Rapid *in vitro* multiplication of *Drosera indica* L., *D. burmanii* Vahl. and molecular diversity in *Oroxylum indicum* Vent. by RAPD analysis: Vulnerable medicinal plants

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DOCTOR OF PHILOSOPHY

By

Jayaram K

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046, India

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Department of Plant Sciences

School of Life Sciences

University of Hyderabad

Hyderabad 500 046, India

CERTIFICATE

This is to certify that **Mr. Jayaram K** has carried out the research work embodied in the present thesis entitled **"Rapid *in vitro* multiplication of *Drosera indica* L., *D. burmanii* Vahl. and molecular diversity in *Oroxylum indicum* Vent. by RAPD analysis: Vulnerable medicinal plants"** for the degree of Doctor of Philosophy under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.

6/6/05

M.N.V. Prasad
Supervisor & Professor

Professor M. N. V. PRASAD
Dept. Plant Sciences
University of Hyderabad
Central University P.O.
HYDERABAD-500 046. INDIA

Head
Department of Plant Sciences

HEAD,
Dept. of Plant Science

Dean
School of Life Sciences

Dean, School of Life Sciences
University of Hyderabad,
Hyderabad-500 134. (India)

Department of Plant Sciences

School of Life Sciences

University of Hyderabad

Hyderabad 500 046, India

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Rapid *in vitro* multiplication of *Drosera indica* L., *D. burmanii* Vahl. and molecular diversity in *Oroxylum indicum* Vent. by RAPD analysis: Vulnerable medicinal plants"** has been carried out by me under the supervision of Professor M.N.V. Prasad in the Department of Plant Sciences, School of Life Sciences. University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

Jayaram K
(Candidate)
Enrol. No: 2KLPPHO7

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Jayaram K

Abbreviations

µg	Microgram
µl	microlitre
µM	micromolar
2,4-D	2,4- Dichlorophenoxy acetic acid
BAP	6-Benzyl Amino Purine
$CHCl_3$	Chloroform
CTAB	Cetyl Trimethyl Ammonium Bromide
dNTP	Deoxy nuclcotide tri-phosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
KN	Kinetin
M	Molarity
MgL^{-1}	milligram/litre
mM	millimolar
MS	Murashige and Skoog
MW	Molecular Weight.
NAA	∝-Naphthalene Acetic Acid
PCR	Polymerase Chain Reaction
PVP	Polyvinyl Pyrrolidone
RAPD	Random Amplified Polymorphic DNA
Taq	*Thermus aquaticus*
TE buffer	Tris EDTA buffer
UPGMA	Unweighted Pairwise Group Matrix for Arithmetic Average.
V/V	Volume/Volume
W/V	Weight / Volume
Z	Zeatin

1. General introduction

India is one of the 12 mega biodiversity centers. It has immense biotic wealth, marked by remarkable ecosystem, species and genetic diversity matched equally by rich cultural diversity and health traditions. Over 7000 species out of an estimated 17,000 higher angiosperms recorded from India are reportedly used for medicinal purposes (Groombridge, 1992). It is estimated that 70-80 % of people worldwide rely chiefly on traditional, largely herbal medicines to meet their primary healthcare needs (Farnsworth et al., 1991; Pei, 2001). India is one of the leading countries exporting medicinal plants contributing to the country's economy. According to an all India ethnobiological survey carried out by the Ministry of Environment and Forests, Government of India, there are about 8000 plants are being used by the people for primary health care needs. Most of the medicinal plants (about 90-95%) are collected from wild and very a few are cultivated (Wakdikar, 2004). Thus, there is an enormous pressure on the plant resources due to escalating demand from the herbal industry.

Today, a large number of medicinal plant species are facing threat due to high demand and destructive collection practices apart from the adverse environmental factors. As on to day, herbal health care product industries are depending on cultivated as well as wild collections of plants. There can be advantages to wild collection over cultivation. From the medical point of view, there is a widespread belief that wild-harvested material is more effective, and attracts higher prices. Thus,

aggressive and in discriminate collection poses a threat to the medicinal and aromatic plants.

Over-harvesting, habitat destruction and fragmentation have restricted an increasing number of plant species to small and isolated populations. Even in undisturbed habitat, plants with healing properties face increased risk because of environmental, demographic and genetic stochasticity (Fisher and Matthies, 1998; Hamilton, 1997). Medicinal plants can have other uses than as sources of medicines, and the threats from over-harvesting may be partly due, to collection for purposes other than medicinal.

There is no absolute estimate for the number of red listed medicinal plants on national and global scale (Schippmann et al., 2002; Vorhies, 2000). This is because populations of many medicinally important species are in best areas oi" populations being destroyed and the extent of many natural habitats are being altered (WWF and IUCN, 1994-1997). There would seem little doubt from theoretical considerations that many medicinal plant species listed as threatened, and indeed others that have not, must be suffering from genetic erosion now, or will do so in the near future (Holsinger and Gottlieb, 1991; Menges, 1991). Thus, conservation efforts are of immediate necessity to protect medicinally important species, without which the species may vanish.

There are two basic conservation strategies, each composed of various techniques that the conservation can adopt to conserve genetic diversity once it has been located. The two strategies are (i) *in situ* and (ii) *ex situ* conservation. Article 2

of the convention on biological diversity (CBD, 1992) provides the definition of these categories (Nigel, 2001). The best approach to prevent extinction is habitat preservation. Unfortunately, habitat preservation is not practicable in many cases. Therefore, micro and macro propagation are essential for multiplication and reintroduction to nature.

The primary objective of nature conservation is the maintenance of genetic diversity. Maintenance of genetic variation is considered essential for the long-term survival of a species (Frankel and Soulé, 1981). Thus, information on the genetic diversity of a species is important in planning its conservation. Small populations are subjected to loss of rare alleles, and this may be important for the long-term response to selection and survival of populations (Allendorf, 1986). Moreover, small populations are prone to extinction from random environmental fluctuations (Goodman, 1987).

For efficient conservation and management of medicinal plant diversity, the genetic composition of species collected from different geographic regions need to be assessed. In order to preserve a species genetic diversity in captivity, it is necessary to carry over much of that diversity from the natural population into the initial captive population. The maintenance of existing levels of genetic diversity is the key issue in conservation biology (Li et al., 2002). Genetic diversity became an issue when Frankel, (1974) postulated that genetic variation is essential for long-term survival of species and for ecological success. If the natural population becomes extinct, reintroduction from *ex situ* conserved population is the only alternative for habitat

restoration. Consequently, the amount of genetic variation in the captive population is critical to assure the success of *ex situ* conservation and subsequent release (Li et al., 2002).

Therefore it is in this context, it is necessary to understand the molecular diversity, which may facilitate adaptation to ever fluctuating environmental factors. Molecular analysis of lntraspecific variation in particular may find application in resolving disputes of taxonomic identities, relations and authentification of the species, developing a comprehensive database of genetic variability in the species for future reference and protection of genetic diversity of the species, identification of useful genotypes that could be developed as cultivars for field trails and sustainable utilization.

There are a few commendable efforts to study the genetic variation within populations of selected medicinal plant species based on isozyme profile in different regions. However, a broad analysis of genetic diversity and within species diversity is rather scanty. Preserving the genetic diversity of a species in captivity and maintenance of the existing levels of genetic diversity are major issues in conservation biology. With the advent of new generation of molecular tools over the last two decades, the entire scenario of biological sciences has been revolutionized. DNA-based molecular markers have acted as versatile tools and have found their own position in all fields of plant biology. The discovery of PCR was a landmark in this effort and proved to be a unique process that brought about a new class of DNA profiling markers. This facilitated the development of marker based gene tags, map

based cloning of agronomically important genes, variability studies, phylogenetic analysis, synteny mapping, marker assisted selection of desirable genotypes, etc. thus giving new dimensions to the topic of genetic diversity of plants. These DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analyzed objectively.

Welsh and McClelland, (1991) developed a new PCR based genetic assay namely randomly amplified polymorphic DNA (RAPD). This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplified range of each other, a discrete DNA product is formed through thermocyclic amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals. However, due to the stochastic nature of DNA amplification with random sequence primers, it is important to optimize and maintain consistent reaction conditions for reproducible DNA amplification. They are dominant markers and hence have limitations in their use as markers for mapping.

The amount of genetic variation in the captive population is critical to the success of *ex situ* conservation. RAPD polymorphism is the reflection of variation of the whole genomic DNA, which would be a better parameter to measure the variation in red listed plant species. In this thesis research findings are presented in two parts.

Part A

Rapid *in vitro* multiplication of *Drosera indica* L. and *D. burmanii* Vahl. Vulnerable medicinal plants.

2. Plants that prey on insects- review of literature

Carnivorous plants have fascinated evolutionary ecologists, botanists and horticulturists for centuries. Early investigators were reluctant to accept that plants could consume small insects and invertebrates (Juniper et al., 1989). Darwin, (1875) provided the first detailed experimental evidence for carnivory in several genera. Since then, approximately 600 species were identified. Which are grouped into 12 angiosperm families, including both monocotyledons, dicotyledons and are sub-classified into 20 genera (http://www.honda-e.com; Juniper et al.. 1989; Albert et al., 1992; http://thecarnivorousplantscociety.org) as follows.

a) Eriocaulaceae [1 genus; *Paepalanthus* (1 species) distributed in Brazil]
b) Bromeliaceae [2 genus; *Brocchinia (2* species) distributed in Coloumbia and Venezuela, *Catopsis* (1 species) distributed in South and North America].
c) Byblidaceae [1 genus *Byblis* (5 species) and endemic to Australia and New Guinea].
d) Cephalotaceae [*Cephalotus follicularis* is the only species and is endemic to Australia].

e) Dioncophyllaceae [*Triphyophyllum peltatum* of western Africa]

f) Droseraceae [3 genus; *Aldrovanda* (1 species) distributed in Africa, South Asia and Australia; *Dionaea* (1 species) endemic to North and South Carolina; *Drosera* (above 170 species) distributed all over the world].

g) Drosophyllaceae (1 genus; *Drosophyllum* (1 species) distributed in Portuguese].

h) Lentibulariaceae [5 genus; *Utricularia* (218 species) distributed all over the world; *Genlisea* (20 species) distributed in Africa, South-North America; *Pinguicula* (77 species) distributed all over the world except in Australia].

i) Nepenthaceae [1 genus *Nepenthes* (92 species) distributed in Southeast Asia, Madagascar, and Australia].

j) Sarraceniaceae [3 genus; *Darlingtonia* (1 species) distributed in North America; *Heliamphora* (10 species) distributed in South America; *Sarracenia* (8 species) distributed in North America].

k) Martyniaceae [2 genus; *Ibicella* (1 species) distributed in South America and Africa; *Proboscidea* (1 species) distribution is Unclear].

1) Roridulaceae [1 genus *Roridula (2* species) distributed in Africa].

The multiple, independent evolution of carnivory in diverse plant families suggests that it is an adaptation to the low nutrient, bright, waterlogged habitats (Givnish et al., 1984). All carnivorous plants can attract, trap, digest and absorb nutrients especially nitrogen and phosphorous from the insect body with the help of digestive enzymes for their metabolic process.

Carnivorous plants are divided into two types based on the trapping mechanism. In the passive type trap, the simplest kind of trap is the pitfall where, the plants create a chamber into which the prey plummets. Digestive enzymes secreted at the bottom of the vase perform the digestion, and the plant absorbs nutrients. The second type is the active type where, leaves have glandular, sticky surfaces adapted to emit either a sugary/fungal smell/glisten in the light to attract insects. When insects lands on these leaves, the legs and wings of the insects become mired and are eventually trapped. The mucus secreted by these leaves coats and suffocates them finally leading to their death. The leaf may even curl over the prey to increase the number of digestive glands that are in contact with the invertebrate morsel.

Many of the insectivorous plants are now endangered because of restricted habitat, medicinal value, botanical curiosity, invasive species and agricultural pollutants. Consumers' interest in conservation of rare plants is a mixed blessing for the industry and the environment. To the commercial growers the demand represents opportunity, but is discouraged by lack of reliable information on the materials and production methods. Some growers may resort to large-scale field collection of certain *Drosera* species, which would result in extinction.

In India three families of insectivorous plants are present in different regions. They are Droseraceae [(2 genera: *Drosera* (3 species) and *Aldrovanda* (1 species)], Nepenthaceae *[(Nepenthes khasiana* (1 species)] and Lentibulariaceae [(2 genera: Utricularia (30 species) and *Pinguicula* (1 species)].

2.1. ***Drosera*** (sundew)

Out of the 453 pages of Darwin's (1875) book on "insectivorous plants", 285 pages are devoted to *Drosera*, to divulge every secret vividly (Clancy and Coffey, 1977). Among the different genera of insectivorous plants, *Drosera* was the first one to be described in detail and its habitat was thoroughly investigated. The *Drosera* commonly called "sundew" consists of approximately 170 species (http://thecarnivorousplantscociety.org), of which about 110 species grow only in the Southern Hemisphere (Mabberley, 1997), with Australia accounting for 54 species (Pietropaola and Pietropaola, 1986), approximately 15 species are native to the Northern Hemisphere and other species are native to the Southern Hemisphere (Trease and Evans, 1978; Marchant et al., 1982; Marchant and Lowrie, 1992; Hoshi and Kondo, 1998). The species of *Drosera* are perennial or annual herbs with hermaphrodite flowers (Chen et al., 1997).

The species of *Drosera* differ enormously in size, habit and show large diversity. The leaf blade may vary in length from as little as 1/20 inch (1.5 mm) to over 2 feet (60 cm). It may be simple or much divided, and can be borne in a rosette at ground level, or singly on a tall or even climbing stem. The upper surface bears tentacles. The root may be either fibrous or fleshy, the stem may shoot from a tuber (Dixon and Pate, 1978; Slack, 1980; Marchant et al., 1982; Pietropaola and Pietropaola, 1986; Pate, 1989). The stem bear single to many flowers. There are generally five petals, and depending on species, form or variety they are variously coloured. They are self or cross-pollinated by means of insect and wind.

The longest tentacles are found around the leaf margin. Generally these tentacles are able to bend only in one direction- towards the center of the leaf and respond to stimuli with greater haste than the inner tentacles. They are thus particularly useful in preventing the escape of larger insects. These tentacles are held outwards, more or less in line with plane of the leaf blade, but they are slightly reflexed. Moving inwards from the margin of leaf, the length of the tentacles gradually diminishes, while at the same time their angle of inclination steepens only in the central zone of the blade, the tentacle are very short and entirely upright. Stimuli may be produced by an irritation, a slight scratch, and a minute weight such as a tiny particle or by the application of either a solid or liquid nutritive substance. However, when tentacles are induced to close over a non-nutritive substance, they usually return to their normal position within 24 hours. The tentacles have glandular tipped stalks of fairly complex structure. The glands themselves are egg shaped, and usually develop a red coloration, especially when exposed to sunlight. They crown the stalks in an upright position, except in the case of the outer tentacles of some species, where the tip of the stalk is flattened into a little spoon in the center of which the gland stands. These glands are quite unique amongst those of other sticky leaved carnivorous plants in possessing three distinct and equally important functions (Slack, 1980; Pietropaola and Pietropaola, 1986).

They not only secrete mucilage, which catches and overpower the prey in the first instance, but also secrete the enzymes like peroxidase, acid phosphatase, esterase, Chitinases and proteases (Mc Nally et al., 1988; Clancy and Coffey, 1977).

They also absorb much of the resultant digested rich of nutrients into the plants system. The trapping action of *Drosera* is an active mechanism, when an insect alights on a leaf and comes into contact with a gland, it is quickly mired in the thick mucilage. As the insect attempts to pull away one of its appendages, the mucilage is drawn out into thin threads. This commotion induces the glands with which it is in contact to produce impulses, which traverse to other tentacles. The impulses trigger the secretion process in other tentacle glands, resulting in the release of additional fluids. Simultaneously, the tentacles commence bending towards the prey (Slack, 1980; Pietropaola and Pietropaola, 1986).

Eventually the flexing tentacles reach the prey, forcing it down to the surface of the leaf where it is bathed in fluids. The prey apparently drowns in these fluids. In many species a lesser or greater part of the leaf blade itself may become tightly folded over the prey. This action does not show signs of starting till the insect is already overcome, and usually takes between 24-48 hours to complete, playing no part in trapping the prey, however some have contradictory view (Slack, 1980). Its main advantage is to increase the surface area of leaf in contrast with prey hence eventually getting more glands into contact with insect. In this way more rapid and efficient digestion will be brought about, and it is not surprising that it occurs most frequently when larger preys are caught. It also plays a useful but secondary role in providing protection from rain, thus preventing the liquid products of digestion from being washed away before being consumed (Slack, 1980; Pietropaola and Pietropaola, 1986).

Species of *Drosera* catch a great proportion of small, crawling arthropods such as ants and springtails and a greater share of large, flying arthropods (Slack, 1980; Pietropaola and Pietropaola. 1986; Joel, 1986; Krafft and Handel, 1991; http://thecarnivorousplantsociety.org). Länger et al. (1995) studied the various shape of the glandular hairs in the genus *Drosera*. On the leaves and sepals of 52 species, representing all sections of the genus *Drosera* except one, 14 different types in total they are arranged in seven groups. The combination of these hairs and the presence of non-glandular hairs confirm the actual classification of the genus. Simple morphological characters on glandular hairs facilitate the identification of species even in the pharmaceutically important cut crude drug.

Almost all species of *Drosera* are found in open, wet, nutrient poor (especially nitrogen, phosphorus), generally acid soils (pH 3-6), usually in peat bogs, predominantly clayey with a small proportion of sand (Roberts and Oosting, 1958; Chandler and Anderson, 1976a; Adams et al., 1979; Givnish et al., 1984; Pietropaolo and Pietropaola, 1986; Givnish, 1989; Juniper et al., 1989; Brewer, 1998; Rice, 2002; Susandarini et al., 2002; Kämäräinen et al., 2003; Kim and Jang, 2004), but in Australia many species have evolved, which have adapted themselves to grow in much drier habitats, which may dry out seasonally or for considerable periods (Slack, 1980).

The most extensive process of carnivorous plants mineral nutrition is photosynthetic fixation of CO_2 by leaves. All carnivorous plants are green and able to fix CO_2 (autotrophy) according to the C_3 scheme of the Calvin cycle (Liittge, 1983).

The relationship between carnivorous plants photosynthetic performance and carnivory is complex and ambiguous (Juniper et al., 1989). Although photosynthetic rate of trap is lower than that of leaves (Knight, 1992; Adamec, 1997), carnivory may increase the plant total photosynthetic rate due to higher leaf biomass and also due to increased rate per unit leaf area (Givnish et al., 1984).

Drosera, is well known for its capacity to attract, capture, digest and use the nutrients derived from its prey for its own benefit, in addition to its usual capacity to photosynthesize as any other green plants (Lloyd, 1942; Chandler and Anderson, 1976a; Piliackas and Barbosa, 1986; Krafft and Handel, 1991; Rebdo-Torstensson, 1994; Brewer, 1998). Insectivory in carnivorous plants is considered to be important to the nutrition of species growing in habitats where nutrients are in limited supply (Darwin, 1896; Chandler and Anderson, 1976a; Watson et al., 1982; Givnish et al., 1984; Karlsson and Carlsson, 1984). The role of carnivory for plants can vary with community structure and also with the availability of different nutrients in the soil (Kraft and Handel, 1991). Various authors have established the abundance of carnivorous plants in moist habitats (Kats, 1941; Dixon and Pate, 1978; Aldenius et al., 1983; Brewer, 1998). According to Swamy and Ram, (1969) and Small et al., (1977) carnivory is more or less facultative for *Drosera* species. The contents of different mineral substances in the soil can significantly influence the characteristics of plants (Chandler and Anderson, 1976a; De-Ridder and Dhondt, 1992; Redbo-Torstensson, 1994).

As carnivorous plants grow together with non-carnivorous plants in their natural habitats, both plant groups are subjected to the same ecological conditions. Carnivory, which developed several times during plant evolution, is only " one of many possible adaptive strategies to unfavourable conditions (Juniper et al., 1989). The role of carnivory in the growth and reproduction of a variety of *Drosera* have been studied both *in vivo* and *in vitro* (Darwin, 1878; Kellerman and Von Raumer, 1878; Büsgen, 1883; Chandler and Anderson, 1976a; Thum, 1986; 1988; Juniper et al., 1989). The nutrient status of most microhabitats colonized by the genus is usually extremely poor, and nitrogen, phosphorus and possibly other elements available through carnivory have been shown to be of nutritional importance under such circumstances (Dixon et al., 1980; Schulz et al., 1991; Adamec, 1997).

There are two hypotheses to explain why carnivorous plants are largely restricted to open habitats. i) Carnivorous plants are especially intolerant of shade because the benefit of producing costly leaf traps declines as light becomes more limiting, and thus photosynthesis becomes less efficient in shady environments (Givnish et al, 1984). ii) The effectiveness of leaf traps to attract prey is severely compromised when obscured by competing vegetation (Gibson, 1983). Regardless of the appropriate cause, both the above hypotheses imply that carnivorous plants are poor competitors in dense vegetation compared to other types of plants.

Many carnivorous plants display considerable spatial separation between their flowers and traps. According to Juniper et al, (1989), Givnish, (1989) and Zamora, (1999) spatial separation in carnivorous plants may have evolved to avoid trapping

potential pollinators; the pollinator protection hypothesis (PPH). However long peduncles may also make flower more attractive to pollinators by placing them in more visible positions (Givnish, 1989; Peakall and Handle, 1993); the pollinator attraction hypothesis (PAH). Short *Drosera* typically trap non-aerial prey, whereas upright forms trap flying prey (Verbeek and Boasson, 1993) and potential pollinators. Thus, it is expected that tall plants would need to separate traps and flowers more han short plants. Short *Drosera* had a greater element of floral- trap separation than tall *Drosera.* Such a relationship is unexpected for plants whose peduncles were evolved to protect their pollinators. Anderson and Midgley, (2001) proposed that flower-trap separation evolved because carnivorous plants are often short and need to project their flowers well above ground level to make them more attractive to pollinators.

Mc Nally et al, (1988) studied the localization of acid phosphatases in the secretary cells of stalked gland tissue of *D. rotundifolia.* Clancy and Coffey, (1977) observed the leaves of the insectivorous plants *D. rotundifolia* produced extra cellular hydrolytic enzymes like acid phosphatses and proteases in response to feeding with gelatin. Chandler and Anderson, (1967) reported the extracts from the leaves and tentacles of field grown plants of *D. wittakeri*, *D. binala* and *D. auriculata* showed acid proteases activity and chitinase activity in field grown plants. Small et al, (1977) demonstrated the enzyme activity of nitrate reductase, nitrite reductase, peroxidases capable of reducing nitrate, glutamate dehydrogenase, and glutamate synthase and glutamine synthetase in cell-free extracts of roots and leaves of *D. aliciae.*

2.2. *In vivo* seed germination

In *Drosera* conventional propagation is achieved through seeds. Sowing seeds are one of the basic techniques used to propagate most *Drosera* species. Unfortunately very few properly documented reports are available on seed germination of individual *Drosera* species.

In *Drosera* the germination of seeds depend on the species. Seeds are to be stored at (2-7°C), be sown the following season. Seeds are sown in moist planting medium under controlled conditions like humidity (60-100 %), temperature (17-35°C). After the seed starts germinating, slowly harden the young seedling by successively increasing ventilation and decreasing the relative humidity to 60-80 %. Increasing temperature is harmful to seeds and young seedlings. Many of the *Drosera* species require stratification, cold or heat treatment for successful germination. Some species require soacking seeds in water in a cold environment. *D. arcturi* seeds germinated uniformly when seeds are laid on damp or water saturated media and are subjected to temperature changes (http://bestcarnivorousplants.org).

Since *Drosera* is a diverse and cosmopolitan genus, the subtropical and tropical *Drosera* seeds can be germinated in a planting medium containing a mix of peat moss and sand (2:1). Temperature should be maintained between 19-25°C and seeds germinate within several days or weeks. After several weeks *D. glanduligera* seeds were germinated at 8-12°C and continued for several months. The group **of forest** sundew *(D. adelae* etc.) can germinate at 28-35°C. South American species seeds may take long time to germinate, usually several months, with germination being **very**

unpredictable. American and European sundews required cold treatments to induce germination. A mixed planting medium (peat moss and sand at 3:1) was used for these species. Seeds usually germinate within several weeks after stratification. *D. binata* does not need stratification if is sown immediately after harvest. For fresh seed of *D. arcturi* stratification at a temperature range of 5-12ºC induces germination within 3 months. Germination of sown seed was 5 % after 5 months. Australian pygmy *Drosera* can be sown on planting medium (peat moss and sand at 2:1) at temperature of 15-25ºC. Thermal stratification or the addition of ash results in more uniform germination rate and is beneficial to *D. lasiantha.* Both procedures can be used for sowing common species as well. Germination is faster and the numbers of the seedling germinated are higher with this method. Seeds of species from the *D. petiolaris* are sown on the surface of the planting medium (peat moss: sand at 3:1 or 3:2) and germination start in a few weeks but sometime is prolonged for several months. It is interesting to note that most species from this group germinate reliably at 16ºC, e.g. *D. caduca*, nevertheless some species for e.g. *D. falconeri* require high temperature near 35ºC to start germination. There are several species of very interesting sundew which have been recently discovered but there is no information regarding their propagation methods (http://bestcarnivorousplants.org).

Recently, there has been considerable interest in germinating seeds of various taxa of Droseraceae. Conran et al. (1997) germinated seeds of about 100 taxa of *Drosera* and recorded number of days from time of sowing until the first seeds germinated and type of seedling morphology. Newly germinated seedlings of

Droseraceae differ with regard to the amount of cotyledonary tissue that emerges from the seed coats (Conran et al., 1997), and these morphological characteristics have been used along with molecular data, to construct cladograms of the family (Williams et al., 1994).

Seeds of *D. alicia* from South Africa required a 24 d period of imbibition at 15/10ºC before any germination occurred, but after 34 d ca. 70 of the seeds had germinated. If seeds were subjected to worm, moist conditions but not if they were stored dry (Ferreira and Small, 1974). In contrast, seeds of *D. rotundifolia* germinated to 30 % in light at 20/15ºC after 4 months of stratification at 5ºC (Grime et al., 1981). Seeds of *D. rotundifolia* mostly germinate in May (spring) in Sweden this indicates that dormancy break occurs during winter (Redbo-Torstensson, 1994). Crowder et al. (1990) obtained 90-95 % germination of *D. anglica, D. intermedia* and *D. rotundifolia* seeds from Saskatchewan, Canada, in light in a glasshouse at 18-22ºC after they had been imbibed in darkness at 10ºC for 8, 16 and 18 weeks respectively. Seeds of *D. anglica, D. intermedia* and *D. rotundifolia,* sown on wet filter paper in petridishes kept out door in Ontaria, Canada, all winter, germinated to 64, 22 and 0 % respectively, the following spring (Crowder et al., 1990). Kinzel, (1909) reported that seeds of *D. anglica, D. intermedia* and *D. rotundifolia* required light for germination. Crowder et al, (1990) also found that light was required for germination of *D. rotundifolia* and *D. anglica* but some seeds of *D. intermedia* germinated in darkness. Baskin et al, (2001) reported the seed dormancy breaking and germination requirements of *D. anglica,* an insectivorous species of the Northern Hemisphere.

Seeds of *D. anglica* collected from Sweden were dormant at maturity in late summer, and dormancy break occurred during cold stratification. Stratified seeds required light for germination, but light had to be given after temperatures were high enough to be favorable for germination. Seeds stratified in darkness at 5/1 °C and incubated in light at 12/12 h daily temperature regimes of 15/6, 20/10 and 25/15ºC germinated slower and to a significantly lower percentage at each temperature regime than those stratified in light and incubated in light.

2.3. Vegetative propagation

Naudin, (1840) was the first to record vegetative propagation of *Drosera*, he described the appearance of buds on the dorsal surface of a mature leaf of *D. intermedia.* Kirschleger, (1855) and Winkler, (1903) made similar observations in case of *D. capensis.* Nitschke, (1860) and Graves, (1897) described vegetative budding for *D. rotundifolia* from leaves and were found most commonly in early fall. Grout, (1898) ascribes the appearance of these buds to excessive moisture conditions. Leavitt, (1899; 1903) was able to propagate *D. filiformis, D. binata* and *D. dichotoma* from cut leaves. He observed that leaves, first formed from such buds in plants of *D. binata*, were orbicular like those of *D. rotundifolia;* while leaves coming from buds on *D. filiformis* were like those of *D. intermedia.*

These observations, together with other studies on reversion (Leavitt, 1903), led him to the conclusion that the leaf of *D. rotundifolia* is the original type of leaf from which those of other species have arisen. Ames, (1899) showed the possibility

of propagating *D. filiformis, D. intermedia, D. rotimdifolia, D. binata* from old leaves cut from mature plants. Dixon, (1901) was able to obtain adventitious buds on leaves of *D. rotimdifolia.* He also found that new plants might arise in the axils of leaves and between the petiole and main axis of the inflorescence as axillary buds. Goebel, (1908) showed appearance of adventitious buds on cut arm leaf of *D. binata.* In *D. rotundifolia* vegetative reproduction takes place when leaf buds form plantlets, or when axillary buds below the rosette form a secondary rosette. As the stem decays, the two separate (Lewis et al., 1928; Crowder et al., 1990). Adventitious plants develop in the autumn. They occur occasionally in the plants grown in green house, possibly due to the presence high humidity (Swales, 1975).

Plantlet regeneration from leaves is widely practiced. Leaf sections only or sections including the petioles are used (Lloyd, 1942). A treatment of leaves with fungicide and phytohormones is recommended. Asexual reproduction is also possible via root cuttings and secondary bulbs (tubers). The most successful means of reproduction of the pygmy varieties is by using " gemmaea", small spherical/ flattish structures at the base of the leaves. These structures form in response to reproduction in photoperiod and temperature. These structures are removed from the plant and propagated as if seedlings (Finnie and Van Staden, 1993). Vegetative reproduction among the perennial pygmy and stilt- form species is commonly accomplished by the seasonal production of rain-drop-distributed gemmae or brood bodies ' Brutknospen' (Goebel, 1908; Lloyd, 1942).

2.4. Napthoquinones and medicinal uses

The Droseraceae are known to contain napthoquinones (Hegnauer, 1966), which are of utmost therapeutic importance (Watt and Breyer-Brandwijk, 1962; Vichkanova et al., 1973; Oliver-Bever, 1986). Plumbagin and 7- methyljuglone are the major napthoquinones reported to occurs in genus *Drosera.* Either one or more of these quinines in genus *Drosera* have been isolated in aerial parts of different species grown *in vitro* and *in vivo* **(Table 1).** Although plumbagin occurs in many species of Droseraceae the compound is also extracted from Plumbaginaceae and Ebenaceae (Veluri and Diwan, 1999).

The species of *Drosera* also contain other napthoquinones and glucosides like Biramentaceone (2,2'-Dimer of 7-methyljiglone), 3-Chloroplumbagin, Droserone (3-Hydroxyplumbagin), Hydroxydroserone (3,8-dihydroxyplumbagin), Ramentone (2-methylnaphtharazin), Droserone-glucoside, Rossoliside (l,4,5-Trihydroxy-7-methylnapthalene-glycoside), 2,3-methoxy-7-methyljuglones, Hydroplumbagin *4-O*-glucoside (Budzianowski, 1995; 1996; 1997; 2000; Finnie and Van Staden, 1993; Nair and Shanmugasundaram, 1990). There are several napthoquinones, which are known only from *Drosera. A* chlorinated napthoquinone unique only to higher plants was isolated from *D. anglica* and *D. inetrmedia.* Droseraone was isolated and characterized in *D. whittakeri* (Asano and Hase, 1943a; 1943b; Rennie, 1887).

Table 1: Distribution of Plumbagin and 7- methyljuglone in *Drosera* species.

Taxon	Napthoquinones	Reference
Drosera indica	Plumbagin	Zenk et al., 1969
D. pygmaea	Plumbagin	Krenn et al., 1995
D. intermedia	Plumbagin	Bonnet et al., 1984
D. rotundifolia	7-methyljuglone	Schölly and Kapetanidis, 1989; Caniato et al., 1989; Krenn et al., 1995; Hook, 2001; Kämäräinen et al., 2003
D. anglica	7-methyljuglone	Krenn et al., 1995
D. capillaris	Plumbagin	Zenk et al., 1969
D. burkeana	7-methyljuglone	Zenk et al., 1969
D. madagascariensis	7-methyljuglone	Zenk et al., 1969
D. cuneifolia	7-methyljuglone	Zenk et al., 1969
D. trinervia	7-methyljuglone	Zenk et al., 1969
D. dielsiana	7-methyljuglone	Krenn et al., 1995
D. capensis	Plumbagin & 7-methyljuglone	Zenk et al., 1969; Pederson, 1978; Crouch et al., 1990; Hook et al., 1997; Hook, 2001
D. ramentacea	7-methyljuglone	Luckner and Luckner, 1970
D. spathulata	7-methyljuglone	Zenk et al., 1969; Blehova et al., 1995
D. binata	Plumbagin & 7-methyljuglone	Zenk et al., 1969; Hook, 2001; Pederson, 1978
D. cistiflora	Plumbagin & 7-methyljuglone	Zenk et al., 1969
D. peltata	Plumbagin	Leclercq and Angenot, 1984; Nair and Shanmugasundaram, 1990.
D. auriculata	Plumbagin	Zenk et al., 1969
D. microphylla	Plumbagin	Durand and Zenk, 1974
D. erythrorrhiza	Plumbagin	Durand and Zenk, 1974
D. whittakeri	Plumbagin	Zenk et al., 1969
D. stolonifera	Plumbagin	Durand and Zenk, 1974
D. natalensis	Plumbagin	Crouch et al., 1990
D. alicia	7-methyljuglone	Zenk et al., 1969
D. dichotoma	Plumbagin	Zenk et al., 1969
D. hamiltoni	7-methyljuglone	Zenk et al., 1969
D. longifolia	Plumbagin	Zenk et al., 1969
D. lunata	Plumbagin	Zenk et al., 1969
D. tracii	7-methyljuglone	Zenk et al., 1969
D. trinervia	7-methyljuglone	Zenk et al., 1969

Napthoquinones are phenolic compounds and they are formed through acetate-malonate and shikimic acid pathways. Napthoquinones of sundew are derived from acetate, which is formed from L-tyrosine most likely by homogentisate ring-cleavage pathway (Durand and Zenk, 1974a). The key enzyme of this ring-cleavage reaction is homogentisate oxidase (Durand and Zenk, 1974b). According to Juniper et al. (1989) homogentisate ring-cleavage pathway is a modification and occurs in the Droseraceae members as a result of low nitrogen availability in the environment. Ramentaceone, which is found in the Droseracea, is also produced by the homogentisate ring cleavage pathway (Durand and Zenk, 1976).

The amount of napthoquinones varies between different *Drosera* species (Bonnet et al., 1984), and different tissues of the plants (Hook et al., 1997; Repčák et al., 2000), different regions and habitat (Kämäräinen et al., 2003). In some sundew species, the concentration of napthoquinones varies during the growing season, but in *D. rotundifolia* the amount is fairly constant (Caniato et al., 1989). In *D. spathulata* the amount of napthoquinones increases with increased level of differentiation during organogenesis *(in vitro)* and the composition of the growth media also has an effect on the production of napthoquinones (Blehová et al., 1995).

One of the earlier reports of the medicinal usage of *Drosera* plants appears in Gerard's *"New Herbal"* in 1633, as an important antitussive for different respiratory diseases, including tuberculosis (Slack, 1980; Schnell, 1984).

Plumbagin exhibits a variety of pharmacological activities like antimicrobial (Van der Vijver and Lotter, 1971; Heble et al., 1974; Ray and Majumdar, 1976;

Krishnaswamy and Purushothaman, 1980; Wurm et al., 1984; Gundidza and Manwa, 1990; Durga et al., 1990; Fujii et al., 1992; Didry et al., 1994; 1998; Samaj et al., 1999; De-Paiva et al., 2003; Ferreira et al., 2004), Bronchial infection, Whooping cough, antiasthma, phthisis and used against old age and arteriosclerosis (Denoel, 1949; Czygan et al., 1989; Schilcher and Elzer, 1993; Blumenthal et al., 1998), Antituberculosis (Heise and Steenken, 1941; Lloyd and Middlebrook, 1944; Denoel, 1949), Antispasmodic (Paris and Quevauvillier, 1947; Gordonoff, 1951; Bezanger-Beauquesne, 1954; Paris and Delaveau, 1959; Juniper et al., 1989; Wagner, 1993), Anticancer (Melo et al., 1974; Krishnaswamy and Purushothaman, 1980; Kreher et al., 1990; Uma Devi et al., 1999; Fujii et al., 1992; Parimala and Sachdanandam, 1993), Antileprosy (Bokemo, 1984), Antifertility, Abortifacient (Bhargava, 1984; Bhargava and Dixit, 1985; Kini et al., 1997), Antimalarial (Nakornchai et al., 1995), Hyperglycemic (Olagunju et al., 1999), Hypolipidemic (Sharma et al., 1991), Imrnunomodulator (Kreher et al., 1990), Cosmetic (Slack, 1980), Aphrodisiac (Finnic and Van Staden, 1993), Chitin synthetase inhibitor, insecticidal (Kubo et al., 1983; Ghosh et al., 1994), Enhances *in vitro* phagocytosis of human granulocytes (Kreher et al., 1990), Extract used in certain sweets (Frenzer, 1980), Leishmanicidal (Chen-Bacab and Peña-Rodriguez, 2001). Plumbagin inhibits the development of insect and parasitic nematodes (Fetterer and Fleming, 1991), Antifeedant (Tokunaga et al., 2004), removal of corns, warts, freckles and sunburns (Ravikumar and Ved, 2000). The napthoquinone juglone is toxic and an effective inhibitor of seed gemination for

many plants, it was shown to be inhibitory also to several insects and to be highly toxic to fungi as well as different fungal pathogens (Seigler, 1998).

The role of 7- methyljuglone for sundew is not quite clear. Plants produce carbon based secondary defence substances in areas where there is a deficiency of nitrogen. Sundews inhabit nitrogen poor locations and presumably they produce carbon-based napthoquinones for defence. These compounds are toxic to certain types of Bacteria and fungi and inhibit their growth. One suggested reason for the production of 7-methyljugolone is that it decreases competetion of nitrogen from captured insects between plants and bacteria and fungi in surface of these insects (Durand and Zenk, 1974a).

Flovonoids are natural compounds shown to exert different biological effects, such as antiviral, anti inflammatory, antimutagenic and anticarcinogenic functions. This activity is reported to result partly from their antioxidants and antiradical properties (Havsteen, 1983; Sugihara et al., 1999).

Cyaniding3,5-di-*O*-glucoside (cyanin), cyaniding *3-0*- galactoside (idaein), cyaniding 3-*O*-glucoside, pelargonidin 3-*O*-galactoside and pelargonidin 3-0-glucoside (callistephin), Quercetin, hyperoside, gossypetin, gossypin (gossypetin 8-*O*-glucoside) and isogossypitrin (gossypetin 7-*O*-α-D-glucoside), have been isolated from different species of *Drosera* (Nair and Shanmugasundaram, 1990; Wang et al., 1998). Cyaniding-glycoside, malvidin-glycosides, pelargonidin-glycoside, quercetin-3-galactoside and quercetin-3-digalactoside (Gascoigne et al., 1948; Paris and Denis, 1957; Paris and Delaveau, 1959; Bienenfeld and Katzmeister, 1966; Bendz and

Lindberg, 1968; Bendz and Lindberg, 1970; Ayuga et al., 1985; Ichishi et al., 1999; Zielińska et al, 2001). Quercetin 3-0- (6''-galloyl) glucoside from *in vitro* culture of *D. aliciae* has pronounced antioxidant activity on human Polymorphonuclear neutrophils (PMNs) and mouse spleen microsomes, with respect to that of quercetin, but latter was much stronger as an inhibitor of lipid peroxidation (Zielińska et al, 2001).

In 1995 there were over 100 medicinal preparations on sale in Germany containing *Drosera* plants. Although there has been experimental cultivation, this is maninly extracted from wild. This species has a high commercial value; the wholesale price in 1996 was US $ 423/kg. 2100 kg of *D. rotundifolia* was collected in Finland in 1994, but only 800 kg would be collected in 1995. The material is exported to Switzerland. This species can only be imported into Germany with a permit proving sustainable harvest in the source country (www.scotland.gov.uk). This is a wild herb, common in humid areas all over Vietnam. In Europe it is used in phytotherapeutic and homeopathic drugs against whooping cough and as an antispasmodicum. World demand is estimated at over 100 tonnes per year. China is exporting this product. The sales price in Hamburg is DM 27/kg (www.giaodiem.com).

