes. The second distinct group had Cr 49 and As 11 at the ends.

DISCUSSION

SSR polymorphism and genetic diversity

Forty percent (40%) of the alleles scored fall outside the range of sizes previously scored and varied by one base pair more than the ones previously confirmed (Schnell et al., 2003). A high level of polymorphism was obtained in most of the loci studied, since 8 of the 10 loci revealed 10 or more alleles in the accessions analysed. The features of the locus of AVAG 21 were not much different from that of an earlier work in which it was reported to be one of the most polymorphic loci after genotyping 258 accessions (Schnell et al., 2003). The average of 11.5 allele / locus in this study was similar to the 10.4 alleles / locus reported by Ashworth and Clegg (2003), where 25 SSRs loci and 180 genotypes were used. However, the similarity obtained in this study is significantly higher than the 37 genotypes obtained in another study (Alcaraz and Hormaza, 2007). This implies that, there is a greater diversity between the avocados studies. In Schnell et al. (2003), 256 alleles were identified from the 14 SSRs loci used, ranging from 8 to 30 per locus and an average of 18.8 from the 14 SSRs was obtained. These results confirmed that the SSRs are highly polymorphic. The difference observed between Schnell et al. (2003) and the present study might be due to differences in the number of the accessions used. The observed heterozygosity (H_{obs}) value calculated from our study ranged from 0.333 in AVAG 10, to 0.6761 in AVAG 21. The average H_{obs} was 0.4765 which is lower than the 0.64 obtained by Schnell et al. (2003) indicating a narrower genetic diversity for the populations analysed. Unique alleles were identified within some samples (Table 2), but their frequencies were too low to allow any meaningful inferences.

Polymorphic information contents (PIC) has been found to be comparable between SSRs RFLPs and Amplified Fragment Length Polymorphisms (AFLPs) or even higher PIC value for SSRs (Smith et al., 1997; Pejie et al., 1998; Menz et al., 2004). The mean PIC and mean number of alleles obtained in our study confirms the findings of Smith et al. (1997), Pejie et al. (1998) and Menz et al. (2004). Almost all the exotic hybrids from CRIG clustered with others from the same parental line. The parental lines of avocado are the Mexican, West Indian, and Guatemalan varieties or hybrids of any two of the three varieties. The introduced hybrids from the CRIG avocado farm used in this study were predominantly of West Indian origin with one (Nabal) from a Guatemalan parent and a few inter hybrid varieties. Some of the accessions from the study area clustered with some of the varieties from CRIG (Figure 1). K'dua was found to be genetically

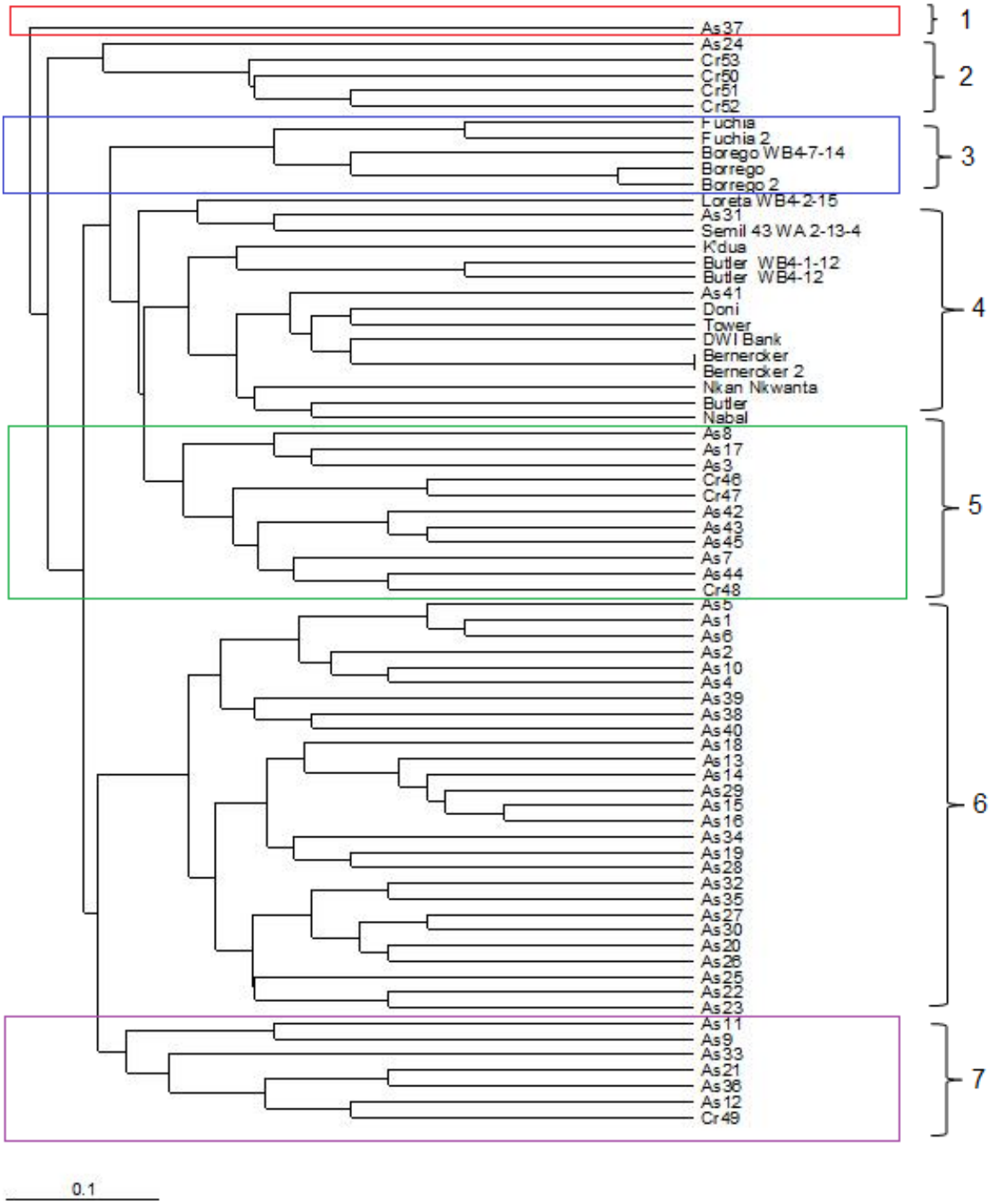

Figure 1. Unweighted Paired Group Method of avocado genotypes, using 10 microsatellite markers. Accessions preceded by "As" are collections from the Ashanti region of Ghana and those preceded by "Cr" were collected from the Central region of Ghana. The Accessions "K'dua" and "Nkan Nkwanta" are collections from the Cocoa Research Institute of Ghana farm. All others are standardised accessions from Israel.

similar to Butler WB4-1-12, Butler WB4-12, DWI Bank and Doni from West Indian parents and developed in the USA. Nabal, a Guatemalan hybrid, was also highly related to the Nkrankwanta variety, As 8 and As 17 from Ghana. This indicates that there are some plants in the study area which are West Indian and Guatemalan hybrids, thus suggesting that there might be some West Indian and Guatemalan hybrids in Ghana as previously

indicated by Taah et al. (2003). Loreta WB4-2-15 was also found to be similar to As 31, whereas As 41 shared similar alleles with Doni, Tower and DWI Bank. These developments suggest that there are a number of unknown accessions of avocados in the study area which should be further studied and analysed. The phylogenetic tree suggests a wide genetic variation among the accessions genotyped. The phylogenetic tree showed highly variable levels of genetic differences between their groups.

The genetic diversity analysis shows that the SSRs used were highly polymorphic in structure. There is a wide range of diversity between the accessions which might have resulted from cross pollination and genetic mutations. Early missionaries to Ghana might have introduced mostly West Indian varieties into the country. However, there are a few Guatemalan hybrids and some hybrid varieties of the West Indian varieties in all areas where plant materials were taken for this study. There is a high probability that, seeds of the same accessions might have been used as propagules by migratory farmers and that the accessions in the Ashanti and Central regions of Ghana might be the same as the ones introduced over a century ago to Ghana by early missionaries.

Conclusion

In conclusion, our study provides insights into the molecular characterisation of avocado in the Ashanti and Central regions of Ghana, paving the way for further molecular genetic investigations to determine the specific avocado accessions found in Ghana.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Fusarium branch blight on highbush blueberry in Argentina

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In Argentina, highbush blueberry (*Vaccinium corymbosum*) is grown in Tucumán, Entre Ríos and Buenos Aires provinces. In the 2010 to 2011 cropping season, a new disease with 10% incidence was observed on the foliage of "Emerald" plants in Concordia, Entre Rios. Symptoms included acropetal blight and premature branch death. Leaves remained attached and showed tan to brown discoloration. A fungal species was isolated from diseased plant parts and identified as belonging to *Fusarium* section Gibbosum. Based on its growth and pigmentation on potato-dextrose-agar, and characters on carnation leaf-piece agar and Spezieller Nährstoffarmer agar, the strain was identified as *Fusarium acuminatum.* Additionally, the internal transcribed spacer (ITS) regions of nuclear ribosomal genes were amplified by PCR, sequenced and the DNA sequence was compared with those in GenBank. The NCBI-Blastn search showed 100% identity of the DNA sequence with GenBank Accession No U85533 sequence for *F. acuminatum*. Pathogenicity was confirmed on wounded branches of potted blueberry plants. To our knowledge, this is the first report of *F. acuminatum* causing branch blight on highbush blueberry in Argentina and worldwide.

