COMPILATION

(Micropropagation Research Papers 1988-2012)

Bulletin No. 37


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Government of Nepal

Ministry of Forests and Soil Conservation

Department of Plant Resources

Thapathali, Kathmandu, Nepal

2014

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2/3. Shoot tip and Protocorm proliferated from shoot-tip explants.
4. Rooting of micropropagated seedling

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Forword

Tissue culture, a new biotechnological tool, offers superior quality plant clone material free of disease in desired quantities. Plant tissue culture is presently of great interest of Molecular Biologist, Plant Breeders, and industrialists. Since the establishment of Tissue Culture Laboratory in 1976 at National Herbarium and Plant Laboratory Godawari, different aspects of micro propagation and micro-shoots rooting in non sterile sand have been carried out in a number of economic plant species. In 2000 A.D. Biotechnology laboratory was established in Department of Plant Resources, Thapathali. The main objective of the Biotechnology laboratory is to use the plant tissue culture technique for the mass production of economically important and endangered plant species, DNA Bar coding of high value medicinal plant species and virus testing of propagated plants.

This book consists of compilation of research works that had been published in different national and international journal. This book is a collection of research works done in both laboratories. Tissue culture laboratory, Godawari and Biotechnology section, Thapathali. The present bulletin (No. 37) consisted of 60 number of articles.

It is hoped that this collections of research articles will be useful for the people those who are engaged in research work. I would like to thanks Ms. Keshari Maiya Rajkarnikar, Scientific Officer, Ms. Sabari Rajbahak, Assistant Scientific Officer Mr. Sisir Panthi Assistant Scientific Officer and Mr. Puskar Basnet for helping in many ways in bringing out this publication.

July 7, 2014

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Introduction

Tissue culture is the development of new plants in an artificial nutrient medium under aseptic condition from explant. The explant is a small piece of plant used to start a culture. The explant can be a very small pieces of plants, such as embryos, seeds, stem, shoot tips, meristem, root tips, callus, single cells and pollen grains.

Plant tissue culture is an important aspect of biotechnology. It has great potential for rapid, large scale and true to type multiplication. The plants from tissue culture are generally clean and healthy. This technique has been applied to several crop species. This technique is of particular interest in case of highly heterozygous species which are virus infected and are generally vegetative propagated.

Since 1963, in vitro culture has been growing rapidly throughout the world. Mass propagation of some ornamentals is one of the most successful examples of commercialization of tissue culture technology. Multimillion dollar industries have been set up in the world to meet the demand for quality plants. Realizing the potential tissue culture technique protocol development for different species are being carried out in different laboratories.

Tissue Culture Laboratory was established in 1976 in Godawari. This laboratory has developed the techniques for micropropagation of economically important plants and elite trees to produce best quality clone plants.

Micro shoots developed from tissue culture can be rooted in non sterile sand. For this the microshoots in flask are acclimatized in glass house for one week and these are individually planted in sun dried clean sand. It is covered with polyethylene sheet to maintain humidity of 80% and temperature range of 25-30^{o} C.

The advantage of the technique is to cut down the cost of production of tissue cultured plant. From this rooting technique, the cost of growth medium, incubation room, electricity and tissue culture manpower is lowered. This rooting technique can be easily done by nursery personnel for the mass production of micro propagated plants. This rooting technique has a great application.

In Tissue Culture Laboratory, micro propagation methods for several plant species have been developed. These methods are simple, effective compared to the conventionally used in vitro cloning methods. In the conventional procedure four steps are involved.

1. Explant establishment,
2. Shoot multiplication,
3. Rooting of multiplied shoots in the medium and
4. Field establishment.

In the step 1. the explant isolation, surface sterilization, washing and establishment in the culture medium is done. In the step 2. the micro shoots are proliferated in a defined culture medium. In the step 3. micro shoots proliferated during incubation are transferred to a rooting medium within the flasks. In the step 4. the micro plants are transferred from the aseptic environment of the laboratory to the natural condition for field establishment.

The steps 3 and 4 are labor intensive and expensive such that the step 3 accounts 30-70% of production cost. And the step 4 encounters the severe problem of low percentage of survival of plants when the plants are transferred from the flask to the field.

In the method developed at Tissue Culture Laboratory, instead of step 3 and 4 the micro shoots are directly rooted in sand under suitable day/night temperature regimes in the ordinary green house conditions. Such a procedure has reduced the production cost. It can be compared with the production cost of seedlings and cuttings.

Micropropagation protocols have been developed in following plant species in Godawari.