Due to alteration of its habitat *D. rotundifolia* as well as the other species of central Europe *(D. anglica, D. intermedia)* are protected by law. Today, it is difficult to collect these species in wild and for this reason, the use of *D.rotundifolia* in medicinal preparations has been to some extent replaced by exotic *Drosera* from the

southern hemisphere, such as *D. ramentacea*, *D. madagascariensis*, *D. burmanii*, *D. indica* and *D. peltata*, which grow in oriental Asia (Japan, China, India, Malaisia, Philippines) in Australia and New Zealand (Le Clercq and Angenot, 1984; Schier et al., 1987; Wawrasch et al., 1993; Länger et al., 1994; Krenn et al., 1995). Several *Drosera* species are included in pharacopoeias and dried plants are marketed as "Herba Droserae and Herba Drosera rotundifoliae", etc. (Länger and Kopp, 1995). Although *D. madagascariensis* is poor in active compounds, it has been accepted in pharmacopoeias. Many *Drosera* species are threatened, because of its medicinal value, and other factors in many countries and they are protecting (Leclercq and Angenot, 1984; Park, 1994; Didry et al., 1998; Nalini and Murali, 2002; Kawiak et al., 2003).

The round leaved sundew is presently not endangerd in Finland (Hämet-Ahti et al., 1998), but the small size of plants makes collection from natural stands laborious and therefore, cultivation possibilities have been studied (Galambosi et al., 1999). Although extensive studies for *in vitro* cultivation and propagation of *D. rotundifolia* were carried out (Blehova et al., 1990; Bobák et al., 1990; Wawrosch et al., 1993), the production of the required quantity of the pharmaceutical ly important crude drug Herba Droserae in this way is not yet possible.

2.5. *In vitro* propagation

Increasing interest in the horticultural and medicinal potential of carnivorous plants in particular *Drosera* has resulted in over harvesting from natural sources.

This, together with a loss of their natural habitats is prompting conservation biologists to investigate these carnivorous plants for rapid propagation in view of their immense biotherapetuic value. The result has been greater research into their micropropagation and the use of *in vitro* grown plants as alternative sources of biomass.

Many species of *Drosera* have been successfully multiplied through *in vitro* by using different explants **(Table 2).** Sterilization of *Drosera* seeds proved difficult because of fungal and bacterial contaminants on the surface of the seeds, and other explants. Sterilization process may vary to different *Drosera* species. Sterilization of seed explants was achieved using CaOCl (3 %) (Simola, 1978a; b) in *D. rotundifolia*, 0.1 % $HgCl_2$ (Small and Hendrikz, 1974) in *D. pygmaea* and *D. aliciae* or NaOCl (Burger, 1961) in *D. intermedia*. 20 % (V/V) commercial bleach and 0.01 % (V/V) Tween-20 for 20 min (Jang and Park, 1999) in *D. rotundifolia,* 0.1% (W/V) Benzalkonium chloride solution for 5 min, 70 % (V/V) ethanol for 30 s (Ichiishi et al., 1999) in *D. spathulata*. 70 % ethanol (10 s) and with 3 % $CaCl_2O_2$ for 20 min (Kawiak et al., 2003) in *D. anglica* and *D. cuneifolia* proved to be effective.

One of the first reports of the *in vitro* culture of *Drosera* was that of Schmid (1912). Burger (1961) reported axenic reduced germination of *D. intermedia* seedlings in a simple nutrient medium. The percentage of germination was 44 %, **when** seeds were incubated in the light, a 15/38ºC alteration in temperature. At 15ºC constant light and dark and alternate light and dark 0 % germination was observed.

Table 2: Summary of *in vitro* propagation of genus *Drosera*

Species	Explants	References
Drosera	Seed	Schmid, 1912.
D. intermedia	Seed	Burger, 1961.
Drosera & D. pygmaea	Seed	Harder, 1964a, b.
D. aliciae	Seed	Small and Hendrikz, 1974; Small et al., 1977
D. rotundifolia	Seed, Axillary shoots, leaves, stem, cell suspensions	Simola, 1978a, b; Bonnet et al., 1984; Van Waes, 1985; Kukulczanka and Czastka, 1988; Kukulczanka and Czastka 1991; Anthony, 1992; Wawrosch et al., 1993; Bobak et al., 1995; Jang et al., 1997; Bobak et al., 1999; Jang and Park, 1999; Hook, 2001; Hirsikorpi et al., 2002.
D. intermedia	Internodes, seeds, axillary bud, leaf	Kukulczanka and Czastka, 1988.
D. hilaria	Leaf fragments	Janssens, 1986.
D. regia	Leaf fragments	Janssens, 1986.
D. natalensis	Leaves, shoots, flower buds, flower stalks, roots	Crouch and Van Staden 1988; Crouch et al., 1990.
D. spathulata	Leaves, callus culture, shoot tip	Bobak et al., 1989; Blehova et al., 1990; Blehova et al., 1992; Bobak et al., 1993; Perica and Berljak ,1996.
D. capensis	Leaves,	Crouch et al., 1990; Anthony 1992; Hook 2001.
D. binata	Whole leaves, shoot tips, leaf, rhizome	Anthony, 1992; Kawiak et al., 2003.
D. peltata	Seeds	Kim and Jang, 2004.
D. anglica	Seeds	Kawiak et al., 2003.
D. cuneifolia	Seeds	Kawiak et al., 2003.
D. indica	Stem segments	Nalini and Murali, 2002.

Whereas at 38°C constant light 2 % germination and in constant dark and alternate light and dark 0% germination was observed. Van Waes (1985) reported of the 16 species used for *in vitro* germination, most seeds germinated after 10 days and the seedlings were ready for transplanting after 4 moths. Small and Hendrikz, (1974) germinated seeds with a photoperiod of 14 h and a day/night temperature of 15/10°C with seeds starting to germinate after 3 weeks; this was confirmed by Kukulczanka and Czastka, (1988). Kawiak et al, (2003) germinated seeds of *D. anglica* and *D. cuneifolia* in 2 % sucrose and 0.7 % agar in different media and after 4-6 weeks the germination frequency estimated at 62 and 71 % respectively. Hirsikorpi et al, (2002), reported that seeds of *D. rotundifolia* were germinated on ½ strength MS medium supplemented with BAP 0.1 mgL^{-1} and NAA 0.05 mgL^{-1}, 2 % sucrose and 100 mgL^{-1} myoinositol, at pH 5.7 were solidified with 0.65 % agar. Jang and Park, (1999) reported that seeds of *D. rotundifolia* germinated within 2-3 weeks on ½ MS medium. The germination rate of seeds was 100 % when the seeds were treated at 4°C for more than 30 days. But the seeds without cold treatment at 4°C for at least 4 weeks did not germinate. Ichiishi et al. (1999) germinated seeds of *D. spathulata* collected from cultivation and inoculated on ½ MS supplemented with 0.8 % sucrose. They were germinated 30-60 days after they were sown on medium. Kim and Jang, (2004) reported the germination rate of *D. pletata* seeds was 70.5 % within 10-15 days on ½ MS medium with seeds that had been stored at 4°C for 30 days. Without cold treatment for at least 4 weeks, the germination rate was very low (about 16.7 %).

Seeds have been used mainly as explants for *D. intermedia* (Burger, 1961), *D. pygmaea* (Harder, 1964a, b), *D. rotundifolia* (Small et al., 1977; Simola, 1978a, b and Kukulczanka and Czastka, 1988) and *D. peltata* (Kim and Jang, 2004).

In vitro culture of *Drosera* explants has been difficult due to fungal and bacterial contaminants on the surface of the leaves as reported in the study by Crouch et al, (1990). In the *in vitro* propagation study done by Anthony, (1992) 95 % of the *D. rotundifolia* cultures started from whole leaves were also contaminated. Root, stem internodes, axillary rosettes, flower buds, and the flower stalks have all been successfully used as explant sources.

Simola, (1978a) reported micropropagtion of *D. rotundifolia* by culture of young seedling explants and studied the effect of several amino acids and some inorganic nitrogen source on the growth of *D. rotundifolia* in long- and shortday conditions. Simola, (1978b) also reported by using same clone of aseptically cultivated *D. rotundifolia*, the growth of *D. rotundifolia* was studied in aseptic cultures with 17 dipeptides as the only nitrogen source. Bobák et al, (1989; 1993) was achieved the regeneration of *D. spathulata* from callus and leaf cultures through organogenesis. Crouch et al, (1990) described the rapid clonal multiplication from leaves taken when mature *Drosera* plants were used as explants of two South African species *D. natalensis* and *D. capensis*. Levels of plumbagin from *in vivo* and *in vitro* grown plants are compared to those present in *Plumbago* roots and stating that the extraction of plumabgin from *Drosera* is not commercially feasible. Intact plants can be readily cultured by *in vitro* propagation methods (Czany et al., 1992) and analysis

have been found to produce the same napthoquinones as naturally grown plants with *D. spathulata* (Budzianowski, 1995; Blehová et al., 1995), *D. rotundifolia* (Bobák et al., 1995), *D. intermedia* (Budzianowski, 1996) and *D. communis* (Reichling et al., 1995). Hook et al, (1997) reported the development and napthoquinone content of *in vitro* culture plants and suspension cultures of *D. capensis.* Kukulczanka and Czastka, (1991) reported the direct regeneration of *D. rotundifolia* from leaf explants. Anthony, (1992) described the *in vitro* propagation of *D. capensis, D. binata* and *D. rotundifolia* by using leaves as explants. Bobák et al, (1993) studied the organogenesis from the callus culture of the *D. spathulata.* Bobák et al, (1995) also studied the direct plantlet regeneration from *D. rotundifolia.* In this study 49 different media were screened to evaluate their effect on regeneration from leaf explants. Perica and Berljak, (1996) reported mass propagation *of D. spathulata* through shoot tip culture on various media. Jang et al, (1997) reported the *in vitro* propagation of *D. rotundifolia* by leaf culture on different strengths of MS medium and different cytokinins. Jang and Park, (1999) established an *in vitro* propagation method of *D. rotundifolia,* the effects of different strengths of MS, various pH, different concentrations of kinetin and BA was evaluated on ½ MS, different concentration of 2,4-D or NAA were evaluated using ½ MS medium. Kawiak et al, (2003) developed the efficient method for the direct regeneration of *D. anglica, D. binata* and *D. cuneifolia* from leaf explants and shoot tips. Kim and Jang, (2004) established an *in vitro* micropropagation method of *D. peltata* (a tuberous sundew) through shoot tip culture. The effects of various media like Murashige and Skoog (MS) (different

strengths), Gamborg B5 medium (B5), Linsmaier and Skoog (LS), and Reinert and Mohr (RM), various pH on ½ MS, different concentration of cytokinins like kinetin, benzyladenine and 3 % (W/V) sucrose were tested on ½ MS medium. The proliferation rate and length of the shoots, the number of tubers, their diameters and fresh weight all were greater on MS medium than on the other media. These data were similar to those of Crouch and Van Staden, (1988). Nalini and Murali, (2002) reported the first successful protocol for *D. indica* from callus using stem segments as explants.

Hook et al, (2001) also reported the napthoquinone contents of *in vitro* cultured plants and cell suspensions of *Dionaea muscipula* and *Drosera rotundifolia, D. binata* var. *binata* and *D. capensis.* In contrast with Wawrosch et al. (1996) the napthoquinone content was higher in these plants. However, Kukulczanka and Czastka (1988) described *in vitro* propagation of *Drosera* species by leaf and axillary bud culture was found to be best on RM medium.

In the genus *Drosera* in the conventional multiplication techniques, leaf explants predominate in usage compared to other explants (Janssens, 1986; Kukulczanka and Czastka, 1988; Crouch et al., 1990). Leaf explants produce numerous adventitious buds on the leaves. Van Waes, (1985) reported the formation of these buds after 8-10 weeks.

D. spathulata is an ornamental, insectivorous plant (Kondo and Kondo, 1983). The plant shows red and green coloration. However, plants with stable red color are horticulturally more desirable than green ones. This red coloration due to anthocyanin pigmentation in leaves of *D. spathulata.* Ichiishi et al., (1999) studied the effect of

five macro-components and sucrose in half strength MS agar medium on red color pigmentation of *D. spathulata* generated from multiple shoots *in vitro.*

The occurrence of extracellular matrix surface network (ECMSN) is reported during proembryo formation in *D. rotundifolia* (Samaj et al., 1995). The ECMSN, which is present exclusively during early stages of embryogenesis, sees to be important for plant regeneration. This ECMSN can serve as an early morphological structural marker on the surface of regeneration competent cells during direct embryogenesis from epidermal leaf cells. Bobak et al, (1999) studied the effects of the microtubule toxins trifluralin and colchicines on the structural organization of ECMSN and extracellular matrix (ECM) layers during somatic embrogenesis in *D. rotundifolia.*

In order to improve napthoquinone production in either tissue cultured or whole plants of *Drosera,* a simple procedure such as *Agrobacterium tumefaciens* mediated transformation and regeneration of leaf explants of *D. routundifolia* was achieved (Hirsikorpi et al., 2002).

2.6. Genomic DNA isolation

Application of molecular technology would increase and facilitate production of secondary metabolites, however the same secondary metabolites may hinder the nucleic acid isolation and interfere with subsequent reactions. A number of DNA isolation protocols are available, unlikely that just one nucleic acid isolation method is suitable for all plants can ever exist (Loomis, 1974). The routine isolation of high

quality nucleic acids from genus *Drosera* leaves turned out to be difficult due to variety of contaminating substances. These substances are thought to originate from the large number of stalked glands on the upper leaf surface that contain viscous mucilage used to trap and immobilize prey. In attempts to evaluate the gene pool of the *Drosera,* Bekesiova et al, (1999) and Pirttilä et al, (2001) individually developed protocols for DNA and RNA by using CTAB based extraction methods for routine isolation of high quality DNA and RNA from small amounts of *in vitro* grown *D. rotundifolia* leaves. The obtained DNA could be analyzed by PCR, restriction endonucleases and DNA gel blotting, and the obtained RNA was of sufficient quality for RT- PCR and RNA gel blotting. Currently, the use of these protocols for other members of the Droseraceae and other species of carnivorous plants is being investigated.

2.7. Genetic transformation and RAPD analysis

Genetic modification of plants using *Agrobacterium tumefaciens* is today a routine procedure for a large number of plant species. The important prerequisite for the method is the possibility to regenerate plants from tissue culture or explants. Genetic engineering by *Agrobacterium* has been used to improve secondary metabolites in medicinal plants (Saito et a1, 1992). Hence Hirsikorpi et al. (2002) developed *A. tumefaciens* mediated genetic transformation method of the carnivorous plant *D. rotundifolia.* The micropropagation conditions of *D. rotundifolia* aseptically germinated seeds were defined and the internal kanamycin resistance was tested.

Transformation was made by co-cultivation of micropropagated *D. rotundifolia* leaves with *A. tumefaciens* strain C58C1 containing a cointegrate plasmid vector with neomycin phosphotransferase and luciferase genes. Transgenic sundews were selected for kanamycin resistance, and viable fully developed plantlets were further assayed by luciferase activity, PCR and southern analysis using *luc-* primers and a *lue-* probe. The transformation efficiency in *D. rotundifolia* was 17%.

To the best of our knowledge there is one report of RAPD analysis have been done to determinate the genetic fidelity in micropropagated plants of *D. anglica* and *D. binata* which were regenerated by adventitious budding from leaf explants and shoot tips and have been concluded that regeneration of plants of *D. hinata* through shoot tip culture is a low risk method for generating genetic variability, whereas material regenerated through leaf explants of *D. anglica* showed 0.08% polymorphism (Kawiak and Lojkowska, 2005).

3. Problem, prospects and objectives

Drosera indica L. and *D. burmanii* Vahl. are annual insectivorous plants, widely distributed in the tropical world, extending to south East Asia to Japan and India, grows in poor, sandy soils and acid, swampy areas as like any other insectivorous plants. *D. indica* and *D. burmanii* exhibit summer dormancy as in the form of seeds and then recommence growth during the cooler winter months.

In Australia, *D. indica* mostly recognize a single variable species with an extensive synonymy, but without any intraspeceific taxa (Van Steenis, 1953; Ohwi, 1965; Obermeyer, 1970; Marchant et al., 1982), although some authors have recognized intraspeceific taxa (Diels, 1906; Walker, 2000) and a number of distinct morphological variants have been noted from northern Australia (Lowrie, 2001a; 2001b).

Susandarini et al, (2002) were identified, three clearly definable morphotypes, within the *D. indica* accessions found across northwestern Australia. It is also reported from field surveys over a number of years, of the co-occurrence of sympatric morphos across wide areas of the Kimberleys, Northern Territory and Northern Queensland. There is no evidence of intergradation between groups in the field (Lowrie, 2001b).

D. indica contains 28 (2n) chromosomes, whereas *D. burmanii* has 20 (2n) chromosomes (Hoshi and Kondo, 1998). In Indo-China maceration of *D. indica* is using for removing corns, warts and cosmetically it is used in the removal of freckles

and sunburns. These insectivorous plants contain valuable secondary metabolites (Zenk et al., 1969; Wang et al., 1998), which are of medicinal value. In India these plants have been placed in vulnerable category according to IUCN (Ravikumar and Ved, 2000; Reddy et al., 2001; Nalini and Murali, 2002).

Because of its medicinal properties, many European countries are importing *D. indica* and *D. burmanii* from oriental Asia for Ayurvedic product named "Herba droserae" instead of other European species which are native and endangered (Didry et al., 1998). Even in Asia the exporters are collecting from wild in destructive manner without adopting adequate measures.

Apart from its collection from wild for herbal industries, the natural habitat of plants is effected due to scanty annual rainfall, irregular monsoon and encroachment by invasive species. Thus, its habitat is getting depleted and isolated, which would influence its life cycle. The above factors are responsible for inbreeding leading to genetic erosion.

In many countries species of *Drosera* have been included in threatened species and are being protected by government environmental regulatory agencies. However in India though the government environmental regulatory agencies have declared *D. indica* and *D. burmanii* as threatened species, stringent conservation measures rather weak.

Hence, we have attempted to establish reliable *in vitro* protocols for rapid multiplication of *D. indica* and *D. burmanii.* The *in vitro* raised biomass would be a reliable source for herbal industry for production of biotherapeutics. Till todate there

is one report of *in vitro* regeneration through callus of stem segments of *D. indica* (Nalini and Murali, 2002), and no reports on *D. burmanii.*

1. To investigate the distribution of *D. indica* and *D. burmanii* from various parts of Andhra Pradesh and threats for these taxa.
2. To examine the seed germination in *D. indica* and *D. burmanii*.
3. To establish *in vitro* protocols for rapid multiplication of *D. indica* and *D. burmanii.*
4. To investigate the seed coat sculpture of *D. indica* and *D. burmanii* accessions by scanning electron microscopy to understand whether there are any infraspecific morphotypes with in these species.
5. Standardization of genomic DNA isolation from *D. indica* and *D. burmanii* for diversity analysis by RAPD markers.

4. Materials and methods

4.1. Field identification and collection of germplasm

Drosera indica was identified in different locations of Andhra Pradesh and 12 accessions were collected. Seeds were stored 4°C in polythene covers, for studies on seed germination and morphological variations. Apart from that habitat and environmental preferences of theses plants were observed during field collection. Similarly 9 accessions of *D. burmanii* were identified, the germplasm was collected and seeds were as stated above for further use.

4.2. Scanning Electron Microscopic investigation of seed coat sculpture

Seed sculptural variations were studied by scanning electron microscopy. Seeds of *D. indica* collected from 12 different locations and of *D. burmanii* collected 9 different locations of A.P were taken up for SEM studies. The seeds were cleaned and mounted on stubs with double sided adhesive sticker, coated with gold carbon in a vacuum evaporator and examined under Philips XL-30 ESEM at 10 KV. Five seeds per accession were examined.

4.3. Seed germination studies

The seeds of *D. indica* and *D. burmanii* were collected from the University of Hyderabad and surrounding Gachibowli area, Andhra Pradesh, India and were stored at 4°C till used. The seeds were treated with 70 % ethanol for 30sec. and 0.1 % $HgCl_2$

for 1 min followed by 3-5 thorough washes using sterile double distilled water. Germination of these sterilized seeds was tried on MS (Murashige and Skoog, 1962) basal medium with 3 % agar at pH 5.7. Seeds were also placed on petriplates containing filter papers, wet with different solutions. These were incubated at 25 ± 2°C under 16/8 h photoperiod with light intensity of 30 $\mu EM^{-2}S^{-1}$.

4.4. *In vitro* shoot multiplication

Shoot tips of *D, indica* and *D. burmanii* were collected from the natural habitat and thoroughly rinsed under tap water. The shoot tips were surface sterilized with 35 % ethanol and 0.05 % of $HgCl_2$ for 30sec. each, followed by 3-5 rinses in sterile double distilled water. Initially shoots were raised on MS basal medium, containing 3 % sucrose, 0.3 % agar at pH 5.7. The cultures were incubated under 25 ± 2ºC under 16/8 hr photoperiod with a light intensity of 30 $\mu EM^{-2}S^{-1}$. Once the cultures were established, further experiments were done by taking explants from these already established cultures. The following factors were investigated as individual experiments.

1. Different concentrations of MS basal medium - ¼, ½, ¾ and full strength
2. Different concentrations of sucrose - 1 %, 2 % and 3 %
3. Different pH - 3.7, 4.7, 5.7 and 6.7

4. Different plant growth regulators like Zeatin - 0.1, 0.5, 1.0 and 2.0 mgL^{-1}, Kinetin - 0.1, 0.5, 1.0 and 2.0 mgL^{-1}, BAP - 0.01, 0.05, 0.1, 0.5, 1.0 and 2.0 mgL^{-1} were tested.

For *D. burmanii* the following cytokinins were used: Kinetin- 0.1, 0.5, 1.0 and 2.0 mgL^{-1}, BAP - 0.1, 0.5, 1.0, 2.0 mgL^{-1}.

4.5. Root induction from shoots

The separated shoots of *D. indica* and *D. burmanii* were cultured on different concentrations of MS basal medium containing 3 % sucrose, 0.3 % agar at pH 5.7. The cultures were incubated under 25 ± 2°C under 16/8 hr photoperiod with a light intensity of 30 $\mu EM^{-2}S^{-1}$ for root initiation.

4.6. Acclimatization of regenerated plants

Regenerated plants having well developed roots were removed from culture bottles and washed free of agar. They were transferred to plastic cups contains sterile soilrite, which is watered in the ratio 1:1 with MS nutrients. If they are watered more, there was a chance of these plants to lodge. They were kept at 25 ± 2°C tightly **covered** with polythene bags. They survived and flowered normally. As these are insect pollinated flowers, there was no seed formation in the given conditions; otherwise plants were healthy and normal with functional leaf glands. Hardening and field transfer of these plants is extremely difficult and they may survive in simulated habitat.

4.7. Isolation of DNA

In order to standardize the genomic DNA extraction of *D. indica* and *D. burmanii* the following modified method of CTAB (Doyle and Doyle, 1987) and plant DNA extraction kit DNA zole (Invitrogen Ltd.) were tested.

5. Results and discussion

5.1. Field observations

The state of Andhra Pradesh is situated in the middle part of the eastern half of the Indian Peninsula, lying between the latitude 12° 37' N and 19° 54'N and longitude 76° 46' E and 84° 46' E. Botanical studies carried out in the state have indicated that Andhra Pradesh is home to 1577 medicinal plants, both native and exotic. These medicinal plants found in the different habitats and ecosystems across the state contribute to the overall floristic diversity of Andhra Pradesh to a larger extent. Andhra Pradesh harbors different insectivorous plants, viz., *Utricularia* and *Drosera indica* and *D. burmanii*. Because *Drosera* species are grown in a restricted natural habitat makes them hard to be found out, because of these factors plants have been included in vulnerable category (Ravikumar and Ved, 2000; Reddy et al., 2001).

D. indica L. (12 accessions) and *D. burmanii* Vahl. (9 accessions) were collected from various parts of Andhra Pradesh **(Figure** 1). In seven areas both *D. indica* and *D. burmanii* were co-existing with their associated species. But in Motugudem (Khammam district, in Andra Pradesh, India) *D. burmanii* alone and in five locations only *D. indica* was observed. Microhabitats of these habitats were recorded **(Table 3).** Field observation reveals *D. indica* and *D. burmanii* are annual insectivorous plants that are restricted to grow in open, marshy, acidic, nutrient poor habitat like any other *Drosera* species **(Figures** 2 a, b). *Drosera* species were mostly

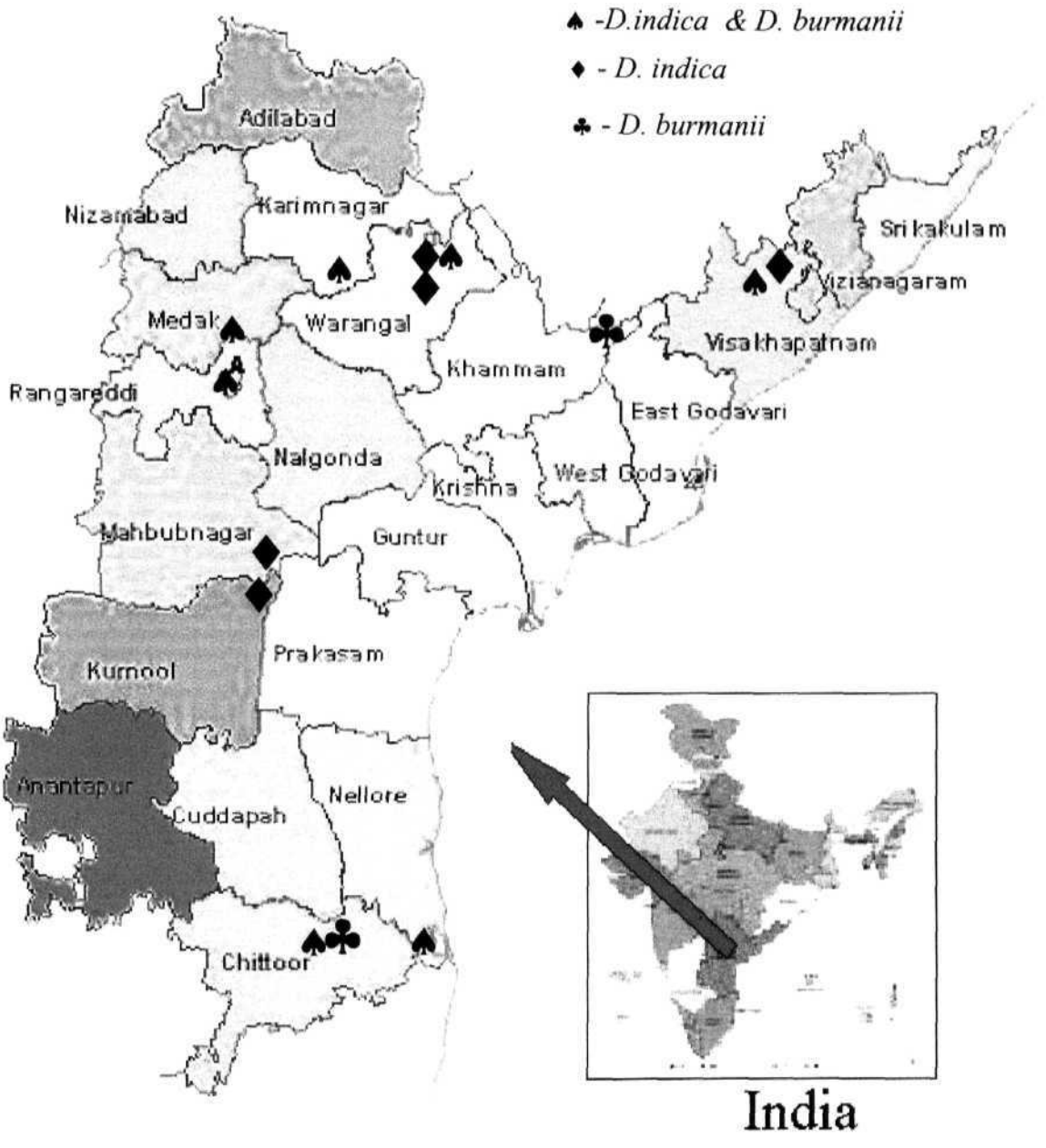

Figure 1: Sampling locations of *D. indica* and *D. burmanii* from different regions of Andhra Pradesh.

Table 3: Sampling locations of *D. indica* and *D. burmanii* and details of micro-habitat and growth conditions. The locality name is followed by districts name (in parenthesis, bold face) of Andhra Pradesh, India.

Place & Districts.	Taxa	Substratum	Microhabitat	Associated plant species	Possible threat factors at the selected sites
Gachibowli **(Rangareddy)**	*D. indica* *D. burmanii*	Sandy, moss and peat	Wet slopes and poorly drained depressions and rock outcrops	*Eriocoluan* *Utricularia*	Urbanization, invasive sps and drought
Narsapur **(Medak)**	*D. indica* *D. burmanii*	Sandy, clay	Rock outcrops and wet slopes and pond edges	*Eriocoluan*	Agro pollutants
Arakuvally **(Visakhapatnam)**	*D. indica*	Clay, peat	Wet slopes at water streams	*Eriocoluan*	Habitat conversion and agro pollutants
Anjodigadda **(Visakhapatnam)**	*D. indica* *D. burmanii*	Clay, moss	Wet slopes at water streams	*Eriocoluan* *Sphagnum*	Soil erosion and habitat conversion
Talakona **(Chittor)**	*D. indica* *D. burmanii*	Sand	Wet slopes with superficial water flow	*Eriocoluan*	Habitat conversion, fragmentation by roads and human activities
Tada **(Nellore)**	*D. indica* *D. burmanii*	Sand	Wet slopes at pulicot lake bed	*Eriocoluan*	Plantations and agricultural fragmentation
Pakhal lake **(Warangal)**	*D. indica*	Sand, clay	Wet slopes with superficial water flow	*Eriocoluan*	Drought
Mulakanuru **(Karimnagar)**	*D. indica* *D. burmanii*	Sand	Rock outcrops and wet slopes	*Eriocoluan* *Utricularia*	Drought
Chelvaye **(Warangal)**	*D. indica*	Sand and clay	Rock outcrops and wet slopes and poorly drained depressions	*Eriocoluan*	Soil erosion and invasive species
Kondaparthi **(Warangal)**	*D. indica* *D. burmanii*	Clay, sand and moss	Poorly drained depressions and wet slopes	*Eriocoluan*	Invasive species
Panchamatalu **(Kurnool)**	*D. indica*	Sand and clay	Wet slopes, rocky out crops	*Eriocoluan*	Soil erosion
Srisailam **(Kurnool)**	*D. indica*	Sand	Wet plateaus	*Eriocoluan*	Drought
Motugudem **(Khammam)**	*D. burmanii*	Sand	Wet slopes and agricultural fields.	*Eriocoluan*	Agricultural activities

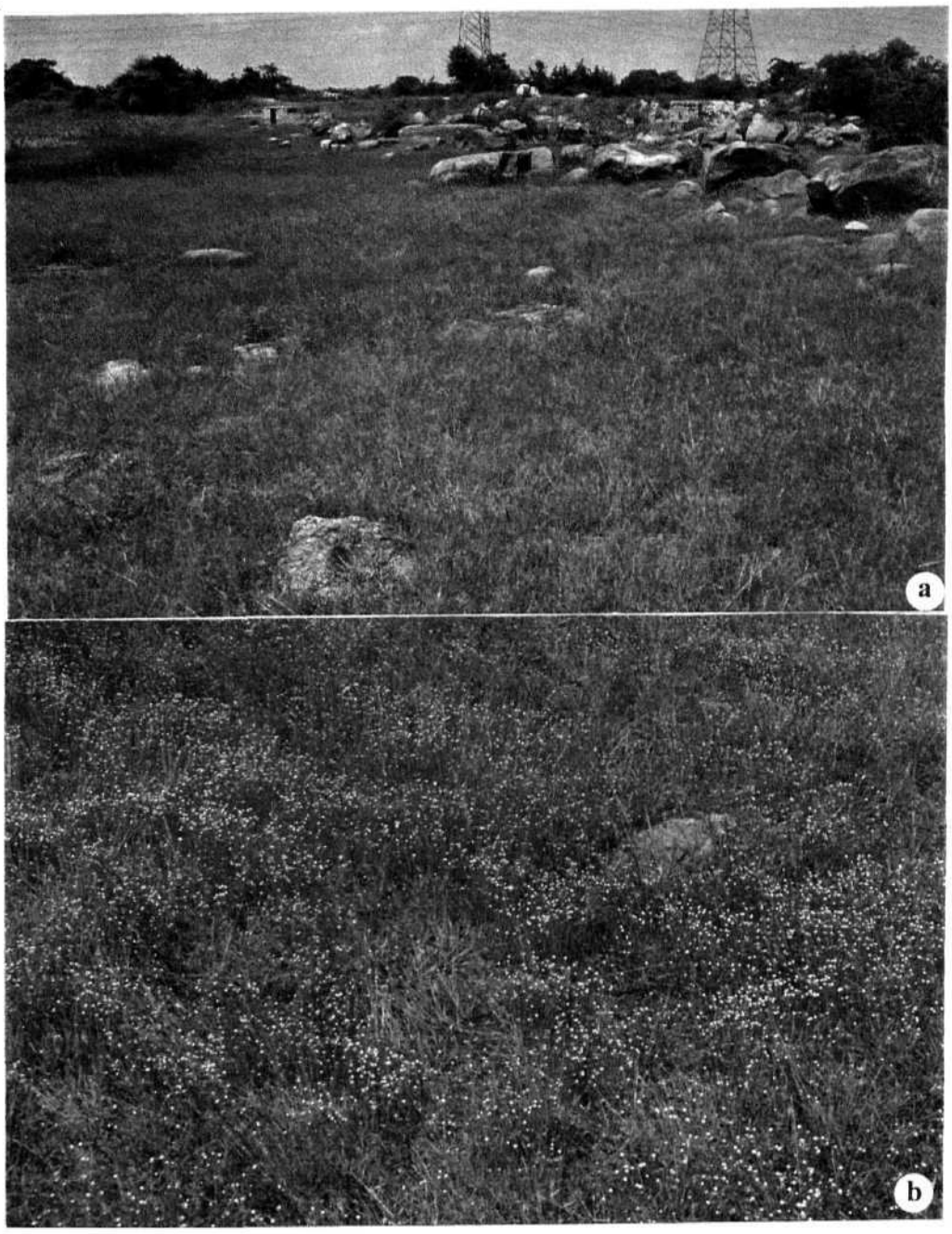

Figure 2: a) Habitat of *Drosera indica* and *D. burmanii* in the University of Hyderabad campus, b) Open and low grasslands are the typical habitat for *Drosera indica* and *D.* burmanii in the University of Hyderabad campus.

seen associated with *Eriocoloun*, *Utricularia* and *Spaghnum* moss and other grasses **(Figures** 3 a, b **and** 4 a, b).

D. indica is small erect herb with long leaf bearing sticky mucilaginous glands on the adaxial surface of the leaf. Some times the plants grow compactly adjacent to rocky outcrops. *D. indica* can attract, trap, and absorb nutrients from various insects. They bear attractive pink flowers **(Figures** 5 a, b). Whereas *D. burmanii* has a rosette-like appearance, with long tentacles on the adaxial surface, along the margin of spathulate leaves **(Figures 6 a-c).** In many places *D. indica* and *D. burmanii* share a common habitat **(Figure** 6 d).

D. indica and *D. burmanii* were collected from Gachibowli area (A.P, India) and their general phenotypic characters were recorded **(Tables** 4 **and** 5). Seeds were stored at 4° C for seed germination studies. The collected plant materials were frozen in liquid nitrogen and were stored at -70°C for DNA isolation and its further use in RAPD analysis.