Key words: Fruit crops, berries, *Vaccinium corymbosum* L., fungi, DNA sequence.

INTRODUCTION

Blueberries are traditionally consumed in North America and European countries which cannot satisfy their demand from October to November (Bañados, 2009). Consequently, fruit production in the Southern Hemisphere constitutes a profitable export activity. In Argentina, highbush blueberry (*Vaccinium corymbosum* L., Ericaceae) crops are mainly located in the provinces of Tucumán, Entre Ríos and Buenos Aires. The district of Concordia, Entre Ríos, reaches 37% of the national planting area (Argentinean Blueberry Committee, 2012). The exportation of fresh and frozen blueberries in 2012 reached 19,993 t with peaks in October and November (Ministerio de Agricultura, Ganadería y Pesca, 2014). Since 1995, researchers of the University of Buenos

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Aires conduct crop health surveys in that area leading to the characterization of several plant pathogens. The list of fungi already identified includes *Pestalotiopsis guepinii* (Desm.)

Steyaert (Wright et al., 1998), *Glomerella cingulata* (Stoneman) Spauld. and H. Schrenk (Wright et al., 1998; Pérez et al., 2012), *Alternaria tenuissima* (Kunze) Wiltshire (Wright et al., 2004), *Botrytis cinerea* Pers. (Vasquez et al., 2007), *Nigrospora sphaerica* (Sacc.) E.W. Mason (syn=*Khuskia oryzae* H.J. Huds.) (Wright et al., 2008), *Bipolaris cynodontis* (Marignoni) Shoemaker (syn=*Cochliobolus cynodontis* R.R. Nelson) (Sisterna et al., 2009), *Sclerotinia sclerotiorum* (Lib.) de Bary (Pérez et al., 2011b), *Fusarium solani* (Mart.) Sacc. (Pérez et al., 2007) and *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach and Nirenberg (Pérez et al., 2011a). *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips was the only pathogen reported to cause acropetal blight (Wright et al., 2012). Recently, unusual aerial symptoms appeared not associated to the above mentioned microorganisms. The objective of this study was to identify the etiology of this disease.

MATERIALS AND METHODS

Collection of samples

Plant disease surveys were conducted in Concordia, located at 31°23′32″S 58°1′1″W, 21 m.a.s.l., with annual rainfall of 1300 mm. Disease incidence was estimated as the proportion of diseased plants. Samples of blighted “Emerald” blueberry branches were collected and taken to the laboratory for further analysis. Small symptomatic branch fragments were surface-disinfected by immersion in ethanol:water (7:3 vol.) for 1 min, 2% (vol.) of Cl as NaOCl for 1 min, rinsed in sterile distilled water, blotted dry, plated on culture medium, and incubated at 22˚C for seven days under fluorescent light with a 12 h photoperiod.

Growth of fungal isolate

The mycelium was sub-cultured by successive transfers of hyphal tips from colony margins onto 2% water agar (WA). The isolate was first identified by biometric and cultural methods, and then subjected to polymerase chain reaction (PCR) assay. The isolate was grown on potato-dextrose-agar (PDA), carnation leaf-piece agar (CLA), and Spezieller Nährstoffarmer agar (SNA) to assess cultural characters by eye and microscopic examinations. On PDA, colony morphology, pigmentation of aerial mycelium and culture media, and mycelium growth at 25 and 35°C were observed. On CLA and SNA, color of sporodochia, shape and size of macroconidia, presence and type of conidiogenous cells (mono or polyphialides), presence/absence of microconidia, chlamydospores and perithecia were recorded (Leslie and Summerell, 2006).

Fungal DNA isolation, amplification and sequencing

Disks of mycelia were excised from a 5-day-old-PDA plate and ground in liquid nitrogen. DNA was extracted using DNeasy Plant Mini kit, quantified and checked by agarose gel electrophoresis. To amplify the internal transcribed spacer (ITS) regions of nuclear ribosomal genes, PCR reaction was carried out in a 20 µl reaction mix containing 25 ηg fungal DNA, 100 µm each of dNTP´s (dATP, dCTP, dGTP, dTTP), 25 µmoles of ITS1 forward (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 reverse (5´-TCCTCCGCTTATTGATATGC-3´) oligonucleotide primers (White et al., 1990) and 1 unit of *Taq* DNA polymerase, in 1X PCR reaction buffer. PCR amplifications were carried out in a thermocycler programmed for initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 30 s, and a 3 min final extension at 72°C. PCR products were checked by electrophoresis on 1.5% agarose gel in TBE buffer. Amplification products of the ITS region were purified with QIAquik PCR purification kit, and sequenced with amplification primers by the dideoxy chain-termination method at INTA Genomic Service Unit (http://www.inta.gob.ar). The sequence was edited manually, and compared with GenBank (http://www.ncbi.nlm.nih.gov/genbank/) database. Similar nucleotide sequences were aligned using Crustal Omega program (Thompson et al., 1994).

Pathogenicity tests

Koch’s postulates were performed on potted two-year-old “Blue Crisp” blueberry grown in INTA-Concordia Experimental Station, as there were no “Emerald” plants available. Market blueberry fruits and plants of different plant species such as carrot *(Daucus carota* var. *sativus* Hoffm.*)*, garlic *(Allium sativum* L.*)* and onion *(Allium cepa* L.) were included in the inoculations. The purified *Fusarium* isolate was increased on culture media at 22°C during seven days. Inoculation methods included: i) conidia suspension prepared by adding sterilized distilled water to PDA slants and shaking for 1 min in a rotary shaker, filtered through sterilized surgical gauze, and adjusted to $3x10^6$ conidia/mL. The suspension was sprayed onto 10 plants with needle-injured branches and foliage. Controls were equally treated but sprayed with sterilized distilled water. The plants were individually enclosed in polyethylene bags for three days and maintained at 22°C in a climatic chamber; ii) plugs of WA with fungal development were placed on needle-wounded branches of 10 plants and attached with pieces of wet cotton. On control plants, WA pieces without the fungus were placed on the wounded branches. The inoculated and control plants were incubated under greenhouse conditions; iii) WA plugs of 4 mm diameter with fungal development were placed on market blueberry fruits which were previously disinfected by immersion in 70% ethanol and 2% bleach, rinsed with distilled water, blotted dry by a laminar flow cabinet, and placed in plastic containers (10 blueberry fruits/container, 4 replicates) in humid chambers or on WA plates (3 fruits/plate, 4 replicates) kept under laboratory conditions. WA plugs without the fungus were used on control fruits, iv) WA plugs of 4 mm diameter with fungal development were placed on surface disinfected carrot taproots and bulbs of garlic and onion, and kept separately in plastic containers under humid conditions in the laboratory. WA plugs without the fungus were placed on control taproots and bulbs. In all cases, inoculated and control plants, fruits, roots and bulbs were monitored regularly for the presence of disease symptoms. The fungus was reisolated from symptomatic inoculated organs after surface disinfection as described.

RESULTS

In October 2010, a new disease was recorded on “Emerald” blueberry plants, with an incidence of 10%. Acropetal blight developed from the base toward the apex of the branches and led to the death of leaves,

Figure 1. Acropetal stem blight on an “Emerald” blueberry plant.

branches and plants. Leaves became tan to brown and remained attached to the branches, which also became tan to brown (Figure 1). A *Fusarium* isolate was obtained from diseased branches. Macroconidia were large, ventrally and dorsally curved with a pronounced foot-shaped basal cell. The isolate was identified as belonging to section “Gibbosum”. On PDA plates, fungal growth was profuse; the aerial mycelium was initially white, developing a rose to burgundy red pigmentation in the center of the colony, and brown to burgundy red pigments in the agar. After two days at 25 and 35°C, colony growth was 15 and 10 mm, respectively. The isolate grew faster at 25°C covering the 9 cm culture plates after 7 days while it required nine days to cover the plate at 35°C (Figure 2). According to this data, it was considered a fast-growing strain at 25°C. On CLA culture medium, macroconidia formed in orange sporodochia were slender, broadly falcate, thick walled, with 3 to 5 septa (mostly 3), a curved elongation in the apical cell and a distinct foot shape, and averaged 48.4 µm (45 to 52 µm) x 3.7 µm (3 to 4 µm). Microconidia were rare, 0 to 1 septate, reniform, born in monophialides. Aseptate conidia were rare, 7.4 µm (6 to 9 µm) x 2 µm while more frequent 1-septate microconidia measured 14.6 µm (13 to 16 µm) x 2 µm. After 5 days on CLA and SNA, round chlamydospores formed abundantly, mainly in chains (2 to 9) averaging 13.2 µm (10 to 15 µm). No perithecia were observed. Based on morphological and cultural characters, the isolate was identified as *Fusarium acuminatum* Ellis and Everh. (teleomorph: *Gibberella acuminata* Wollenw.) (Nelson et al., 1983; Crous et al., 2004; Leslie and Summerell, 2006). The isolate was deposited in the IMYZA Microbial Collection as INTA-IMC582. The 507 bp DNA sequence was deposited in GenBank with the accession number KF250347. The Blastn analysis of the ITS region of nuclear ribosomal DNA showed 100% identity with the DNA sequence for *F. acuminatum* (teleomorph *Gibberella acuminata*), accession No U85533 (O'Donnell, 1997). This sequence was cited as reference for *F. acuminatum* by Leslie and Summerell (2006). Figure 3 shows the results of agarose gel electrophoresis of the strain.

