



RESEARCH PAPER

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Genetic characterization and *in vitro* propagation of three medicinal plants collected from high altitude sites

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Key words: Medicinal plants, *Ficus cordata*, *Ficus palmata*, *Pulicaria vulgaris*, axillary bud explants, micro propagation, shoot initiation, multiplication, RAPD-PCR.

<http://dx.doi.org/10.12692/ijb/6.6.37-46>

Article published on March 22, 2015

Abstract

Characterization is the first step in the conservation and utilization of indigenous genetic resources. A protocol for micro propagation of three important high altitude medicinal plant species (*Ficus cordata* ssp. *salicifolia* (vahl) C.C. Berg, *Ficus palmata* Forsk and *Pulicaria vulgaris* Gaertner) was established using nodal segments harboring axillary buds as explants. RAPD-PCR technique was used to characterize the three studied medicinal plants. *In vitro* stages of shoot initiation and shoot multiplication were studied by culturing the explants on Murashige and Skoog medium (MS). MS medium was supplemented with different concentrations and combinations of benzyl aminopurine, kinetin, α -naphthalene acetic acid and 2-isopentyladenine. For *Ficus cordata* ssp. *salicifolia* (vahl) C.C. Berg, the highest percentage of shoot initiation (86.6%) and the maximum average number of multiplied shoots (3.25) were observed on MS medium containing 0.5 mg/ l BAP + 1 mg / l 2iP. For *Ficus palmata* Forsk, the highest percentage of shoot initiation (92%) was observed on MS medium supplemented with 2.5 mg/ l BAP + 1.0 mg/ l Kn, whereas maximum average number of multiplied shoots (3.25) was produced on MS medium with 2.0 mg/ l BAP. For *Pulicaria vulgaris* Gaertner, the highest percentage of shoot initiation (93.3) was achieved on MS medium containing 0.1 mg/ l Kn + 3.0 mg / l 2iP with an average of (3.75) shoots / explants. Fourteen RAPD primers have successful PCR amplification and produced informative and distinct electrophoretic banding pattern. Different DNA fragments were produced and utilized for characterizing the studied plants. RAPD markers are yield different information and detect differences along the entire genome. Different studies are needed to collect, characterize and propagate other important medicinal plants from Taif high altitude regions.

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Introduction

Since ancient times, mankind has been dependent on plants for food, flavours, medicinal and many other uses. Ancient written records of many civilizations (i.e. Egyptian, Roman, Chinese) give strong evidence regarding use of medicinal plants (Cowan, 1999), for example ayurveda documents record the use of medicinal plants to cure many ailments (Micke *et al.*, 2009; Patwardhan *et al.*, 2005).

The flora of Saudi Arabia is one of the richest biodiversity areas in the Arabian Peninsula and comprises very important genetic resources of crop and medicinal plants. In addition to its large number of endemic species, the components of the flora are the admixture of the elements of Asia, Africa and Mediterranean region (Atiqur Rahman *et al.*, 2004). The greatest species diversity has been observed in Asir, Hijaz and the western mountainous area of the Kingdom, which borders the Red Sea. This is due to a greater rainfall and range of altitude from sea level to 9300 ft at Jabal Sawdah, near Abha (Collenette, 1998).

Ficus palmata is a small tree belonging to family *Moraceae* (Khan *et al.*, 2011). This plant is distributed in Nepal, Somalia, South Egypt, Peninsula and India (Srivastava *et al.*, 1996; Bhatt *et al.*, 2010).

F. palmata is used as fuel wood and traditionally used for the effective treatment of many diseases, viz skin diseases, ringworm, wound infections and haemorrhoid (Manandhar, 1995; Sabeen and Ahmad, 2009). The fig fruit (*Ficus palmata*) is very nourishing food and used in industrial product under various forms, i.e. fresh, dried and canned, stuffed with nuts, covered with chocolate or aromatized in different ways (Guasmi *et al.*, 2006; Palopoli, 1990). *Ficus cordata* Thunb (*Moraceae*) is a savana tree of around ten meters height found in Senegal, Angola, South Africa and Cameroon (Sabatie, 1985). The leaves of this plant are used against hyperaesthesia, ataxia, muscle tremor and padding motions and can kill heifers 48h after ingestion (Poumale *et al.*, 2008). Additional ethnopharmacological investigations

showed that the stem bark of this plant is used by some western Cameroonian traditional healers for the treatment of *jaundice*; who can be a symptom of several related liver diseases (Donfack, 2011).