In both species though abundant seeds were developed, most of them failed to germinate due to water limitation. On the other hand during heavy rains the minute seeds get carried away from their natural habitat to unfavorable conditions. *D. indica* and *D. burmanii* seeds exhibit summer dormancy. Flowering and fruiting season may vary due to difference in timings of monsoons in different regions within Andhra Pradesh. In many areas plants have great threats from invasive species like *Hyptis suaveolens* and *Parthenium hysterophorous.* Agriculture, urbanization, nutrient rich

Figure 3: a) *D. indica* in flowering (pink flower) associated with *Eriocaulon* (white globose inflorescence), b) *D. indica* thicket with slender leaves

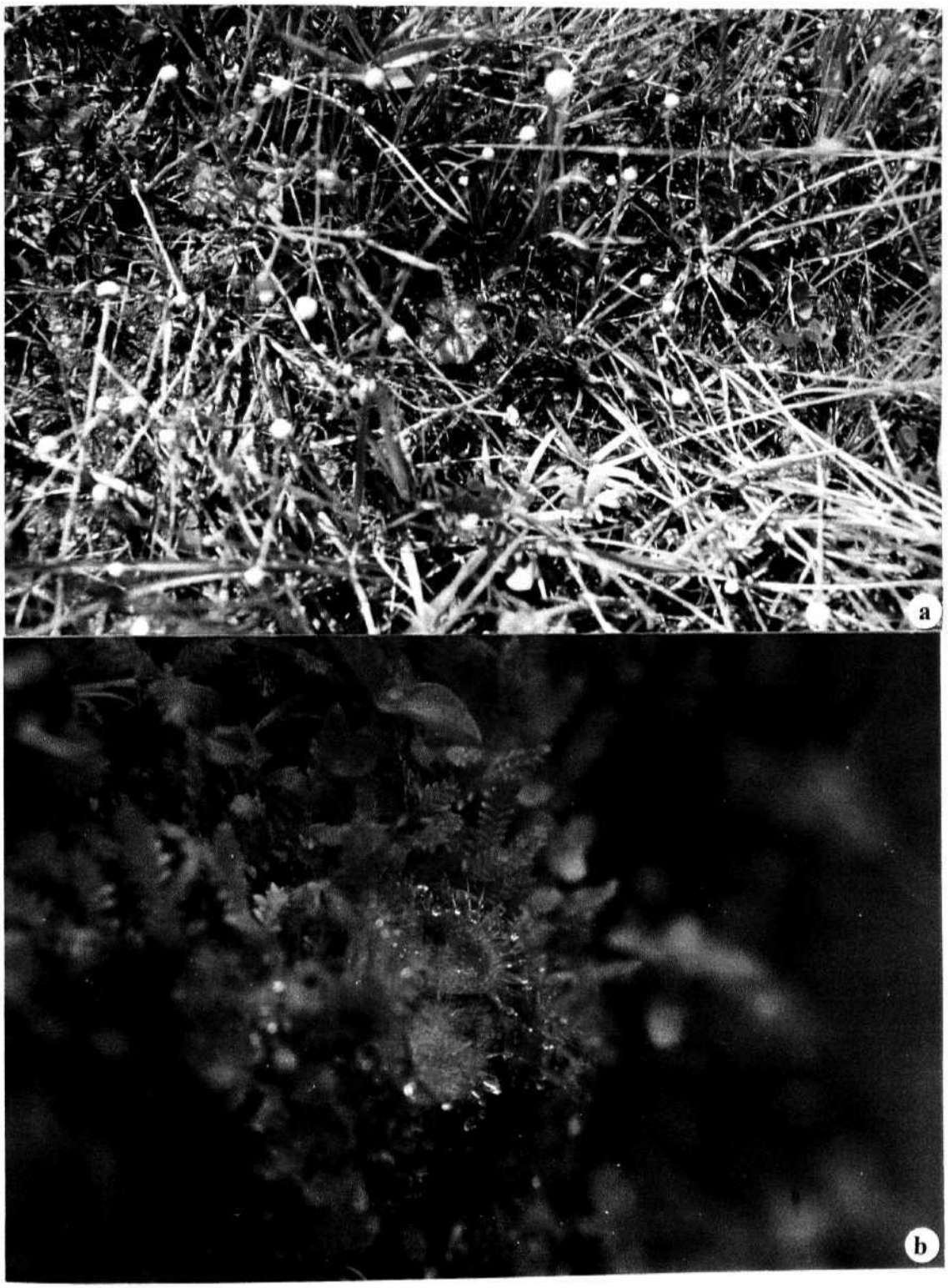

Figure 4: a) Habitat *oi'D. burmanii* in the University of Hyderabad campus.
b) *D. burmanii* associated with *Sphagnum.*

Figure 5: *D. indica* preying on insects with the help of its sticky leaf tentacles a) Houseflies trapped by leaf tentacles, b) Butterfly

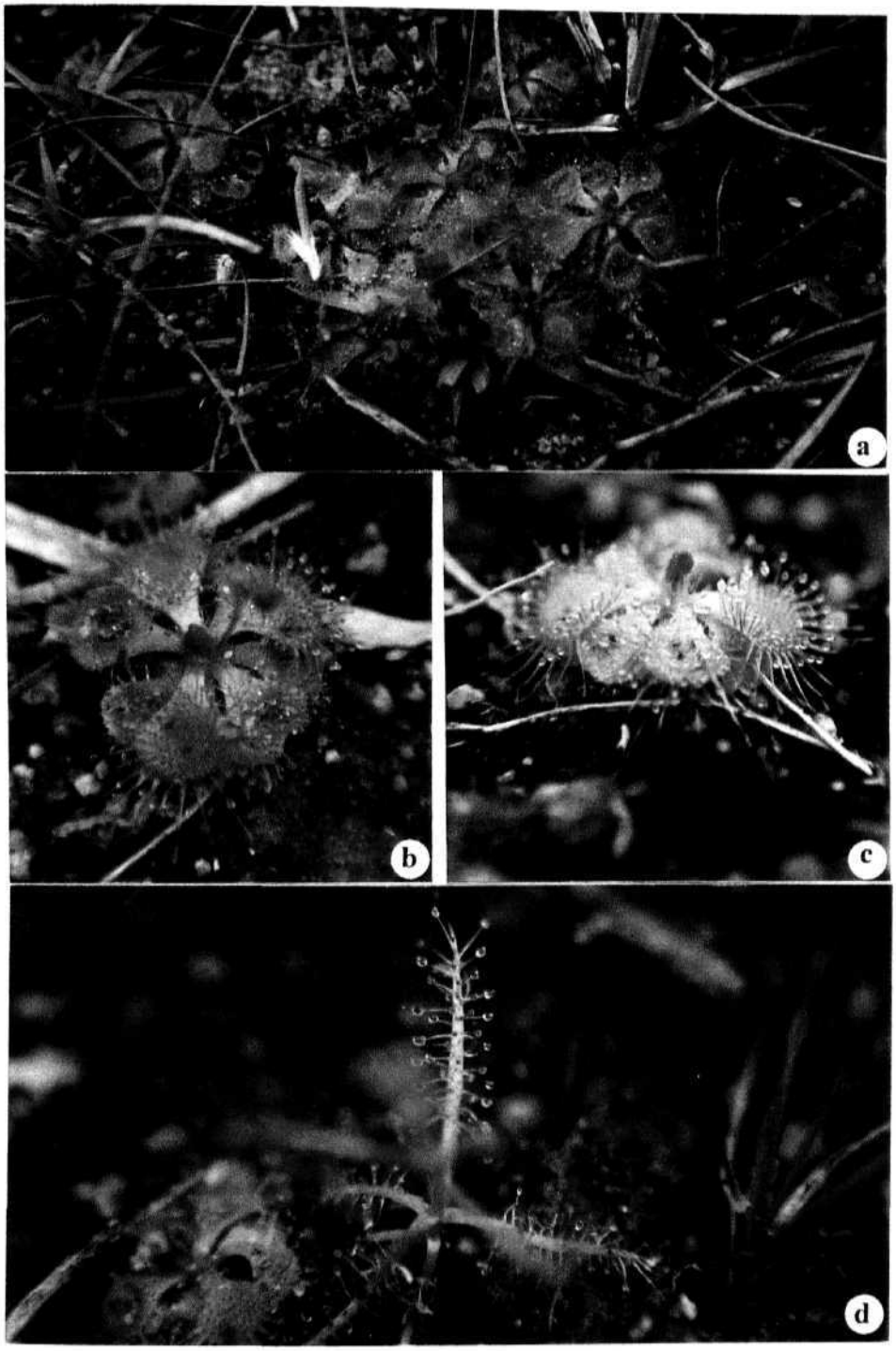

Figure 6: Habit of *D. burmanii*
a) Densely grown rosette in natural habitat
b) Enlarged view of plant
c) Enlarged view with leaf tentacles
d) Associated with *D. indica* (leaves with sticky mucilaginous gland)

Table 4: Phentotypic parameters of *D. indica*

Phentotypic parameters	Phentotypic parameters Mean ± S.E
Length of the plant	17.3 ±1.8
Length of the inflorescence	4.9 ±0.5
Number of inflorescence/ plant	2.7 ±0.5
Number of flowers / plant	20.8 ± 1.3
Number of seeds/ capsule	68.3 ±8.8

Table 5: Phentotypic parameters of *D. burmanii*

Phentotypic parameters	Phentotypic parameters Mean± S.E
Width of the plant	2.5 ±0.1
Length of the inflorescence	13.1 ±0.5
Number of inflorescence/ plant	2.4 ± 0.4
Number of flowers / plant	30.3 ±2.1
Number of seeds/ capsule	174.4 ±15.7

agricultural wastes and draining of wetlands apart from their naturally restricted habitat add to the adverse conditions for their natural growth and propagation **(Figures 7 a-d).** Above-mentioned factors prompted us to investigate this topic of research.

5.2. Seed germination studies

The genus *Drosera* is extremely large, and diverse in their habit and reproduction according to which the cultural requirement also varies. Conventional propagation can be achieved through seeds. Unfortunately very few properly documented reports are available on seed germination of individual *Drosera* species. Recently, there has been considerable interest in germinating seeds of various species of Drosera.

Initial germination experiments using seeds of *D. indica* and *D. burmanii* were done in controlled glass house conditions, where the temperature was maintained at 30±5°C. The seeds of *D. indica* and *D. burmanii* stored at $4^{o}C$ were subjected to different treatments with various concentrations of giberllic acid, cold and heat shock to break the dormancy and were sown in soil collected from the natural habitat along with untreated seeds as control. But seeds of both species did not germinate even after 30 days **(Table 6).** In nature *D. indica* and *D. burmanii* are seed propagated. Once monsoon starts the seeds of *D. indica* and *D. burmanii* imbibe water, and germination takes place. Mechanism of seed germination in *D. indica* and

Figure 7: a) Marshy habitat of *D. indica* around rocky outcrop, b) Enlarged view of leaf tentacles, c) Urbanization and land use alteration resulting in the habitat loss for *D. indica* and *D. burmanii* in University of Hyderabad campus, d) *D. indica* and *D. burmanii* - habitat fragmentation to biotic interference.

Table 6: Seed germination studies of *D. indica* and *D. burmanii*

Different treatments	No. of seeds	No. of days	Observations
GA3 5 ppm	50	30	No
10 ppm	50	30	-
15 ppm	50	30	-
20 ppm	50	30	-
25 ppm	50	30	-
50 ppm	50	30	-
100 ppm	50	30	-
Hot water treatment	50	30	-
Tap water	50	30	-
24 hours cold water treatment	50	30	-
On MS basal medium	50	30	-

D. burmanii in nature is complex and difficult to presume. Many of the *Drosera* species seeds need a cold treatment (4°C) for breaking the dormancy and germination. However even after cold stratification seeds of *D. indica* and *D. burmanii* did not germinate. Conran et al. (1997) germinated seeds of about 100 taxa of *Drosera* and recorded number of days from time of sowing until the first seeds germinated and seedling morphology. Seeds of *D. alicia* Rayn-Hamet from South Africa required a 24 d period of imbibitions at 15/10°C before any germination occurred, but after 34 d ca. 70 of the seeds germinated. If seeds were subjected to warm, moist conditions but not if they were stored dry (Ferreira and Small, 1974). In contrast, 30 % seeds of *D. rotundifolia* germinated in light at 20/15°C after 4 months of stratification at 5°C (Grime et al., 1981). Seeds of *D. rotundifolia* mostly germinate in May (spring) in Sweden this indicates that dormancy break occurs during winter (Redbo-Torstensson, 1994). Crowder et al. (1990) obtained 90-95 % germination of *D. anglica, D. intermedia* and *D. rotundifolia* seeds from Saskatchewan, Canada, in light in a glasshouse at 18-22°C after they had been imbibed in darkness at 10°C for 8, 16 and 18 weeks respectively. Seeds of *D. anglica, D. intermedia* and *D. rotundifolia*, sown on wet filter paper in plastic petridishes kept out door in Ontaria, Canada, all winter, germinated to 64, 22 and 0 % respectively, the following spring (Crowder et al., 1990). Kinzel, (1909) reported that seeds of *D. anglica, D. intermedia* and *D. rotundifolia* required light for germination. Crowder et al. (1990) also found that light

was required for germination of *D. rotundifolia* and *D. anglica* but some seeds of *D. intermedia* germinated in darkness.

Baskin et al. (2001) reported that *D. anglica* were dormant at maturity in late summer, and Dormancy break occurred during cold stratification and stratified seeds required light for germination, but light had to be given after temperatures were high enough to be favorable for germination. All these varied results show that germination of *Drosera* species differ according to the local environmental conditions in native territory. Unfortunately there are not many reports on *Drosera* species regarding seed germination and propagation methods from India.

Seeds have been used mainly as explants for *D. intermedia* (Burger, 1961), *D. pygmaea* (Harder, 1964a, b), *D. rotundifolia* (Small et al., 1977; Simola, 1978a, b and Kukulczanka and Czastka, 1988) and *D. peltata* (Kim and Jang, 2004).

Sterilization of *Drosera* seeds and other explants proved difficult because of fungal and bacterial contaminants on the surface of the seeds. Sterilization process may vary from species to species. Sterilization of seed explants was achieved using CaOCl (3 %) (Simola, 1978a; b) in *D. rotundifolia*, 0.1 % $HgCl_2$ (Small and Hendrikz, 1974) in *D. pygmaea* and *D. aliciae*, NaOCl (Burger, 1961) in *D. intermedia*. 20 % (V/V) commercial bleach and 0.01 % (V/V) Tween-20 for 20 min (Jang and Park, 1999) in *D. rotundifolia*, 0.1 % (W/V) Benzalkonium chloride solution for 5 min, 70 % (V/V) ethanol for 30 s (Ichiishi et al., 1999) in *D. spathulata*

and 70 % ethanol 10 sec and with 3 % $CaCl_2O_2$ for 20 min (Kawiak et al., 2003) in *D. anglica* and *D. cuneifolia* proved to be effective.

One of the first reports of the *in vitro* culture of *Drosera* is that of Schmid (1912). Burger (1961) reported axenic reduced germination of *D. intermedia* seedlings in a simple nutrient medium. The percentage of was germination 44%, when seeds were incubated in the light with a 15/38ºC alternation in temperature. At 15°C constant light and dark and alternate light and dark, 0 % germination was observed. Whereas at 38°C and constant light 2 % germination and in constant dark and alternate light and dark, 0 % germination was observed. Seeds of *D. indica* and *D. burmanii* cultured on MS basal medium containing 3 % agar at pH 5.7 seeds did not germinate under the photoperiod of 16/8h and in dark conditions even after 30 days (Table 6). This can be probably due to toxic effect of sterilant, used during sterilization and seeds might require constant exposure to light, temperature, and dormancy breakers like GA_3. Though the experiments were repeated several times there was no germination observed.

But Van Waes (1985) reported that of the 16 species used for *in vitro* germination, most seeds germinated after 10 days and the seedlings were ready for transplanting after 4 months. Small and Hendrikz (1974) germinated seeds with a photoperiod of 14h and day/ night temperature of 15/10ºC with seeds starting to germinate after 3 weeks; as confirmed by Kukulczanka and Czastka (1988). Kawiak et al., (2003) germinated seeds of *D. anglica* and *D. cuneifolia* in 2 % sucrose and 0.7 % agar in different media and after 4-6 weeks the germination frequency estimated as

62 and 71 % respectively. Hirsikorpi et al, (2002), reported that seeds of *D. rotundifolia* were germinated on ½ strength MS medium supplemented with BAP 0.1 mgL^{-1} and NAA 0.05 mgL^{-1}, 2 % sucrose and 100 mgL^{-1} myoinositol, at pH 5.7 were solidified with 0.65 % agar. Jang and Park (1999) reported that seeds of *D. rotundifolia* germinated within 2-3 weeks on ½ MS medium. The germination rate of seeds was 100% when the seeds were treated at 4°C for more than 30 days. But the seeds without cold treatment at 4°C for for less than 30 days did not germinate. Ichiishi et al. (1999) germinated seeds of *D. spathulata* Kanto type collected from cultivation and inoculated on ½ MS supplemented with 0.8 % sucrose. They were germinated 30-60 days after they were inoculated on medium. Kim and Jang, (2004) reported the germination rate of *D. peltata* seeds was 70.5 % within 10-15 days on ½ MS medium with seeds that had been stored at 4°C for 30 days. Without cold treatment for at least 4 weeks, the germination rate was very low (about 16.7 %).

5.3. *In vitro* propagation

Insectivorous plants are being depleted from the natural environment due to large-scale collection of the plants and urbanization of locations that formerly supported carnivorous plant population (Campbell, 1983; Juniper et al., 1989). Although now there are reputable dealers of carnivorous plants who do not collect plants from the wild, some species are already considered threatened or endangered (Ayensu and DeFilipps, 1978). If these plants are to be continuously made available to the public without danger to existing natural stands, it is imperative that alternative

methods of production be investigated. *In vitro* propagation of carnivorous plants is indicated because many plants may be rapidly produced from small amount of tissue, thereby minimizing collection from natural populations.

Since seeds were not suitable for explant preparation due to lack of germination, shoot tips of *D. indica* and *D. burmanii* were taken as explants. The collected plants of *D. indica* and *D. burmanii* from natural habitats with natural soils were maintained in an air conditioned room at 20±2ºC with 80 % humidity until it is used for *in vitro* culture under laboratory conditions. Nalini and Murali, (2002) grown the *D. indica*, with sterilized *Sphagnum* peat moss and sand combination (3:1) used as substratum and light intensity of 1000 lux.

In vitro propagation of *Drosera* plants has been proved to be difficult due to fungal and bacterial contaminants on the surface of the leaves (Crouch et al., 1990; Anthony, 1992). Even in *D. indica* and *D. burmanii*, sterilization of the shoot explants when done using 40-70 % concentration of ethanol and 0.1 % $HgCl_2$, even if it was for brief period of time (1 min.) deteriorated the tissue. Very low concentrated solutions viz. 35 % ethanol and 0.05 % $HgCl_2$ for 30-45 sec. proved effective for elimination of contaminants without harming the explants.

Leaf and nodal explants were also tried but due to necrosis of tissue during sterilization made it difficult to continue. Nalini and Murali (2002) reported that the tender stem segments (about 1 to 2 cm) were sterilized using 0.01 % (W/V) $HgCl_2$ for

5 min. followed by 0.05 % (V/V) polysorbate-20 for 2 min was effective, but this protocol was not reproducible in our lab conditions.

5.4. Influence of MS medium strength

Since *Drosera* plants grow normally in nutrient poor habitats, the effects of the strength of MS medium on shoot proliferation and growth were tested. In case of *D. indica,* the shoot proliferation was not much influenced by different strengths of MS medium **(Table** 7). The number of shoots was almost same in all the tested strength of MS medium, but the number of roots was more in ¼ strength MS but length of roots were similar in all the tested strengths of MS medium. Roots were black, unbranched and entirely covered with root hairs. When plantlets were separated and transferred to MS medium, they survived well **(Figures** 8 a-f). Whereas in case of *D. burmanii*, the shoot proliferation was not influenced by the strengths of the MS medium **(Table** 8) and in all the tested MS concentrations rooting also was observed. The number of shoots was almost same in all the strength of MS medium, but number of shoots induced from the axil of bracts was more when full strength MS medium was used. These shoots developed roots from axils of leaves. Roots were black, unbranched and completely covered with root hairs and flowering was also observed. When plantlets were separated and transferred to MS medium, they survived well **(Figures** 9 a-g). However some reports show that shoot proliferation was greatly influenced by strength of MS medium used for culture (Jang et al., 1997; Anthony, 1992; Jang and Park, 1999; Kim and Jang, 2004). But

Table 7: Influence of MS medium strength on shoot proliferation from shoot of *D. indica* after 8 weeks culture.

Con. of medium	No. of explants	No. of multiple shoots/ explants Mean ± S.E	No. of roots/ explants Mean ± S.E	Length of roots/ explants Mean ± S.E
25%	40	4.1 ±1.2	10.9±2.5	1.9 ±0.3
50%	40	3.4 ±0.8	7.1 ± 1.3	1.2 ±0.3
75%	40	3.3 ± 1.1	7.8 ±1.7	1.7 ±0.4
100 %	40	3.2 ± 0.8	6.4 ±1.5	1.6 ±0.4

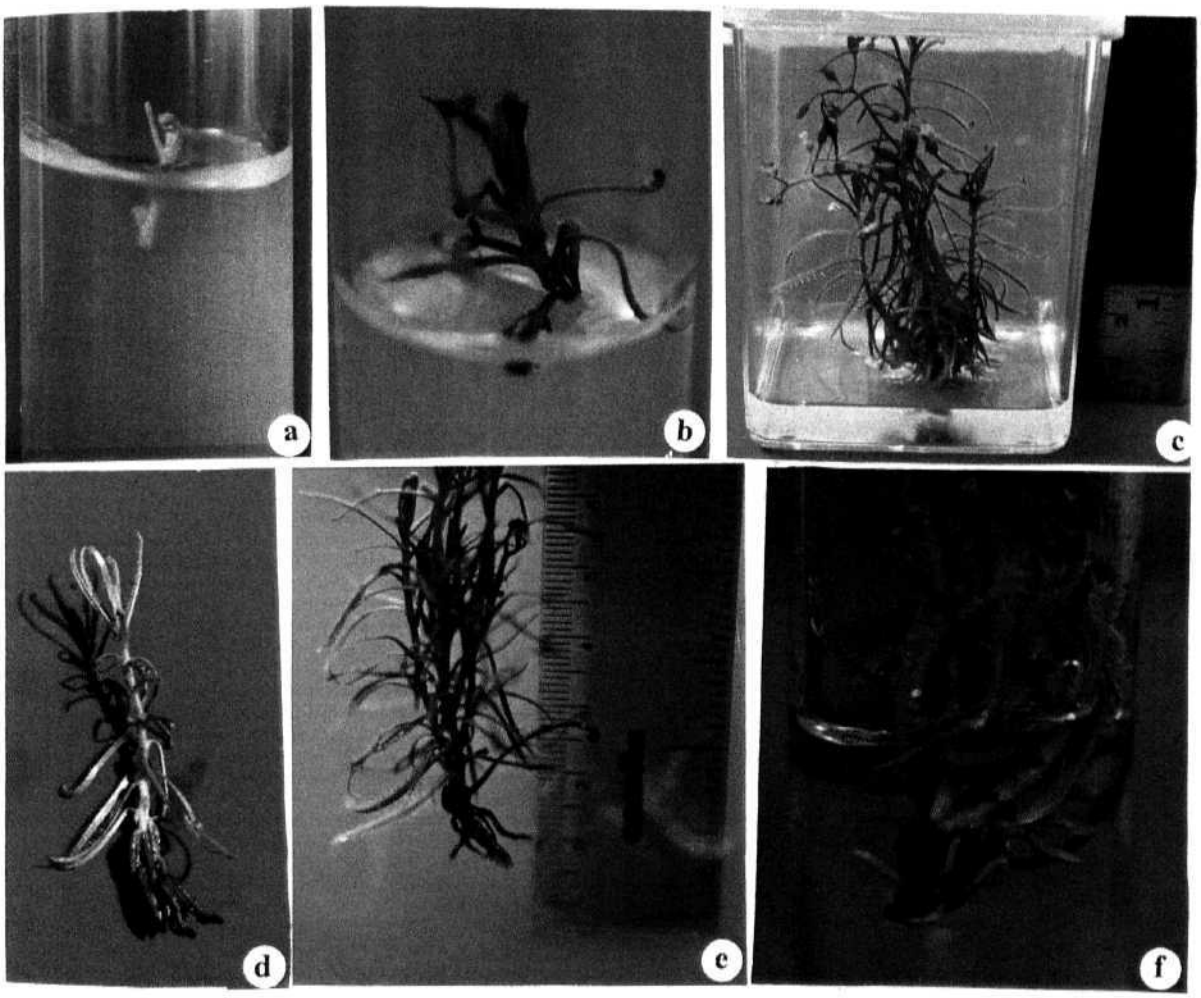

Figure 8: *D. indica* regeneration from shoot tip
a) Shoot cxplant on MS basal medium.
b) Shoot cxplant on MS basal medium after 10 days.
c) Fully grown *in vitro* plant with *in vitro* llowcring on MS basal medium.
d) *In vitro* raised plantlet on ½ MS medium.
e) Rooting on MS Basal medium.
f) Enlarged view of *in vitro* rooting

Table 8: Influence of MS medium strength on shoot proliferation from shoot of *D. burmanii* after 8 weeks culture.

Diff. con. MS Medium	No. of multiple shoots Mean ± S.E	No. Of shoots induced from the axils of bracts Mean ± S.E	% Of respons
25%	1.2 ±0.2	3.2 ± 1.0	76
50%	2.2 ±0.9	5.8 ±1.7	70
75%	1.3 ± 0.4	5.1 ±1.6	63.3
100%	1.5 ±0.6	5.1 ±1.5	53.3

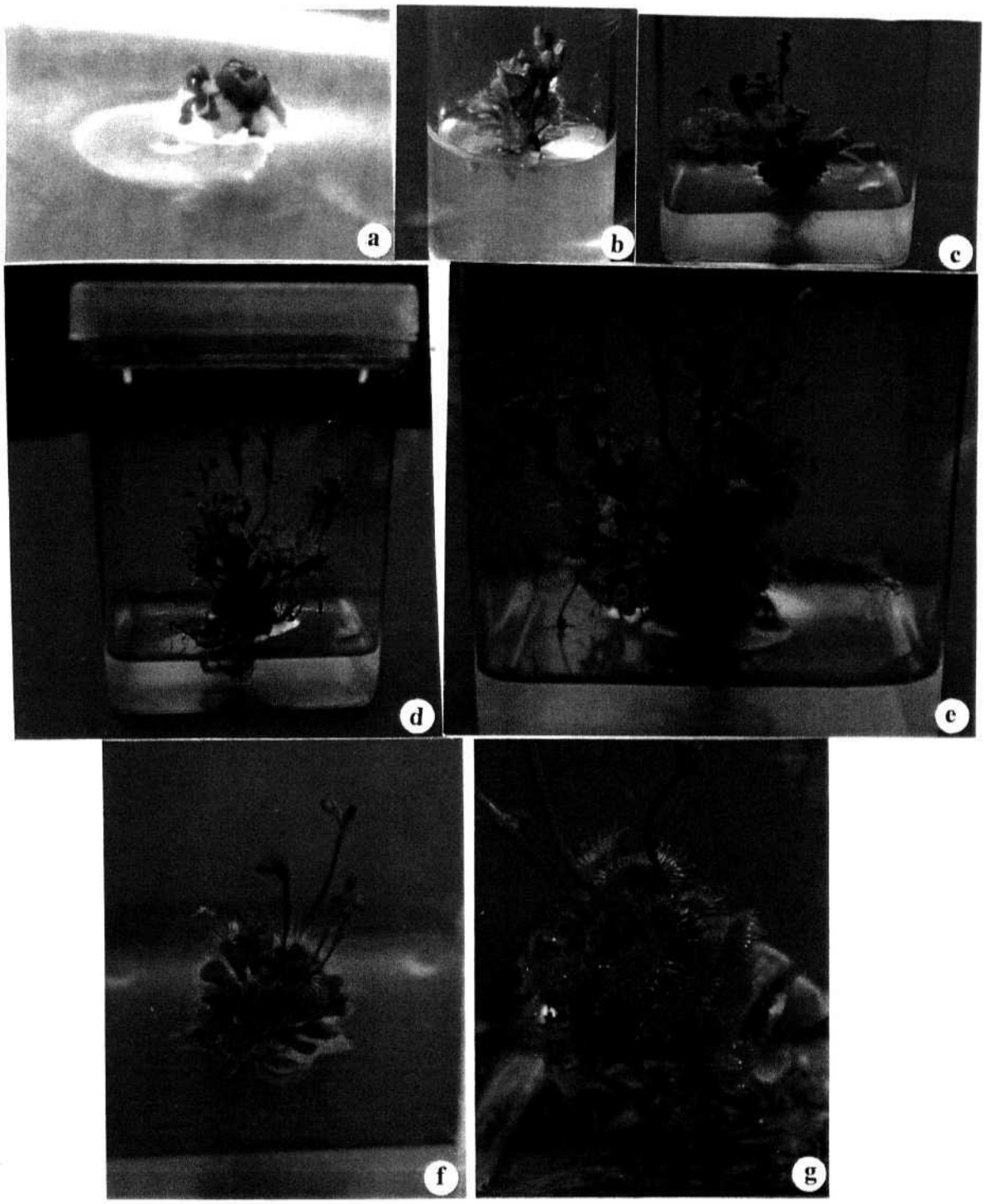

Figure 9: Regeneration of *D. burmanii* from shoot tip

a) Shoot explant on MS basal medium.
b) Shoot explant on MS basal medium after 10- 15 days.
c) Fully grown *in vitro* plant.
d) Fully grown *in vitro* plant with good rooting.
e) Regeneration of plantlets from axils of the bracts
f) Induction of *in vitro* flowering.
g) Close view of *in vitro* cultured plants with its leaf tentacles.

according to Crouch and Van Staden (1988) and Perica and Berljak (1996) the best basal medium for tissue culture of *Drosera* genus was MS medium.

5.5. Role of sucrose concentration

For *in vitro* cultures the main source of carbohydrate being sucrose the effects of sucrose concentration on shoot proliferation of *D. indica* and *D. burmanii* were examined. There was no significant effect of different concentrations of sucrose on shoot proliferation for *D. indica.* Almost all shoots produced roots also at their base. But percentage of response for rooting was found better at 3 % sucrose. Roots were black, unbranched and totally covered with root hairs **(Table** 9). Same was the observation on *D. burmanii* also. The roots developed also resembled to those developed on *D. indica* **(Table 10).**

5.6. Influence of pH on shoot proliferation

The insectivorous plants grow in a low pH soil, which ranges from 3 to 5 (Pietropaolo and Pietropaolo, 1986). Since pH affect nutrient uptake and shoot proliferation (Parliman et al., 1982), the effect of pH levels on shoot proliferation of *D. indica and D. burmanii* were examined.

In case of *D. indica* the shoot proliferation was not much influenced by different pH. But percentage of rooting and number of roots and length of roots were good at pH 5.7. In all the pH tested rooting was observed. Roots were black, unbranched and wholly covered with root hairs **(Table 11)**. In case of *D. burmanii*

Table 9: Role of sucrose on shoot proliferation from shoots of *D. indica* after 8 weeks cultures on MS medium.

Diff. conc. of sucrose	%of rooting	No. of multiple shoots/explant Mean ± S.E	No. of roots/explant Mean ± S.E	Length of the roots Mean ± S.E
1 %	76.0 %	1.5±0.4	4.4 ±0.8	1.1 ±0.3
2%	93.9%	2.7 ±0.5	6.3 ± 1.5	0.9 ±0.2
3%	100%	2.7 ±0.8	6.3 ± 1.3	0.6 ±0.1

Table 10: Role of sucrose on shoot proliferation from shoots of *D. burmanii* after 8 weeks cultures on MS medium.

Diff. con. of sucrose	No. of multiple shoots Mean ± S.E	No.of shoots induced from the axils of bracts Mean ± S.E	% Of response
1%	1.2±0.3	4.0 ±1.2	23.3
2%	1.0 ±0.05	4.0 ±1.0	76.6
3%	1.7±0.7	5.3 ±1.3	83.3

Table 11: Influence of pH on shoot proliferation from shoots of *D. indica* after 8 weeks cultures on MS medium.

Different conc. of pH	%of rooting	No.of multiple shoots/explant Mean ± S.E	No.of roots/explant Mean ±S.E	Length of roots/explant Mean ±S.E
3.7	74.0 %	1.9±0.6	4.2 ±1.2	0.3 ± 0.0
4.7	85.0 %	2.0 ±0.5	5.4 ±1.3	0.3 ± 0.05
5.7	100%	2.7 ±0.8	6.3 ±1.3	0.6 ±0.1
6.7	85.0%	2.1 ±0.5	4.7 ±1.2	0.3 ± 0.05

the shoot proliferation from the shoot tip explants was not influenced by different pH but as the pH decreased, there was a gradual increase in the number of shoots developed from the axil of bracts though the percentage of response was low. These shoots also developed roots from the axils of leaves. Roots were black, unbranched and entirely covered with root hairs. When these were separated and transferred to MS medium, they survived and continued growth. In all the pH tested rooting was observed **(Table 12).** In *D. peltata* and *D. rotundifolia* the effect of different pH greatly influenced on shoot proliferation (Jang and Park 1999; Kim and Jang 2004) and it was severely inhibited in more acidic media. Perica and Berljak (1996) reported that *in vitro* plant growth of *D. spatulata* was strongly affected by medium pH. Simola (1978) cultured the young seedling of *D. rotundifolia* on medium with pH 6.0, which grew well.

5.7. Effect of cytokinins

Cytokinins generally inhibit root development and promote shoot growth (Pennazio, 1975). In *D. indica* among the tested cytokinins, Zeatin at 0.1, 0.5, 1.0 and 2.0mgL^{-1} (mean 19 shoots/ explant), Kinetin at 0.5 and 1.0mgL^{-1} (mean 14 shoots/ explant) and BAP at 0.05 and 0.1 mgL^{-1} (mean 14 shoots/explants) proved to be effective in developing multiple shoots within 50-60 days of culture **(Table 13) (Figures 10 a-d and 11 a-d).** Even roots were also observed except at higher concentrations, but they were thin compared to the roots developed on MS basal medium. Roots were black, unbranched and with root hairs all over. Increasing the

Table 12: Influence of pH on shoot proliferation from shoots of *D. burmanii* after 8 weeks cultures on MS medium.

Different con.of pH	No. of multiple shoots Mean ± S.E	No.of shoots induced from the axils of bracts Mean ± S.E	% Of response
3.7	1.5± 0.6	5.2 ±1.4	63.3
4.7	1.3 ±0.4	5.0 ±1.3	80
5.7	1.3 ±0.3	4.4 ±1.5	70
6.7	1.3 ±0.4	3.3 ±0.8	70

Table 13: Effect of cytokinins on shoot proliferation from shoot of *D. indica* after 8 weeks culture on MS medium.

Different con. of cytokinins in mgL^{-1}	No. of shoots/explants Mean ± S.E
MS ±0.1 Z	17.2 ±5.3
MS + 0.5 Z	19.6 ±4.5
MS + 1 Z	18.6 ±4.6
MS + 2 Z	15.6±7.1
MS ±0.1 KN	6.5 ±1.7
MS ± 0.5 KN	14.0 ±5.3
MS + 1 KN	12.1 ±4.2
MS + 2 KN	7.6 ±1.5
MS + 0.01 BAP	8.3 ±2.1
MS ± 0.05 BAP	7.8 ±2.4
MS ±0.1 BAP	14.0 ±5.4

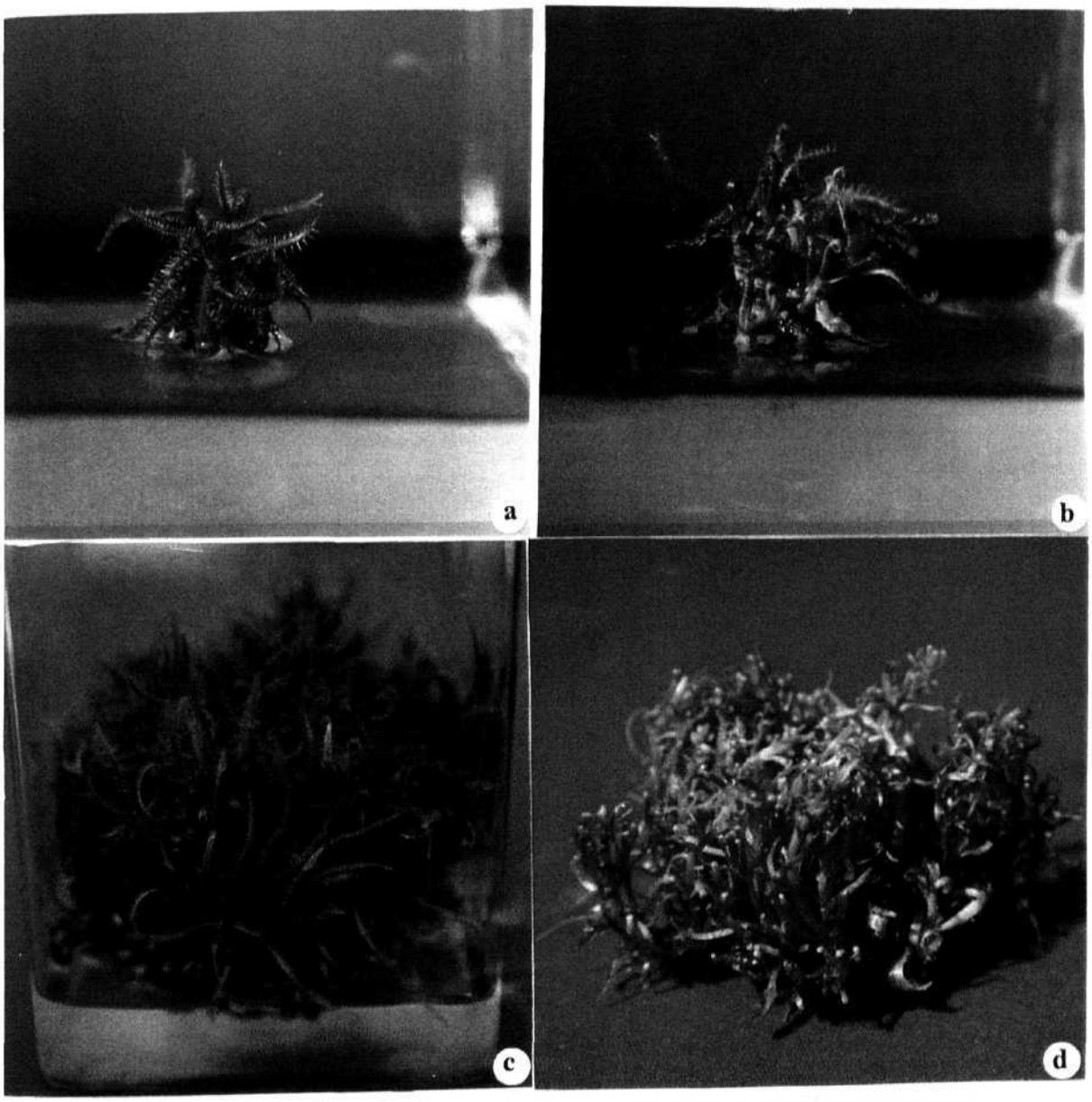

Figure 10: Induction of multiple shoots of *D. indica* on MS medium with

0.5 mgL^{-1} kinetin after 2 weeks.

0.5 mgL^{-1} zeatin after 2 weeks.

0.5 mgL^{-1} kinctin after 6 weeks.

0.5 mgL^{-1} zeatin after 6 weeks.

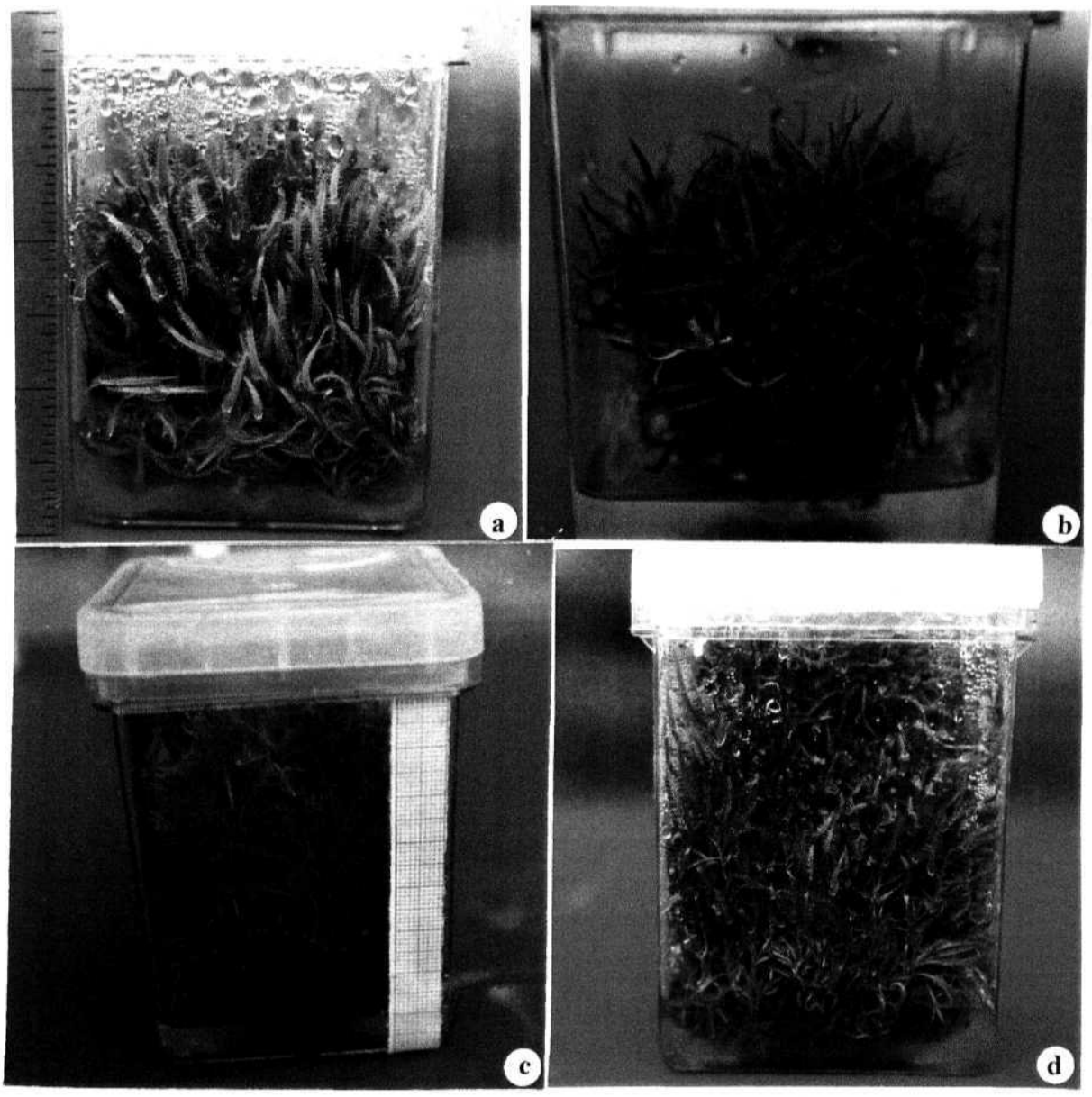

Figure 11: Magenta boxes filled with rapidly multiplied shoots of *D. indica* produced on MS medium with different cytokinins after 6 weeks.

a) 0.5 mgL^{-1} kinetin

b) 1.0 mgL^{-1} kinctin.

c) 1.0 mgL^{-1} zeatin

d) 0.5 mgL^{-1} zeatin

cytokinin concentrations shoot growth retardation was observed. In *D. burmanii* KN at 1.0 and 2.0 mgL^{-1} (mean 8.6 shoot/explant) and BAP at 0.5, 1.0 and 2.0 mgL^{-1} (mean 8.7 shoots/explants) individually developed multiple shoots within 50-60 days **(Table 14) (Figures 12 a-c).** But above 0.5 mgL^{-1} concentrations both growth regulators showed retarded growth of the developed shoot buds. And they were so compact to distinguish and separate individual shoots. There was no significant effect of Z at any tested concentration. Roots were also observed except at higher concentrations, but they were thin compared to the roots developed on MS basal medium. Roots were black, unbranched and entirely covered with root hairs. Increasing the cytokinins concentrations shoot growth retardation and red pigmentation was observed. Nalini and Murali (2002) stated in *D, indica* that higher concentrations of BAP or NAA are not desirable and generally induces a red pigmentation and necrosis. Kim and Jang (2004) reported that in addition of BA or KN in culture media greatly inhibited shoot and tuber formation of *D. peltata*. The higher the BA or KN concentration, the stronger the suppression, especially if BA was used. Similar results were obtained by Anthony (1992) with leaf explants of *Drosera* spp. grown on ½ MS. Jang and Park (1999) also reported in *D. roiundifolia* that addition of KN or BA in ½ MS medium was strongly suppressed shoot proliferation. The suppression of shoot proliferation was more effective in BA supplemented. However, Kukulczanka and Czastka (1988) found that *in vitro*

Table 14: Effect of cytokinins on shoot proliferation from shoot of *D. burmanii* after 8 weeks culture on MS medium.