In the pathogenicity tests, symptoms on plants sprayed with conidia suspensions (i) appeared 15 days after the treatment. Stem blight developed acropetally from the wounds. The response varied greatly among and within plants. At day 21, 20% of them had died, and the others showed branch blight that ranged between 1 cm long and branch death (Figure 4a). In 30 days, 70% of the plants had died. Six days after inoculation with WA plugs (ii), the plants showed 0.6 x 0.4 cm dark brown necrotic lesions on the branches. Symptoms were not observed on control plants. After 15 days, necrotic lesions longer than 1 cm developed acropetally on branches (Figure 4b). Plant death was observed at 21 days. Control plants did not show disease symptoms. The fungus developed on the surface of inoculated blueberry fruits (iii) after 14 days (Figure 4c). After 9 days, inoculated carrots (iv) showed dark red discoloration with rotten areas of 2.5 cm diameter covered with profuse mycelia (Figure 4d). No symptoms were recorded on the controls, and inoculated garlic and onion bulbs. *F. acuminatum* was reisolated from inoculated organs that developed disease symptoms.

DISCUSSION

The presence of a new species of *Fusarium* is reported on blueberry in this paper. There was a coincidence of the morphological traits and growth at 25 and 30°C of our strain with the descriptions for *F. acuminatum* (Crous et al., 2004; Leslie and Summerell, 2006; Nelson et al., 1983). Besides, the GenBank DNA sequence comparison of ITS region of ribosomal genes indicated complete identity with the sequence U85533 for *F. acuminatum* (O'Donnell, 1997), which was cited as a reference for this species by Leslie and Summerell (2006). Williams et al. (2002) developed a species-specific primer set to identify *F. acuminatum* such as FAC-F (5´-GGGATATCGGGCCTCA-3´) and FAC-R (5´-GGGATATCGGCAAGATCG-3´). However, Kikot et al. (2011) reported that this primer set was non-specific and showed cross-reaction with *F. graminearum*. Harrow et al. (2010) differenciated *F. acuminatum*, *Fusarium*

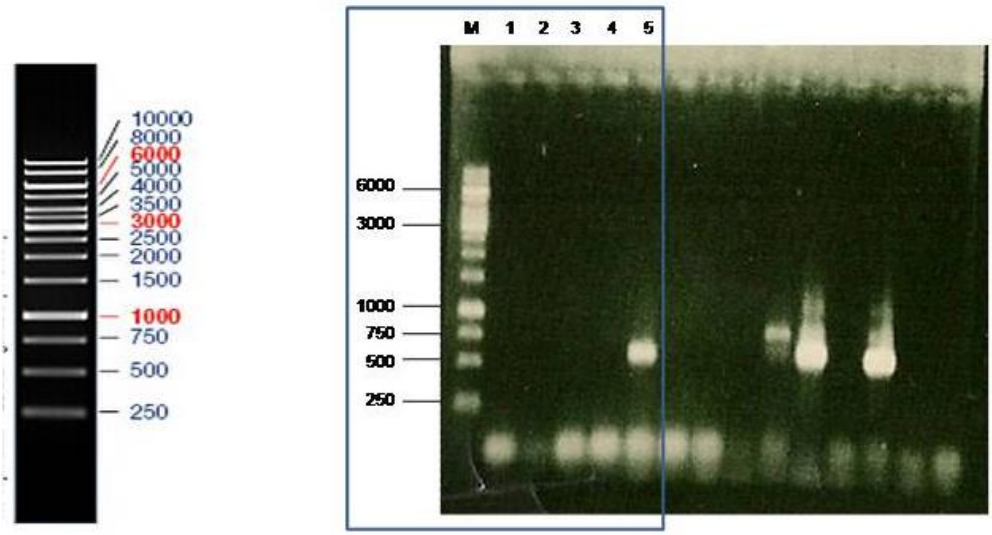

Figure 3. Agarose gel electrophoresis of F. acuminatum ribosomal ITS region amplified by PCR with primers ITS1 and ITS4 (White et al., 1990). M: molecular weight marker GeneRuler 1 kb DNA Ladder (thermo scientific).

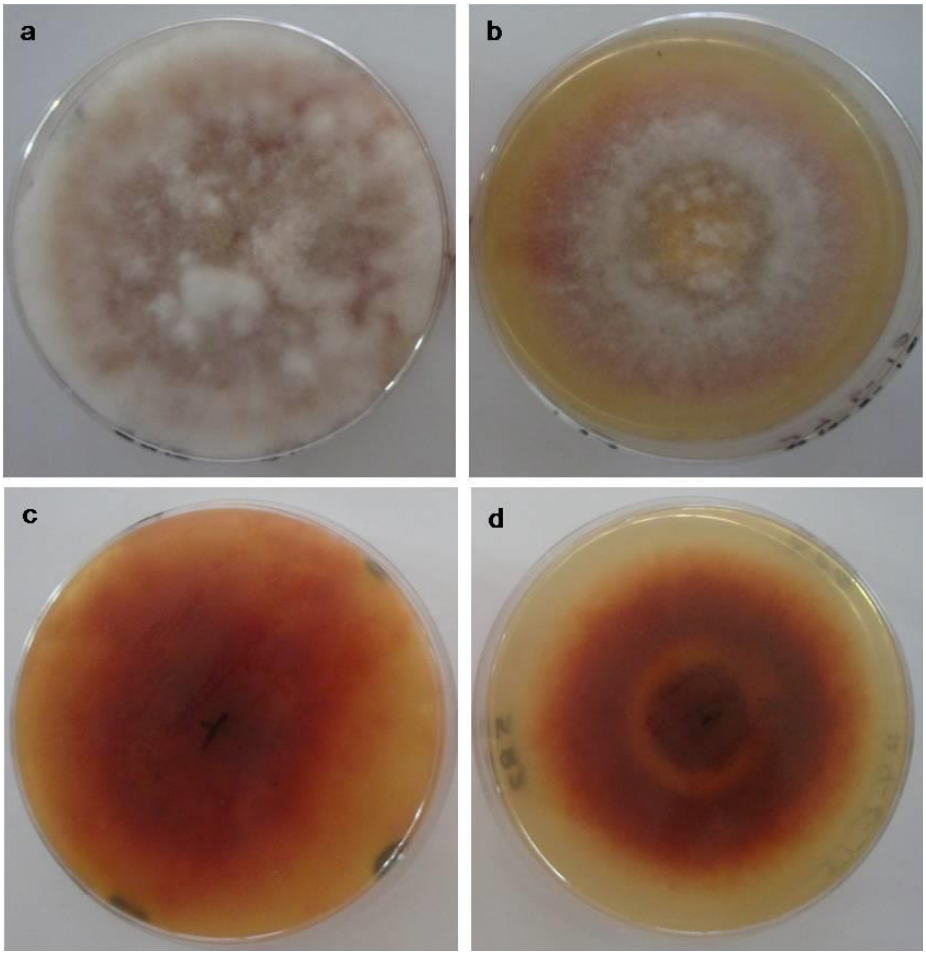

Figure 2. Growth of *Fusarium acuminatum* after 7 days on PDA at different temperatures. **(a)** Colony at 25˚C. **(b)** Colony at 35°C. **(c)** Reverse at 25˚C. **d)** Reverse at 35˚C.

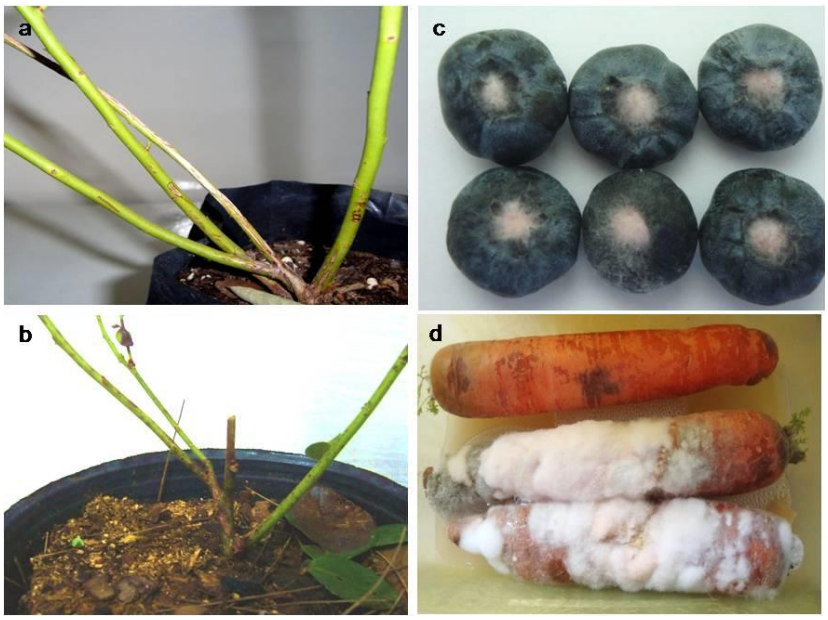

Figure 4. Results of target inoculations with *F. acuminatum.* **(a, b)** Necrotic spots and blight on blueberry branches. **(c)** *F. acuminatum* growth on blueberry fruits. **(d)** Rot and pathogen development on carrots.

avenaceum and *Fusarium tricinctum* isolates using βTUB and EF1α. The identification of *Fusarium* species is important to predict the potential mycotoxigenic risk of a strain (Sampietro et al., 2010, Wing et al., 1993; Altomare et al., 1997; Visconti et al., 1989). As some *F. acuminatum* isolates may be toxin-producers (Logrieco et al., 1992; Marín et al., 2012), it deems necessary to continue research on this subject. We observed that the isolate of *F. acuminatum* was able to colonize blueberry fruits and carrot taproots. However, fruit infection was not observed either in field conditions or after harvest.