The genus *Pulicaria* is rich in phenolic compounds and monoterpene hydrocarbons and comparatively low in sesquiterpene hydrocarbons. The essential oil obtained from steam distillation of aerial parts contain (+) carvotanacetone, beta-linalool and thymol as major constituents. The oil exhibited activity against Gram-positive and Gram negative bacteria (Mossa *et al.*, 1987; El-Kamali *et al.*, 1998; El-Kamali *et al.*, 2009; El-Kamali *et al.*, 2010).

Medicinal plants represent an important health and economic component of biodiversity. It is essential to make the complete inventory of the medicinal component of the flora of any country for conservation and sustainable use. The conservation of the threatened and endangered medicinal species in the wild is indispensable (Mossa *et al.*, 2000).

Gene bank is a collection of seeds and other plant reproductive material, primarily of cultivated plants and their wild relatives. The mandate of a gene bank is to secure the conservation of these collected plant genetic resources and provide access to them. In addition, a gene bank is responsible for registering, studying, describing, and documenting its collection, and making both information and plant material available to researchers and other interested users (FAO, 2013).

Although field gene banks provide easy access to conserved material for use, it is run the risk of destruction by natural calamities, pests and diseases. For this reason, safety duplicates of the living collections are established using alternate strategies of conservation and it is in this area that biotechnology contributed significantly by providing complementary *in vitro* conservation options through tissue culture techniques. *In vitro* conservation also offers other distinct advantages. For example, the material can be maintained in a pathogen-tested

state, thereby facilitating safer distribution. Moreover, the cultures are not subjected to environmental disturbances (Withers and Engelmann, 1997).

The *in vitro* propagated medicinal plants furnish a ready source of uniform, sterile, and compatible plant material for biochemical characterization and identification of active constituents (Wakhlu and Bajwa, 1986; Miura *et al.*, 1987). In addition, compounds from tissue cultures may be more easily purified because of simple extraction procedures and absence of significant amounts of pigments, thus possibly reducing the production and processing costs (Chang *et al.*, 1994).

Characterization and evaluation of genetic diversity is the first step in the conservation and utilization of indigenous medicinal plant species. Moreover Estimation of genetic diversity is a prerequisite for improving of any species or genetic material. Molecular markers have been utilized in order to characterize medicinal plants and evaluation genetic diversity within and among medicinal plants genotypes using different PCR techniques based on nuclear and /or mitochondrial genomes (Akbulut *et al.*, 2009; De Masi *et al.*, 2005).

Random amplified polymorphic DNA (RAPD) is a polymerase chain reaction (PCR) based fingerprinting technique that amplifies random DNA fragments with single short primers of arbitrary nucleotide sequence under low annealing stringency (Williams *et al.*, 1990).

RAPD technique has an extra advantage that it does not require any sequence information on the target genome. The RAPD markers have been described as a simple and easy method to use for species characterization (Guasmi *et al.*, 2006; Poeaiml *et al.*, 2012). Several studies have been conducted to characterize *Pulicaria* species and at molecular level in different countries (De Masi *et al.*, 2005; Guasmi *et al.*, 2006; Akbulut *et al.*, 2009; El-Kamali *et al.*, 2010; Poeaiml *et al.*, 2012).

This study was carried out to develop micro propagation protocols for three important medicinal plant species *Ficus cordata* sp. *salicifolia* (vahl) C.C. Berg, *Ficus palmata* Forsk and *Pulicaria vulgaris* Gaertner as well as genetic characterization of these plant species. The present work is preliminary step towards establishment of medicinal plant gene bank at Taif University, KSA.

Materials and methods

Media preparation

Full strength MS medium supplemented with 3% (w/v) sucrose, 0.7% (w/v) phytoagar and different concentrations and combinations of BAP, Kn, NAA and 2iP were used for shoot initiation, multiplication and elongation stages. The pH of all media was adjusted to 5.7 using 1.0 N potassium hydroxide (KOH) and 1.0 N hydrochloric acid (HCl), before adding 0.7% (w/v) phytoagar. Media were autoclaved for 20 min at 121°C and 1.5 k/cm² pressure.