Diff. con. KN & **BAP** medium mgL^{-1}	No. of multiple shoots Mean±S.E	No.of shoots induced from the axils of bracts Mean ± S.E	% Of response
MS+0.1KN	1.9 ±0.8	4.0 ±1.2	40
MS+0.5 KN	4.6 ± 1.9	4.8 ±1.7	53.3
MS+1.0 KN	7.5 ±2.4	3.1 ±1.5	63.3
MS+2.0 KN	8.6 ±1.9	3.3 ±0.9	46.6
MS+0.1 BAP	2.5 ±0.8	2.7 ±0.6	36.6
MS+0.5 BAP	6.3 ±2.0	2.0 ±0.8	33.3
MS+1.0 BAP	8.7 ±2.1	1.7 ±0.4	23.3
MS+2.0 BAP	7.2±1.1	2.2 ±0.6	16.6

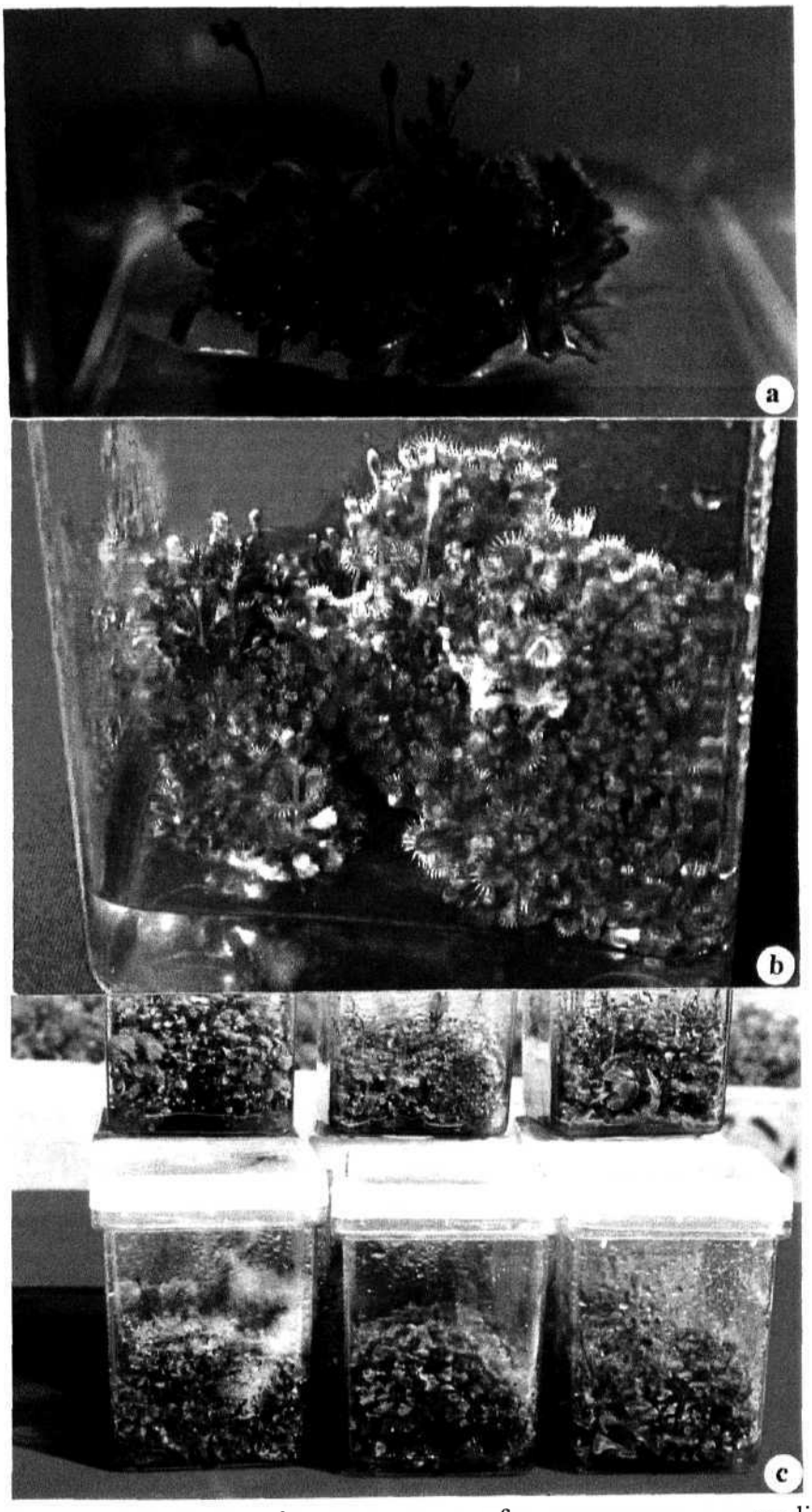

Figure 12: Initiation of multiple shoots of *D. bwmanii* on MS medium with

a) 2.0 mgL^{-1} kinetin after 2-3 weeks.

b) 2.0 mgL^{-1} kinetin after 6 weeks.

c) Magenta boxes filled with rapidly multiplied shoots

propagation of *Drosera* sps. by axillary bud culture was best on RM medium with 1.0 ppm BA. Perica and Berljak (1996) reported that full strength MS medium without growth regulators was optimal for regeneration and multiplication of *D. spatulala* by shoot tip culture. Nalini and Murali, (2002) also reported in *D. indica* that higher concentration of BAP and NAA are not desirable and generally induces a red pigmentation and necrosis. Kawiak et al. (2003) reported that higher concentrations of growth regulators lead to the morphological deviation of regenerated plants. This effect was also observed by Królicka et al. (1998) who reported the regeneration of deformed shoots from *D. ramentacea* explants.

In *D. burmanii* when the plantlets showing retarded growth were transferred to MS medium elongation was observed. When stunted inflorescences were cut and cultured on MS basal medium or lower concentrations of BAP or KN (0.1 and $0.5 mgL^{-1}$) multiple shoots developed from the axils of bracts, which appeared to develop better than from shoot tips and the number of multiple shoots was also very high after 50-60 days.

Shoot regeneration from the axil of bracts was observed occasionally in *D. indica,* in all the concentrations especially in prolonged cultures (60-90 days) and except at higher concentration of BAP and KN (<1.0 mgL^{-1}) (Figures 13 a-d). They developed roots in the same medium and when these plantlets were transferred to MS medium they grew well. There was no significant effect of AH on the explants with

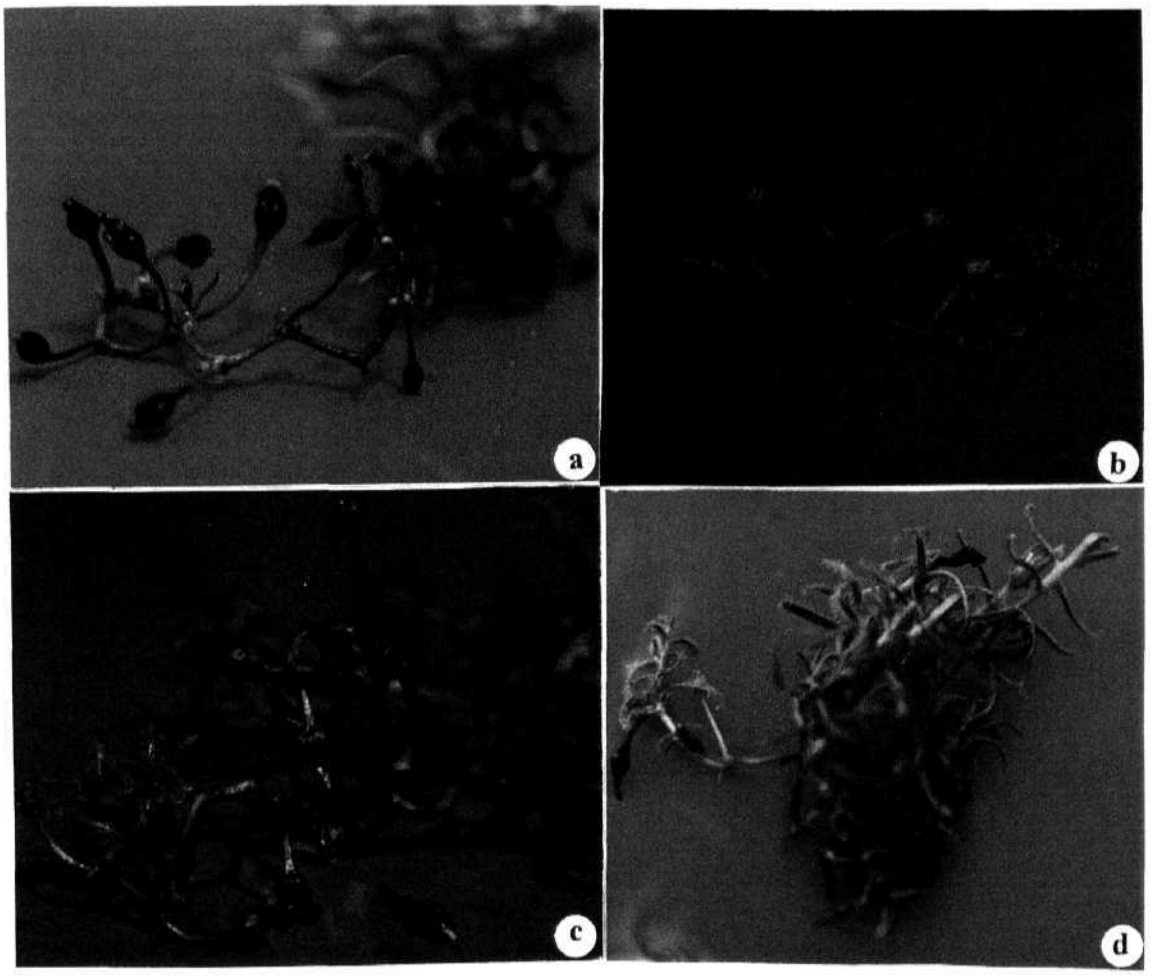

Figure 13: Regeneration of *D. indica* plantlets from axils of bracts on MS medium with ≤0.1 mgL^{-1} of kinetin - a) after 1 week, b) 2 weeks, c) and d) after 3 weeks.

regards to multiplication or elongation. But medium-containing GA3 assisted shoot elongation and, 2,4-D explants did not survive more than 10 days.

Occasionally plantlet regeneration was observed from the base of the flower stalks of *D. burmanii* at lower concentrations of BAP (0.5 and 1.0 mgL^{-1}) **(Figures** 14 a, b). In *Dionaea muscipula* flower stalk explants was considered a good alternative to initiate *in vitro* cultures (Teng, 1999). When the unrooted shoots were separated and transferred to MS medium rooting was observed. Roots were black in colour with dense root hairs.

Direct plantlet regeneration from somatic tissue is a rare phenomenon and has been described in a few species such as *Ranunculus sceleratus* L. (Konar and Nataraja, 1965), *Daucus carota* L. (Mc William et al., 1974), *Dactylis glomerata* L. (Conger et al., 1983), *Lycopersicum* (Young et al., 1987), *Cichorium* (Dubois et al., 1991), *Nicotiana tabacum* L. (Stolarz et al., 1991) and *D. rotundifola, D. natalensis, D. capensis, D. anglica, D. cuneifolia* (Simola, 1978; Crouch et al., 1990; Kukulczanka and Czastka, 1991; Anthony, 1992; Jang and Park, 1997; Bobak et al., 1995; Kawiak et al., 2003). The growth retarded plants of *D. burmanii* when transferred to MS medium or lower concentration of KN or BAP (< 0.5 mgL^{-1}) they regained normal growth and directly started developing numerous shoots on leaves without a callus interference they started developing roots after 6-8 weeks **(Figures 15 a-e; 16 a-c and 17 a, b).**

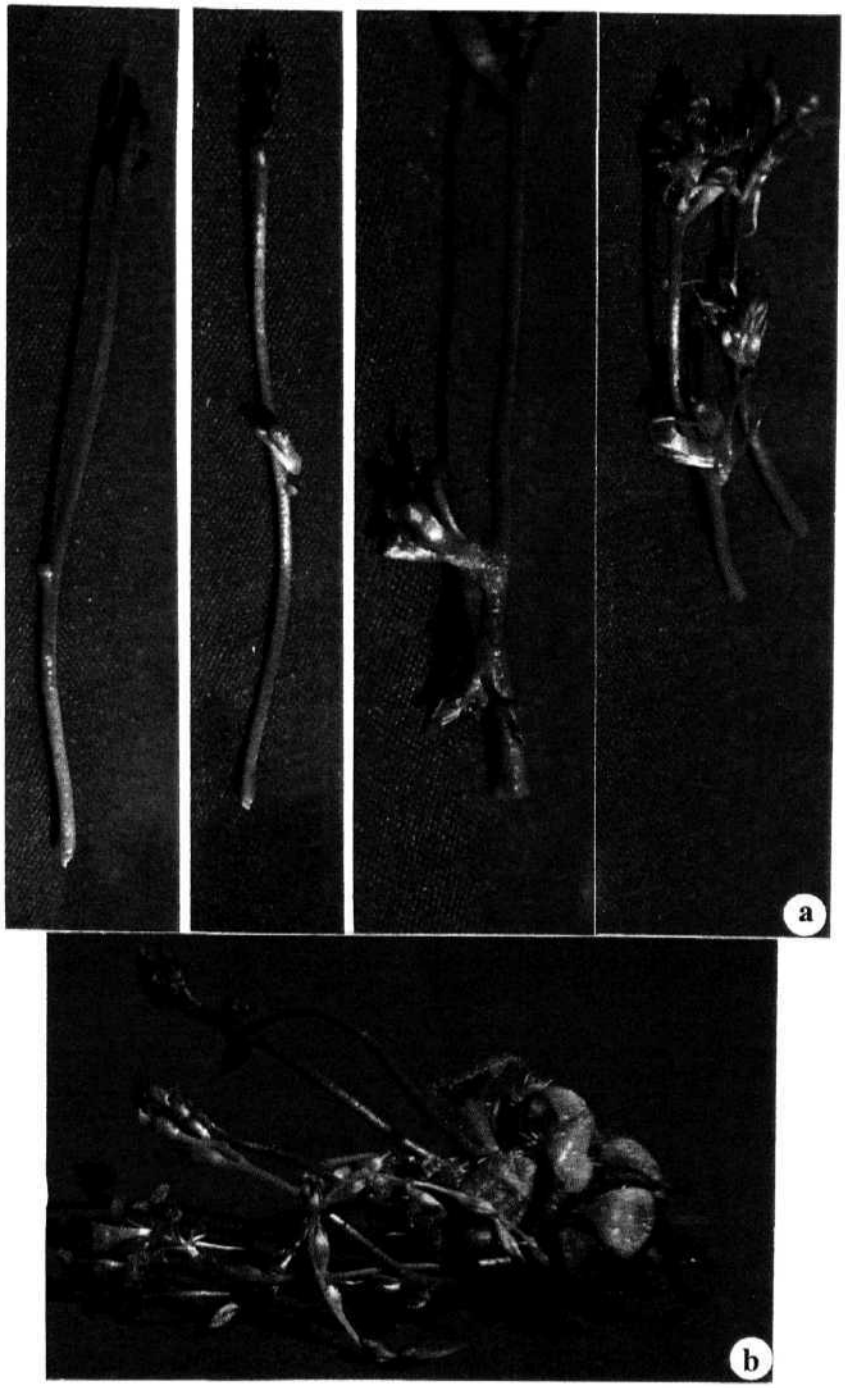

Figure 14: a) Regeneration of plantlets from the peduncle of *D. burmanii* on 0.1 mgL^{-1} of kinctin. b) Well established *in vitro* plant of *D. burmanii* with innorescence and plantlet from axil of bract.

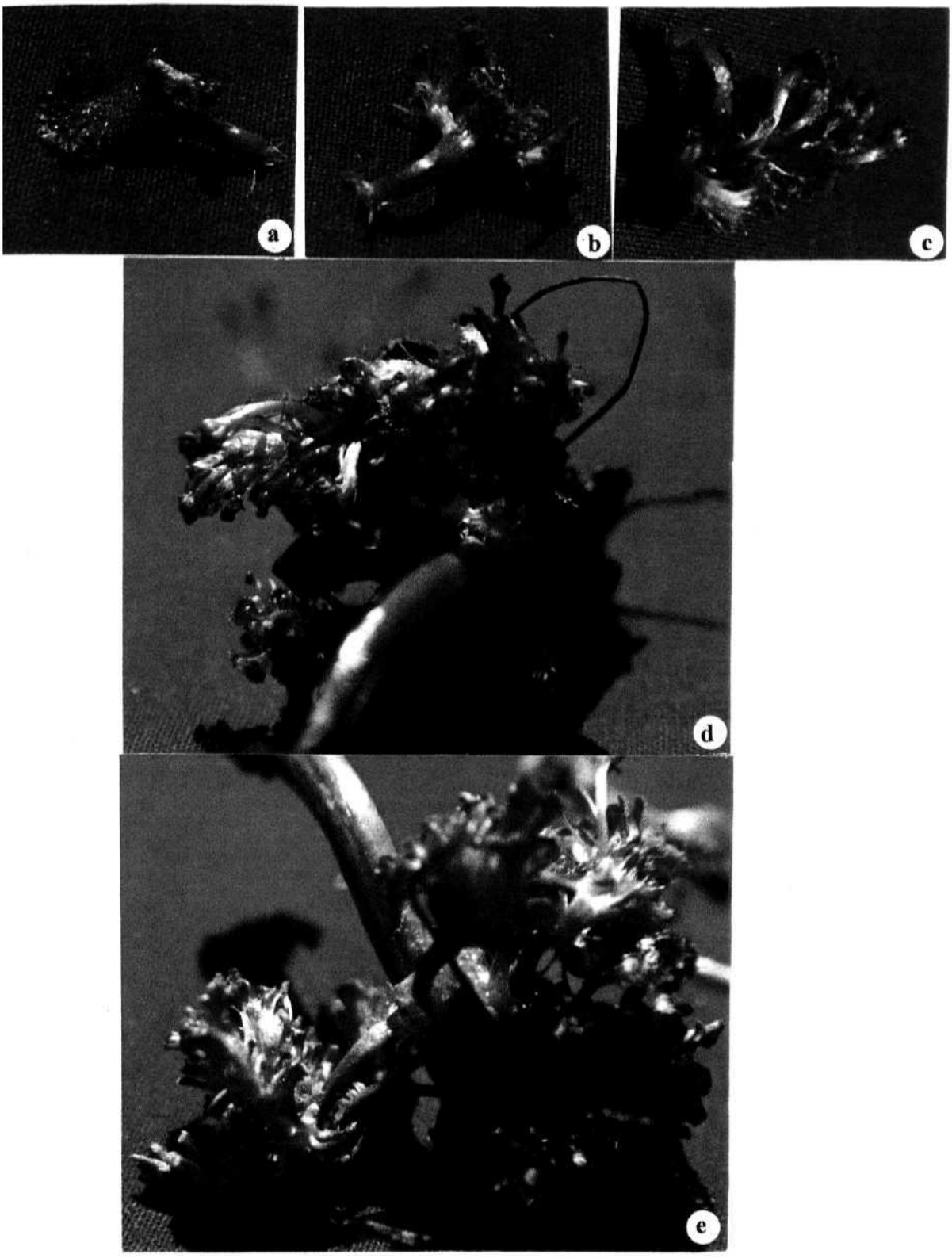

Figure 15: Different stages of characteristic multiples shoot initiation on leaf surface of *D. burmanii*

a) Initiation of multiple shoots after 1 week.
b) Induction of shoots after 3 weeks.
c) Induction of shoots after 4 weeks'.
d) Plant bearing multiple shoots on leaf surface.
e) Close of view of multiple shoots on abaxil of leaves.

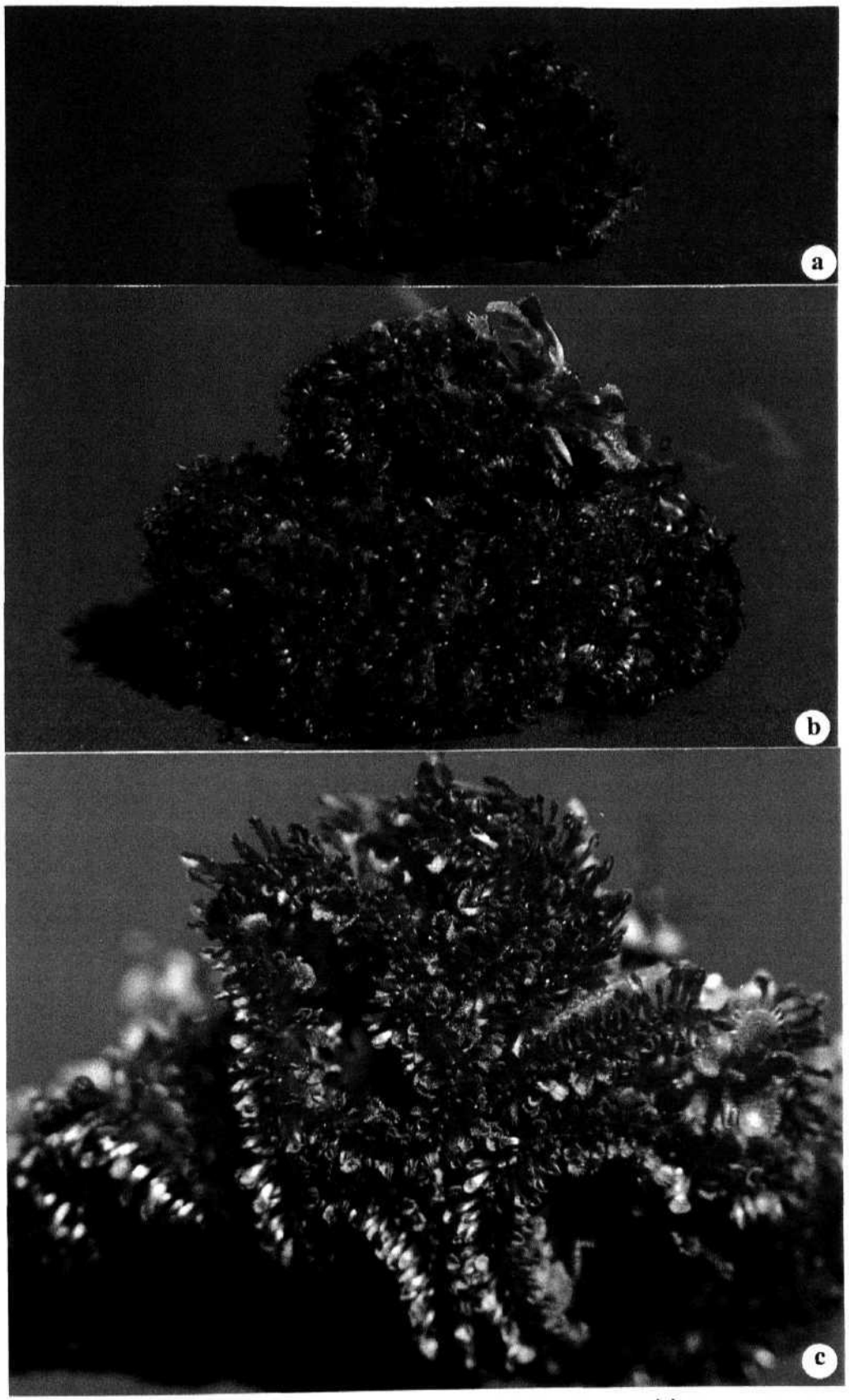

Figure 16: Different stages of characteristic multiples shoot initiation on leaf surface of *D. burmanii*

a) Induction of shoots after 6 weeks.
b) After 8 weeks.
c) Magnified view of characteristic clusters of multiple shoots.

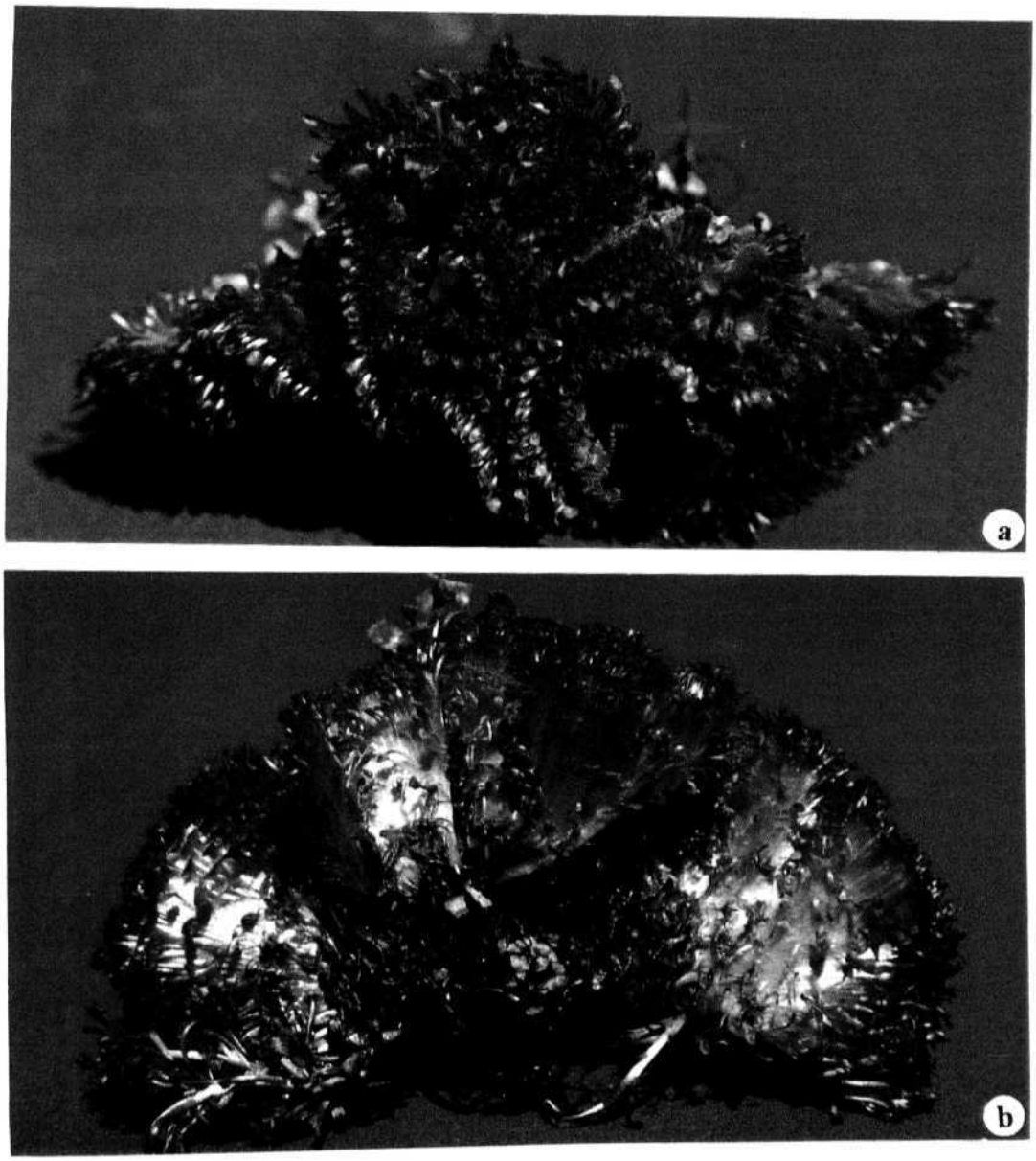

Figure 17: a) Characteristic multiple shoots on leaf surface of *11 burmaniii* after 8 weeks, b) Shoots bearing roots on basal region

The ability of explants to develop flowers *in vitro* depends on internal and external, chemical and physiological factors that interact in inter-dependent, complex and unpredicted ways (Tran than van, 1973; Scorza and Janick, 1980; Croes et al., 1985; Compton and Vielleux, 1992). Combinations of genetic and environmental factors also play a role in flowering response *in vitro* (Tisserat and Galletta, 1993). Various factors such as carbohydrates, growth regulators, light and pH of the culture medium are playing a major role in flowering (Heylon and Vendrig, 1988). Flowering *in vitro* has been reported in some woody species (Scorza, 1982). Induction of *in vitro* flowering is mainly due to stimulation of endogenous cytokinins by the growth regulators provided (Singh et al., 2000). But in *D. indica* and *D. burmanii* with out providing cytokinins also induction of *in vitro* flowering was observed in all the tested concentrations of MS medium, sucrose and in various pH values **(Figures 18 a-d).** This clearly indicates that induction of *in vitro* flowering is not due to exogenous cytokinin stimulations alone. In *D. indica* at lower concentration of cytokinins like BAP (0.01 mgL^{-1}), KN (0.1, and $0.5mgL^{-1}$), Z (0.1 and 0.5 mgL'^{1}), AH and GA3 (0.1, 0.5, 1.0 and 2.0 mgL'^{1}) *in vitro* flowering was observed but at higher concentrations of KN (1.0 and 2.0 mgL''^{1}*, BAP (0.1 mgL''^{1}) and Z (1.0 and 2.0 mgL''^{1}) an increase in the number of multiple shoots occurred albeit showed retarded growth and failed to induce *in vitro* flowering. Whereas in *D. burmanii* lower concentration of cytokinins like KN (0.1 and 0.5 mgL''^{1}) and BAP

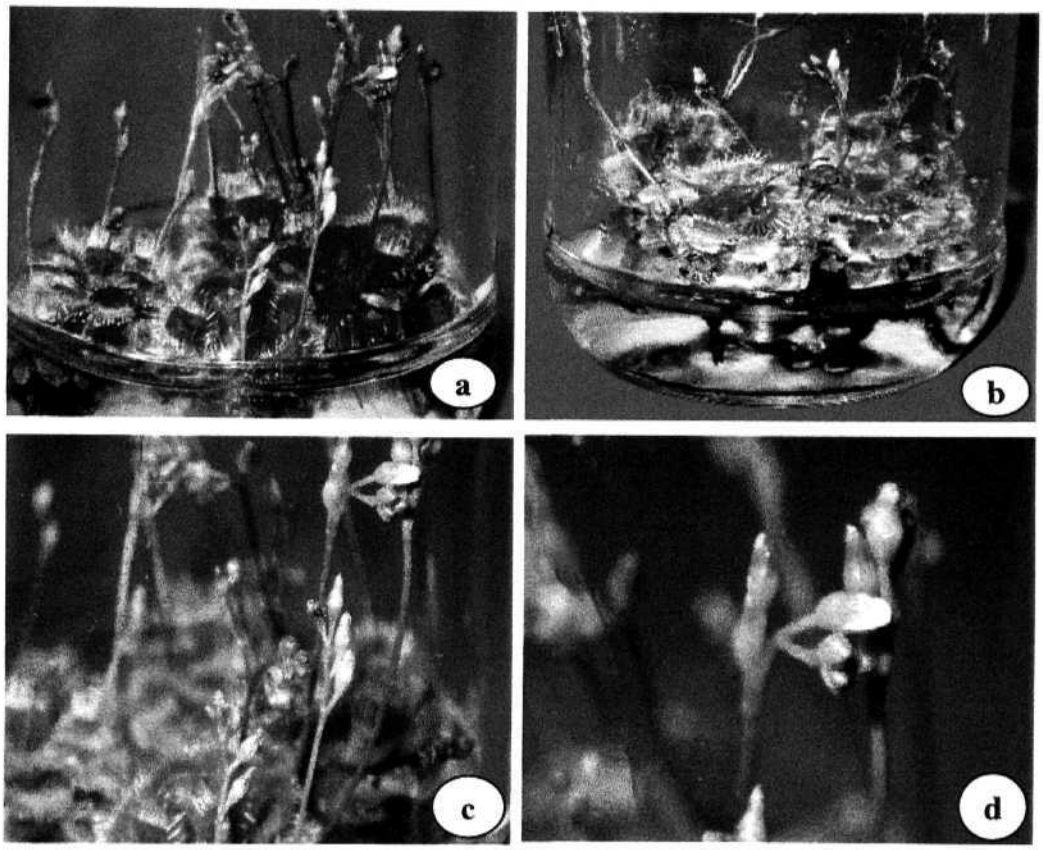

Figure 18 : a) Plants of *D. burmanii* growing on ½ MS.

b) Plants of *D. burmanii* growing on MS basal medium.

c) *In vitro* flowreing of *D. burmanii*

d) *Magnified view of D, burmanii*

(0.1 and 0.5 mgL^{-1}), *in vitro* flowering was observed but at higher concentrations of KN (1.0 and 2.0 mgL^{-1}) and BAP (1.0 and 2.0 mgL^{-1}) an increase in the number of multiple shoots occurred albeit showed retarded growth of plantlets and inflorescence also. Important findings in this study are plantlet regeneration from the axils of bract was observed at lower concentration of KN (0.1 and 0.5 mgL^{-1}) and BAP (0.1 and 0.5 mgL^{-1}) and also these plantlets developed roots from their base. This probably indicates that cytokinins influence induction of *in vitro* flowering, and that it depends on the endogenous level of the hormone, which is so high to induce flowering that it develops flowers in culture conditions even without providing an external stimulation. When these retarded plantlets were transferred to normal MS medium they regained normal growth and induced flowering. Explants were collected from almost matured, one month old plants. This also adds to the list of factors influencing *in vitro* flowering. Hence age of the tissue is also an endogenous factor that controls *in vitro* flowering, which in turn is the physiological state of the explant. In *D. indica* and *D. burmanii* the *in vitro* flowers failed to produce seeds though the flowers are opened. In nature the genus is insect or self-pollinated. When compared to other insect pollinated species of the genus, the morphology of *D. indica* and *D. burmanii* depicts more of insect/wind-pollinated characters. Hence the absence of seed set is probably due to the lack of pollinating insects or wind in the culture vessels. This also shows that *D. indica* and *D. burmanii* is strictly a self- incompatible species. Bobak et al. (1995) reported that the regenerated plants produced flowers in *D.*

rotundifolia. Jang et al. (1997) was also stated about *in vitro* flowering in *D. rotundifolia* on 1/3 MS medium. Jang and Park, (1999) reported that about 90 % of the plantlet produced flowers on *Vz* MS medium without hormones.

But the plantlet cultures on ½ MS medium with hormones did not produce flowers in *D. rotundifolia.* Simola (1978a) found that, in contract to other carnivorous plants, *D. rotundifolia* is able to grow and flower on medium with inorganic nitrogen as the sole source of nitrogen. Anthony, (1992) reported that about one-half of the *D. rotundifolia* and *D. capensis* cultures produced flowers on ½ MS with or without hormones, and 90 % of the *D. binata* cultures produced flowers on ½ MS containing hormones. *D. binata* cultures on medium without hormones did not produce flowers. Harder, (1964a) and Harder and Zemlin (1967) reported that *D. pygmaea* and *D. rotundifolia* was able to flower when cultured on inorganic medium. Simola, (1978a) observed some of the flowers of *D. rotundifolia* opens at 25-29°C.

5.8. *In vitro* rooting

Rooting of *D. indica* and *D, burmanii* were tried on MS medium with out any growth regulators. 4-5 unbranched black thick roots with dense root hairs developed from each shoot within 15-20 days of culture **(Figures** 19 **a-d).** But Nalini and Murali (2002) reported in *D. indica* for root initiation NAA and NOA was effective. Whereas in *D. regia* there was little need for hormones are required for root formation (Van Waes, 1985; Janssens, 1986). Roots were black, unbranched and entirely covered with root hairs. Many authors however, have reported root induction in a growth

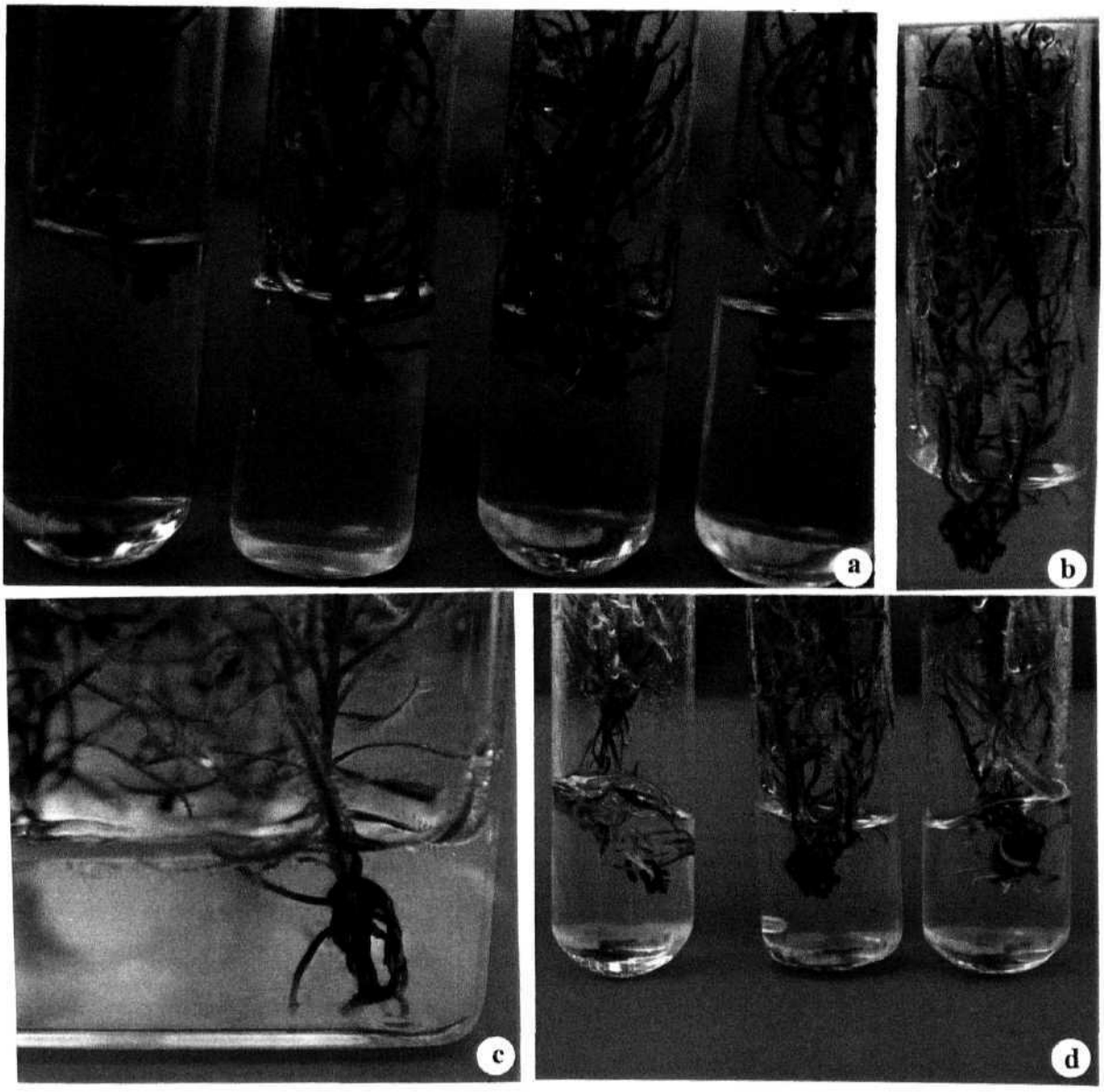

Figure 19: *In vitro* rooting of *D. indica*
a) MS basal medium
b) ½ MS medium
c) 3 % sucrose.
d) 1 %, 2 %, 3 % sucrose (left to right)

regulator free MS medium as in the case of *D. rotundifolia* (Simola, 1978b; Bobak et **al,** 1995). Anthony, (1992) reported that all subcultured *D. rotundifolia* and *D. capensis* plantlets produced extensive root systems after 6-8 weeks, in contrast to the generally week rooted plants found in the wild (Juniper et al., 1989). Only 10 % of the *D. hinata* plantlets rooted on medium without hormones, whereas 30 % rooted on ½ MS containing BA and NAA.