The fungus *F. acuminatum* has been cited in temperate regions usually as a soil saprophyte or associated with root and crown diseases on numerous plants (Leslie and Summerell, 2006). A total of 235 species has been included in its host list by Farr and Rossman (2014). Additionally, 83 and 11 records were added for the synonym *Fusarium scirpi* var. *acuminatum* and the teleomorph stage *Gibberella acuminata*, respectively. This pathogen has been isolated from roots, stems, leaves, flowers, seeds and fruits (Esquivel, 1991; Elmer, 1996; Farr and Rossman, 2014). Although it has been mainly associated to root and collar rot (Rai and Singh, 1981; Mathew et al., 2010; Lazreg et al., 2013; Borrego-Benjumea et al., 2014), there are several reports on aerial organs of plants such as banana (Esquivel, 1991), durum wheat (Fakhfakh et al., 2011), onion (Parkunan and Jin, 2013), pumpkin (Elmer, 1996), and pigeonpea (Sharma et al., 2014). In spite of the wide host range of *F. acuminatum*, highbush blueberry (*V. corymbosum*) has not been previously cited as a host. Consequently, this is the first report of *F. acuminatum* as pathogen of *V. corymbosum* in Concordia, Argentina, and to our knowledge, the first citation worldwide. *Amaranthus caudatus* var. *mantegazzianus* (Pass.) Hanelt, *Aspidosderma quebracho-blanco* Schltdl., *Cucurbita ficifolia* Bouché, *C. maxima* Duchesne ex. Lam. subsp. *maxima*, *Glicine max* (L.) Merrill, *Hordeum distichon* L., *Olea europea* L., *Pinus elliottii* Engelm., *Pinus ponderosa* Dougl ex Lawson and P. Lawson, and *Pinus taeda* L. are the species recorded as hosts of *F. acuminatum* in Argentina (Nome Huespe et al., 2014). As blueberry is grown in this country near vegetable, cereal and oil crop fields which are susceptible to *F. acuminatum* as cited in foreign literature, this new host may be important as inoculum source. In addition, even though no natural infections have been reported in Argentina, the isolate was able to infect carrots, causing rot.

F. acuminatum has been reported as one of the *Fusarium* species with ice-nucleating activity (INA) which can be important for the frost damage to the host before infection (Pouleur et al., 1992). This INA characteristic may have intrinsic host origin, or be caused by extrinsic ice (frost, snow, frozen soil or parts of plants or INA microorganisms (Pouleur et al., 1992; Richard et al., 1996; Humphreys et al., 2001; Lundheim, 2002). The INA of intrinsic origin has been studied *in vitro* in blueberry cultivars (Kishimoto et al., 2014). Also, INA has been associated with bacteria, lichens, free-living fungi, among others (Pouleur et al., 1992; Richard et al., 1996; Lundheim, 2002). The species *Fusarium acuminatum*, *F. avenaceum*, *F. moniliforme*, F. *oxysporum*, and *F. tricinctum* have been reported as ice nucleator fungi (Pouleur et al., 1992; Richard et al., 1996; Humphreys et

al., 2001; Seifi et al., 2014). Also, it has been suggested that beside morphological traits, INA characteristic may add useful data to identify *Fusarium* species (Richard et al., 1996). The INA ability of some strains of *F. acuminatum* may be of significance as frost damage of plants may facilitate pathogen infections and disease development under cold weather conditions. The studied disease was detected during the spring. Winter surveys and additional research will be needed in case disease symptoms are observed after cold weather, associated or not to INA activity of the pathogen.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Short Communication

Comparative study on early detection of sugarcane smut (*Sporisorium scitamineum)* by polymerase chain reaction (PCR) and microscopy

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Sugarcane smut caused by *Sporisorium scitamineum* has the potential to result in substantial tonnage loss and significant reduction in sucrose recovery. As early and precise diagnosis is an integral component in the successful management of sugarcane smut, the present study was undertaken to accurately determine the presence of pathogen employing PCR-based methods supplemented with microscopy. Healthy sets of sugarcane cultivars *viz.,* Co 96007 (Susceptible) and Co 6806 (Resistant) were challenge inoculated by hypodermal injection with teliospore suspension of *S. scitamineum* (containing 1 x 10^6 teliospores/ml) and planted in sterile soil with appropriate uninoculated controls. Actively growing meristem of the plantlets was sampled at different time points for examination with microscopy and PCR using primers *bE4* and *bE8* of mating type genes. The whole experiment was conducted for eight weeks and meristem tissue was sampled weekly starting from three weeks post inoculation. Our results show that the PCR assay is more sensitive in early detection of the pathogen (3^{rd} week) in both susceptible and resistant cultivars as compared to microscopic observations of the meristem samples stained with lactophenol cotton blue. However, the pathogen could not be detected from the 4th week onwards in resistant variety Co 6806. In microscopy assay, mycelial colonization was evident only from the 5^{th} week onwards in the susceptible cultivar Co 96007, but not in the resistant cultivar Co 6806 at any of the time intervals until 8 weeks post inoculation. Results of this study suggest that, for the early and precise detection of smut pathogen in sugarcane, the PCR-based assay should be considered as a suitable diagnostic tool rather than microscopy. This could add to effective sugarcane quarantine and successful management of sugarcane smut.

Key words: Smut pathogen, *Saccharum officinarum*, cultivars, pathogen detection, light microscopy, host resistance.

INTRODUCTION

Sugarcane smut is caused by the basidiomycete fungus *Sporisorium scitamineum* (syd.) (Piepenbring et al., 2002 (Syn: *Ustilago scitaminea* H. and *P.* Sydow)) that belongs to the fungal sub-class *Ustilaginaceae*. *S. scitamineum*

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was first noted in 1877, in the Natal region of South Africa (Martin, et al., 1961) and has been a serious threat for sugarcane cultivation in India and other parts of the world for many years. It can devastate susceptible sugarcane varieties by quick spreading and considerable reduction in yield (Fletcher, 2013). In the 1930's, *S. scitamineum* caused severe problems in India and since then it became widespread in most of the sugarcane growing states in the country (Viswanathan et al., 2009).

The life cycles of the smut fungi are similar for all species and involve transitions between three cell types. Diploid teliospores are the resting cell type and are disseminated mainly by wind or rain splashes. They germinate by forming a probasidium on which, following meiosis, four sporidia emerge. The haploid sporidia represent the second cell type. They grow by budding, and compatible (opposite mating-types/plus and minus) sporidia fuse to give rise to the dikaryotic pathogenic third cell type which exhibits mycelial growth (Alexopoulos, 1962). Karyogamy takes place in the dikaryotic mycelium and diploid teliospores are formed within the host tissues (Bakkeren and Kronstad, 1993). The life cycle is regulated by the *a* and *b* mating-type loci within the sporidia. *a* has two alleles which encode a pheromone and a receptor whilst *b* is multiallelic and appears to control pathogenicity and sexual development (Bakkeren et al., 1992). With the use of primers based on the *U. maydis b*E mating-type gene, (Albert and Schenck, 1996) the corresponding gene was sequenced in *U. scitaminea*. Molecular detection of the smut pathogen in sugarcane has since become possible by using PCR to amplify the *b*E mating-type gene of *U. scitaminea*

To control the disease, sugarcane seeds are treated with hot water and breeding for resistance is perfomed; all of which increases the costs for production. Hence, early and accurate diagnosis of plant disease is a crucial component of *S. scitamineum*-sugarcane as well as other pathogen-management systems. To detect meristem colonization of sugarcane with *S. scitamineum,* previous studies performed histopathological examinations of the infected stalk (Alexander and Ramakrishan, 1980; Waller, 1970). However, this has implications on the accuracy of the prediction as microscopy is insufficient to distinguish between different fungal pathogen species. In the recent years, molecular biology techniques like PCR involving specific primers is aiding significantly in early detection and evaluation of plant diseases. The objective of this study was to evaluate the accuracy and sensitivity of the diagnostic tools - PCR with pathogen-specific primers and microscopy to detect smut.

MATERIALS AND METHODS

Healthy plants of susceptible Co 96007 and resistant Co 6806 cultivars were placed for three to four days in moistened gunny bags for sprouting. Sprouted buds were de-scaled and subjected to hypodermal syringe inoculation with *S. scitamineum* teliospore suspension containing 1×10^6 spores/ml, without damaging the buds. Inoculated buds were planted in pots along with un-inoculated sprouted buds (syringe inoculated with sterile water) which served as mock-control and the pots were maintained under ideal glass house conditions. After germination, meristem from the control and inoculated plantlets were sampled at weekly intervals from three to eight weeks post inoculation (wpi) and were subjected to the PCR based assay and microscopic examination. DNA was extracted from the meristem of the plantlets and subjected to PCR using *bE4* and *bE8* primers (Albert and Schenck, 1996). The reaction mixture also contained 0.2 mM dNTPs, 0.5 mM Mg Cl_2 and 1.5 unit μL^{-1} Taq DNA polymerase. The reaction was run for 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 1 min (Ali Moosawi-Jorf and Mahin, 2007). For the microscopic study, meristem was longitudinally cut into ultra-thin sections and fixed with formalin acetic acid (FAA), mounted on a glass slide, stained with 0.1% lacto phenol cotton blue (Lloyd and Naidoo, 1981) examined microscopically and photographed under low (100x) and high (1000x).