Source of the explants

Nodal explants containing lateral buds were selected and excised from shoots of medicinal plant species *Ficus cordata* ssp. *salicifolia* (vahl) C.C. Berg, *Ficus palmata* Forsk and *Pulicaria vulgaris* Gaertner. The plant materials were collected from Wadi Zee Ghazal, Al-Shafa at Taif governorate. The nodal explants were cut in 3-4 cm length segments and rinsed using tap water to remove the superficial dust followed by a detergent for 3 min and surface sterilized by dipping in 70% ethanol for 1 min, then they were surface-disinfested for 10 min using Clorox solution 20% (5.25% NaOCl) containing 2 drops of Tween-20 emulsifier to aid wetting. The sterilized explants were washed 2-3 times with sterile distilled water (s.d. H₂O) to remove disinfecting solution. They were trimmed down to 1 cm long prior to transferring on shoot initiation medium.

In vitro culture

Sterilized nodal cuttings containing axillary bud explants were cultured for four weeks on shoot initiation media containing full strength MS medium with various concentrations and combinations of BAP

, Kn, NAA and 2iP were used for shoot initiation as shown in table 1. For shoot multiplication, induced shoots were subcultured on the same media for another four weeks. MS medium without growth regulators was used as a control for all the *in vitro* cultures treatments. The number of explants initiating shoots and the average number of shoots per explant were recorded. All the *in vitro* cultures were incubated at $26 \pm 2^{\circ}\text{C}$ in a growth room on a 16/8 hour light/dark and 3,000 lux light intensity provided by cool-white fluorescent light.

DNA extraction and RAPD PCR

Genomic DNA was extracted according to manufacturer's instructions of Plant tissue DNA extraction kit (Favorgen, National Biotechnology Park, Taiwan). Briefly, Plant tissues were grinded in liquid nitrogen and lysed by buffer containing detergent. The tissue debris in lysate was removed by provided filter column. In the presence of a chaotropic salt, the genomic DNA in the lysate binds to the glass fiber matrix in the spin column. After washing off the contaminants, the purified DNA was eluted by low salt elution buffer or water. Spectrophotometric determination of DNA concentration at A260 was done. DNA integrity was checked by agarose gel electrophoresis 1%. PCR reactions were conducted using 2x superhot green PCR Master Mix (Promega, USA) with 10 Pmol of each 20 different arbitrary 10-mer primers (Operon Technologies, Inc.) and 25-50ng/ul DNA template.. The names and sequences of successful oligoprimers are listed in Table 2. RAPD amplifications were performed in a Eppendorf Master cycler using the following PCR program: 1 cycle at 94°C , 2.5 min; 40 additional cycles consisting of 94°C 45 sec, 35°C 30 sec, and 72°C 2 min. After the amplification, the PCR reaction products were eletrophoresed with 100 bp ladder marker (Fermentas, Germany) on 10 x 14 cm 1.5%-agarose gel (Bioshop; Canada) for 30 min using Tris-borate-EDTA Buffer. The gel was stained with 0.5 µg/ml of ethidium bromide (Bioshop; Canada).

RAPD gels analysis

All gels were visualized and documented using a

GeneSnap 4.00-Gene Genius Bio Imaging System (Syngene; Frederick, Maryland, USA). The digital image files were analyzed using Gene Tools software from Syngene. Each band was scored as: present (1); or absent (0), and pair wise comparisons between samples were used to calculate the Jaccard's Coefficient of genetic similarity matrix. Hierarchical cluster analysis to produce a dendrogram was performed using the unweighted pair-group method with arithmetical (UPGMA).

Results

In vitro propagation

Higher germination rate is an important factor for establishing plant tissue culture and be particularly useful when there is a need to submit a uniform set of seedlings to a treatment (Sakhanokho *et al.*, 2001). As shown in (Table 1), Nodal explants cultured on MS medium supplemented with different concentrations and combinations of (BAP, Kn, NAA and 2iP) induced more axillary shoot proliferation when compared with the MS medium without growth regulators (control medium). For *Ficus cordata* ssp. *salicifolia* (vahl)C.C. Berg, the highest percentage of shoot initiation (86.6%) and the maximum average number of multiplied shoots (3.25) were observed on MS medium containing 0.5 mg/ l BAP + 1 mg / l 2iP (Figure 1). For *Ficus palmata* Forsk, the highest percentage of shoot initiation (92%) was observed on MS medium supplemented with 2.5 mg/ l BAP + 1.0 mg/ l Kn, whereas maximum average number of multiplied shoots (3.25) was produced on MS medium with 2.0 mg/ l BAP (Figure 2). For *Pulicaria vulgaris* Gaertner, the highest percentage of shoot initiation (93.3) was achieved on MS medium containing 0.1 mg/ l Kn + 3.0 mg / l 2iP with an average of (3.75) shoots / explants (Figure 3).