5.9. Hardening of *in vitro* raised plants and an attempt to field transfer

Rooted plants were washed in tap water and transferred to plastic pots containing sterilized soilrite. Pots were temporally covered with transparent plastic boxed to ensure high humidity and gradually opened during acclimatization period of 2 weeks. The growth chamber was maintained at 23±25°C with light intensity of 30 $\mu EM^{-2}S^{-1}$ on a 16/8 h photoperiod **(Figures 20 a-c).** The plants were maintained with liquid sterile MS medium. In this process plant survival rate is more than 60 percent. *In vitro* root systems continued to grow, indicating their normal viability and function. Acclimatized plants were then transferred to the green house. The survival rate was satisfactory depending on temperature (should be 20 ±2°C) and humidity (>60).

To our knowledge there are no reports on field transfer of *in vitro* raised *Drosera* species. Nalini and Murali (2002) stated that reintroductions of *D. indica* plantlets were not successful but they were presently underway into reintroduction to natural environment in the forest. Though an attempt was made to reintroduction to

Figure 20: *In vitro* raised plants of *D. indica* transferred to glasshouse
a) Well-established plants after transfer to soilrite in glasshouse.
b&c) Close view of plants with its functional leaf tentacles in glasshouse.

natural habitat of the *in vitro* produced plants of *D. indica* and *D. burmanii* was not satisfactory inspite of transfer through simulated assembly of standing pots in trays of water (transit between *in vitro* and field conditions) **(Figure 21).** Probably this could be due to semiarid tropic conditions in Deccan region. Moreover *D. indica* and *D. burmanii* are completing their entire life cycle within 50-80 days in field if conditions are favorable if not plantlets get dried. Even in *in vitro* condition both the *Drosera* species are attaining to maturation within 40-60 days except in production of seeds. Hence *in vitro* cultures of *D. indica* and *D. burmanii* can be used for a major source of production of plumbagin and other important secondary valuable secondary metabolites.

It is for the first time a detailed and reproducible protocol for the multiplication of *D. indica* and *D. burmanii* from the shoot tips have described. Hundreds of the valuable plants can be produced in a short period of time with out much using costly hormones and from single shoot, suggesting the feasibility of *in vitro* large scale production for valuable secondary metabolite **(Figures** 22 a-c **and** 23 a-d). Most of the research on the tissue culture of sundew until now has stressed micropropagation. The main goals were have been the optimization of culture conditions (Simola, 1978; Anthony, 1992), leading to the higher production of important secondary metabolites *in vitro* (Blehová et al., 1992). Bonnet et al., (1984) have reported that the content of active secondary substances is up to six-fold higher in cultures of *D. rotundifolia* than in plants.

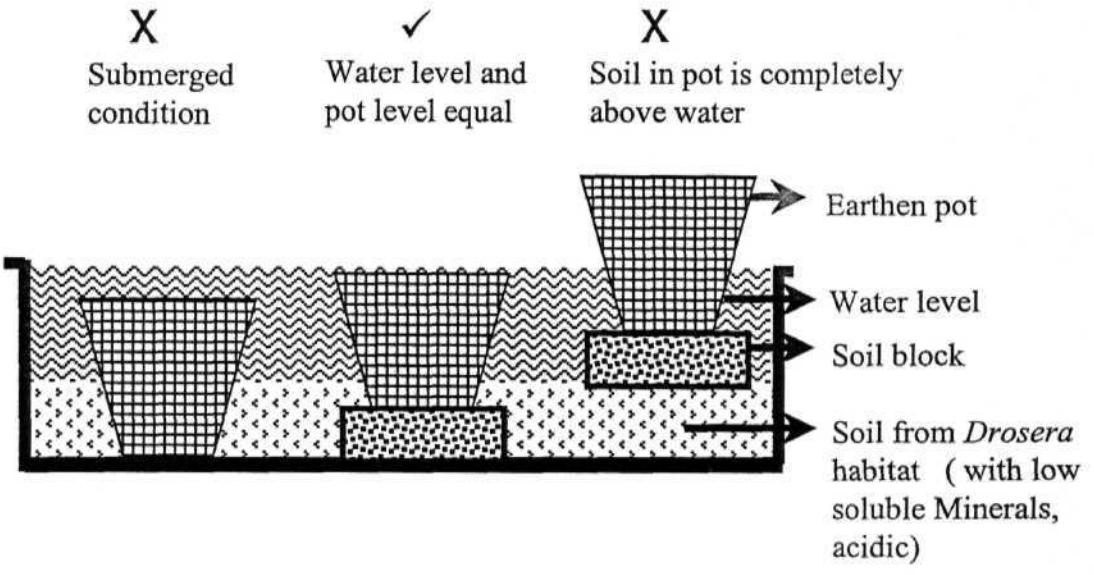

Figure 21: Simulated habitat for transfer of *in vitro* rised *D. indica* and *D. birmanii* to field condition.

Figure 22: Culture racks filled with Magenta boxes showing rapidly multiplied *D. indica* on MS basal medium supplemented with cytokinins

a) Different concentrations of zeatin.
b) Different concentrations of kinetin.
c) Different concentrations of 6-benzyl amino purine.

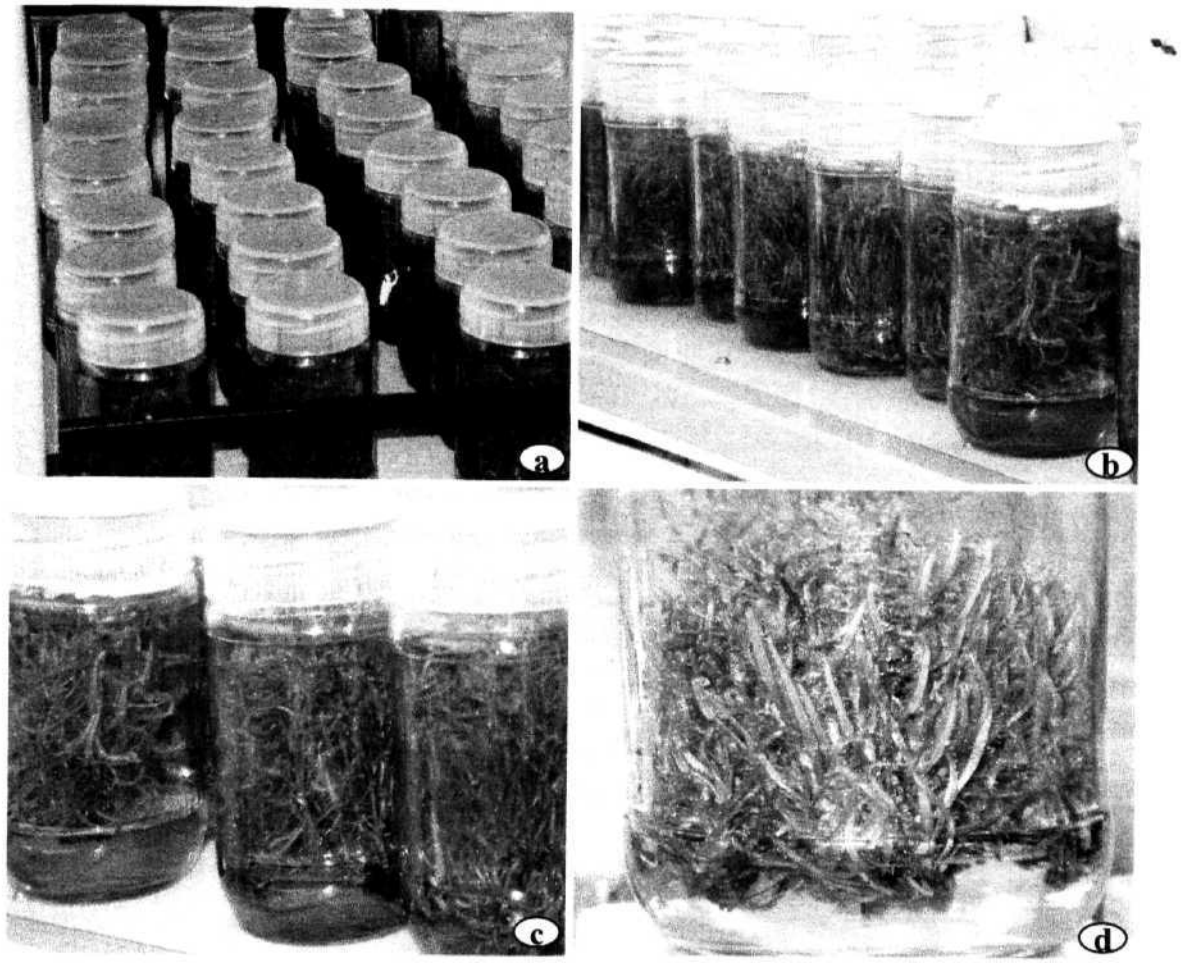

Figure 23: Culture racks filled with rapidly multiplied *D. indica.* a) Glass bottles showing rapidly multiplied on MS basal medium supplemented with different cytokinins. b) *In vitro* repository after 6-8 weeks on MS medium supplemented with KN. c) Rapid *in vitro* production *D. indica* plant lets d) Magnified view of *in vitro* biomass.

5.10. Scanning Electron Microscope investigation of seed coat sculpture

D. indica exhibits considerable morphological variation in plant size, petiole development, stem, leaf and flower colour, filament form and seed features (Susandarini et al., 2002). Taxonomic treatments mostly recognize a single variable species with an extensive synonymy, but without any intraspecific taxa (Van Steenis, 1953; Ohwi, 1965; Obereyer, 1970; Marchant et al., 1982), although some authors have recognized intraspecific taxa (Diels, 1906; Walker, 2000) and a number of distinct morphological variants have been noted from northern Australia (Lowrie, 2001a; 2001b).

The collected and stored seeds of *D. indica* (12 accessions) and *D. burmanii* (9 accessions) from different parts of Andhra Pradesh, India were subjected for scanning electron microscopy for sculptural variation. Seeds of *D. indica* and *D. burmanii* are not showed any variations on seed surface, all the accessions of *D. indica* seeds were showed transversely hexagonal reticulate **(Figures 24-26),** whereas all the accessions of *D. burmanii* were showed tetragonal elongated reticulates **(Figures 27 and 28).** Susandarini et al, (2002) reported morphological variation within the *D. indica* in northern Australia. They were differentiated into three distinct morphotypes mainly on the basis of seed and stamen characters. These morphotypes occurred sympatrically at a number of localities over a wide area across northern Australia, but without obvious evidence of intergradations. This identified morphotypes represents cryptic species within the *D. indica* and which are needed to

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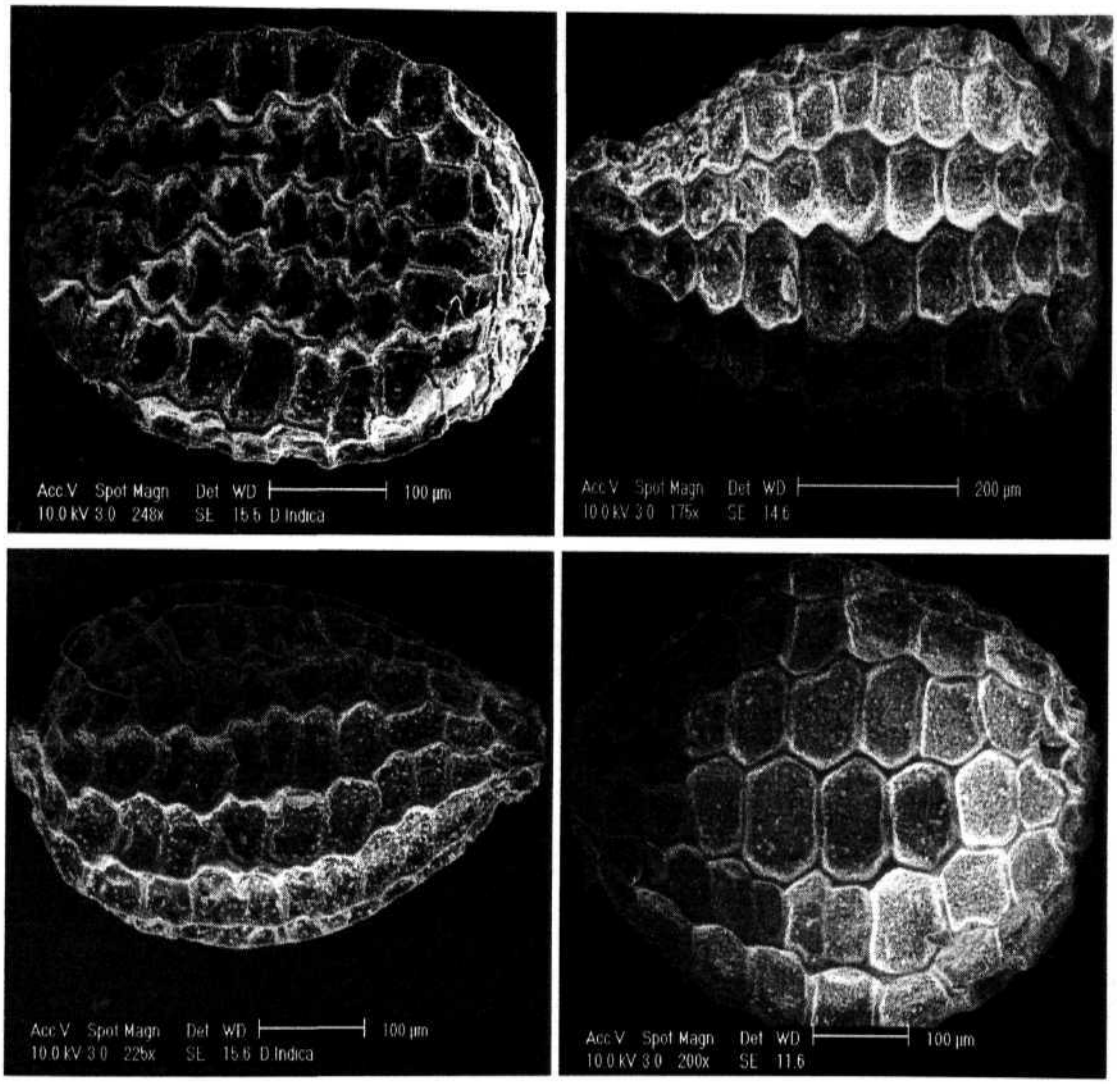


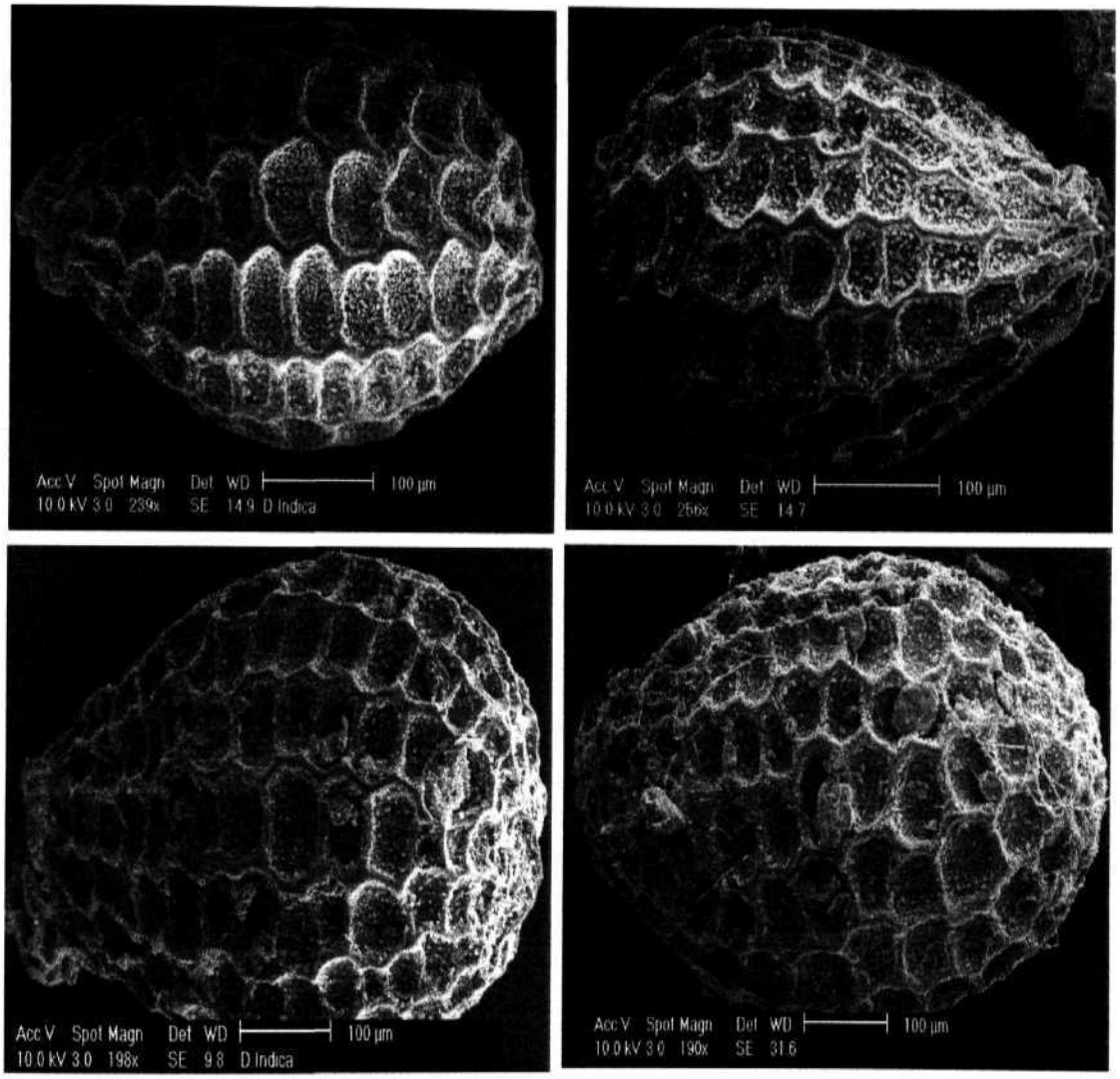
CHELVAYA

GACHIBOWLI

Figure 24: Scanning electron micrographs of different accessions of *D. indica* seed ornamentation.

KONDAPARTHI

MULAKANURU



NARSAPUR

PAKHAL

Figure 25: Scanning electron micrographs of different accessions of *D. indica* seed ornamentation.

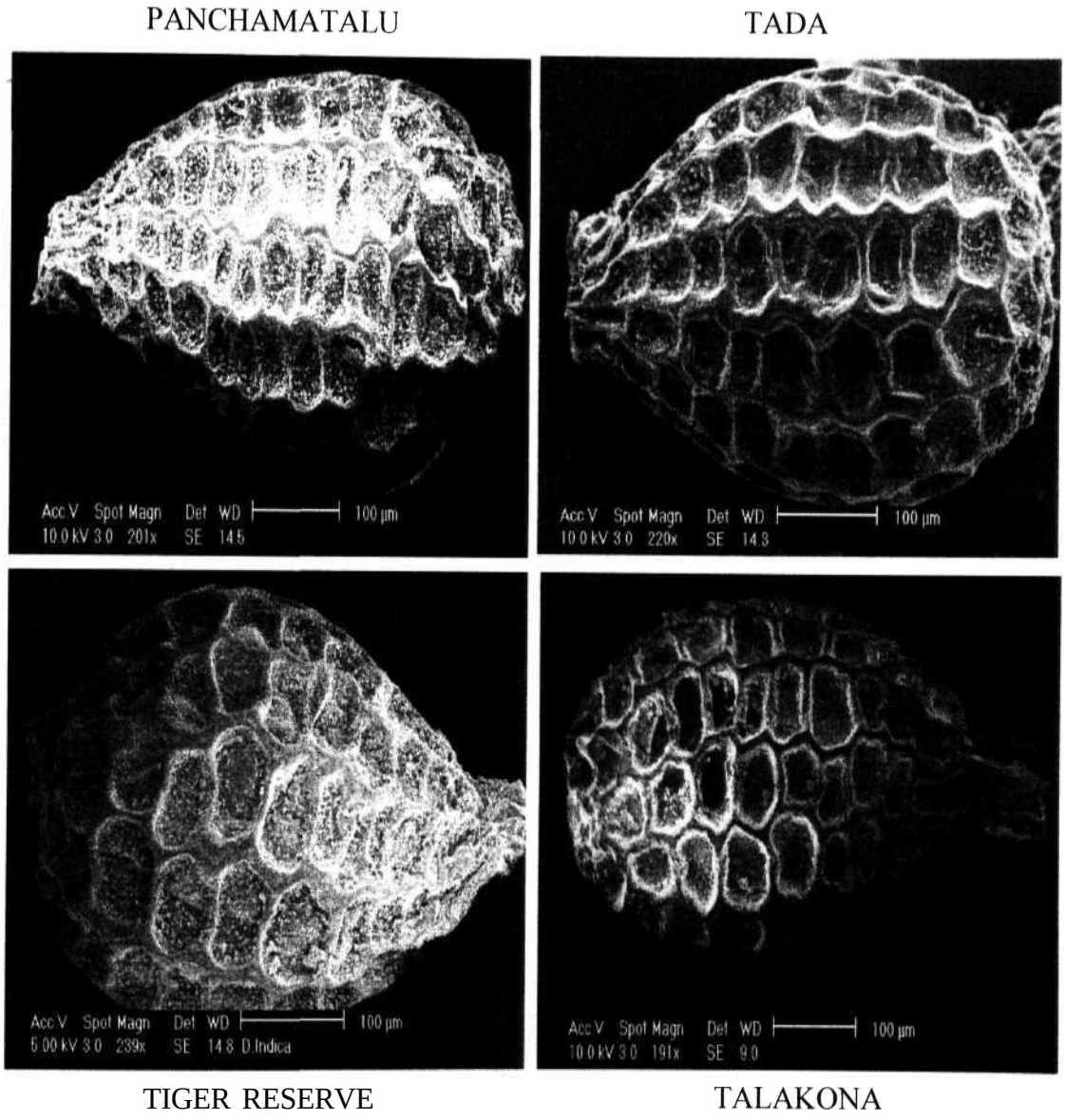


Figure 26: Scanning electron micrographs of different accessions of *D. indica* seed ornamentation.

GACHIBOWLI

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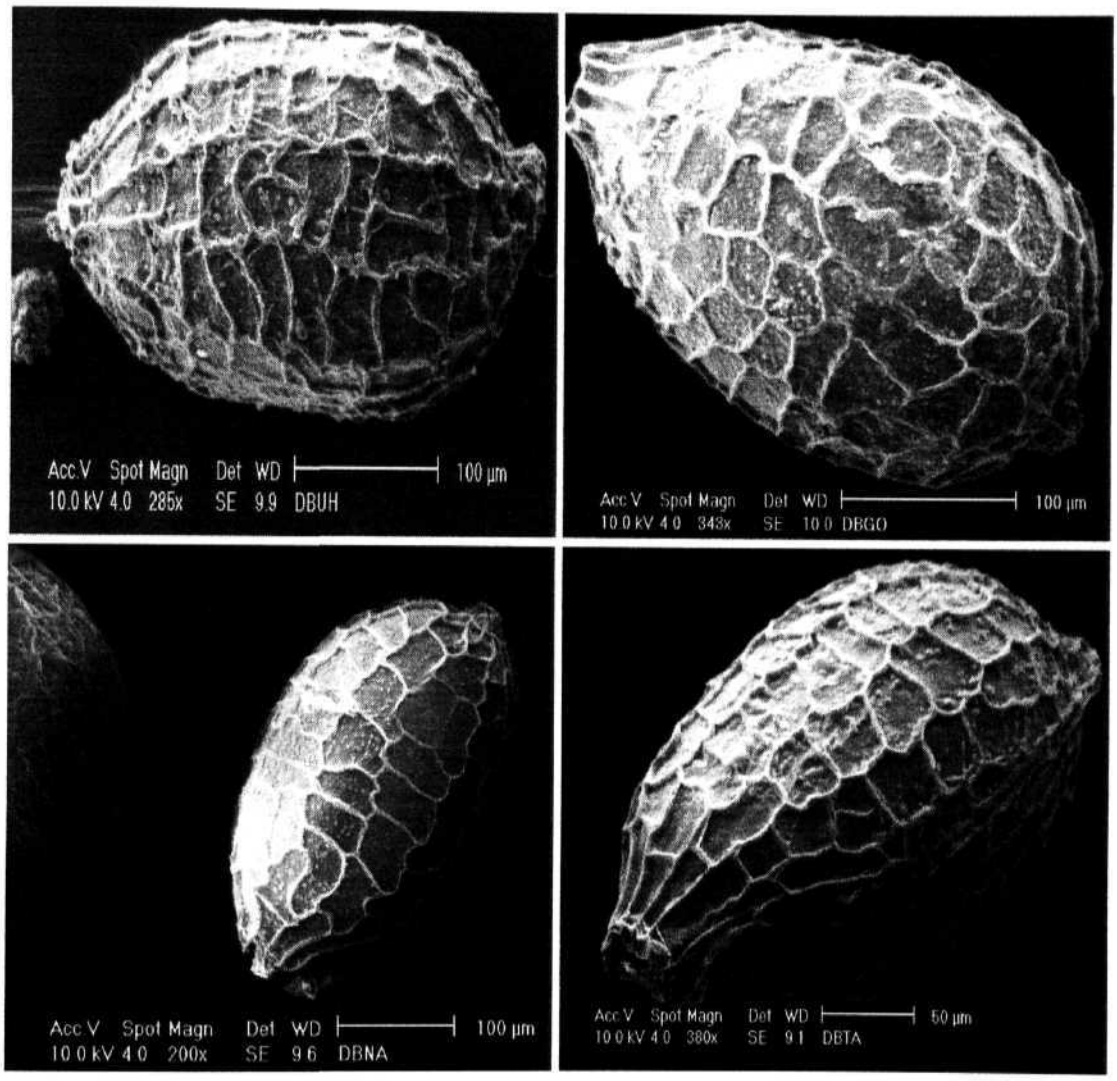

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TALAKONA

Figure 27: Scanning electron micrographs of different accessions of *D. burmanii* seed ornamentation.

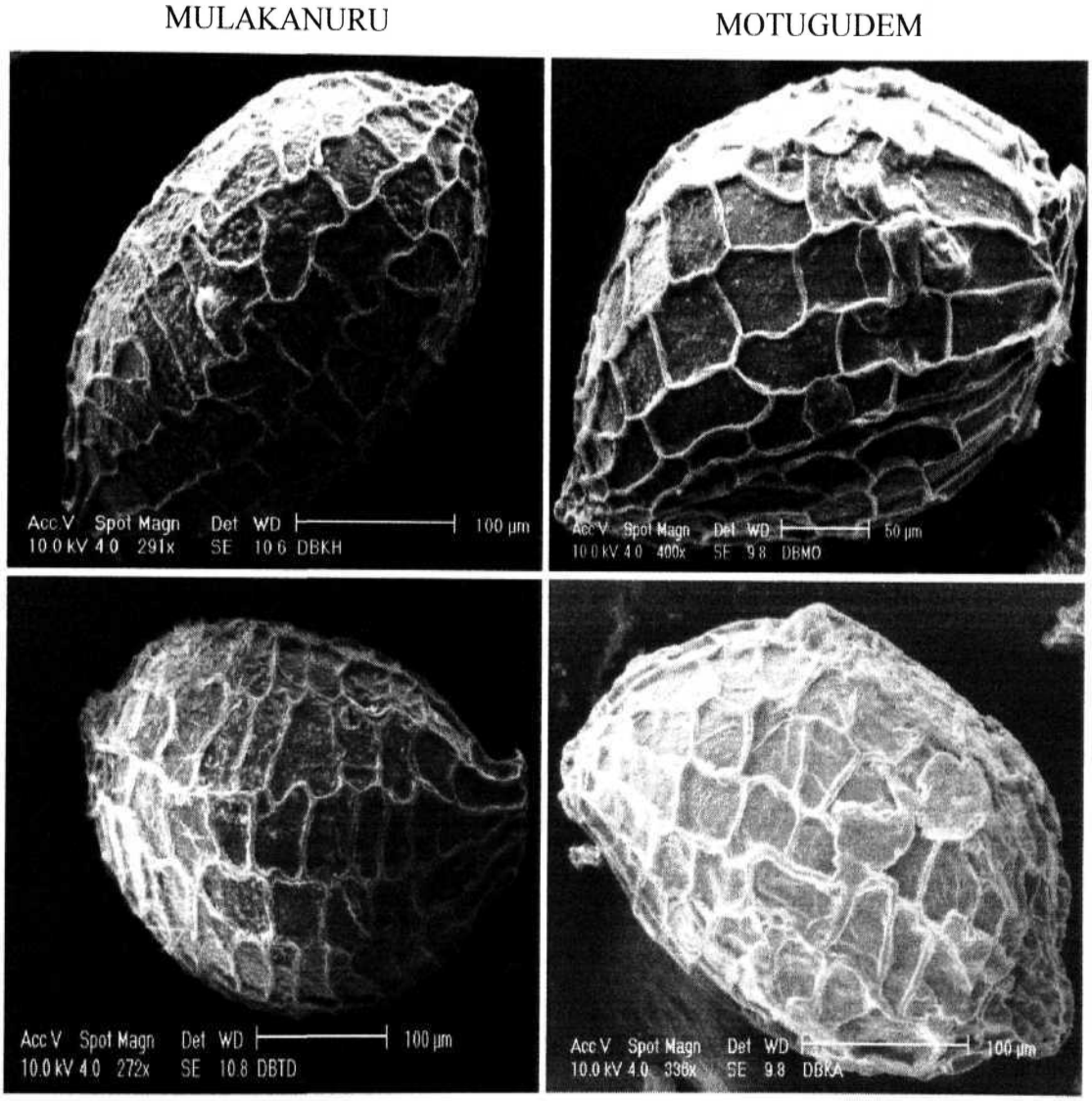

Figure 28: Scanning electron micrographs of different accessions of *D. hurmanii* seed ornamentation.

establish the number of possible taxa within *D. indica.* Hence plants of *D. indica* and *D. burmanii* growing across A.P not showing any considerable morphotypes, though there was marked variations in terms of morphology of plants.

5.11. RAPD analysis

For efficient conservation and management of medicinal plant diversity, the genetic composition of species collected from different phytogeographical regions needs to be assessed. Discontinuous distribution and restricted habitats of *D. indica* and *D. burmanii* makes small isolated populations in A.P. In isolated small fragmented populations, genetic drift, inbreeding may eventually reduce gene flow and reduced fitness because of inbreeding depression can thereby cause a reduction of genetic variation within populations and promote the evolution of genetic differentiation between them (Wright, 1951; Lacy, 1987; Lande and Barrowclough, 1987; Ellstrand and Elam, 1993; Frankham, 1996; Richards, 2000; Kéry, 2000; Newman and Tallmon, 2001). Reproduction of plants in small populations may also be negatively affected by reduction in the diversity of incompatibility alleles (Byers, 1995; Frankham, 1995). Populations with low genetic variability also have reduced potential to adapt to environmental changes (Ellstrand and Elam, 1993). **In small** isolated populations, the accumulation of mildly detrimental mutations may further reduce fitness (Lande, 1995; Lynch et al., 1995). Genetic diversity within populations is considered to be of great importance for possible adaptation to environmental changes and consequently, for long-term survival of species (Vida, 1994). Without an

appropriate amount of genetic diversity, species are thought to be unable to cope with changing environments and evolving competitors and parasites (Van Valen, 1973). Attempts to isolate genomic DNA of *D. indica* and *D. burmanii* for RAPD analysis by different standard protocols was not successful. The quality and quantity of isolated DNA was not adequate for RAPD analysis. This might be due to phenolic compounds and other polysaccharides, which are known to present in genus *Drosera* (Bekesiova et al., 1999; Pirttilä et al., 2001). However, in genomic DNA was successfully isolated from *D. rotundifolia* and evaluating the use of these protocols for other members of the Droseraceae and other species of carnivorous plants to analyze and evaluate the gene pool of insectivorous plants with the full toolkit of plant molecular biology, such as cDNA and genomic library construction and various subtractive techniques (Bekesiova et al., 1999; Pirttilä et al., 2001).

To our best knowledge there is only one report dealing with the RAPD analysis to determine the genetic fidelity of *in vitro* raised plants of *D. anglica* and *D. binata* which were regenerated by adventitious budding from leaf explants and shoot tips. It has been concluded that regeneration of plants through shoot tip culture is a low risk method for generating genetic variability, whereas material regenerated through leaf explants requires further verification (Kawiak and Lojkowska, 2004).

6. Conclusion

The habitat of *D. indica* and *D. burmanii* is unique. Moreover environmental effects factors like scanty rainfall, irregular monsoon and temperature fluctuations, rapid conversion of fragile ecosystems fror urbanization, indiscriminate collection for various medicinal uses, the population is rapidly vanishing. Hence, there is an urgent need to conserve these vulnerable species.

During an extensive field survey was carried out throught Andhra Pradesh, in many locations it was observed that these populations which were vanishing due to urbanization, agricultural fertilizers, drought and invasive species.

Hence plant tissue culture technique opens a new vista to conserve these valuable medicinal plants, where shoot tips were used as explants in both the species, which were more effective when compared to other explants.

It is for the first time a detailed and reproducible protocol for the multiplication of *D. indica* and *D. burmanii* from the shoot tips is described, will be useful for propagation and *in vitro* conservation of germplasm. Though field transfer was not satisfactory due to various environmental conditions in Hyderabad (Andhra Pradesh).

Long-term storage of *in vitro* cultures is an excellent way of germplasm conservation of the threatened plants and for use of plant material for further studies. As the *in vitro* developed *D. indica* and *D. burmanii* plants were healthy for more than 45 days, this can be taken advantage in long term *in vitro* conservation of these red listed medicinal plants.

Reintroduction of the *in vitro* produced plants to natural habitat was not satisfactory inspite of transfer through simulated assembly of standing pots in trays of water (transit between *in vitro* and field conditions). It does not make any difference in utilizing the *in vitro* produced huge amount of biomass for commercial purpose to exporting to European countries to prepare " Herba droserae" or to domestic herbal industries. Therefore, one of the feasible strategies to utilize this abundantly produced *in vitro* fresh material of *D. indica* and *D. burmanii* would for bioprospection of plumbagin and other useful metabolites. Further, procurement of large quantities of fresh material from nature would be uncertain/impossible. More over production of this large amount can be done with small amount of tissue according to the tissue culture protocol designed. Utilization of *in vitro* produced *Drosera* would be an alternative viable strategy for production of plumbagin and other invaluable photochemical to fetch profitable international market.

Part B

Ex situ conservation and molecular diversity in *Oroxylum indicum* Vent. by RAPD analysis: Vulnerable medicinal plant.

7. Review of literature

7.1. Habit and distribution

Oroxylum indicum Vent., is a small or medium-sized deciduous tree belonging to Bignoniaceae, present in India and Sri Lanka eastwards through Southeast Asia to Philippines and Indonesia. It is distributed throughout India upto an altitude of 1200 m, usually in ravines, moist habitats in deciduous and in evergreen forests of Southern India; rarely seen in dry, western area. It has been listed as **a** vulnerable plant, both globally and regionally (Ravikumar and Ved, 2000; Sumy et al., 2000; Reddy et al., 2001; Dalai and Rai, 2004). All parts of the plant are used for medicinal purposes. This plant is an important herbal medicine in many Asian countries and is used in folk medicine as a cure for various diseases (Biswas and Ghosh, 1994).

7.2. Medicinal uses of plant parts

Leaves are emollient containing anthraquinone and aloe-emodin (Dey et al., 1978), and are used for the treatment of ulcers and headache (Ravikumar and Ved, 2000). Decoction of the leaves is used in Malaysia for stomachache, rheumatism and used externally to cure the enlarged spleen. Among the tribal inhabitants of Maharastra (India) the crushed leaves are applied to relieve joint pains (Parrotta, 2001). Wall et al, (1988) reported significant antimutagenic activity against 2-aminoanthracene in the C_2H_5OH-CH_2Cl_2-soluble fraction prepared from twigs and leaves of *O. indicum*.

The fruits are used as expectorant, purgative and bitter tonic (Kirtikar and Basu, 1996). The tender fruits are used to treat cough, bronchitis, indigestion, leucoderma, stomache and as carminative, refreshing and antihelmintic. It is a major ingredient in Ayurvedic medicine, used to treat heart, throat and ailments of piles. Mature fruits are used in the treatment of intestinal worms, bronchitis and bleeding piles (Ravikumar and Ved, 2000; Sumy et al., 2000; Parrotta, 2001; Dalai and Rai, 2004). In North and North Eastern areas of Thailand, the fruits (called *peh-gaa*) and flowers of the plant are consumed commonly. Two flavones have been identified from fruits namely, oroxylin A and chrysin and a triterpene carboxylic acid, urosolic acid (UA), known to act as inhibitors of O_2^- generation in Xanthine (XA)/Xanthine oxidase (XAO) system. These compounds also showed marked inhibitory effects on the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced O_2^- generation in dimethylsulfoxide (DMSO)-differentiated HL-60 cells (Jiwajinda et al.,

2002). The methanol extract from the fruits inhibited the mutagenicity of Trp-P-1 in an Ames test and strong effect on the *in vitro* anti tumor promoting activity according to the Epstein-Barr virus activation assay (Na-Thalang et al., 2000; Nakahara et al., 2001). Twigs, fruits and flowers of *O. indicum* contained a high concentration of baicalein, flavonoid exhibiting antimutagenic and antibacterial activities (Nakahara et al., 2002).

The seeds contain flavonoids such as chrysin (5,7-dihydroxyflavone), oroxylin A (5,7-dihydroxy-6-methoxyflavone), baicalein (5,6,7-trihydroxyflavone) and baicalein glycosides, benzoic acid and fatty acids (Nakahara et al., 2001; Chen et al., 2003). Apart from the above-mentioned compounds, seeds are purgative and show the presence of terpenes, alkaloids, and saponins (Bhattacharje and Das, 1969), and their extract exhibits antimicrobial activity (Rasadah et al., 1998). The seeds are used as expectorant, purgative, bitter tonic. Powdered seeds boiled with mustard oil are used as a rub to reduce joint pain in southern Bihar (Kirtikar and Basu, 1996; Parrotta, 2001).

In Hindu medicine the roots, bark, stem, and leaf are prescribed for snakebite, diarrohea and dysentry (Bhattacharya, 1980; Ghani, 1998).

The powdered bark of stem is given to treat fever among tribal inhabitants of Southern Bihar; and combined with the powdered bark of *Azadirachta indica* and *Enatada rheedii* and leaves of *Radermachera xylocarpa,* is prescribed for leprosy by the inhabitants of Gandhamardan Hills in Orissa (Parrotta, 2001), contains flavones, namely oroxylin A, chrysin and scutellarin-7-rutinoside, traces of alkaloids

(Subramanian and Nair, 1972), tannic acid, sitosterol and galactose, and is used for treating rheumatism (Dalai and Rai, 2004). The stem bark also displayed antiproliferative activity on human breast tumor cell lines (MCF7 and MDA-MB-231) (Lambertini et al., 2004), and antifungal activity against dermatophytes and wood rot fungi. Ali et al, 1998 stated that the activity is probably due to lapachol and β-lapachone, present in the plant extract.

Root bark contains flavones, namely oroxylin A, chrysin and scutellarin-7-rutinoside, traces of alkaloids (Subramanian and Nair, 1972), tannic acid, sitosterol and galactose, and is used for treating rheumatism (Dalai and Rai, 2004). The fresh root bark is a major ingredient of the compound formulation "Dasamoola (ten roots)" a well-known Ayurvedic medicine. It is an anti-inflammatory and effective in bronchitis, cough, headache, weakness, drowsiness and digestive problems (Manonmani et al., 1995; http://www.himalayahealthcare.com) **(Table 15).** It is considered to be hot, bitter, astringent, anti-inflammatory, appetizing, antiperiodic, anthelmintic, sprains, hiccough, cough, asthma, bronchitis, gout, leucoderma, carminative, diuretic, stomachic, aphrodisiac and as tonic, curing fevers, for treating diarrhoea and dysentery, abdominal pain, rheumatism, thirst, vomiting, anorexia, worms, leprosy. Boiled in sesame oil, it is recommended for otorrhoea. Its decoction is used by the tribal inhabitants of Sundargarh district in Orissa State of India to treat dropsy and eruptive fevers (Parrotta, 2001). Two flavonoids namely 2,5-dihydroxy-6,7-dimethoxyflavone and 3,7,3',5'-tetramethoxy-4-hydroxy flavones have been identified from root bark of *O. indicum*. These two compounds are found to be

Table 15: Composition of Dasamoola (Manonmani et al., 1995; www.himalayahealthcare.com)

Plant name	Family	Composition (mg)
Aegle marmelos Corr.	Rutaceae	10
Gmelina arborea L.	Verbenaceae	10
Caesalpinia bonducella Fleming	Caesalpiniaceae	10
Clerodendon phlomodies L.	Verbenaceae	10
Oroxylum indicum Vent.	Bignoniaceae	10
Desmodium gemgeticum DC.	Papilionaccae	10
Uraria picta Desv.	Fabaceae	10
Solanum indicum L.	Solanaceae	10
Solanum xanthocarpum Schard and wendil.	Solanaceae	10
Tribulus terrestris L.	Zygophyllaceae	10

cytotoxic (Uddin et al., 2003). Recently Mao (2002) reported that the decoction of root bark is effective on nasopharyngeal cancer, and for curing gastric ulcer, while the paste of the bark is applied to mouth cancer, scabies, and tonsil pain.