Trees:

Artocarpus lakoocha, Artocarpus heterophyllus, Dalbergia sisoo, Eucalyptus camaldulensis, Eucalyptus citriodora, Eucalyptus terecticornis, Ficus auriculata, Ficus carica, Ficus elastica, Ficus lacor, Ficus nemoralis, Ficus nerifolia, Ficus memicordata, Morus alba, Populous ciliate, Elaeocarpus sphaericus, Daphne papyracea, Paulownia tomentosa, Azadiracha indica, Acacia auriculiformis, Santalum album

Horticultural crops:

Brassica oleracea var. capitata, Citrus limon, Citrus sinensis, Fortunella spp., Musa spp., Poncirus trifoliate, Solanum tuberuson, Saccharum spp., Fragaria ananassa, Zinziber officinale. Stevia rebaudina bertoni, Citrus aurantifolia, Actinidia deliciosa, Solanum lacinatun

Medicinal Plants:

Chrysanthemum cinerarifolium, Solanum laciniatum, Piper longum Withinia somnifera, Aloe vera, Swertia chirita, Amonum subulatum, Rauvolfia serpentina, Valeriana jatamansi, Rheum emodi, Cephaelis ipecacuanhe, Swertia ciliata Neopicrorhiza scrophulariifolia

Ornamental Plants:

Chrysanthemum morifolium, Chrysanthemum spp., Dianthus caryophyllus, Gerbera, Gladiolus, Lilium nepalensis, Lilium longiflorum, Rosa rosa, Saintpaulia ionatha, Spathiphyllum wallisii, Syngonium, Asiatic Lily, Primula obconica, Begonia tuberhybrida, Fuschia Hybrida, Antirrhium majus,

Orchids:

Cymbidium giganteum, Cymbedium grandiflorum, Cymbidium longiflorum, Dendrobium densiflorum, Dendrobium fimbriatum, Vanda teris. Dendrobium amoenum Cymbidium aloifolium, Vanilla planifolia,

Bamboo:

Dendrocalamus hamiltonii, Dendrocalamus strictus.

Anther Culture:

Allium fistulosum, Nicotiana tabacum, Oryza sativa.

Content

The in vitro Proliferation of Forest Trees Dalbergia sissoo Roxb. ex Dc

B. Suwal, A. Karki[1] and S. B. Rajbhandary[2]
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Abstract

Multiple shoots were induced on cotyledonary node culture of Dalbergia sissoo in the presence of benzylaminopurine (BAP) at 1.0 mg/l and naphtaleneacetic acid (NAA) at 0.1 mg/l. These shoots continued to proliferate at a sustained rate of 10 15 microshoots over two years of 8 weekly sub-cultures in the basic medium, with the supplement of BAP at 0.25 mg/l. Such microshoots rooted readily in non sterile sand beds with subsequent successful field establishment.

Key words : *Dalbergia sissoo,* cotyledonary culture, multiple shoots, non sterile rooting, field establishment.

Zusammenfassùng

Bei Kotyledonen Nodien Kulturen von *Dalbergia sissoo* wurden durch die Anwendung von Benzylaminopurin (BAP) (1.0 mg/l) und Napthalenessigsäure (NAA) (0.1 mg/l), multiple Sprosse induziert. Diese Sprosse pflanzten sich weiterhin mit einer ununterbrochenen Rate von 10 15 Sprossen über 2 Jahre fort, wobei die Subkulturen 8 Wochen dauern, bei Verwendung des Basismediums unter Zugabe von 0.25 mg/l BAP. Solche Mikrosprosse wurden in nicht sterilem Sand leicht bewurzelt und erfolgreich in Feldversuche ausge-bracht.

Introduction

Dalbergia sissoo is a multipurpose native species which is extensively used in afforestation in the Nepal Terai for production of fodder, fuel and timber. Since there is considerable phenotypic variation between trees in provenance trials of *D. sissoo* raised from seed, genetic improvement will depend on the selection of individual trees with superior characteristics and propagation of these by clonal means. However, cloning of superior material through the use of conventional vegetative cuttings resulted in uneven growth, as only tip cuttings gave rise to straight growing plants, whereas branch cuttings produced trees which retained their characteristics as branches (plagiotrophic growth) (K. White, personal communication). Moreover only 25 percent of the cuttings were successfully rooted (Singh, 1982). An alternative to vegetative propagation by cuttings is to use the tissue culture method of multiplying clones using meristem or shoot tips or buds as the explants.

To develop a process for cloning elite candidate trees of *Dalbergia sissoo* it was thought convenient first to devise a method of producing tissue culture plants using explants from seedlings grown in culture. Once a successful proliferation medium has been established it can then easily be tested for cloning superior individuals through meristem or shoot tip or bud culture.

We report here the shoot proliferation, followed by non-sterile rooting of these shoots, and subsequently field establishment of rooted plants, by culturing *D. sissoo* cotyledons excised from *in vitro* grown seedlings.

Maierials and Methods

Seeds of *D. sissoo* were obtained from the Afforestation Division, Hattisar, Kathmandu. The

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seeds were kept in running water for one hour, followed by a brief dip in water containing teepol, at the rate of two drops of teepol in 100 ml of water. The seeds were then washed five times in distilled water. Finally they were sterilized in 0.1 percent $HgCl_2$ solution fo 20 minutes, and subsequently washed five times in sterilized distilled water. The seeds were transferred to culture flasks (100 ml conical flasks containing 40 ml solidified medium), when the seedlings germinated within four days. Cotyledons excised from the seedlings were cultured on Murashige and Skoog medium (1962) (MS), with the addition of benzylaminopurine (BAP) at 1.0 mg/l and naphtaleneacetic acid (NAA) at 0.1 mg/l, and 1000 mg/l caseinhydrolysate. The medium was solidified with 0.6 percent bacteriological agar with pH adjusted to 5.8 before sterilization. It was sterilized by autoclaving at 15 lb/sq. in for 15 minutes. The cultures were incubated at 25±4°C under a 16 h photoperiod, with light provided by fluorescent tubes at ca 3000 lux.