RESULTS AND DISCUSSION

In the PCR based method, specific amplicons (459 bp) were observed in all the six intervals (from 3 to 8 wpi) in susceptible variety Co 96007 (Figure 1). In contrast, the resistant variety Co 6806 revealed *bE* amplicons only for 3 wpi. This may result from the restriction of pathogen colonization probably by the host resistance mechanism. The non-inoculated plant samples from both cultivars revealed no PCR products in all the six intervals. In the microscopic study, pathogen colonization in the susceptible variety Co 96007 was examined from 3 wpi. However, evident colonization was observed only from 5 wpi. Interestingly, the presence of the pathogen in the resistant variety Co 6806 could not be detected in any of the intervals (Figure 2). In confirmation with the PCR approach non-inoculated controls were also negative in the microscopic study. Table 1 depicts the results obtained with both diagnostic tools. The accuracy of the microscopic examination is limited and time-consuming. As discussed by Ali Moosawi-Jorf and Mahin (2007), microscopic detection of the sugarcane smut fungus may not be accurate. Moreover, detection and discrimination become difficult at early stages of plant colonization both in field and laboratory conditions, because in the infected tissues smut hypha cannot be discriminated morphologically from other fungal hypha.

Results of this study suggest that the PCR based assay is more sensitive, rapid and accurate compared to microscopic examination of infected plant tissue. The *bE* mating-type gene used in this study for detection is specific for *S. scitamineum*, and the results of PCR were validated using appropriate positive controls from DNA sourced out from the dikaryotic mycelia confirming our results obtained by PCR amplification. Similar study was observed by Toth (1998) the mating-type (*bE*) gene to detect the smut pathogen is more specific and extremely sensitive in deduction of pathogen. Dalvi et al. (2012) finding similar observation during field and PCR screening to evaluate the clones to confirm smut infections whip production is the most reliable symptom of smut disease

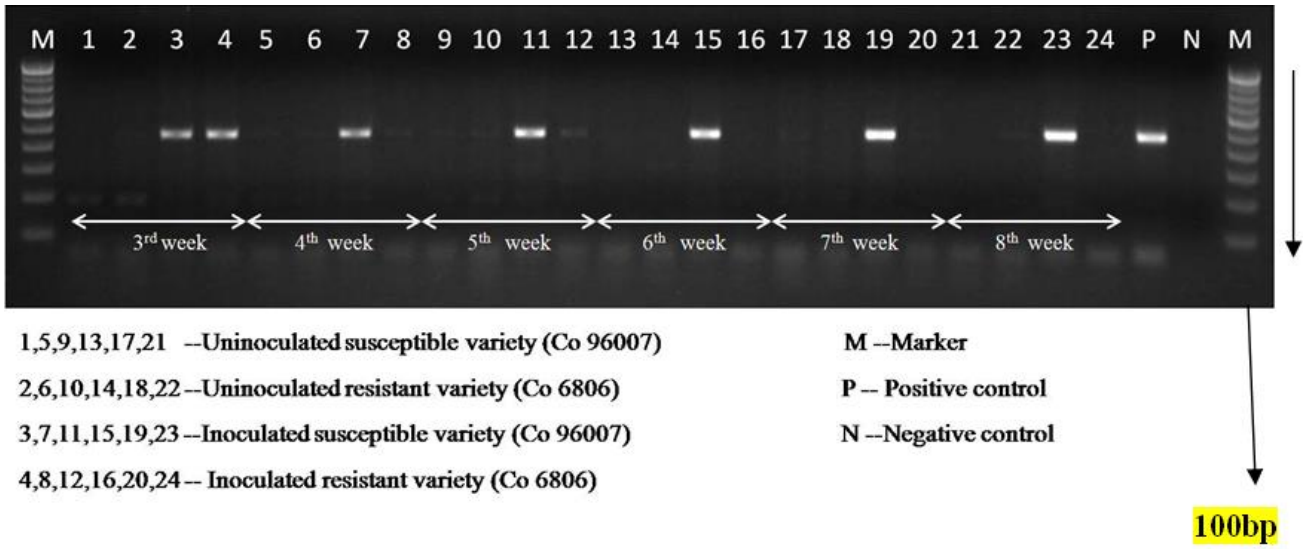

Figure 1. Profile of the detection of *Sporisorium scitamineum* by PCR 100 bp.

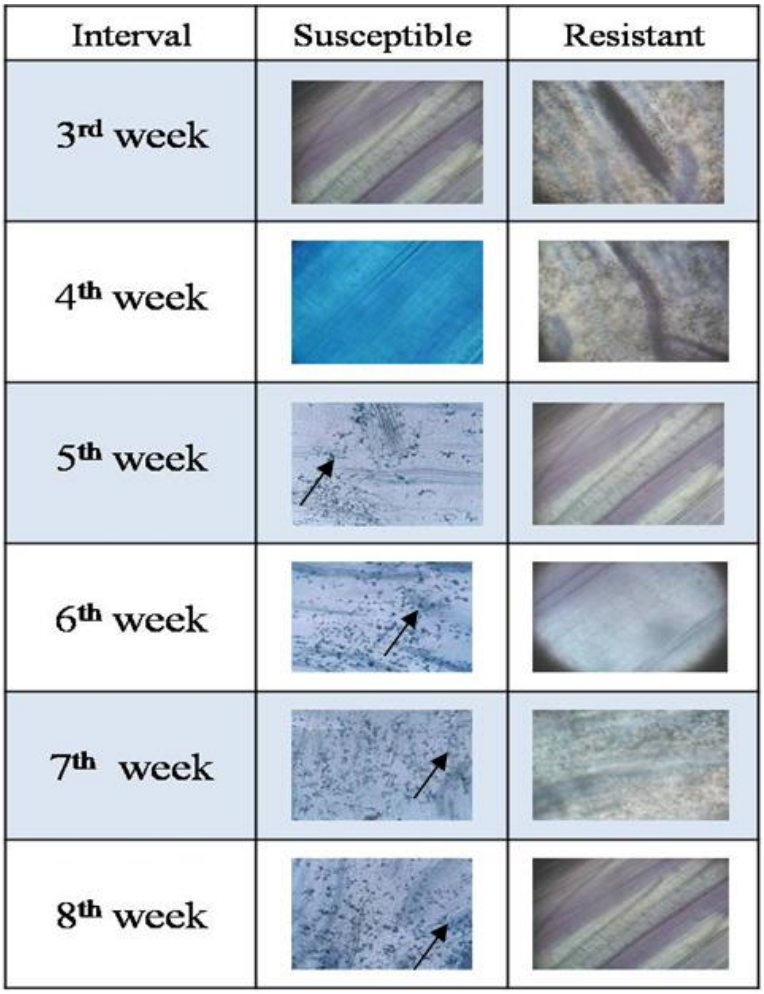

Figure 2. Detection of *Sporisorium scitamineum* by Light Microscopy.
→ - indicates the pathogen colonization and intensity.

Table 1. Detection of *Sporisorium scitamineum* by PCR and microscopic study.

Variety	Diagnostic technique	Result at different time intervals					
		3rd week	4th week	5th week	6th week	7th week	8th week
Co 96007	PCR	+ve	+ve	+ve	+ve	+ve	+ve
(Sensitive)	Microscopy	-ve	-ve	+ve	+ve	+ve	+ve
Co 6806	PCR	+ve	-ve	-ve	-ve	-ve	-ve
(Resistant)	Microscopy	-ve	-ve	-ve	-ve	-ve	-ve

in sugarcane but when there is no whip expression due to environmental conditions, the infested sugarcane plants, often tiller profusely with the shoots being more spindly and the leaves being more upright and narrow emerging from the shoots following infection. Similar study was reported by Singh et al. (2004).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Variants of NAT2 polymorphisms: Intra and inter-ethnic differences

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***N-Acetyltransferase 2* (*NAT2*) gene is known for its polymorphism. The genetic variations leads to the change in the N-acetylation activity and these differences in the acetylation activity leads to the classification of the population into various groups such as rapid, intermediate and slow acetylators. In the present study, we identified different mutations and alleles by sequencing a stretch of 927 bp which covered exon 2 of *NAT2* gene and is reported to have all the allelic variants reported till date. Previously identified mutations and some new allele sub-type were detected for *NAT2* gene in the present study. Overall, we were able to classify 94 individuals into two distinct groups as slow and intermediate acetylators based on their genotype. Out of all the reported alleles, *NAT2*4*, **5*, **6*, **7* and **12* alleles were found in the studied population (n=94) and two new allele subtype *NAT2*5P* and *NAT2*7C* were detected in the studied population which have not been reported earlier. We did not observed any gender differences in the present study based on NAT2 acetylation activity.**

Key words: N-Acetyltransferase 2 (NAT2), polymorphisms, slow acetylators, rapid acetylators.