7.3. Commercial value and *in vitro* propagation

The root bark contains the drug "Syonaaka" that is sold, under the trade name "Shyonaaka mool chaal or Sonaapaathaa mool chaal". The material is sometimes adulterated with stem bark of the same species. Roots were priced at Rs. 3/Kg (Ravikumar and Ved, 2000). Twigs are traded in India and the region, at Rs. 9/kg (about US 20 cents/kg). Its extracts on the international market fetch Rs. 500,000/kg (US$15,000/kg) (Sahai, 2000).

The plant species is naturally propagated by seeds, which have a short viability period. Certain limitations of conventional propagation methods, such as being labor-intensive, the need for large areas for ex *situ* conservation, out-breeding, a long juvenile phase, and seasonal dependence, make it necessary to apply tissue culture techniques. To our knowledge, *In vitro* propagation of *O. indicum* using cotyledonary nodal explants was achieved (Dalai and Rai, 2004).

8. Problem, prospects and objectives

Being medicinal, not commercially cultivable species, due to Jhum (Podu) cultivation and lack of awarness about these valuable medicinal plants in tribal people, these plant population size is reduced and in many areas within Andhra Prasad, India plants hence with due course of time the plants may become isolated populations.

As isolated, small sized population has great threat due to genetic drift and other environmental factor it is essential to check the initial genetic diversity among the collected populations for long term *ex situ* conservation of *O. indicum*. Hence RAPD is one of the molecular techniques, which facilitates to know the diversity among the individual populations collected from various locations of Andhra Pradesh.

1. Field identification and collection of *O. indicum* from various locations of Andhra Pradesh.
2. *Ex situ* conservation of collected germplasm in field experimental site and seed bank.
3. Assessment of molecular diversity among different accessions of *O. indicum* by RAPD analysis.

9. Material and methods

9.1. Field identification and *ex situ* conservation

Total of 8 accessions of *Oroxylum indicum* in the form of plants and seeds were collected from various parts of Andhra Pradesh, India **(Figure** 29). Total 273 plants are maintained in the field experimental site **(Table 16; Figure 30)** and seeds were stored at room temperature for germination studies.

9.2. Seed viability

Mature dried pods of *O. indicum* were collected during February and March (2003-2004). Seeds were removed from the pods and dried in open sunlight, and were soaked in tap water for 4-6 hr, followed by the dibbling of seeds in loose soil, surface was covered with hay and irrigated periodically twice a day to ensure high humidity and moisture in field experimental site.

93. DNA isolation

Total genomic DNA was extracted following the protocol of Khanuja et al., (1999) with minor modifications. 0.1 gm of leaf material was powdered in liquid nitrogen, and immediately transferred to a microcentrifuge tube containing extraction buffer (100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 1.5 M NaCl, 2 % CTAB, 0.2 % β-mercaptoethanol (V/V)(added immediately before use) and 1 % PVP (W/V)(added immediately before use) and mixed well to form a slurry and incubated at 65°C for

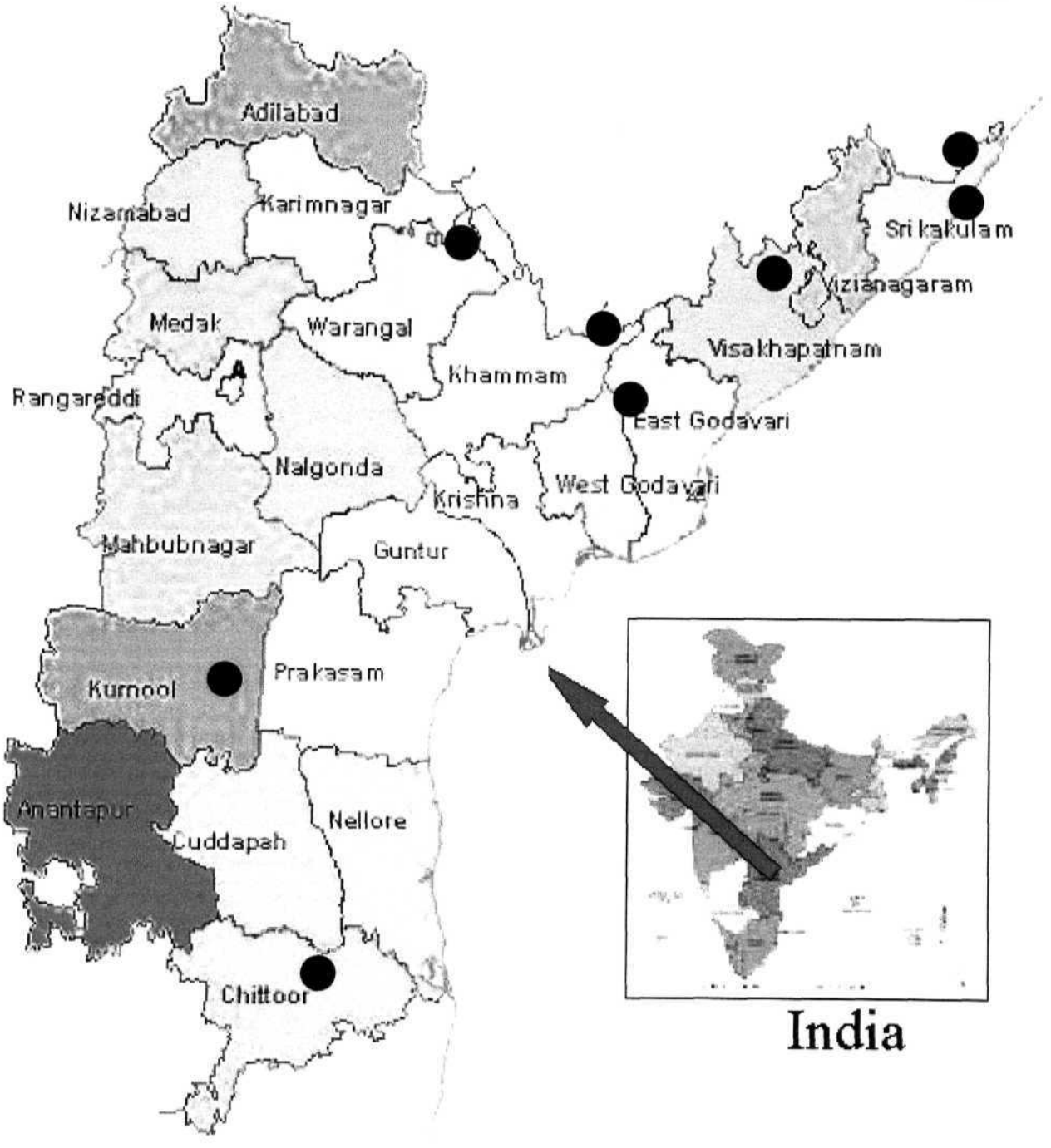

Figure 29: sampling locations of *O. indicum* collected from various regions of Andhra Pradesh, India.

Table 16: Population of *Oroxylum indicum* used as sources of DNA:

Locality	Type of germplasm Collected		No. of plants in Field experimental site
Mahandragiri hills (Srikakulam Dist.)	Plants	Seeds	25
Bendi gate (Srikakulam Dist.)	Plants	-	49
Araku vally (Visakhapatnam Dist.)	Plants	Seeds	21
Maredumilli (East Godavari Dist.)	Plants	Seeds	59
Sukumamidi (Khammam Dist.)	Plants	Seeds	105
Mallur (Warangal Dist.)	Plants	-	3
Ahobilam (Karnool Dist.)	Plants	-	4
Talakona (Chittor Dist.)	Plants	-	7

Figure 30: *Oroxylum indicum* introduced to experimental field (3 years old) a) Accession from Sukumamidi (Khammam) and b) Accession from Talakona (Chittoor) in Andhra Pradesh, India.

60-90 min with slow shaking for every 10 min. 3 µl of RNase (10 µg/ml) was added to the supernatant and incubated for lhr at 37°C, proteins were extracted twice with chloroform:iso-amylalcohol (24:1), by centrifuging at 10,000 rpm for 12 min at RT followed by extraction of DNA by adding equal volumes of isopropanol and incubated at ½ hr at -20°C than the mixture was centrifuged at 12,000 rpm for 12 min the pellet was washed twice with 80 % ethanol and air dried and was dissolved in 100 µl of Tris-EDTA buffer (0.5 M Tris-HCl and 0.5 M EDTA pH 8.0).

Approximate DNA concentrations were determined by running samples on 0.8 % agarose gel or by taking the absorbance at 260 nm.

9.4. PCR amplification

DNA amplification was performed in a MJ Research Inc. thermalcycler using the arbitrary decamers. The 40 primers were selected from the primer kits OPA and OPC (QIAGEN, Operon, Germany). 50 ng of DNA was amplified via the PCR reaction using 25 µl reactions under the following conditions: 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 3 mM $MgCl_2$ (Invitrogen Ltd.), 0.2 mM dNTP mix (Genetix), 0.5 µM of single primer and 1U *Taq polymerase* (Invitrogen Ltd). Programmed for an initial denaturation of 3 min. at 94°C followed by 30 cycles of 45 sec at 94°C, 1 min at 37°C and 1 min at 72°C and finally a 7 min extension at 72°C **and a** hold temperature at 4°C negative controls without template DNA were also run in the experiments. All the experiments were repeated twice to ensure the reproducibility.

9.5. Agarose gel electrophoresis

Amplified DNA fragments were separated by electrophoresis at 60V in 1X TBE buffer for 3-4 hr on 2 % agarose gels stained with 2µl of ethidium bromide (10 µg/ml) and photographed by the Gel documentation system (LTF Labortechnik). Molecular weights of fragments were estimated using 100-3000 bp DNA ladder.

9.6. RAPD data analysis

RAPD is a dominant marker. Therefore amplified fragments were scored for the presence (1) and absence (0) of homologous bands. Genetic similarity between individuals was estimated using the Dice co-efficient (Dice, 1945). Cluster analysis using the UPGMA was performed using NTSYS version 2.02j software (Rolhf, 1998).

10. Results

During field survey plants and seeds of *Oroxylum indicum* were collected from various locations of Andhra Pradesh. Total 273 plants of *O. indicum* are being maintained in field experimental site for *ex situ* conservation. The collected seeds were germinated in *in vivo* condition under the shade and around 80 % germination was observed.

The isolation of genomic DNA and conditions for PCR were standardized. A total of 387 bands were scored for 40 RAPD primers from a range from 4 to 20. Maximum loci were observed with primer OPC-05, minimum loci were observed with primer OPC-01, corresponding to an average of 9.6 bands per primer, and 49.61 % (188) of these were polymorphic **(Figures 31-70).** Percentage of polymorphic bands for each primer ranged from 0 % to 100 % **(Table 17).** Genetic distance lies between 0.80-0.95 **(Figure 71).** The size of the amplified bands range from 200-1800 bp. Based on the cluster analysis, by using unweighted pairwise group matrix for arithmetic average (UPGMA) analysis, two major groups of *O. indicum* have been found within the collected accessions from various geographical locations. Plants collected from Araku valley (ORAK) and Maredumilli (ORMM) were genetically 100 % similar, and formed one sub group with plant collected from Sukumamidi (ORSM). They share above 90 % genetically similarity. Plants collected from Mahandragiri hills (ORMH) and Mallur (ORML) formed one subgroup and share above 90 % genetic similarities. Plants collected from Palasa (ORPS) and Talakona

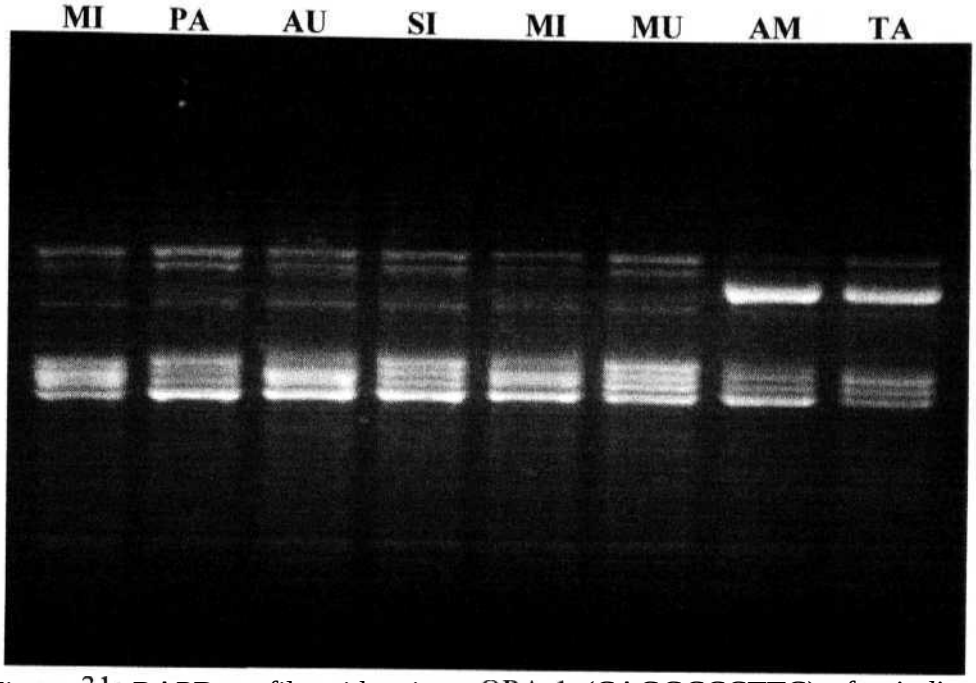

Figure 31: RAPD profile with primer OPA-1 (CAGGCCCTTC) of *O. indicum.* Total 8 amplified bands were generated among which 3 are polymorphic.

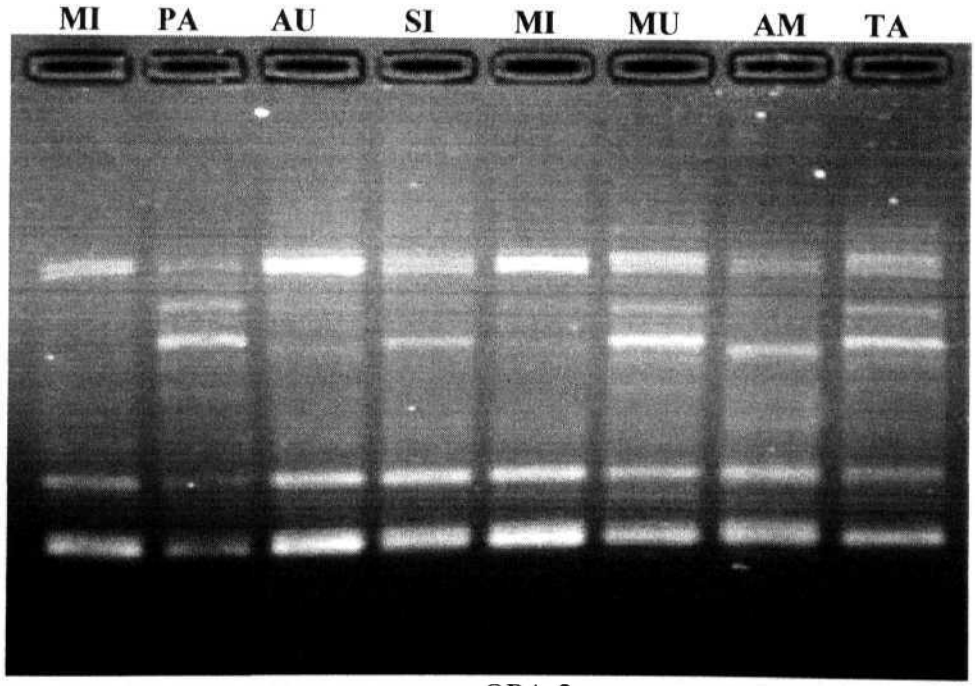

Figure 32: RAPD profile with primer OPA-2 (TGCCGAGCTG) of *O. indicum.* Total 5 amplified bands were generated among which 3 are polymorphic.

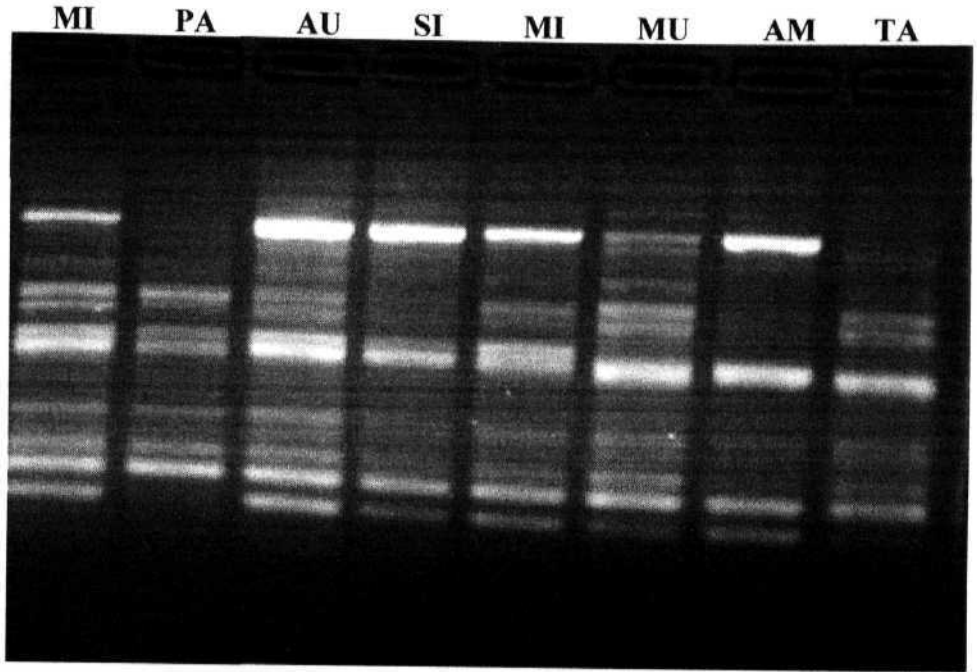

Figure 33: RAPD profile with primer OPA-3 (AGTCAGCCAC) of *O. indicum.* Total 9 amplified bands were generated among which 5 are polymorphic.

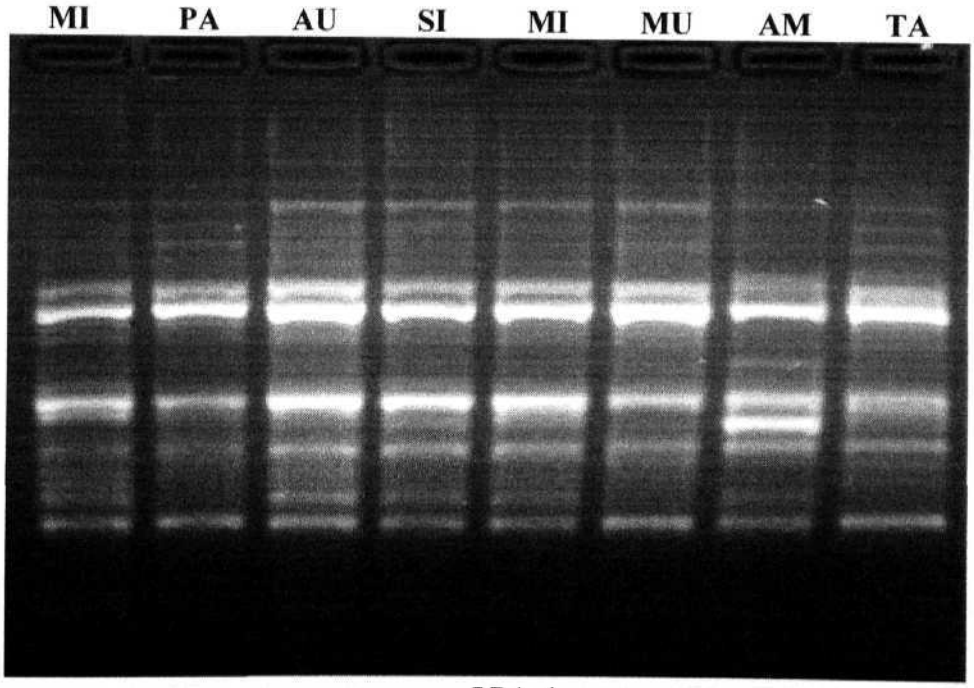

Figure 34: RAPD profile with primer OPA-4 (AATCGGGCTG) of *O. indicum.* Total 13 amplified bands were generated among which 7 are polymorphic.

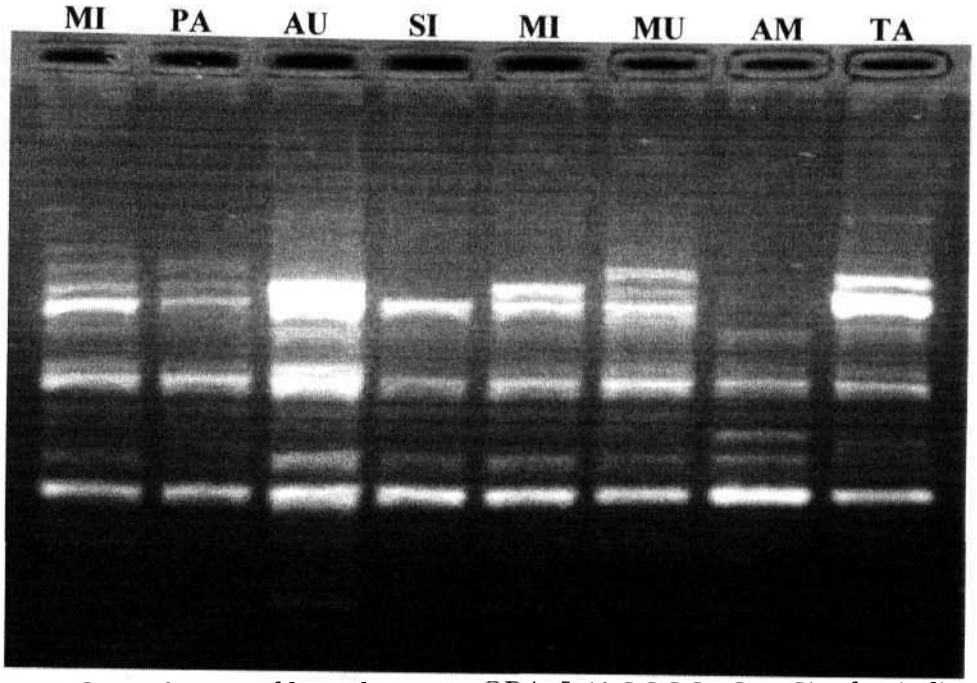

Figure 35: RAPD profile with primer OPA-5 (AGGGGTCTTG) of *O. indicum.* Total 13 amplified bands were generated among which 10 are polymorphic.

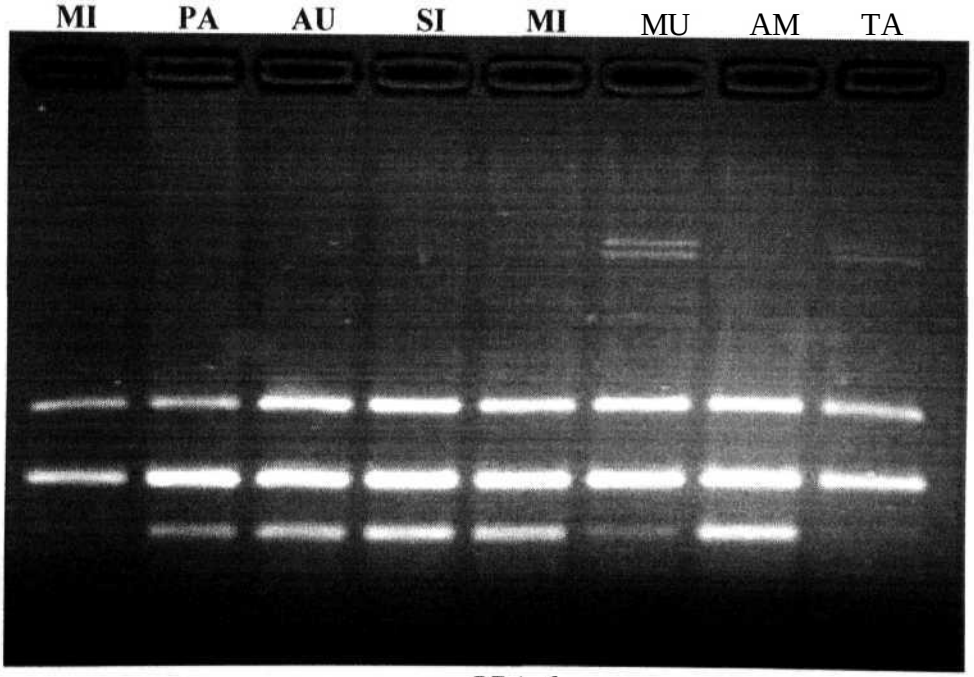

Figure 36: RAPD profile with primer OPA-6 (GGTCCCTGAC) of *O. indicum.* Total 5 amplified bands were generated among which 3 are polymorphic.

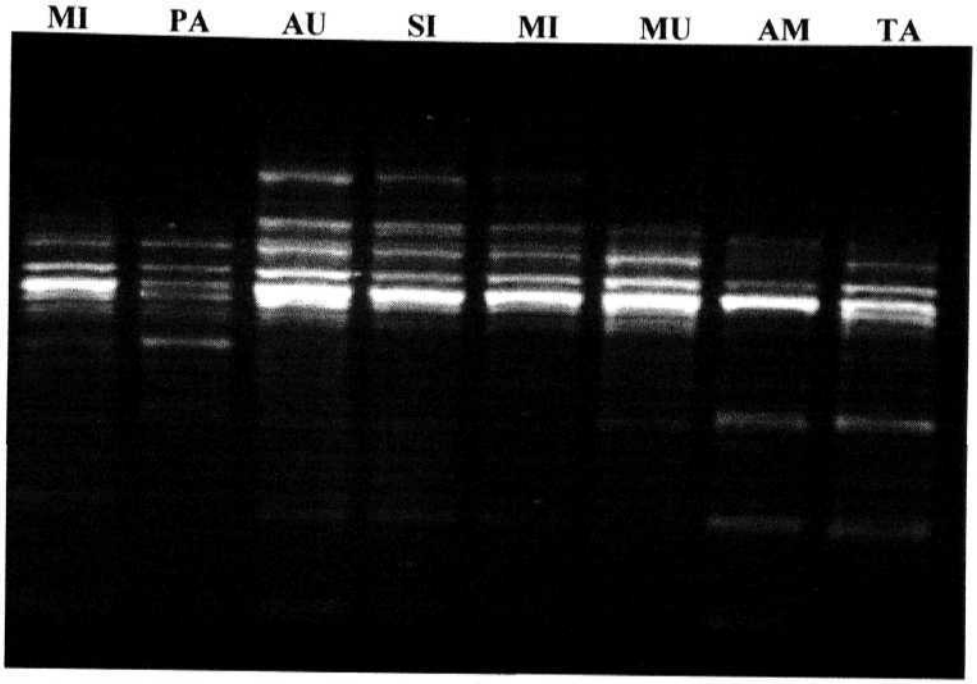

Figure 37: RAPD profile with primer OPA-7 (GAAACGGGTG) of *O. indicum.* Total 10 amplified bands were generated among which 6 are polymorphic.

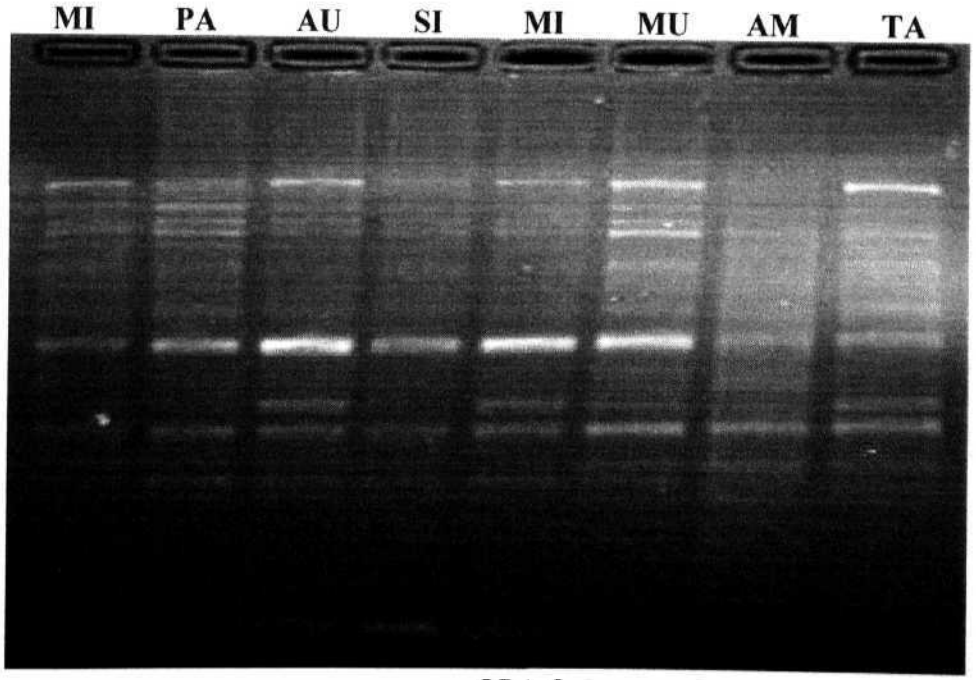

Figure 38: RAPD profile with primer OPA-8 (GTGACGTAGG) of 0. *indicum.* Total 11 amplified bands were generated among which 8 are polymorphic.

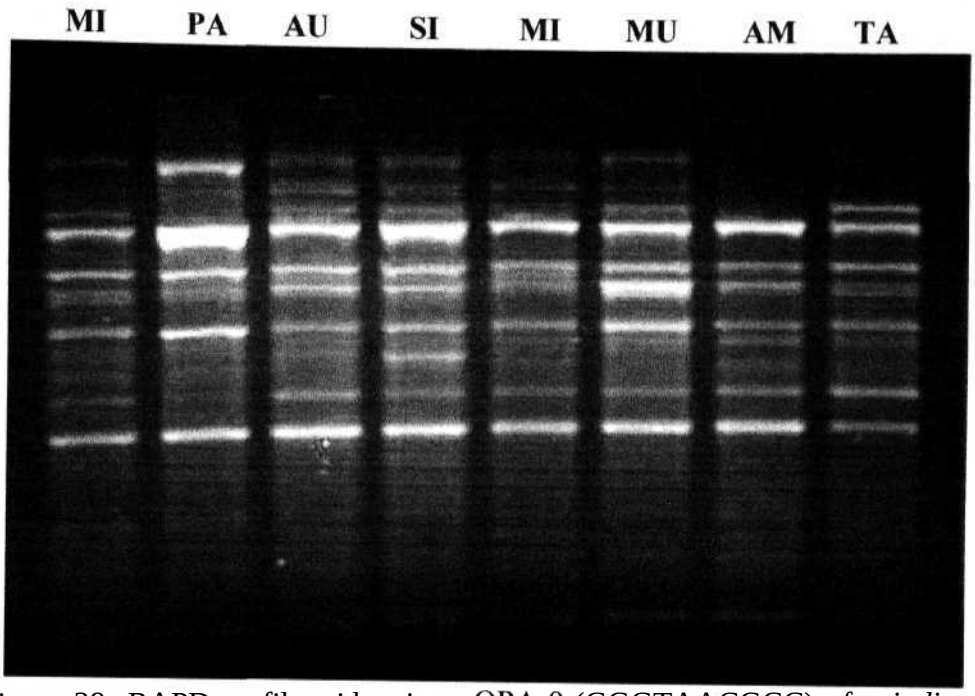

Figure 39: RAPD profile with primer OPA-9 (GGGTAACGCC) of *O. indicum.* Total 11 amplified bands were generated among which 9 are polymorphic.

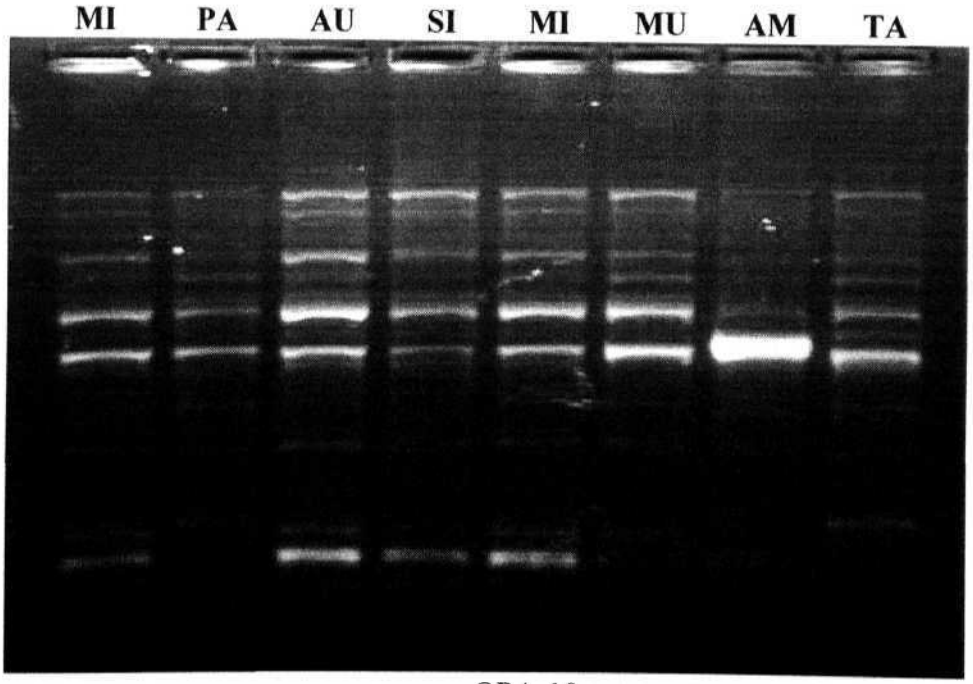

Figure 40: RAPD profile with primer OPA-10 (GTGATCGCAG) of *O. indicum.* Total 11 amplified bands were generated among which 10 are polymorphic.

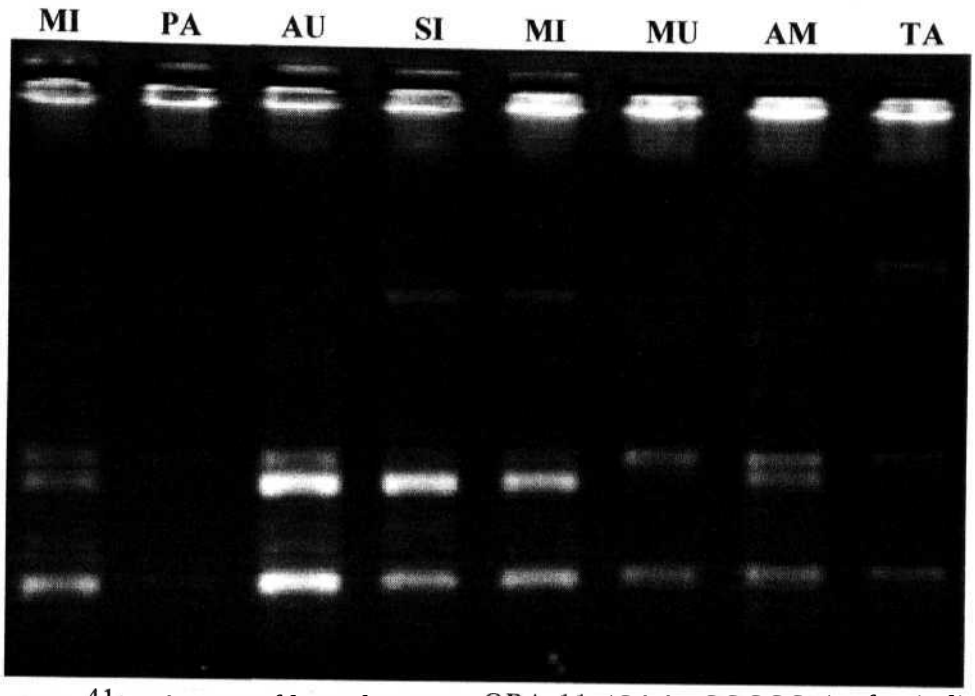

Figure 41: RAPD profile with primer OPA-11 (CAATCGCCGT) of *O. indicum.* Total 6 amplified bands were generated among which 4 are polymorphic.

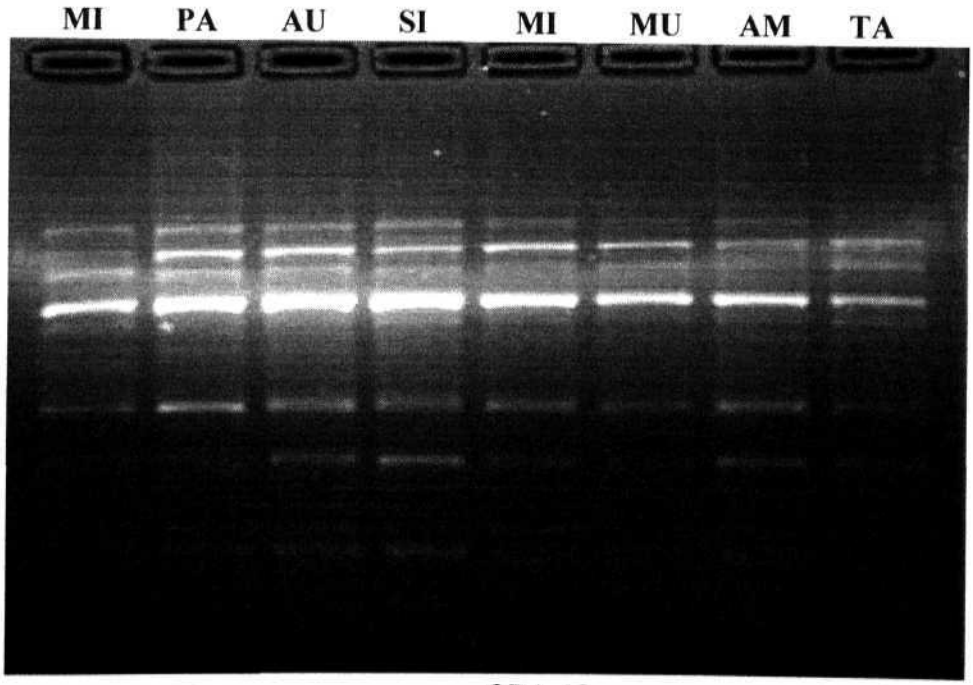

Figure 42: RAPD profile with primer OPA-12 (TCGGCGATAG) of *O. indicum.* Total 8 amplified bands were generated among which 2 are polymorphic.