Genetic polymorphism

Twenty RAPD primers were tested in the present study. Of these, 14 primers which are listed in (Table 2) were produced good amplification products and chosen for RAPD analysis. The remaining 6 RAPD primers did not produce amplification products and storable bands. Among the different primers used,

173 bands were amplified. Of these, 151 were polymorphic (87.3%) and 22 were monomorphic (12.7%). The total number of amplified bands through all primers and plants was 233. Variations in the size and number of amplified fragments from each primer were detected. The size of amplified fragments ranged

from approximately 150 bp in primer A3 and C3 (in sample 8) to approximately 3000 bp in primer A4 in all studied samples. The maximum number (33 fragments) was amplified with primer F3 and the minimum number (7 fragments) was amplified with primer B2.

Table 1. Effect of different concentrations and combinations of BAP, Kn, NAA and 2iP on shoot initiation and multiplication from auxiliary buds explants of *Ficus cordata* salicifolia, *Ficus palmata* Forsk and *Pulicaria vulgaris* Gaertner.

Name of species	Growth regulators (Mg/l)				NE	NIS	IS%	ANS/E
	BAP	Kn	NAA	2ip				
<i>Ficus cordata</i> ssp. salicifolia	0.0	0.0	0.0	0.0	30	7.0	23.3	0.00
	0.5	0.0	0.0	1.0	30	26	86.6	3.25
	1.0	0.0	0.0	0.5	30	12	40.0	2.25
	2.0	0.0	0.0	0.1	30	18	60.0	2.75
<i>Ficus palmata</i> Forsk	0.0	0.0	0.0	0.0	25	5.0	20.0	0.00
	1.0	0.3	0.1	0.0	25	15	60.0	2.75
	2.0	0.0	0.0	0.0	25	20	80.0	3.25
	2.5	1.0	0.0	0.0	25	23	92.0	1.75
<i>Pulicaria vulgaris</i> Gaertner	0.0	0.0	0.0	0.0	30	8.0	26.6	0.00
	0.0	1.0	0.0	1.0	30	23	76.6	1.25
	0.0	0.5	0.0	2.0	30	25	83.3	2.75
	0.0	0.1	0.0	3.0	30	28	93.3	3.75

NE: Number of explants. NIS: Number of initiated shoots. IS%: Percentage of initiated shoots.

ANS/E: Average number of shoots/explants.

Table 2. List of primers, their nucleotide sequences and total number of bands for each plant produced by 14 primers.

Primer code	Primer sequence	plants			Total bands	Amplified bands	Polymorphic bands	Monomorphic bands
		3	8	9				
OP A-1	CAGGCCCTTC	4	6	3	13	9	8	1
OP A-2	TGCCGAGCTG	8	7	6	22	11	7	4
OP A-3	AGTCAGCCAC	1	3	7	11	10	10	0
OP A-4	AATCGGGCTG	7	8	5	20	13	11	2
OP B-2	TGATCCCTGG	1	3	3	7	5	5	0
OP C-3	GGGGGTCTTT	4	10	4	18	12	11	1
OP E-2	GGTGC GGGA	6	9	10	24	16	15	1
OP E-4	GTGACATGCC	3	7	5	15	12	12	0
OP E-6	AAGACCCCTC	3	10	5	17	12	12	0
OP E-7	AGATGCAGCC	6	14	13	17	19	16	3
OP F-3	CCTGATCACC	9	8	6	33	16	15	1
OP F-7	CCGATATCCC	6	5	3	22	12	12	0
OP G-2	GGCACTGAGG	6	8	9	14	10	6	4
OP H-5	AGTCGTCCCC	9	11	10	21	16	11	5
Total		73	109	89	233	173	151	22

Discussion

In vitro propagation

Cytokinin is one of the plant hormones crucial for plant growth and development and is known to promote cell division and differentiation (Dello *et al.*, 2007). The effect of different plant growth regulators

especially cytokinins on multiple shoot formation has been reported (Ganesan and Jayabalan, 2006; Hussain *et al.*, 2008; Sivanesan *et al.*, 2008; Aruna *et al.*, 2012; Sonali *et al.*, 2013). According to the responses of nodal explants of *Ficus cordata* ssp. salicifolia and *Pulicaria vulgaris* Gaertner as shown

in (Table1).