INTRODUCTION

N-Acetyltransferase 2 (NAT2) is involved in the metabolism of arylamines and hydrazines. The substrates of NAT2 mainly include drugs, such as isoniazid (INH), as well as chemicals and carcinogens (Weber, 1997; Grant et al., 1997; Gross et al., 1999). Thus, N-acetylation activity is associated with drug effects or toxicities and susceptibility to various cancers. The ability of NAT2 to N-acetylate arylamines is subject to genetic polymorphisms in the *NAT2* gene. *NAT2* genotype and its acetylation rate are different among various populations. Acetylation polymorphism causes inter-individual variation in the biotransformation of different drugs, pro-carcinogens and other xenobiotics which have a primary aromatic or hydrazine structure. This enzyme therefore plays an important role in the detoxification and potential metabolic activation of numerous xenobiotics (Rodrigues-Lima and Dupret, 2002; Anitha and Banerjee, 2003). The *NAT2* acetylation

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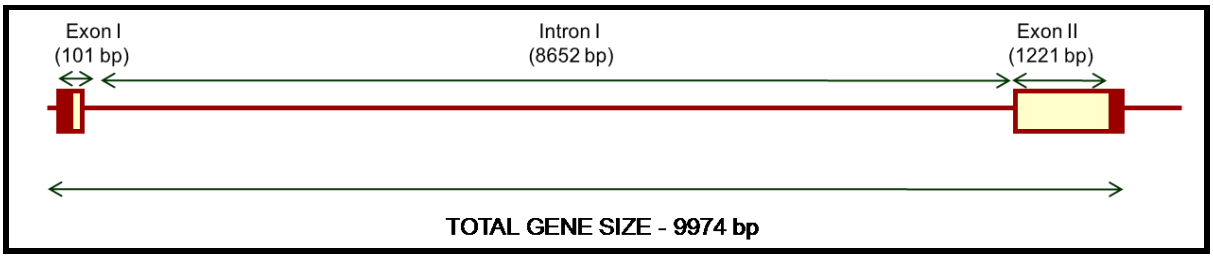

Figure 1. *NAT2* gene map (http://asia.ensembl.org/index.html).

polymorphism has been associated with higher incidences and/or severity of adverse drug reactions (ADRs) to isoniazid, hydralazine, procainamide and sulfamethoxazole (Evans, 1989). Differences in acetylation activity among individuals and in populations of diverse racio-geographic origin have led to the phenotypic classification of humans as rapid (normal activity), intermediate and slow (reduced activity) acetylators (Hein et al., 2000; Lee et al., 2002). Such phenotype/genotype variations have been observed and reported for many drugs. For example, isoniazid is one of the important drugs used in the treatment of tuberculosis and sulphamethoxazole is used in the treatment of secondary infections in AIDS patients. The homozygous occurrence of *NAT2* inactivating mutations results in a slow-inactivator phenotype. Such a phenotype, due to accumulation of the drug, leads to increased dose-dependent toxicity such as hydralazine-induced lupus, isoniazid-induced neuropathies and sulfonamide-induced hypersensitivity reactions in some ethnic groups. It is well established that slow acetylators are more likely to suffer side-effects when prescribed isoniazid, although there is also evidence that these individuals' overall response to therapy may be better as a result of being exposed to higher drug levels for a longer duration (Das et al., 1973; Evans and Relling, 1999; http://nat.mgb.duth.gr).

The *NAT2* polymorphism may also modulate risk of lung, bladder, breast and colon cancer as a result of NAT2 acetylating aromatic amines found in tobacco smoke and cooked foods. Epidemiological studies show an association between *NAT2* acetylation polymorphism and both urinary bladder and colorectal cancers (Eichholzer et al., 2012; Fontana et al., 2009; Brockton et al., 2000). Slow acetylators, who smoke tobacco, have an increased risk of development of bladder cancer compared to rapid acetylators, which are at more risk to colorectal cancer (Osian et al., 2006; Huang et al., 2007; Gu and Wu, 2011). This difference is due to their inability to detoxify aromatic amines in tobacco smoke. The slow acetylator phenotype has also been implicated as a factor in individual susceptibility to immunoglobulin E-mediated food allergy (Woosley et al., 1978; Daly, 1995).

NAT2 gene is located on chromosome 8p22 (OMIM_612182) and spans approximately 9 kb length with two exons (http://www.ensembl.org/index.html). To date, approximately 25 different alleles have been identified for *NAT2* gene (Daly, 1995; Lee et al., 2002; http://nat.mgb.duth.gr). In Caucasians, three variant alleles *NAT2*5*, *NAT2*6* and *NAT2*7*, were among the majority of slow acetylator groups which may vary in other ethnic groups. The frequency of slow acetylators also varies with ethnic origin, ranging from 90% in North Africans to less than 10% in many Asian populations (Evans, 1989). The present study was focused on detecting the known and the unknown mutations *NAT2* gene in Indian population.

MATERIALS AND METHODS

Volunteers

Healthy Indian male (65) and female (29) volunteers (n=94) of age 18 to 55 years were recruited for the present study with their consent.

DNA isolation

Peripheral blood samples (5 ml) were drawn from the volunteers, in sterile tubes containing EDTA as anticoagulant. DNA was extracted by the standard phenol-chloroform method, after digestion with Proteinase K (Sambrook et al., 1989; Sistonen et al., 2009).

Detection of NAT2 variants

NAT2 is 9974 bp gene, consisting of two exons (Figure 1) (http://asia.ensembl.org/index.html). All the alleles reported to date comprise of mutations located on exon 2 (http://nat.mgb.duth.gr). Thus, a fragment of 1393 bp was amplified from genomic DNA which covered the exon 2 (1221 bp) of *NAT2* gene. A region of 927 bp was sequenced from the pre-amplified PCR product which covered all the known alleles. Sequencing was carried out by the sequencing services provided by Polymorphic DNA Technologies, Alameda, California. A bidirectional high throughput capillary sequencing based on the Sanger's dideoxy chain-termination DNA sequencing method, was used for detection of *NAT2* variants (http://nat.mgb.duth.gr). Sequencing analysis detected the known and unknown mutations. The 927 bp sequence, located on Chromosome 8: 18257447-18258373, which was sequenced from the pre-amplified PCR product, is as follow:

CACACGAGGAAATCAAATGCTAAAGTATGATATGTTTTTATGTT
TTGTTTTTCTTGCTTAGGGGATCATGGACATTGAAGCATATTTT

Table 1. *NAT2* Allele frequency in the studied Indian population.

Allele	Frequency (%)			Enzyme activity	R/S
	Total (n=94)	Male (n=65)	Female (n=29)		
**4*	9.6	10.0	8.6	Normal	R
**5*	34.6	36.2	31.0	Almost none	S
**6*	23.4	20.0	31.0	Almost none	S
**7*	5.3	6.2	3.5	Almost none	S
**12*	27.1	27.7	25.9	Normal	R

R-Rapid acetylators, S-slow acetylators

GAAAGAATTGGCTATAAGAACTCTAGGAACAAATTGGACTTGG
AAACATTAACTGACATTCTTGAGCACCAGATCCGGGCTGTTCC
CTTTGAGAACCTTAACATGCATTGTGGGCAAGCCATGGAGTTG
GGCTTAGAGGCTATTTTTGATCACATTGTAAGAAGAAACCGGG
GTGGGTGGTGTCTCCAGGTCAATCAACTTCTGTACTGGGCTCT
GACCACAATCGGTTTTCAGACCACAATGTTAGGAGGGTATTTT
TACATCCCTCCAGTTAACAAATACAGCACTGGCATGGTTCACC
TTCTCCTGCAGGTGACCATTGACGGCAGGAATTACATTGTCGA
TGCTGGGTCTGGAAGCTCCTCCCAGATGTGGCAGCCTCTAGA
ATTAATTTCTGGGAAGGATCAGCCTCAGGTGCCTTGCATTTTCT
GCTTGACAGAAGAGAGAGGAATCTGGTACCTGGACCAAATCA
GGAGAGAGCAGTATATTACAAACAAAGAATTTCTTAATTCTCAT
CTCCTGCCAAAGAAGAAACACCAAAAAATATACTTATTTACGCT
TGAACCTCGAACAATTGAAGATTTTGAGTCTATGAATACATACC
TGCAGACGTCTCCAACATCTTCATTTATAACCACATCATTTTGT
TCCTTGCAGACCCCAGAAGGGGTTTACTGTTTGGTGGGCTTCA
TCCTCACCTATAGAAAATTCAATTATAAAGACAATACAGATCTG
GTCGAGTTTAAAACTCTCACTGAGGAAGAGGTTGAAGAAGTGC
TGAGAAATATATTTAAGATTTCCTTGGGGAGAAATCTCGTGCCC
AAACCTGGTGATGGATC

RESULTS

Sequence analysis of NAT2 gene

The sequencing data was analyzed for the presence of known and unknown polymorphisms. Mutations at positions 282C>T, 341T>C, 481C>T, 590G>A, 803A>G and 857G>A were detected from the sequence analysis of 94 individuals which lead to the detection of *NAT2*4*, **5*, **6*, **7* and **12* alleles. Allele not carrying any mutation was classified as *NAT2*4*, rapid acetylators (R). Alleles *NAT2*5*, **6* and **7* were classified as slow acetylators (S), while *NAT2*12* did not lead to any change in *NAT2* enzyme activity (R) (http://nat.mgb.duth.gr).