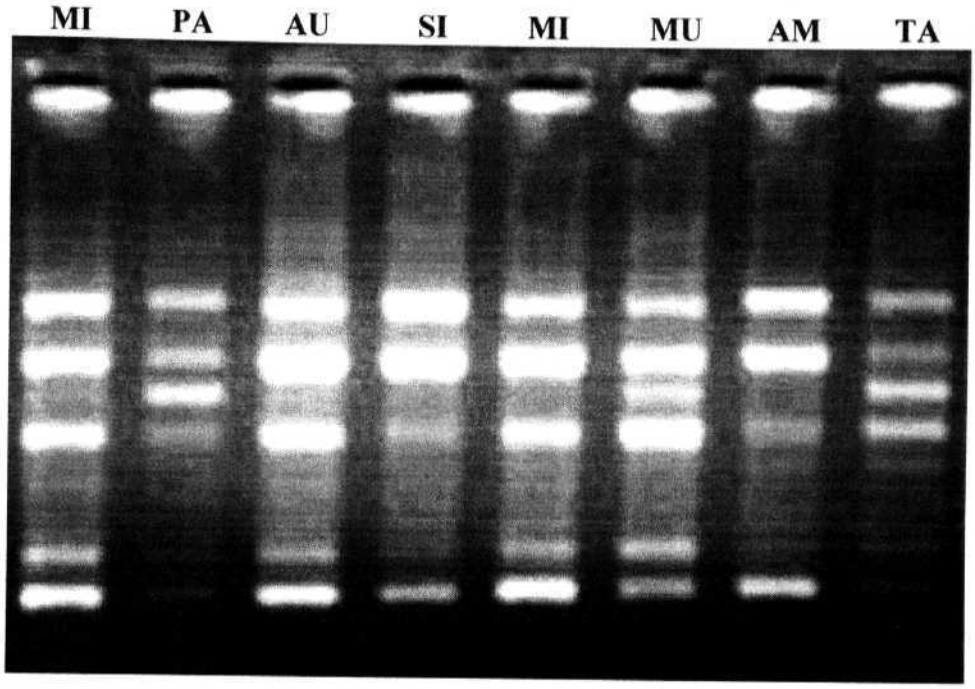

Figure 43: RAPD profile with primer OPA-13 (CAGCACCCAC) of *O. indicum.* Total 6 amplified bands were generated among which 2 are polymorphic.

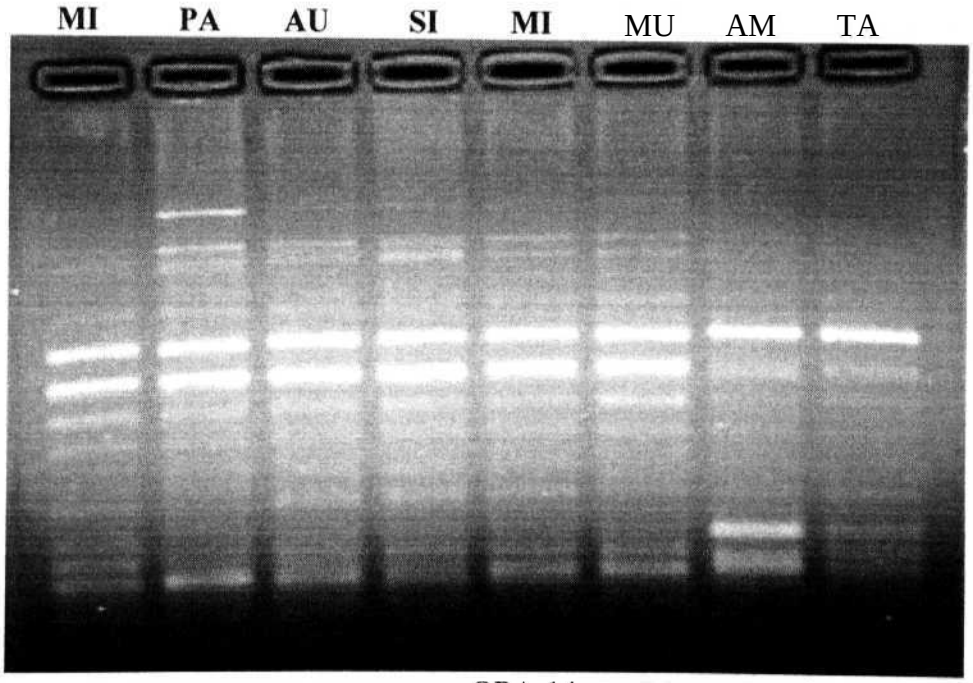

Figure 44: RAPD profile with primer OPA-14 (TCTGTGCTGG) of *O. indicum.* Total 11 amplified bands were generated among which 8 are polymorphic.

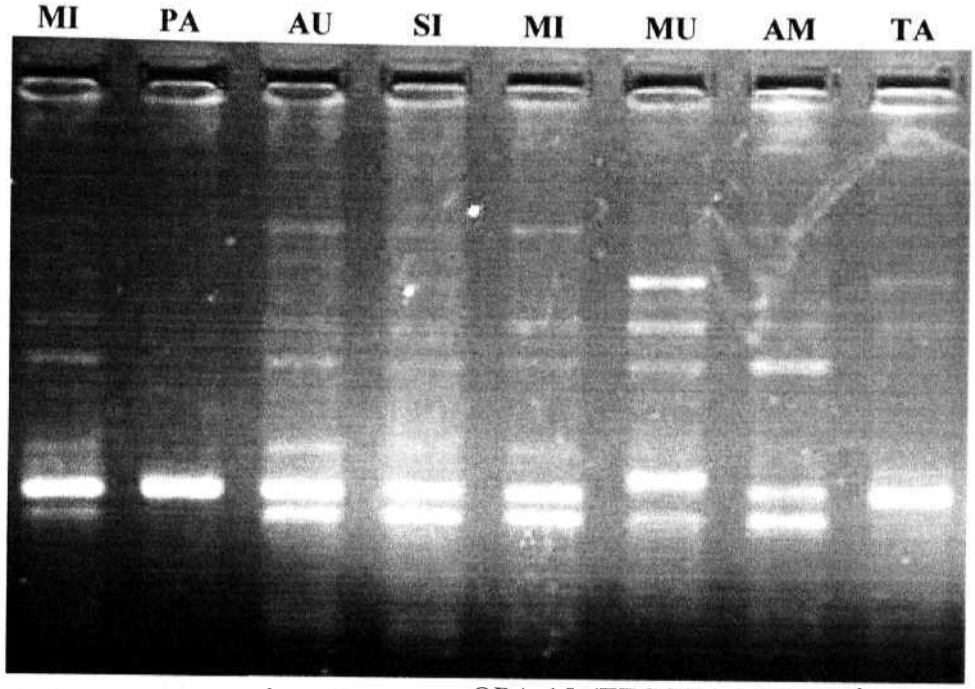

Figure 45: RAPD profile with primer OPA-15 (TTCCGAACCC) of *O. indicum.* Total 7 amplified bands were generated among which 6 are polymorphic.

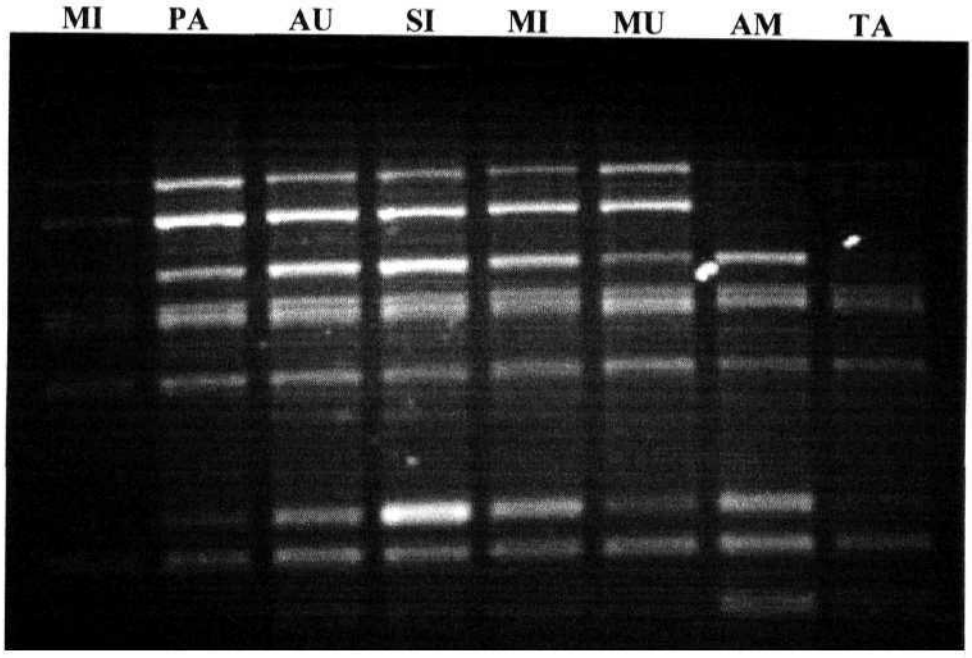

Figure 46: RAPD profile with primer OPA-16 (AGCCAGCGAA) of *0. indicum.* Total 7 amplified bands were generated among which 2 are polymorphic.

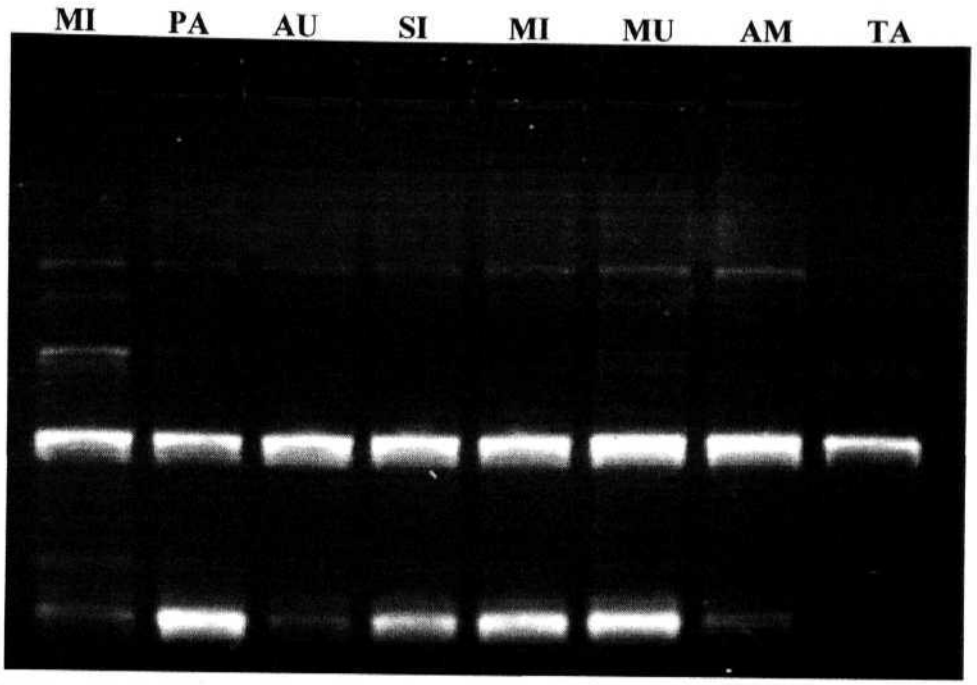

Figure 47: RAPD profile with primer OPA-17 (GACCGCTTGT) of *Cl indicum.* Total 7 amplified bands were generated among which 4 are polymorphic.

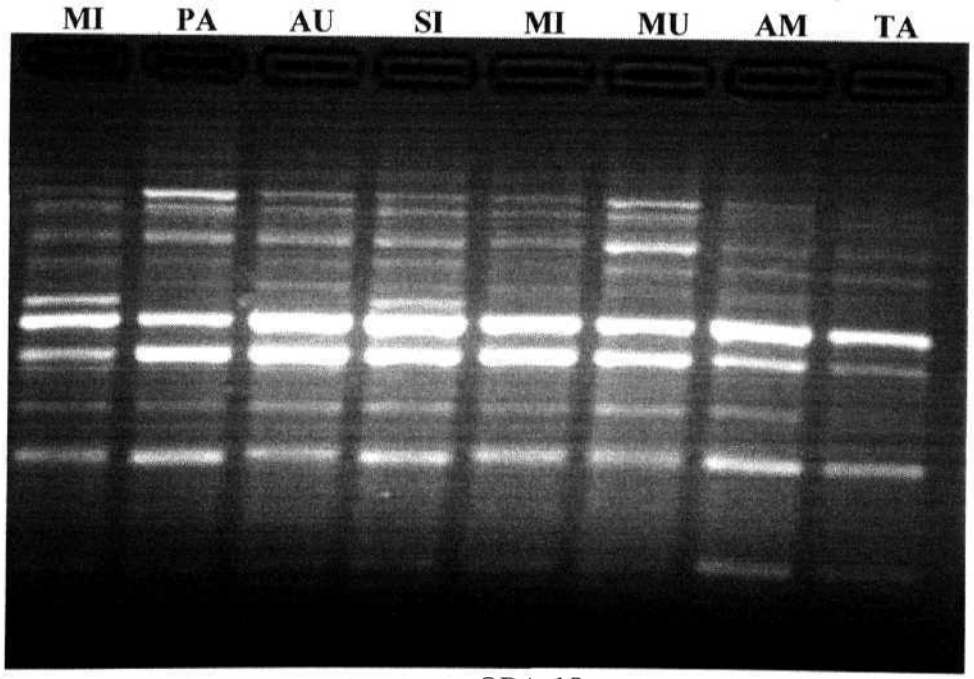

Figure 48: RAPD profile with primer OPA-18 (AGGTGACCGT) of *O.indicum.* Total 11 amplified bands were generated among which 1 are polymorphic.

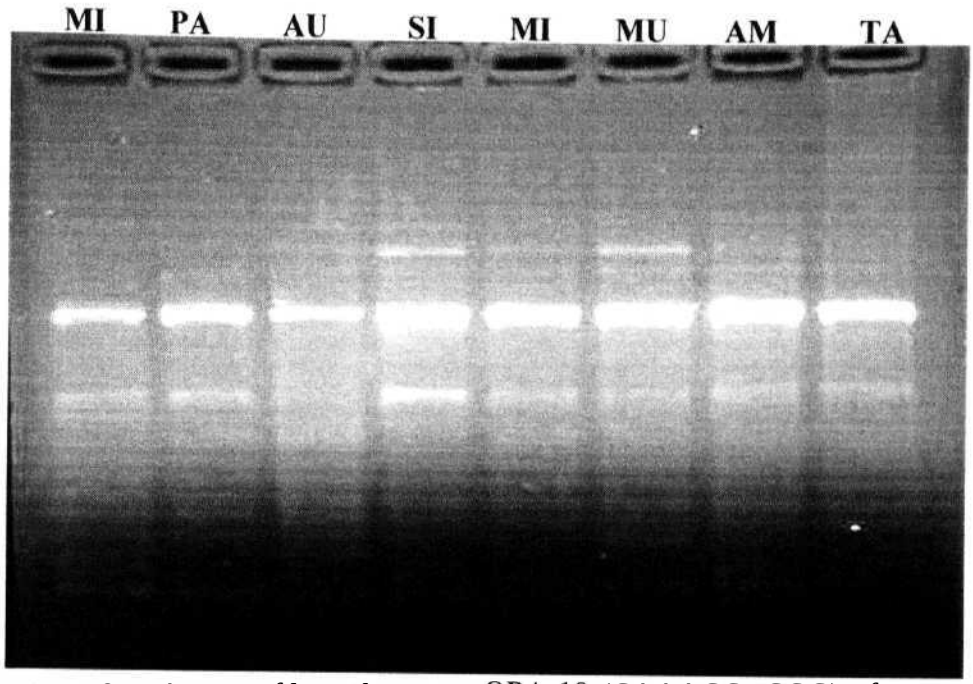

Figure 49: RAPD profile with primer OPA-19 (CAAACGTCGG) of *O.indicum*. Total 5 amplified bands were generated among which 3 are polymorphic.

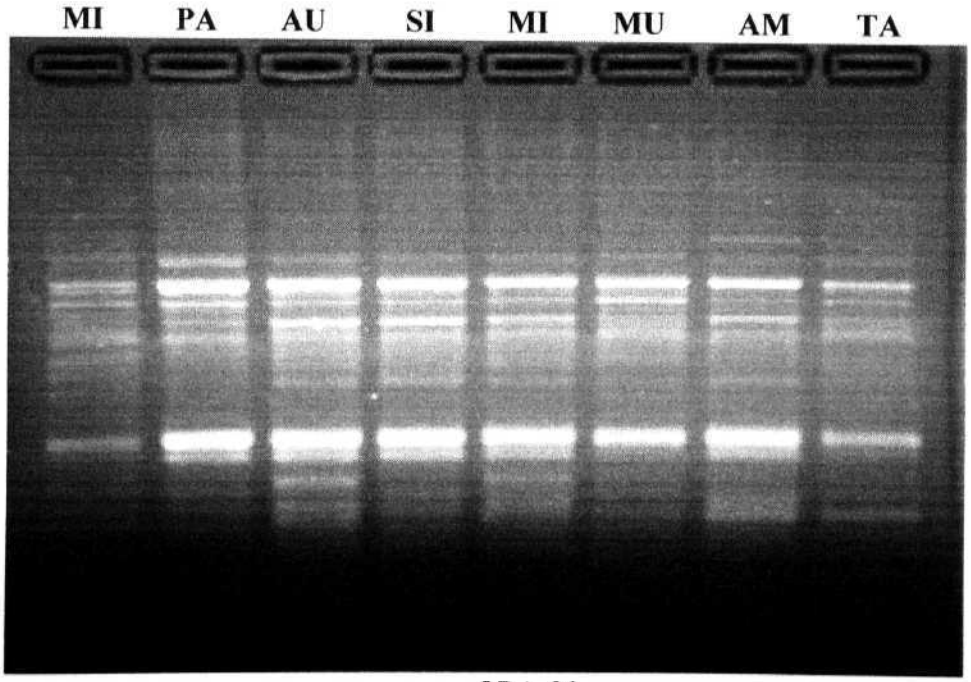

Figure 50: RAPD profile with primer OPA-20 (GTTGCGATCC) of *O. indicum.* Total 11 amplified bands were generated among which 8 are polymorphic.

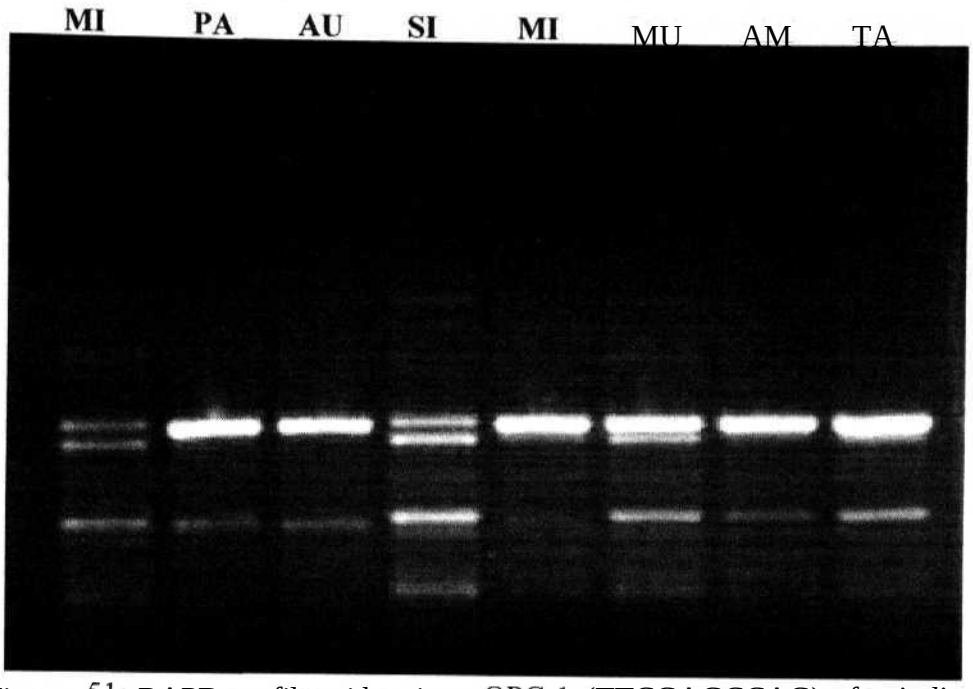

Figure 51: RAPD profile with primer OPC-1 (TTCGAGCCAG) of *O. indicum.* Total 4 amplified bands were generated among which 2 are polymorphic.

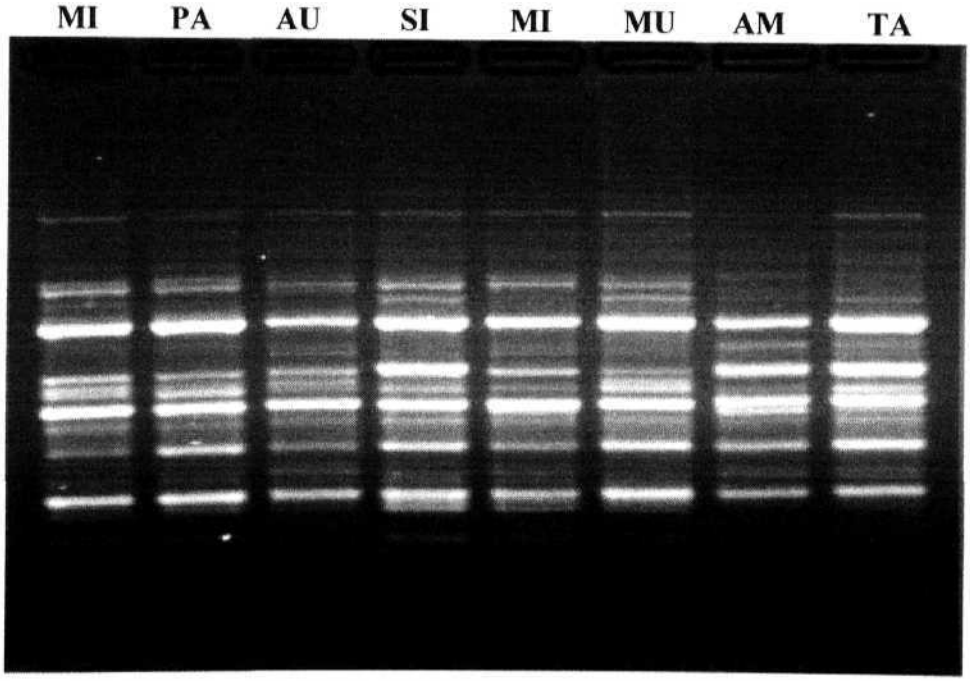

Figure 52: RAPD profile with primer OPC-2 (GTGAGGGGTC) of *O. indicum.* Total 15 amplified bands were generated among which 9 are polymorphic.

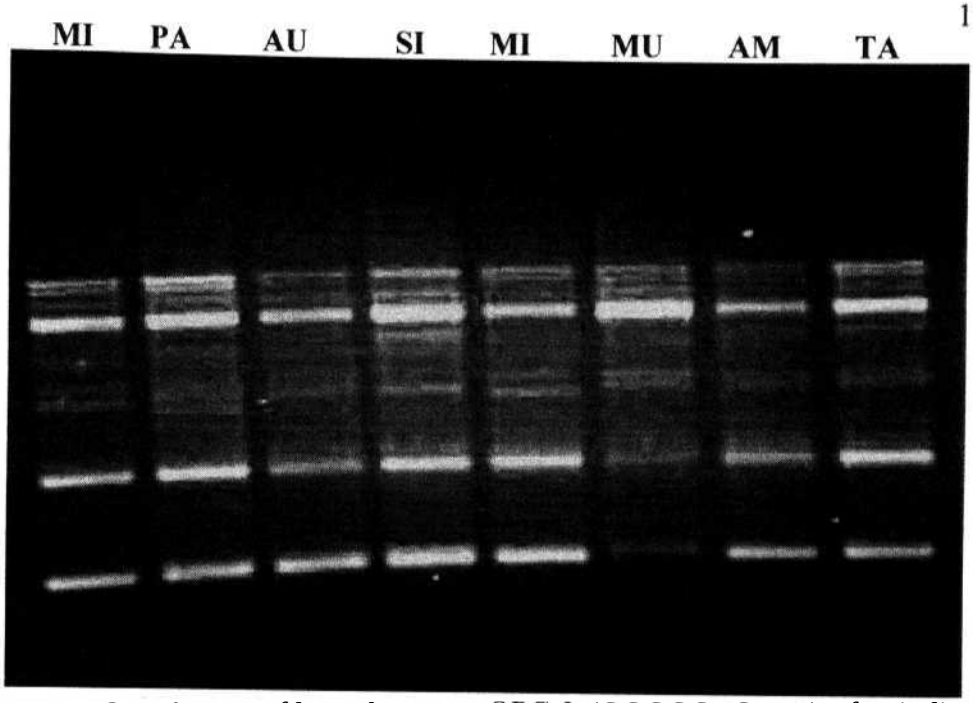

Figure 53: RAPD profile with primer OPC-3 (GGGGGTCTTT) of *O. indicum.* Total 8 amplified bands were generated among which 3 are polymorphic.

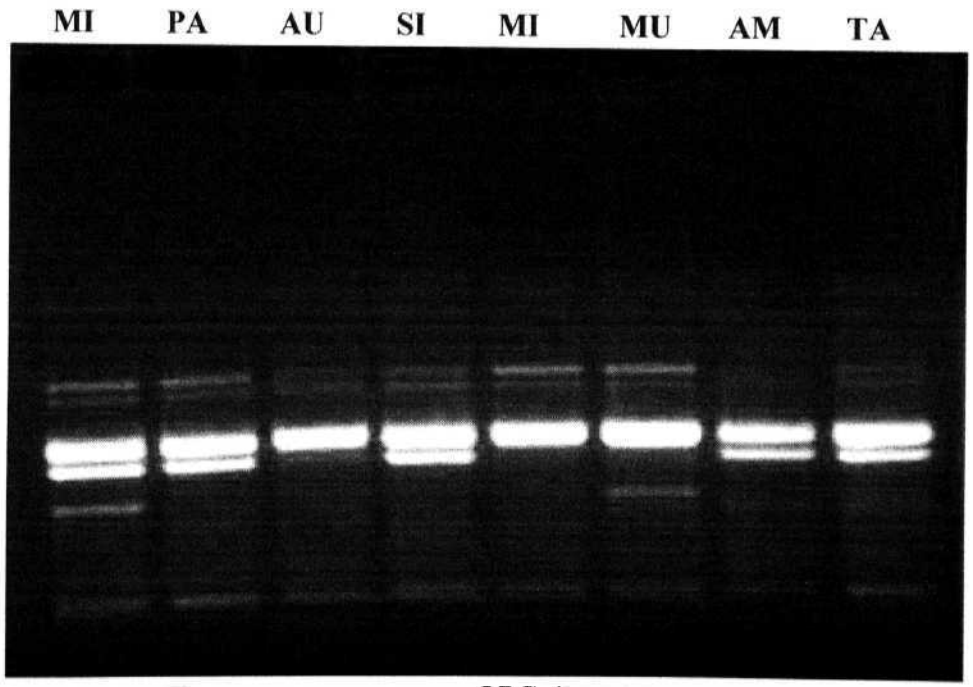

Figure 54: RAPD profile with primer OPC-4 (CCGCATCTAC) of *O. indicum.* Total 6 amplified bands were generated among which 3 are polymorphic.

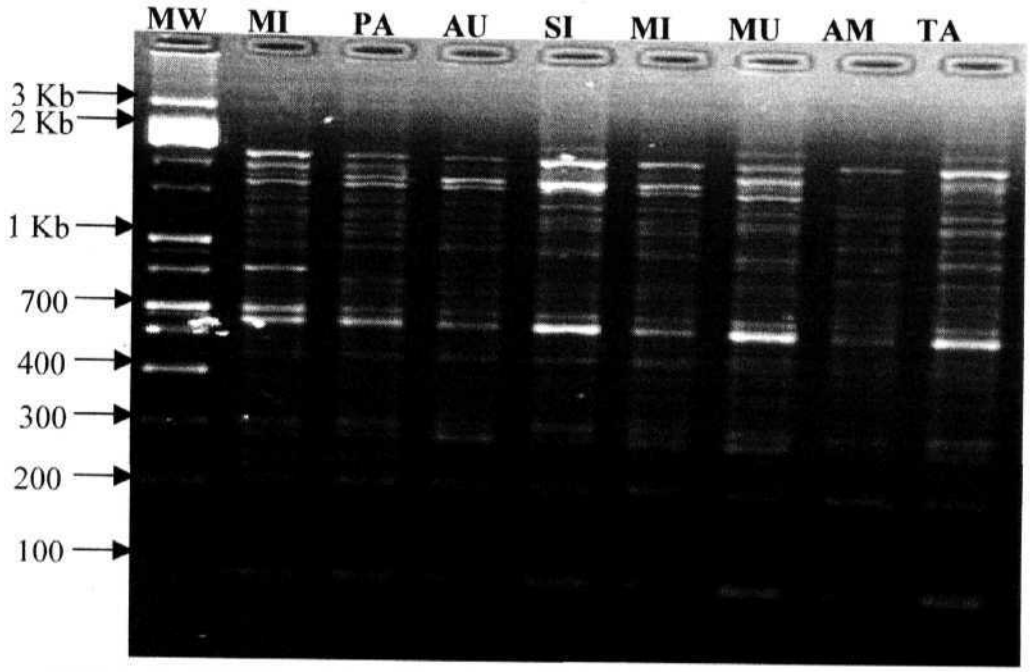

Figure 55: RAPD profile with primer OPC-5 (GATGACCGCC) of *O.indicum.* Total 20 amplified bands were generated among which 9 are polymorphic.

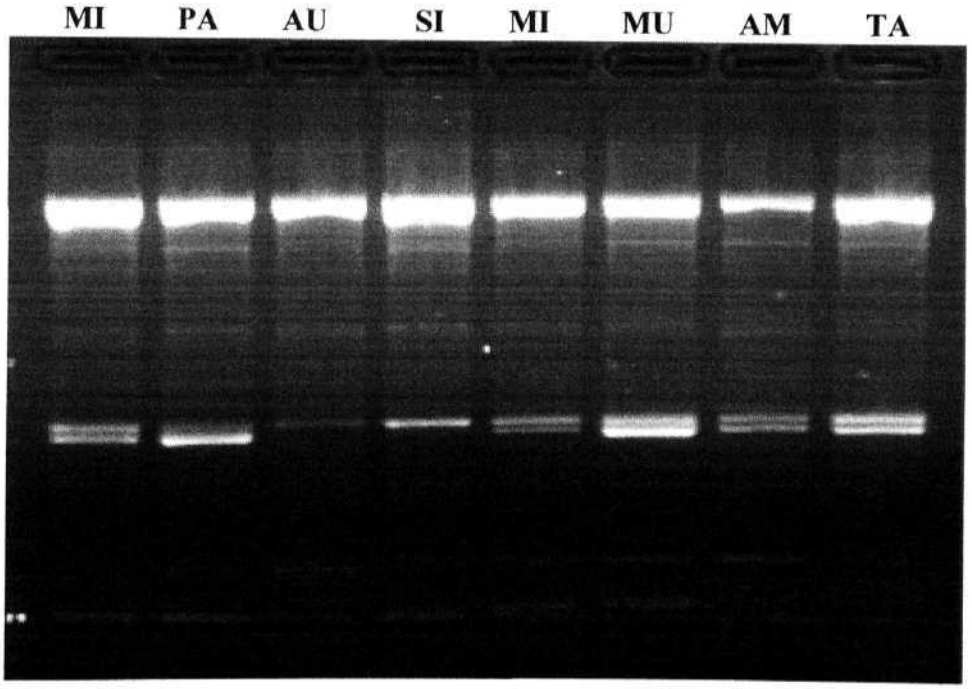

Figure 56: RAPD profile with primer OPC-6 (GAACGGACC) of *O.indicum.* Total 8 amplified bands were generated among which 2 are polymorphic.

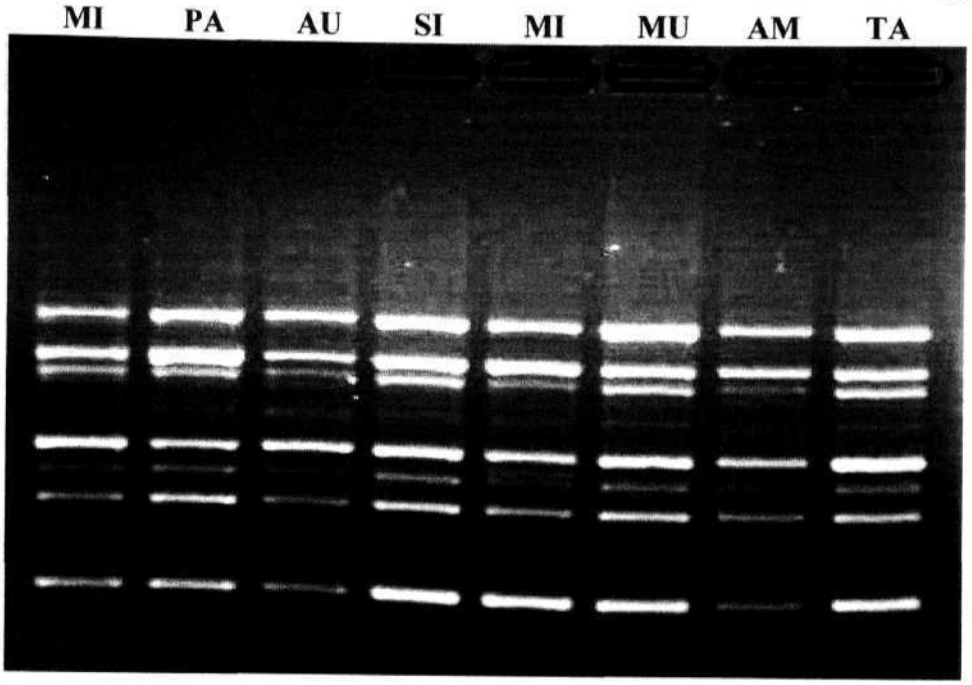

Figure 57: RAPD profile with primer OPC-7 (GCCCGACGA) of *O. indicum.* Total 10 amplified bands were generated among which 1 are polymorphic.

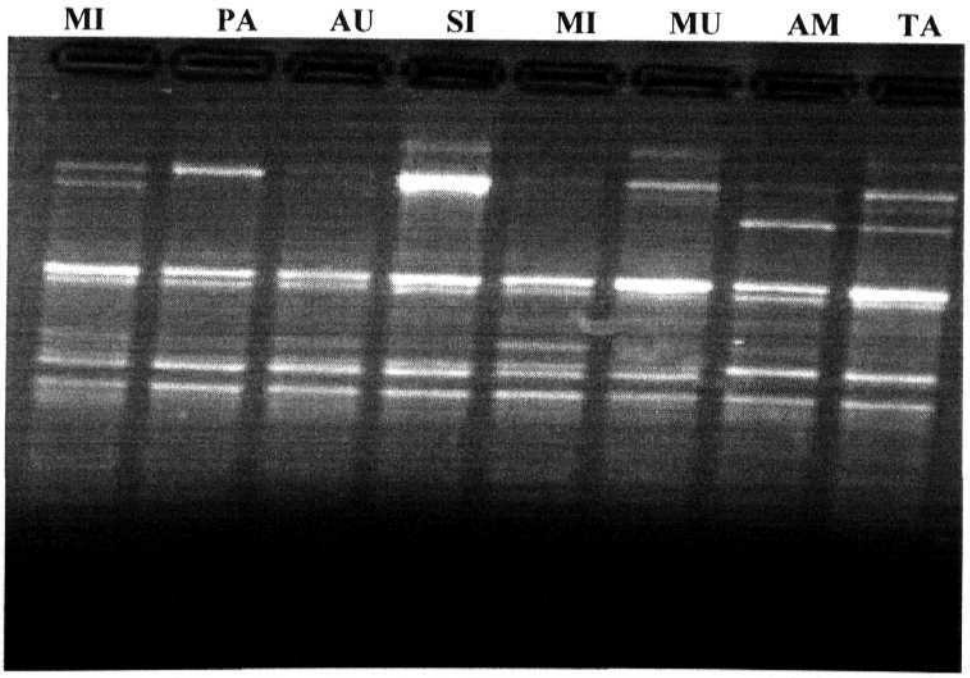

Figure 58: RAPD profile with primer OPC-8 (TGGACCGGTG) of *O. indicum.* Total 10 amplified bands were generated among which 6 are polymorphic.

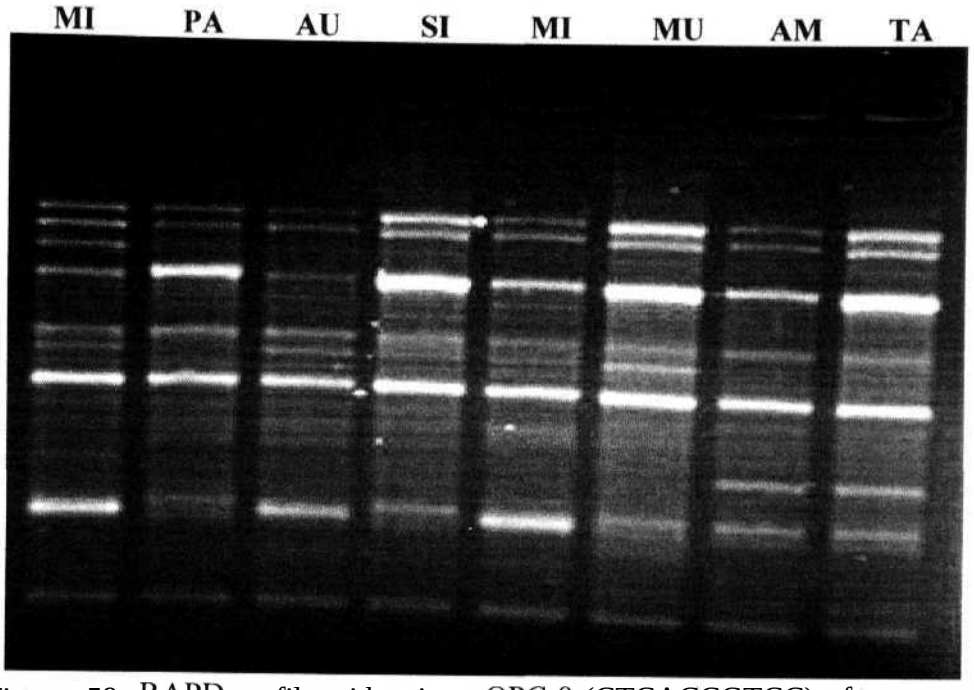

Figure 59: RAPD profile with primer OPC-9 (CTCACCGTCC) of *O.indicum*. Total 10 amplified bands were generated among which 3 are polymorphic.

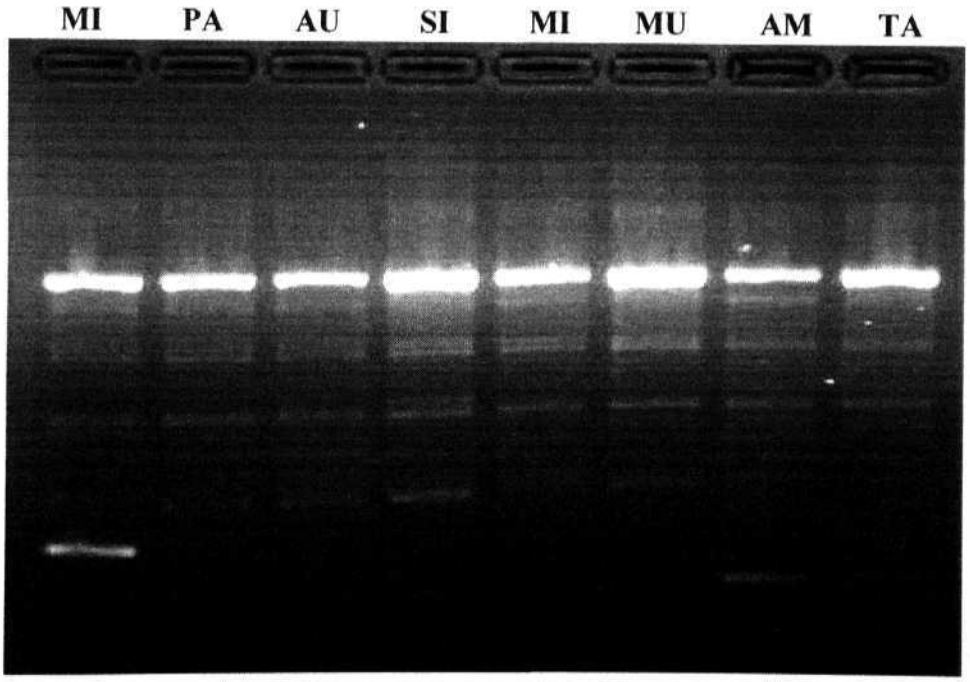

Figure 60: RAPD profile with primer OPC-10 (TGTCTGGGTG) of *O. indicum.* Total 9 amplified bands were generated among which 5 are polymorphic.