Fig. 1. Shoot multiplication stage of a medicinal plant *Ficus cordata* ssp. *Salicifolia*.



Fig. 2. Shoot initiation and multiplication stages of a medicinal plant *Ficus palmata* Forsk.

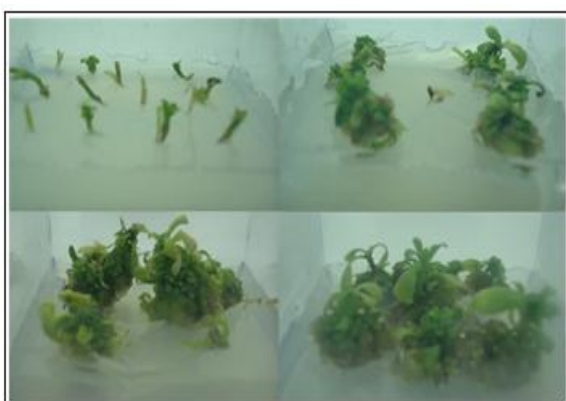


Fig. 3. Shoot initiation and multiplication stages of a medicinal plant *Pulicaria vulgaris*.

Although an increase in BAP concentration to 2 mg/l with low concentration of 2iP 0.1 mg/l gave an increase in shoot initiation percentage (60%) but an increase in 2iP concentration to 1 mg/l and decrease in BAP concentration to 0.5 mg/l was more efficient

on shoot initiation percentage (86.6%). These results are relatively similar with the results reported on the effect of cytokinins on *in vitro* multiplication of *Sophora tonkinensis* by Sonali *et al.* (Sonali *et al.*, 2013), an increase in 2iP concentration from 0.5 to 2.0 μ mol resulted in an increase in the induction of axillary shoots. Maximal shoot regeneration (75%) was achieved on the MS medium containing 2.0 μ mol 2iP with an average of 5.0 shoots per explants. The results of the responses of nodal explants of *Ficus palmata* Forsk in (Table1) showed that, an increase in BAP concentrations from 1.0-2.5 mg/l resulted gradually increased in shoot initiation percentage while an increase of BAP 2.0- 2.5 mg/l resulted in decrease of an average number of shoots (3.25-1.75) per explants. The effect of BAP on shoot multiplication has been reported in some medicinal plants (Martin, 2000 ; Mulwa and Bhalla, 2000; Sasikumer *et al.*, 2009).It was demonstrated that, the use of high cytokinin levels was one of the most effective methods to reduce shoot and leaf growth and promote the formation of meristematic clusters (Lobna *et al.*, 2008). According to the results in (Table 1), the combination of (BAP and Kn) was more efficient on shoot induction than the combination of (BAP, Kn and NAA). The effect of auxin on shoot induction has been reported (Gulati and Jaiwal, 1992; Khalaflla and Hattori, 2000; Ishag *et al.*, 2009), it was reported that the addition of NAA to medium containing cytokinin did not improve shoot multiplication rate.

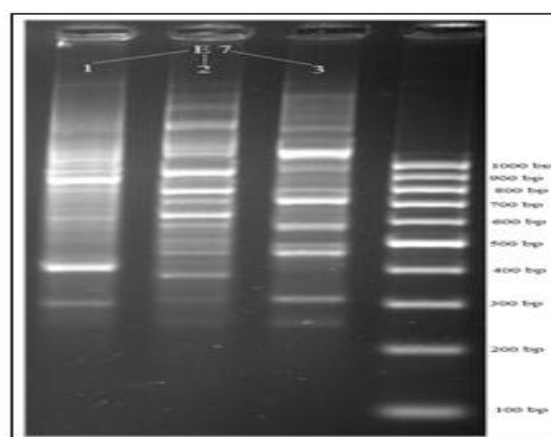


Fig. 4. RAPD electrophoretic banding pattern produced by OP-E-7. Lane M: Standard DNA Ladder 100bp; Lane 1: *Pulicaria vulgaris*; Lane 2: *Ficus cordata*; Lane 3: , *Ficus palmate*.