New alleles in Indian population

A mutation at position 578C>T was detected in a single individual, in the studied population (n=94), along with other known mutations at 282C>T, 341T>C, 481C>T, 590G>A, 803A>G. This mutation leads to a substitution of threonine amino acid to methionine amino acid at 193 position (T193M), which along with other mutation may lead to change in NAT2 enzyme activity. Thus, this new combination of mutations has been classified as *NAT2*5P* allele by the Arylamine N-acetyltransferase Gene Nomenclature Committee. The mutation combination leads to identification of a new allele specific to the Indian population as this combination is presently not reported in other populations. A new sub-type of *NAT2*7* allele was also found in studied population. It is being termed as *NAT2*7C*, which comprised 282C>T, 803G>A and 857G>A mutations, which may lead to the slow acetylation phenotype (http://nat.mgb.duth.gr). The frequency of this allele was found to be 8.5% (10.8% in males, 3.4% in females). The genotype observed were NAT2*6C/*7C (slow acetylation), NAT2*7C/*12A (intermediate acetylation).

NAT2 genotype and allele frequencies

The alleles *NAT2*4*, **5*, **6*, **7* and **12* were detected from the sequencing data in the studied population. The allelic frequency for these alleles of *NAT2* gene ranged from 5.3 to 34.6% (Table 1). *NAT2*5* was found in 34.6% of individuals. Individuals were classified into three categories as rapid acetylators (R), intermediate acetylators (I) and slow acetylators (S) depending on the alleles and their reported enzyme activities. Based on this classification, the allele frequency of the rapid acetylators was found to be 36.7% and slow acetylators were 63.3% in the studied population (n=94). The genotype frequency of *NAT2* is shown in Table 2. *NAT2*6/*12* genotype dominated the studied population, while no individual was found to have *NAT2*4/*4* genotype, which is wild type genotype. The genotype frequency of rapid acetylators was 5.4%, intermediate acetylators was 62.7% and slow acetylators was 31.9%. No significant difference was observed between males and females in the studied population.

NAT2 inter-ethnic differences

A comparison of the allele frequencies of *NAT2* with other populations is shown in Table 3, and Figure 2. The majority of the studied population had *NAT2*5* (34.6%),

Table 2. *NAT2* Genotype frequency in the studied Indian population.

Genotype	Frequency (%)			Enzyme activity	R/I/S
	Total (n=94)	Male (n=65)	Female (n=29)		
**4/*5*	16.0	15.4	17.2	Decreased	I
**4/*6*	2.1	3.1	0.0	Decreased	I
**4/*12*	1.1	1.5	0.0	Normal	R
**5/*5*	14.9	13.8	17.2	Almost none	S
**5/*6*	2.1	3.1	0.0	Almost none	S
**5/*7*	2.1	1.5	3.4	Almost none	S
**5/*12*	19.1	24.6	6.9	Decreased	I
**6/*6*	9.6	7.7	13.8	Almost none	S
**6/*7*	3.2	4.6	0.0	Almost none	S
**6/*12*	20.2	13.8	34.5	Decreased	I
**7/*12*	5.3	6.2	3.4	Decreased	I
**12/*12*	4.3	4.6	3.4	Normal	R

R, Rapid acetylators; I, Intermediate acetylators; S, Slow acetylators.

Table 3. Comparison of *NAT2* allele frequency in the studied Indian population (%) with other populations (Cascorbi et al., 1995; Aynacioglu et al., 1997; Martinez et al., 1998; Jorge-Nebert et al., 2002; Loktionov et al., 2002; Anitha and Banerjee, 2003; Agúndez, 2003; Tanira et al., 2003; Belogubova et al., 2005; Deguchi et al., 2005; Srivastava and Mittal, 2005; Patin et al., 2006; Rabstein et al., 2006; Al-Yahyaee et al., 2007; Teixeira et al., 2007; Agúndez et al., 2008; Yuliwulandari et al., 2008; Sabbagh et al., 2011).

Allele	Studied population (n=94)	South Indians (n=166)	Caucasians (n=1034)	Omanis (n=127)	Japanese (n=175)	Tswana (n=101)	Korean (n=1000)	Rio de Janeiro (n=298)	Ngawbe (n=105)	Nicaraguans (n=137)	Sudanese (n=127)	Spanish (n=1312)	Turk (n=303)	German (n=844)	Russian (n=364)	Kyrgyz (n=290)	Indonesian (n=212)	R/S
**4*	**9.6**	11.7	22.0	17.7	68.6	13.4	66.1	17.3	72.4	41.6	8.7	22.2	23.1	22.7	23.5	39.8	37.3	R
**5*	**34.6**	25.9	45.4	42.4	2.0	32.2	1.6	40.1	2.4	35.8	47.3	45.6	41.7	46.4	45.6	19.3	9.0	S
**6*	**23.3**	36.7	26.2	26.4	19.8	18.8	20.1	26.3	0.0	17.4	28.7	26.7	30.5	27.8	27.2	26.5	36.8	S
**7*	**5.3**	22.3	1.2	3.9	9.6	0.0	11.5	4.2	23.3	0.0	3.1	1.2	4.5	1.3	3.1	12.1	15.3	S
**11*	**0.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	S
**12*	**27.2**	2.0	2.6	5.2	0.0	20.8	0.3	4.4	0.0	3.6	8.3	2.6	0.2	0.0	0.5	0.5	0.9	R
**13*	**0.0**	2.1	0.0	2.4	0.0	6.4	0.1	3.0	1.9	1.1	0.1	0.3	0.0	1.5	0.0	0.0	0.7	R
**14*	**0.0**	0.0	1.0	0.0	0.0	8.4	0.0	4.7	0.0	0.4	3.2	1.4	0.0	0.1	0.0	0.0	0.0	S

R, Rapid acetylators; S, slow acetylators.

*NAT2*6* (23.3%) and *NAT2*12* (27.2%) allele while, most of the other populations mentioned in Table 3, were predominated by *NAT2*5*, *NAT2*6* or *NAT2*7* alleles. Significant differences were observed when the allele frequency of slow acetylators from the present study were compared

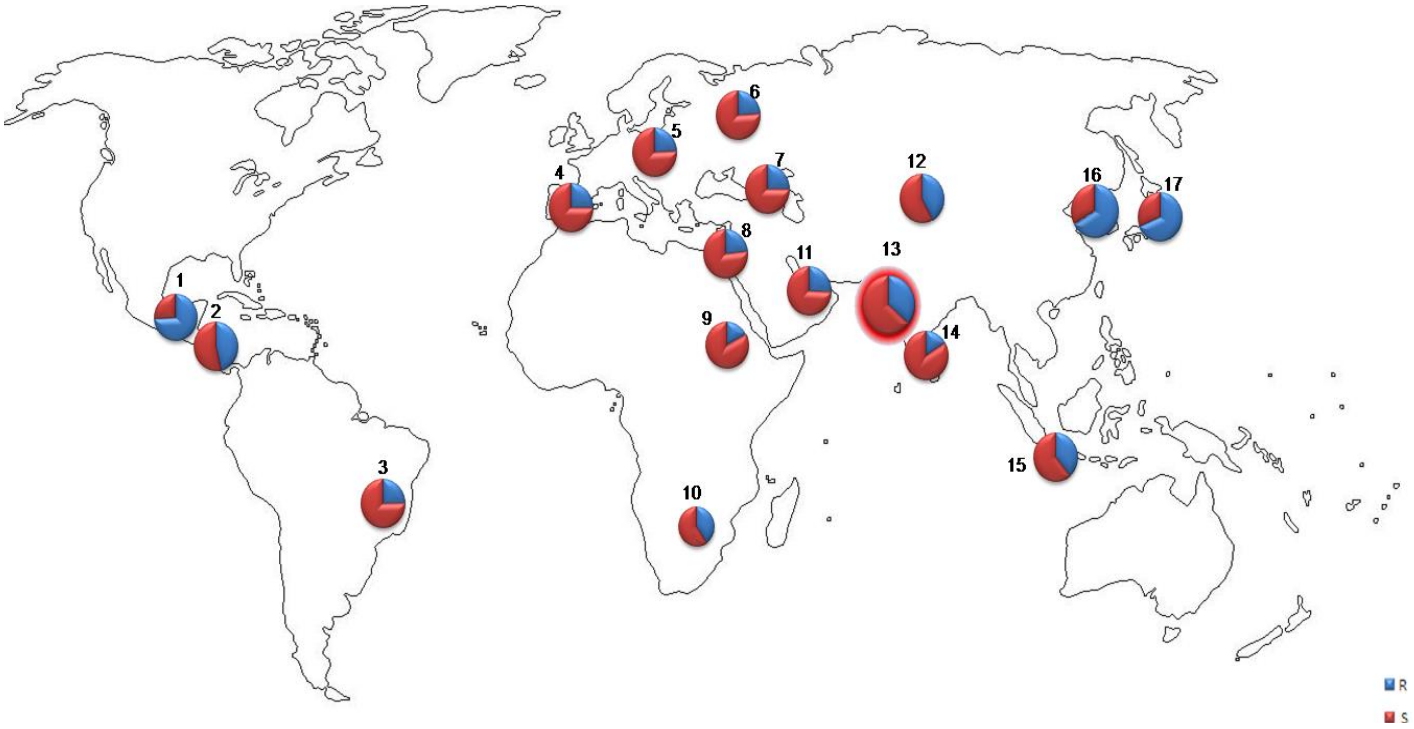

Figure 2. Comparison of *NAT2* allele frequency in different populations (R-Rapid acetylators, S-Slow acetylators): 1, Nicaraguans (n=137); 2, Ngawbe (n=105); 3, Rio de Janeiro (n=298); 4, Spanish (n=1312); 5, German (n=844); 6, Russian (n=364); 7, Caucasians (n=1034); 8, Turk (n=303); 9, Sudanese (n=127); 10, Tswana (n=101); 11, Omanis (n=127); 12, Kyrgyz (n=290); 13, Studied population (n=94) ; 14, South Indians (n=166); 15, Indonesia (n=212); 16, Korean (n=1000); 17, Japanese (n=175) (Cascorbi et al., 1995; Aynacioglu et al., 1997; Martinez et al., 1998; Jorge-Nebert et al., 2002; Loktionov et al., 2002; Anitha and Banerjee, 2003; Agúndez, 2003; Tanira et al., 2003; Belogubova et al., 2005; Deguchi et al., 2005; Srivastava and Mittal, 2005; Patin et al., 2006; Rabstein et al., 2006; Al-Yahyaee et al., 2007; Teixeira et al., 2007; Agúndez et al., 2008; Yuliwulandari et al., 2008; Sabbagh et al., 2011).