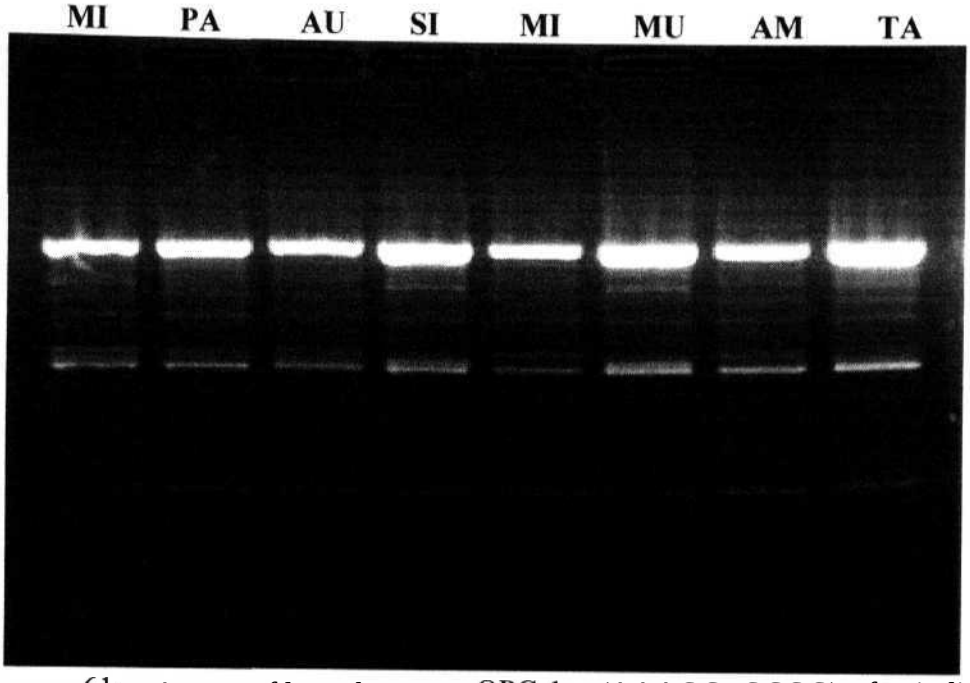

Figure 61: RAPD profile with primer OPC-11 (AAAGCTGCGG) of *O. indicum.* Total 9 amplified bands were generated among which 5 are polymorphic.

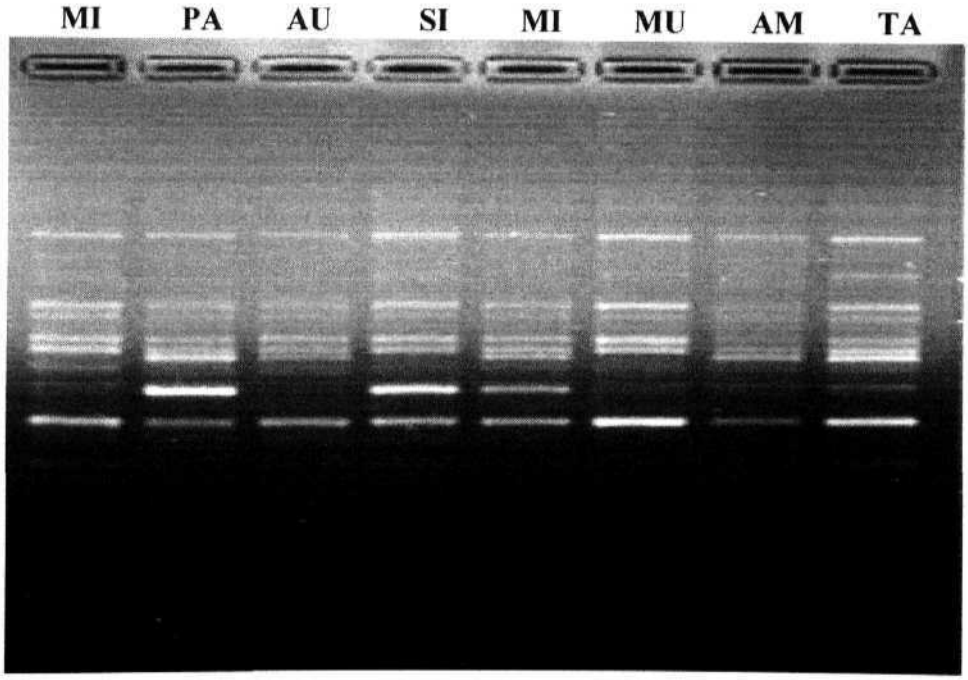

Figure 62: RAPD profile with primer OPC-12 (TGTCATCCCC) of *O. indicum.* Total 13 amplified bands were generated among which 4 are polymorphic.

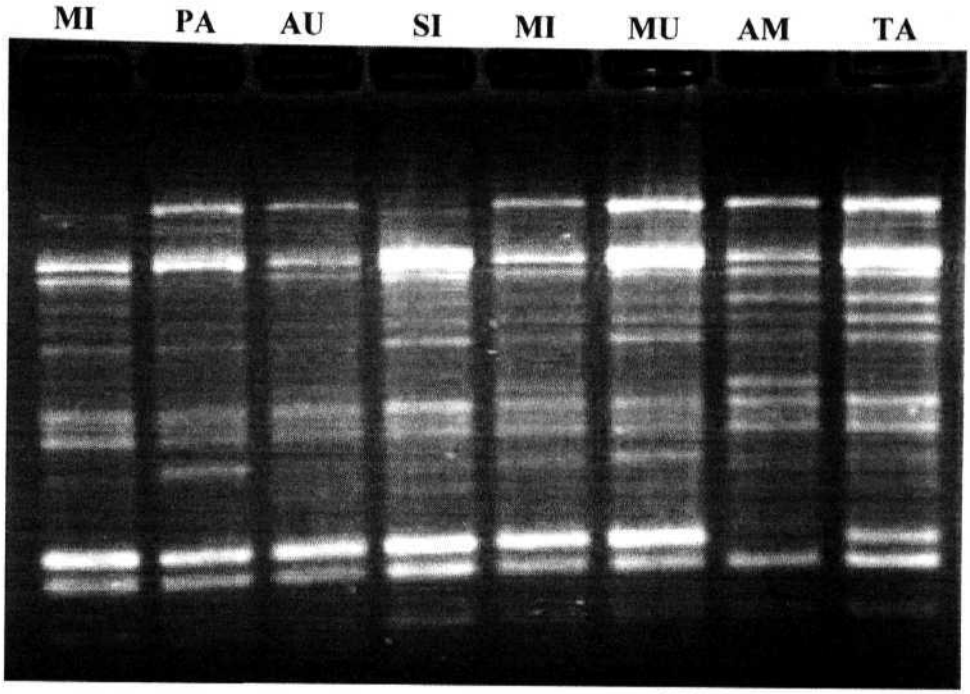

Figure 63: RAPD profile with primer OPC-13 (AAGCCTCGTC) of *O.indicum.* Total 14 amplified bands were generated among which 7 are polymorphic.

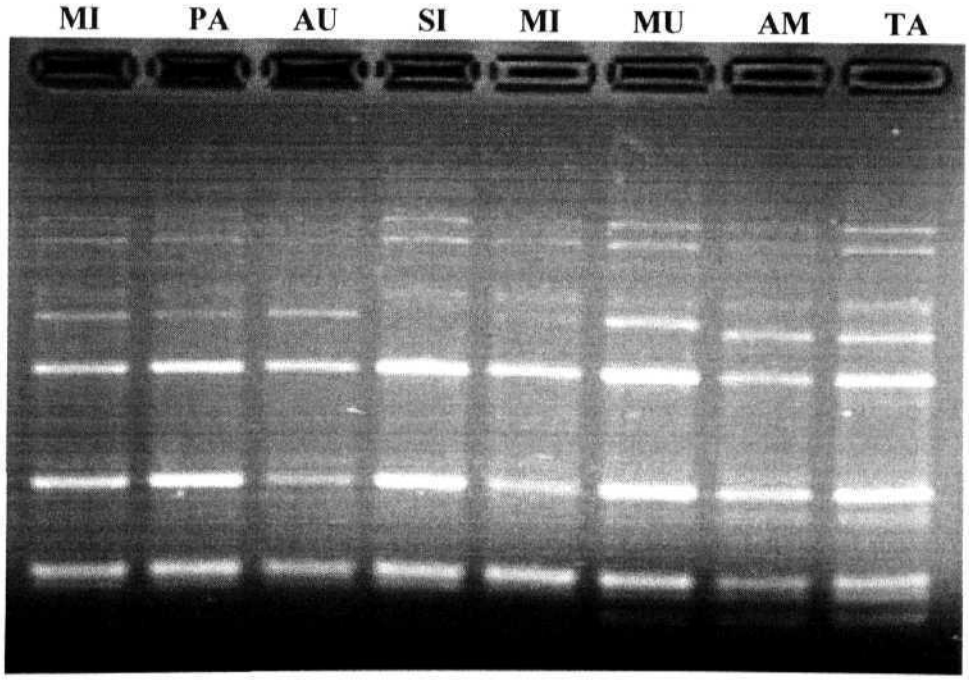

Figure 64: RAPD profile with primer OPC-14 (TGCGTGCTTG) of *O. indicum.* Total 9 amplified bands were generated among which 3 are polymorphic.

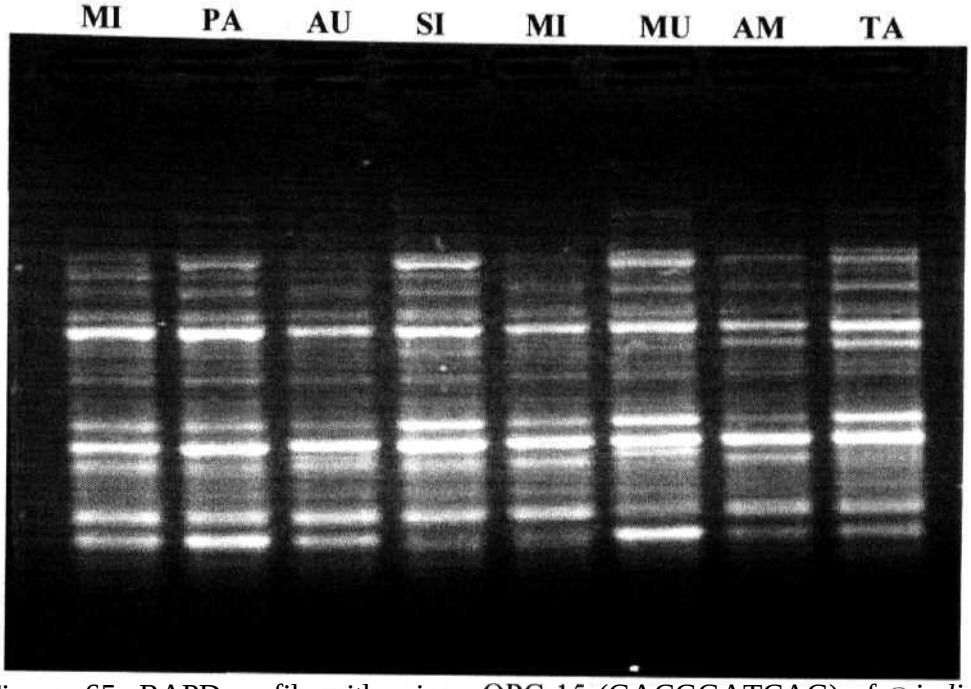

Figure 65: RAPD profile with primer OPC-15 (GACGGATCAG) of *O. indicum.* Total 15 amplified bands were generated among which 4 are polymorphic.

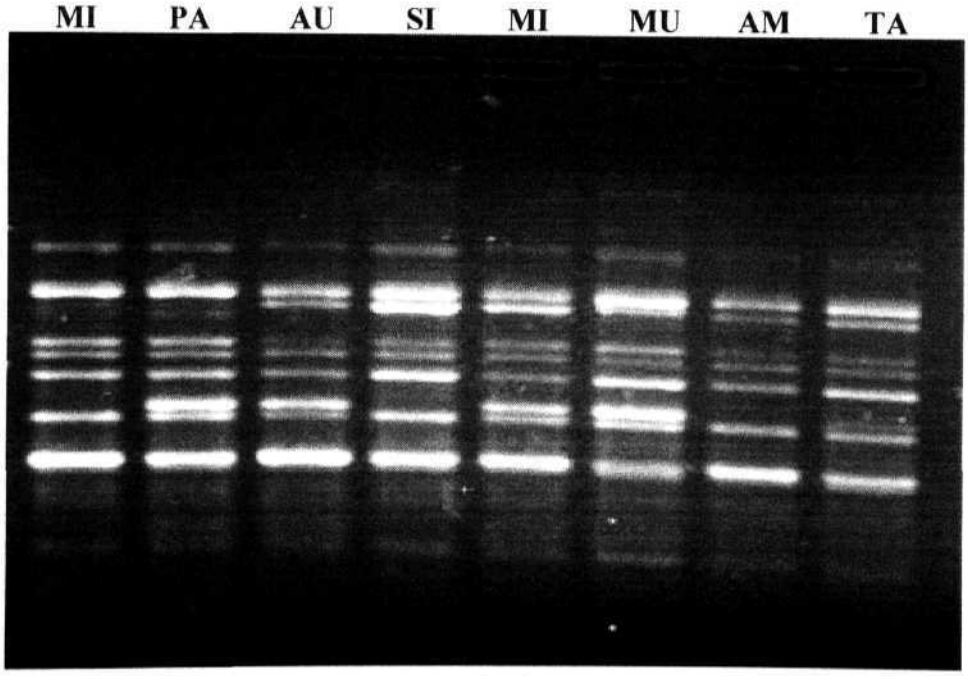

Figure 66: RAPD profile with primer OPC-16 (CACACTCCAG) of *O. indicum.* Total 11 amplified bands were generated among which 3 are polymorphic.

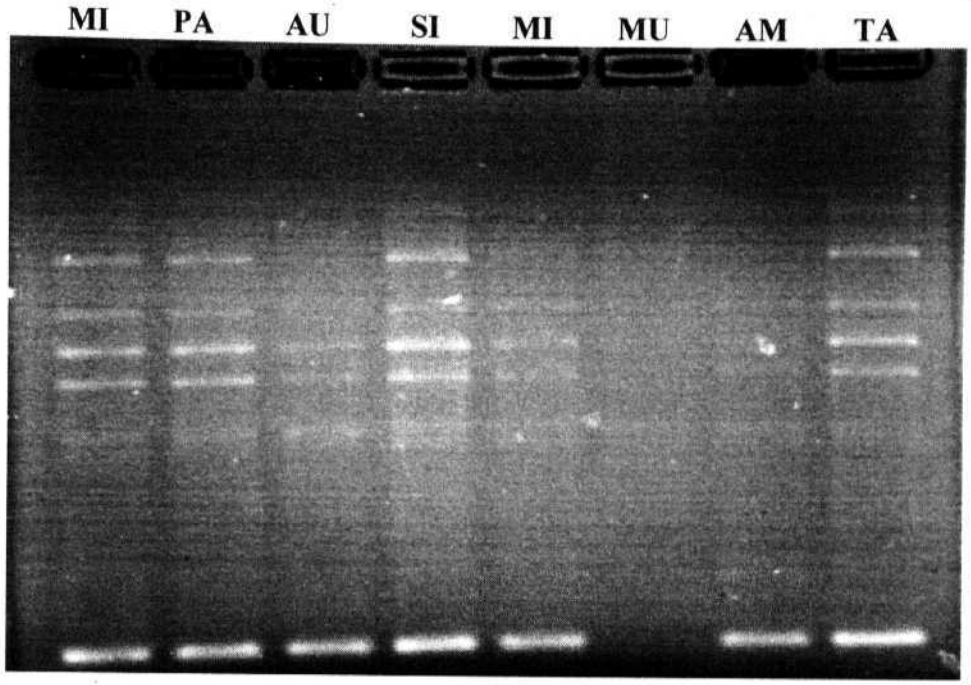

Figure 67: RAPD profile with primer OPC-17 (TTCCCCCCAG) of *O.indicum*. Total 6 amplified bands were generated among which 6 are polymorphic.

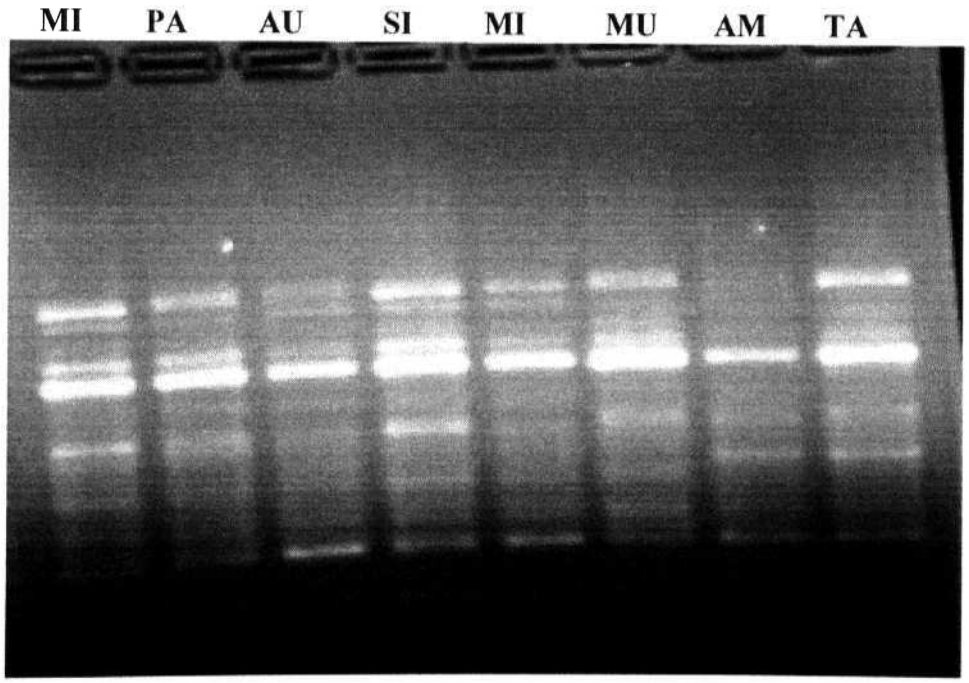

Figure 68: RAPD profile with primer OPC-18 (TGAGTGGGTG) of *O. indicum*. Total 10 amplified bands were generated among which 4 are polymorphic.

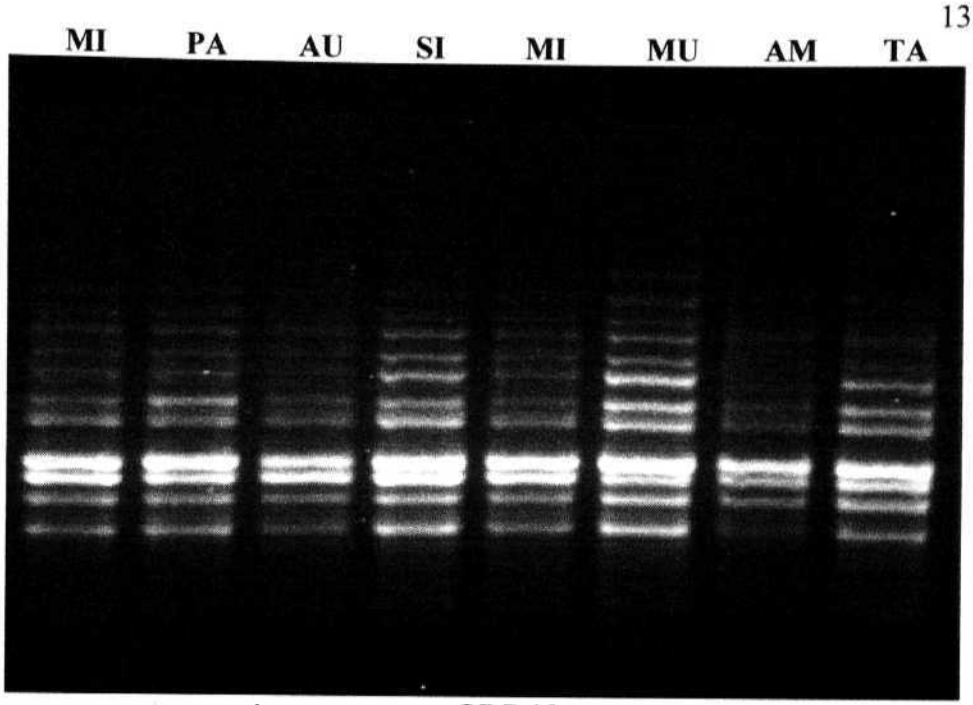

Figure 69: RAPD profile with primer OPC-19 (GTTGCCAGCC) of *O.indicum*. Total 12 amplified bands were generated.there are no polymorphic bands generated.

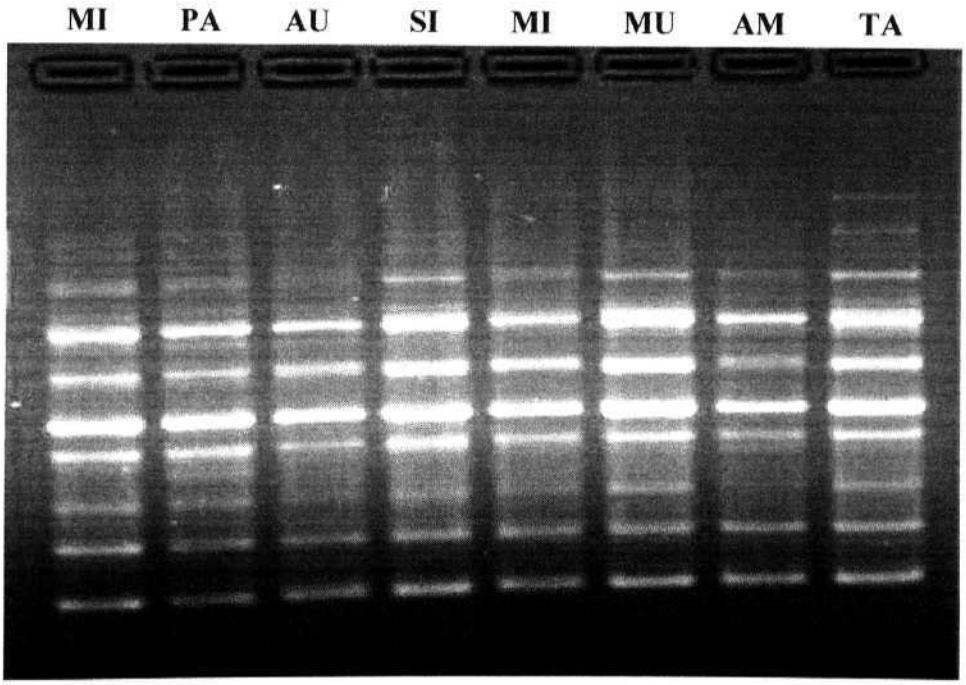

Figure 70: RAPD profile with primer OPC-20 (ACTTCGCCAC) of *O. indicum.* Total 13 amplified bands were generated among which 5 are polymorphic.

Table 17: Summery of the RAPD profile and number of scored amplification products for the 40 primers used in the genetic analysis of *O. indicum*

Code	5' to 3'	Number of bands	Number of polymorphic bands	% Of polymorphism
OPA-01	CAGGCCCTTC	8	3	37.5
OPA-02	TGCCGAGCTG	5	3	60
OPA-03	AGTCAGCCAC	9	5	55.5
OPA-04	AATCGGGCTG	13	7	53.8
OPA-05	AGGGGTCTTG	13	10	76.9
OPA-06	GGTCCCTGAC	5	3	60
OPA-07	GAAACGGGTG	10	6	60
OPA-08	GTGACGTAGG	11	8	72.7
OPA-09	GGGTAACGCC	11	9	81.8
OPA-10	GTGATCGCAG	11	10	90.9
OPA-11	CAATCGCCGT	6	4	66.6
OPA-12	TCGGCGATAG	8	2	25
OPA-13	CAGCACCCAC	6	2	33.3
OPA-14	TCTGTGCTGG	11	8	72.7
OPA-15	TTCCGAACCC	7	6	85.7
OPA-16	AGCCAGCGAA	7	2	28.5
OPA-17	GACCGCTTGT	7	4	57.1
OPA-18	AGGTGACCGT	11	1	9.0
OPA-19	CAAACGTCGG	5	3	60
OPA-20	GTTGCGATCC	11	8	72.7
OPC-01	TTCGAGCCAG	4	2	50
OPC-02	GTGAGGCGTC	15	9	60
OPC-03	GGGGGTCTTT	8	3	37.5
OPC-04	CCGCATCTAC	6	3	50
OPC-05	GATGACCGCC	20	9	45
OPC-06	GAACGGACTC	8	2	25
OPC-07	GTCCCGACGA	10	1	10
OPC-08	TGGACCGGTG	10	6	60
OPC-09	CTCACCGTCC	10	3	30
OPC-10	TGTCTGGGTG	9	5	55.5
OPC-11	AAAGCTGCGG	9	5	55.5
OPC-12	TGTCATCCCC	13	4	30.7
OPC-13	AAGCCTCGTC	14	7	50
OPC-14	TGCGTGCTTG	9	3	33.3
OPC-15	GACGGATCAG	15	4	26.6
OPC-16	CACACTCCAG	11	3	27.2
OPC-17	TTCCCCCCAG	6	6	100
OPC-18	TGAGTGGGTG	10	4	40
OPC-19	GTTGCCAGCC	12	0	0
OPC-20	ACTTCGCCAC	13	5	38.4

	1	2	3	4	5	6	7	8
ORMH	1.0000							
ORPS	0.8637	1.0000						
ORAK	0.8928	0.8585	1.0000					
ORSM	0.8971	0.8527	0.9060	1.0000				
ORMM	0.9027	0.8724	0.9575	0.9096	1.0000			
ORML	0.8987	0.8519	0.8849	0.8957	0.8917	1.0000		
ORAH	0.8227	0.8077	0.8542	0.8448	0.8683	0.8135	1.0000	
ORTK	0.8621	0.8600	0.8376	0.8417	0.8511	0.8828	0.8590	1.0000

Figure 71: Similarity matrix generated from Dice coefficient estimate based on the number of shared fragments of *O. indicum* collected from various parts of Andhra Pradesh, India.

(ORTK) formed one subgroup and 85 % genetically similar. These seven accessions formed one major group and Ahobilum (ORAH) accession formed one individual group and showed 84 % genetic similarities with other major group **(Figure 72).** There is no relation observed between geographical distance and genetic similarity among the populations, which indicates the high outcrossing nature of these populations.

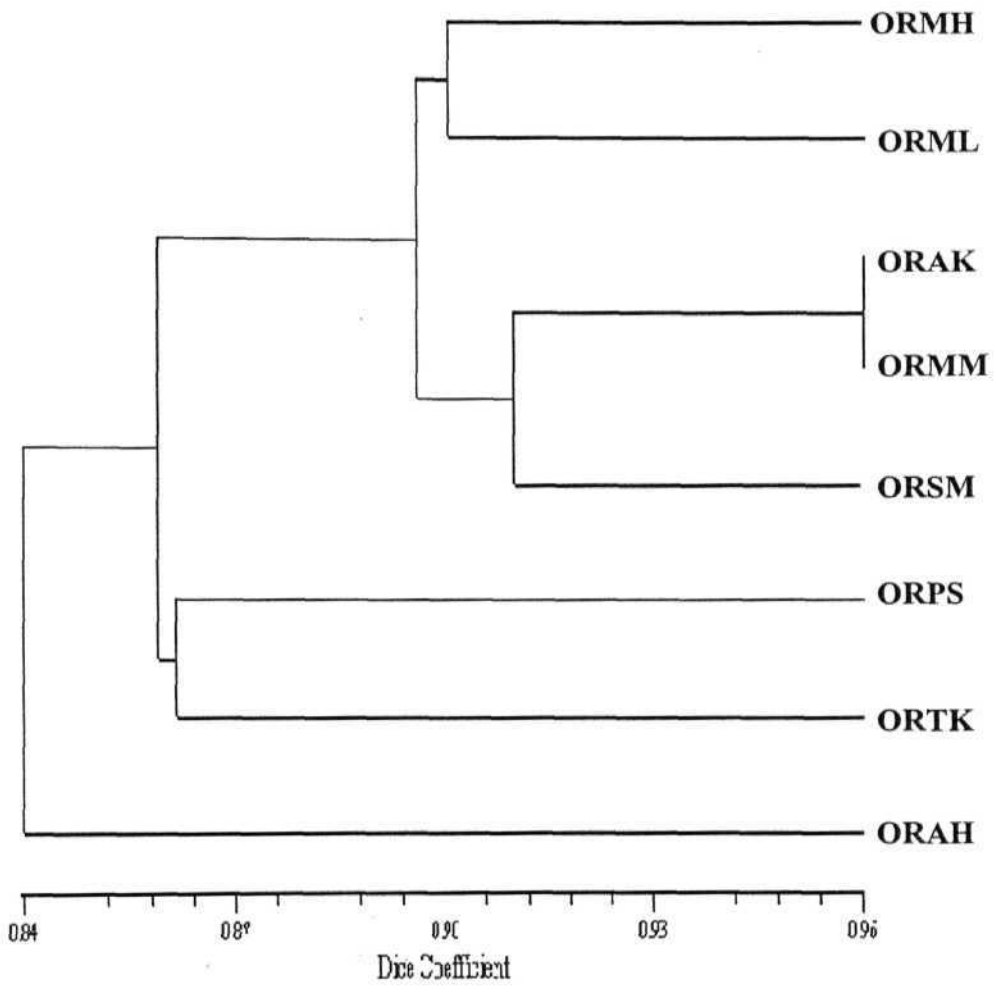

Figure 72: UPGMA (Unweighted pairwise group method analysis) cluster analysis of RAPD data for 8 different populations of *O. indicum.*

11. Discussion

Based on field survey, apart from natural environmental factors, eight natural populations of *O. indicum* are dwindling because of "Jhum cultivation and other human activities". Though seed production is more, mostly dispersed by wind, monsoons affects seed germination and seedling establishment. Therefore, any method of *ex situ* conservation that anticipates reintroduction must optimize the survival of seedlings to assure the viability of these populations. Specifically we propose an *ex situ* conservation strategy based on wild seed harvest, propagation, and reintroduction. Seed collection is easy for *O. indicum* compared to many other threatened species, and although the ratio of seed production to germination in nature is uncertain, because of water limitation. Hence *O. indicum* mostly can be observed near water source. Hence shady and good water irrigation above 80 % germination was observed.

The overall genetic diversity of a taxon has great implications for its long-term survival and continued evolution (Avise and Hamrick, 1996). Therefore, knowledge of the levels and distribution of genetic diversity is important for designing conservation strategies for threatened and endangered species (Hamrick, 1983; Hamrick and Godt, 1989; Francisco-Ortega et al., 2000). Habitat destruction and fragmentation have restricted an increasing number of plant species to small and isolated populations. Even in intact habitat remnants, plant populations face a greater

risk of extinction because of environmental, demographic and genetic stochasticity (Fisher and Matthies, 1998). Apart from environmental factors, being medicinal plant *O. indicum* populations are slowly decreasing in natural habitat. For efficient conservation and management of medicinal plant diversity, the genetic composition of species collected from different phytogeographical regions needs to be assessed. Hence vulnerable medicinal plant of *O. indicum* collected from different parts of Andhra Pradesh were subjected for RAPD analysis to check the diversity with in the collected accessions.

Since RAPD technology was first introduced by Williams et al., 1990, it has become a widely used method of assessing genetic variability in many medicinal plants (Lay et al., 2001; Li et al., 2002; Li et al., 2002; Fu et al., 2003; Qiu et al., 2004). Limitations of RAPDs include sensitivity to reaction conditions, lack of band reproducibility, and possibility of co-migration of different amplification products, but these can be largely overcome by applying uniform procedures to minimize reaction variability and carring out control and replicate runs as a test for artifactural bands (Williams et al., 1990; Howland and Arnau, 1992; Hadrys et al., 1992; Lamboy, 1994; Roderick, 1996). Another consideration is paralogous (genes generated through gene duplication) rather than orthologous (genes derived via genetic divergence) relationship among assumed homologous fragments (Rieseberg, 1996). This occurrence among RAPD fragments decreases with increasing genetic relatedness and is therefore less severe when working with closely related populations or clonally reproducing organisms (Rieseberg, 1996).

Since *O. indicum* is not a cultivable and widely using in medicinal preparations these plant populations are day-by-day reducing their population size. The fragmentation and isolation of small populations, which increases genetic drift and reduces gene flow, can thereby cause a reduction of genetic variation within populations. Small populations are subject to loss of rare alleles, and this may be important for the long-term response to selection and survival of populations (Allendorf, 1986; Newman and Tallmon, 2001). In this context the extent to which a species may be inbred may have a profound effect on amounts of population differentiation (Charlesworth and Charlesworth, 1995; Hamrick and Godt, 1996; Hendrix and Kyhl, 2000; Barrett, 2002). Moreover, small populations are more prone to extinction from random environmental fluctuations (Goodman, 1987).

In general, the higher the number of amplified products, the better the discrimination of population. Nei, (1978) suggested that a minimum number of 50 different loci should be used for estimating genetic distances. Hence the number of primers used is critical in genetic analysis. In the present study, which was used 188 polymorphic loci or bands were studied to determine the genetic relatedness among eight accessions of *O. indicum.*

The existing levels of genetic diversity and the maintenance of these levels of diversity are major issues in conservation biology. The existing levels of diversity in *O. indicum* are low, as the percentage of polymorphic bands of *O. indicum* (48.67 %) of RAPD in the species was higher than in other endangered plants, eg. *Lactoris fernandeziana* (Lactoridaceae) 24.5 % (Brauner et al., 1992), *Cathaya argyrophylla*

32 % (Wang et al., 1996), *Paeonia suffruticosa* 22.5 and *P. rockii* 27.6 % (Pei et al., 1995), and *Dacydium pierrei* 33.3 % (Su et al., 1999). This shows that the species' genetic diversity is not low, and it should be able to fit the environmental variation. Genetic diversity became an issue when Frankel, (1974) postulated that genetic variation is essential for long-term survival of species and ecological success.

If the natural population becomes extinct, *ex situ* conserved population will maintain the evolutionary process of the species, and can be released back to nature until habitat restoration. Consequently, the amount of genetic variation holding in the captive population is critical to assure the success of *ex situ* conservation and subsequent releasing. Hence the collected germplasm of *O. indicum* from various parts of Andhra Pradesh can be reintroduced back to natural habitat, with out mixing with the genetically varied populations to the natural population when its becomes rare.

12. Conclusions

Oroxylum indicum is a medicinally very important plant and as it is used in many countries in Ayurvedic preparations (Dasamoola) apart from its usage of fruit as a vegetable especially in Thailand. Moreover it is not a cultivable species and the herbal industries are depending on wild populations for collecting roots, which costs Rs 3/Kg and twigs Rs 9/Kg in Indian market. Due to indiscriminate collection, natural population is dwindling and hence, there is the need to conserve this vulnerable plant.

During extensive field survey, germplasm from 8 locations of Andhra Pradesh, in the form of seeds and seedlings were collected and are being maintained in the field experimental site and seed bank. As on date 273 plants are surviving and adapted to local edaphic and climatic conditions. Irrigation facilities and shade (50%) are favourable for the growth of seedlings. Though it naturally propagates by seeds, due to adverse environmental conditions survival in natural habitat was comparatively less.

In order to conserve the plant species under *ex situ* conditions for longer and to know the variations among collected accessions, diversity anlysis was carried out. Since there were no morphological variations observed among the collected accessions from various locations, analysis was done at molecular level by using RAPD technique.

O. indicum collected from various locations in Andhra Pradesh showed a high level of polymorphism. RAPDs revealed approximately 50 % polymorphism among these accessions. This can be justified since, it is a bat pollinated tree species. Considering the grouping of accessions in dendrogram, it can be suggested that the *in situ* conservation measures must be initiated in all those locations. Seeds and seedlings from each of the areas are also need to be takenup for *ex situ* conservation as was done in this study.

An important conservation management condition however is that, since population size also effects the genetic diversity, in *O. indicum* which is dependent on many biotic and abiotic factors there is no specific population size either small/large, which might have a relative impact on genetic variation, there was no distinction between geographical and genetic distance. Seed dispersal also plays an integral part in genetic diversity studies as it was observed in *O. indicum* due to the lightweight and winged seeds.

There are reports that regional, habitat, photoperiod and temperature can affect secondary metabolite production (Kämäräinen et al., 2003; Voirin et al., 1990; Jensen et al., 1995). Since germplasm of *O. indicum,* is significant in medicinal and phytochemical, as used in ayurvedic preparations, in according to the genetic variations detected by RAPD analysis, collected from various part of Andhra Pradesh may show some variation in terms of secondary metabolites content, which need to be further studied.

From the conservation point of view, each population of *O. indicum* collected from various regions of Andhra Pradesh should be conserved individually since they are genetically different according to UPGMA cluster analysis.

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14. Summary

Identification of *D. indica* and *D. burmanii* is difficult because of their habit and unique habitat preference, but due to the existence of associated genus like *Eriocaulon*, *Utricularia* and *Sphagnum* since they share the common habitat, it was easier to identify the plants. In all the collected accessions of *D. indica* seed ornamentation is transversely hexagonal in shape, whereas in *D. burmanii* seed ornamentation is tetragonal reticulate in shape. In *D. indica* and *D. burmanii* no germination was observed after subjecting to various treatments even after 30 days in both *in vitro* and *in vivo.*

In *D. indica* production of multiple shoots was efficient at 0.5 mgL^{-1} of KN, Z and 0.1 mgL^{-1} BAP. *In vitro* flowering was observed in different strengths of MS medium, MS with various concentration of sucrose, MS with various pH values and MS medium supplemented with lower concentrations of cytokinins (Z, KN and BAP). Regeneration of plants from the bracts of inflorescence was observed on MS with lower concentrations of cytokinins, which were also used as explants for multiple shoot production. Plant growth was retarded at higher concentrations of KN, Z and BAP but these plants regained their normal growth when transferred to MS basal or MS with lower concentrations of cytokinins. Though multiple shoot production was observed in different strengths of MS media, MS with various pH values and various concentrations of sucrose, it was comparatively lesser on MS with cytokinins. Rooting was successfully observed on MS basal medium.

In *D. burmanii,* production of multiple shoots was efficient on 2.0 mgL^{-1} of KN and 1.0 mgL^{-1} BAP. *In vitro* flowering was observed in all the tested conditions except in higher concentrations of cytokinins (above 0.5 mgL^{-1} of KN and BAP). Characteristic multiple shoots were observed from leaves, after transferring from higher concentration of BAP (2.0 mgL^{-1}) to MS basal medium. Occasionally regeneration of plantlets from the pedicel was observed on lower concentrations of KN (1.0 mgL^{-1}), which were successfully used as explants for regeneration of multiple shoots. Plantlet regeneration was observed from axils of bracts of inflorescence at different strengths of MS medium, different concentrations of sucrose and various pH values in *D. burmanii.* Rooting was successfully observed on MS basal medium. Field transfer of *in vitro* raised plants require a transition through a simulated habitat to achieve success.

For RAPD analysis of the collected accessions of *D. indica* and *D. burmanii,* DNA isolation was tried using different protocols but, the quality and quantity of DNA obtained was very poor, because of variety of contaminating substances, which are thought to originate from the large number of stalked glands on the upper leaf surface that contain an highly viscous mucilage used to trap and immobilize insects. Hence molecular diversity could not be studied in *D. indica* and *D. burmanii.* The only DNA isolation protocol, which was standardized till now in insectivorous plants was in *D. rotundifolia,* which is being currently in use for evaluating other members of the Droseraceae and other insectivorous plants (Bekesiova et al., 1999).

A total of 273 plants of *O. indicum* are being maintained in field experimental site for *ex situ* conservation. Isolation of genomic DNA and conditions for PCR were standardized. A total of 387 bands were scored for the 40 RAPD primers for a range from 4 to 20, corresponding to an average of 9.6 bands per primer, and 49.61% (188) of these were polymorphic. Percentage of polymorphic bands for each primer ranged from 0% to 100%. Genetic distance lies between 0.80-0.95. The size of the amplified bands range from 200-1800bp. Based on the cluster analysis, by using Unweighted Pairwise Group Matrix for Arithmetic average (UPGMA) analysis, two major groups of *O. indicum* have been found within the collected accessions from various geographical locations. There is no relation observed between geographical distance and genetic similarity among the populations.

15. Research communications

K. Jayaram and M.N.V.Prasad (2005). Rapidly *in vitro* multiplied *Drosera* as reliable source for plumbagin bioprospection. Current Science (Accepted).