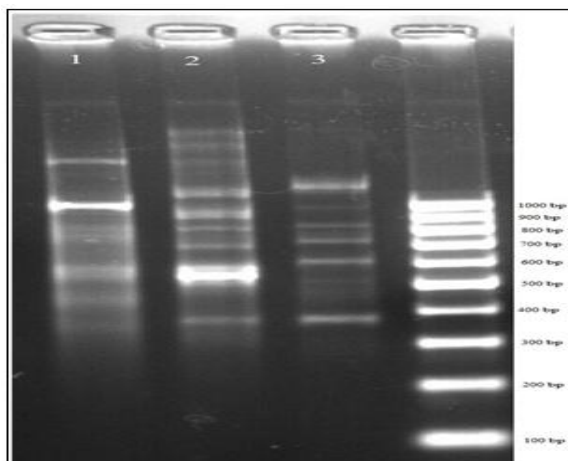


Fig. 5. RAPD electrophoretic banding pattern produced by OP-E-4. Lane M: Standard DNA Ladder 100bp; Lane 1: *Pulicaria vulgaris*; Lane 2: *Ficus cordata*; Lane 3: , *Ficus palmate*.

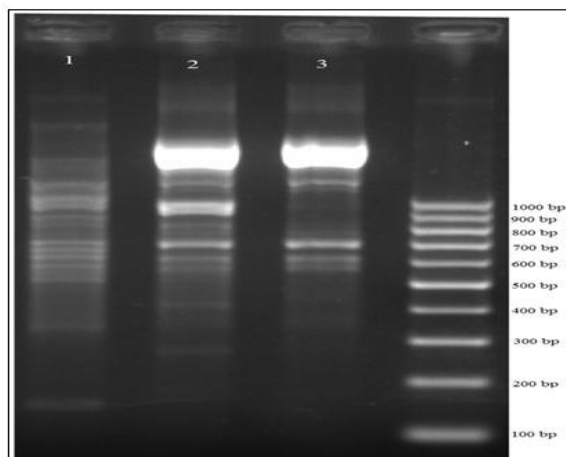


Fig. 6. RAPD electrophoretic banding pattern produced by OP-H-4. Lane M: Standard DNA Ladder 100bp; Lane 1: *Pulicaria vulgaris*; Lane 2: *Ficus cordata*; Lane 3: , *Ficus palmate*.

During present work 14 primers have successful PCR amplification and produced informative and distinct electrophoretic banding pattern. The size and number of produced DNA fragments was varied with different primers which indicate random pattern of amplification and its might be due to nucleotide changes at the primer annealing site or due to addition or deletion between two priming sites (Sharma *et al.*, 2001). This pattern of amplification indicates a genetic heterogeneity between the studied plant species and random pattern of amplification (Kamaleldin *et al.*, 2003). The maximum number (33fragments) was amplified with primer (F3) and

the minimum number (7 fragments) was amplified with primer (B2). Therefore the markers used in the present investigation proved to be quite powerful in the characterization of the studied genotypes. Moreover the RAPD technique is to be applicable for genotype identification. RAPD-PCR markers yield different information, since they analyze different sequences and detect different types of variations in the plant DNA. RAPD-PCR detects differences along the entire genome, not only in particular sequences (Ortiz-Herrera *et al.*, 2004).Therefore RAPD markers have been utilized to characterizing large number of medicinal plants such as *Pulicaria* sp. (Miura *et al.*, 1987); *Juniperus* (Kasaian *et al.*, 2011); *Ficus* sp.(De Masi *et al.*, 2005; Akbulut *et al.*, 2009; Basheer-salimia *et al.*, 2012).

Abbreviations

BAP, Benzyl aminopurine; Kn, kinetin; MS, Murashige and Skoog medium; NAA, α - naphthalene acetic acid; 2iP, 2-isopentyladenine ;s.d.H₂O, sterile distilled water; RAPD-PCR, Random amplified polymorphic DNA- polymerase chain reaction.

Conclusion

In this study a micro propagation protocols and RAPD PCR technique were adopted for three important medicinal plant species *Ficus cordata* ssp. *salicifolia* (vahl) C.C. Berg, *Ficus palmata* Forsk and *Pulicaria vulgaris* Gaertner. These protocols will help for characterization as well as mass propagation of these species for pharmaceutical industries and *in vitro* germplasm conservation.

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