with reported frequency for South Indian, Japanese, Korean, Ngawbe and Spanish (*p<0.0001*); Sudanese (*p<0.005*), whereas the difference between our data, Caucasians, Tswana, Rio de Janeiro and Omanis (*p<0.1*); Nicaraguans, Turk, German, Russian, Kyrgyz and Indonesian (*p>0.1*) was not significant (Cascorbi et al., 1995; Aynacioglu et al., 1997; Martinez et al., 1998; Jorge-Nebert et al., 2002; Loktionov et al., 2002; Anitha and Banerjee, 2003; Agúndez, 2003; Tanira et al., 2003; Belogubova et al., 2005; Deguchi et al., 2005; Srivastava and Mittal, 2005; Patin et al., 2006; Rabstein et al., 2006; Al-Yahyaee et al., 2007; Teixeira et al., 2007; Agúndez et al., 2008; Yuliwulandari et al., 2008; Sabbagh et al., 2011).

Comparison of the genotype frequencies of *NAT2* among Asians is shown in Table 4 and Figure 3. *NAT2*6/*12* genotype predominated the studied population, while this combination was not found in South Indians, Omanis and Japanese. The frequency of *NAT2*5/*6* was found as low as 2.1% in the present study but among the South Indians and Omanis, was somewhere around 19% and was not detected in Japanese population (Anitha and Banerjee, 2003; Agúndez, 2003; Tanira et al., 2003; Deguchi et al., 2005).

DISCUSSION

NAT2 previously identified mutations and new allele sub-types were detected in the present study, by sequencing a stretch of 927 bp covering exon 2, which is reported to have all the allelic variants of *NAT2* gene (Evans, 1989; http://asia.ensembl.org/index.html). *NAT2*4*, **5*, **6*, **7* and **12* alleles were found in the studied population (n=94) in which alleles *NAT2*5*, **6* and **7* are classified as slow acetylators (S) (http://nat.mgb.duth.gr). The wild type allele which did not have any mutation was classified as *NAT2*4*, the rapid acetylator (R). Individuals grouped as slow acetylators usually do not respond to drugs like isoniazid and may show signs of ADRs.

Furthermore, NAT2 is also responsible for detoxification of many aromatic amines found in tobacco smoke, etc which, on accumulation in the body might develop ADRs or diseases like cancer (Hirvonen and Vineis, 1999; Anitha and Banerjee, 2003; Chauhan et al., 2007; Sistonen et al., 2009). Thus, as observed in the present study, 31.9% of the total population might be at a risk of developing such ADRs or may be susceptible to certain diseases. Individuals classified as intermediate acetylators were found to be 62.7% in the studied

Table 4. Comparison of *NAT2* genotype frequency in the studied Indian population (%) with other populations (Anitha and Banerjee, 2003; Tanira et al., 2003; Deguchi et al., 2005).

Genotype	Studied population (n=94)	South Indians (n=166)	Omanis (n=127)	Japanese (n=175)	R/I/S
**4/*4*	0.0	0.0	2.4	46.5	R
**4/*5*	16.0	2.0	12.6	1.7	I
**4/*6*	2.1	10.2	11.8	30.2	I
**4/*12*	1.1	1.0	1.6	0.0	R
**4/*7*	0.0	9.1	2.4	12.2	I
**4/*13*	0.0	1.0	0.8	0.0	R
**5/*5*	14.9	9.2	22.9	0.6	S
**5/*6*	2.1	19.3	19.7	0.0	S
**5/*7*	2.1	11.1	3.1	1.2	S
**5/*12*	19.2	1.0	8.7	0.0	I
**6/*6*	9.5	13.3	10.3	3.5	S
**6/*7*	3.2	17.2	0.0	2.3	S
**6/*12*	20.2	0.0	0.0	0.0	I
**6/*13*	0.0	0.0	1.6	0.0	I
**7/*12*	5.3	2.0	0.0	0.0	I
**7/*13*	0.0	3.1	0.8	0.0	I
**7/*7*	0.0	1.0	0.0	1.7	S
**12/*12*	4.3	0.0	0.0	0.0	R
**13/*13*	0.0	0.0	0.8	0.0	R

R, Rapid acetylators; I, intermediate acetylators; S, slow acetylators.

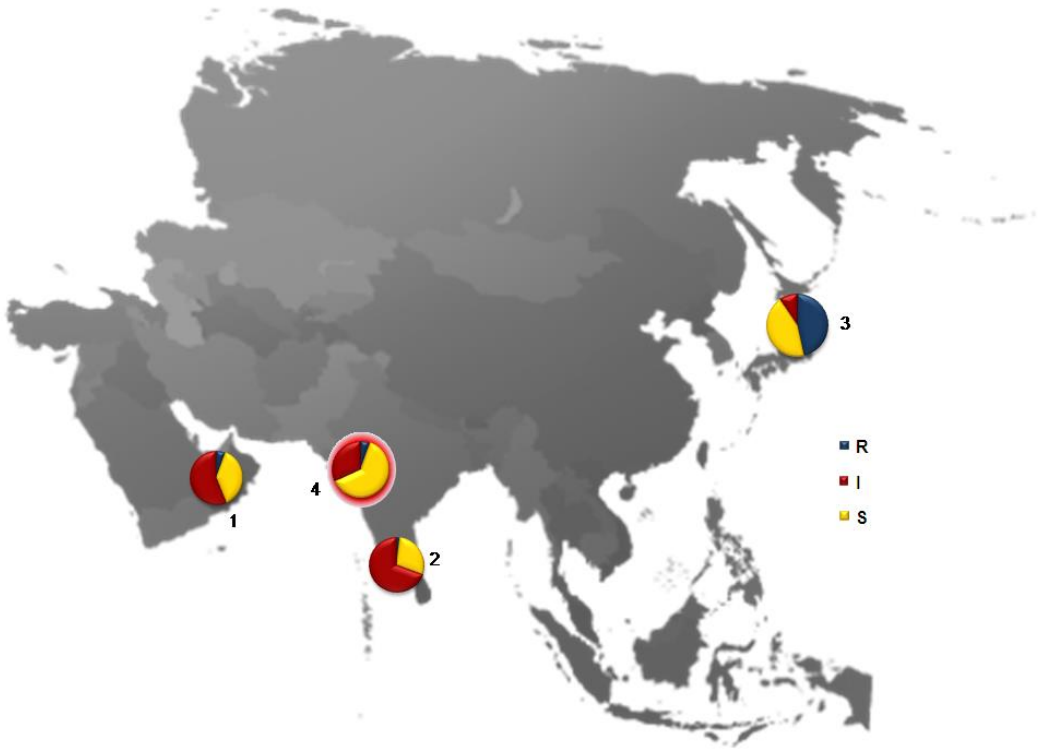

Figure 3. Comparison of *NAT2* genotype frequency among Asians (R-Rapid acetylators, I-Intermediate acetylators, S-Slow acetylators): 1- Omanis (n=127), 2- South Indians (n=166), 3- Japanese (n=175), 4- Studied population (n=94) (Anitha and Banerjee, 2003; Tanira et al., 2003; Deguchi et al., 2005).

population. The present study, thus reflects that the entire population can be divided into slow or intermediate acetylators which might display partial or very low acetylation activity (Table 4) (Anitha and Banerjee, 2003; Tanira et al., 2003; Deguchi et al., 2005). A new sub-type of *NAT2*5* and *NAT2*7* allele were detected in the studied population which has not been reported earlier. *NAT2*5* sub-type consisting of 282C>T, 341T>C, 481C>T, 578C>T, 590G>A, 803A>G mutation has been named as *NAT2*5P* allele by the Arylamine N-acetyltransferase Gene Nomenclature Committee. Whereas, *NAT2*7* allele sub-type has been termed as *NAT2*7C* allele having mutations at position 282C>T, 803G>A and 857G>A (http://nat.mgb.duth.gr). Among the studied population, 31.9% individuals were estimated to be slow acetylators for *NAT2* from the genotype frequency; whereas 71.1% South Indians and 9.3% Japanese were reported as individuals showing slow acetylation phenotype for *NAT2* (Table 4) (Anitha and Banerjee, 2003; Tanira et al., 2003; Deguchi et al., 2005). All these differences suggest diversity among various ethnic groups and within Indian population for *NAT2* gene.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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