Under these conditions 2-5 shoots developed from the nodal region of the cotyledon after seven days. These shoots were used in a number of experiments using different concentrations of BAP (0.01 to 10 mg/l) and kinetin (2-4 mg/l), either alone or in combination with NAA (0.01-0.1 mg/l). The cytokinin concentration that supported a high rate of shoot proliferation was selected for further subculture.

Fig. 1 : Development of shoots from cotyledonery node on MS supplemented with 1 mg/l BAP + 0.1 mg/l NAA, and 1000 mg/l caseinhydrolysate

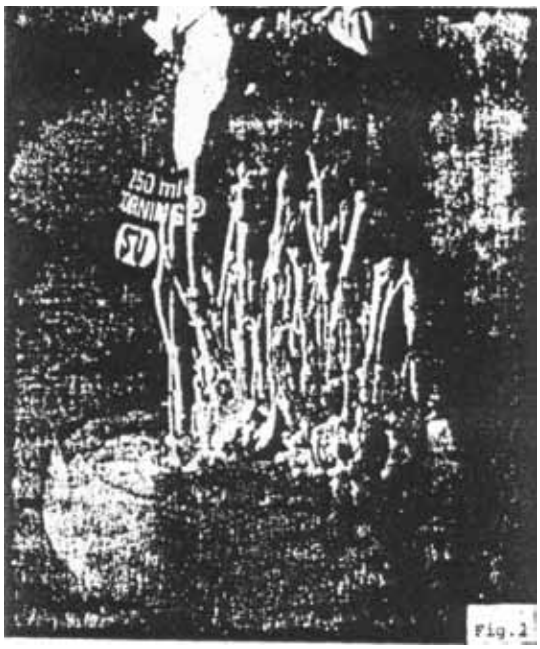

Fig. 2 : Multiple shoot formation on MS supplemented with 0.25 mg/l BAP and 1000 mg/l caseinhydrolysate. Eight weeks in culture.

The sterile rooting step was skipped, and the multiplied shoots were separated out treated in 0.01 percent indoleacetic acid for 5 minutes and transplanted, through the year 1985, to sand beds in a greenhouse. To facilitate rooting relative humidity was maintained at 70-80 percent.

Rooted plants, 10-20 cm high, were transplanted into the soil in the greenhouse, followed by planting out in the field.

Results

When the multiple shoots developed on cotyledonary node (Fig. 1) were transferred to the media supplemented with both BAP at 10-0.01 mg/l and NAA at 0.1 mg/l, callus formation increased with the increase in BAP concentration. At the higher concentrations of BAP (2-10 mg/l), with 0.1 mg/l NAA, brown callus tissues were formed on the parts of the explant in contact with the medium and no development of shoots occurred. BAP at or below 10 mg/l with NAA at 0.1 mg/l induced tile formation

of callus as well as shoots and roots. With 0.01 mg/l NAA, BAP at 1.0 mg/l or below this level also produced callus shoots and roots.

Fig. 3 : Rooted plantlets after 4 weeks in the sand

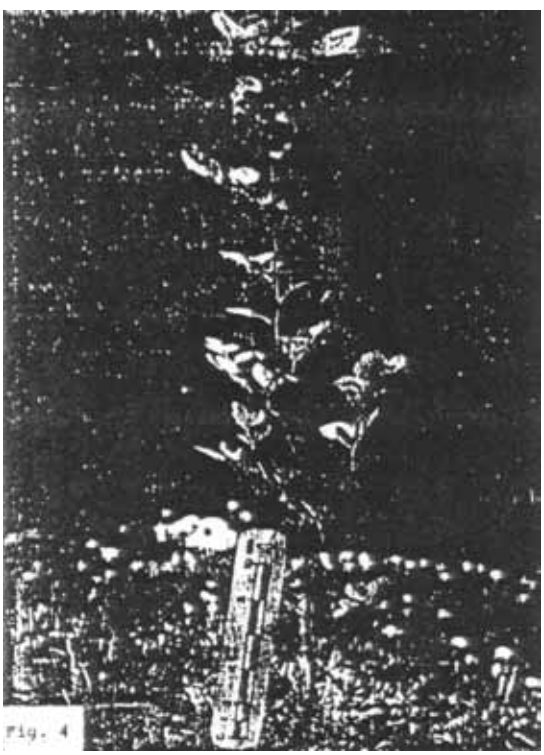

Fig. 4 : Field established plant. Growth of plant in 12 weeks after the transfer.

BAP at 1.0, 0.5, 0.25, and 0.12 mg/l induced the formation and development of shoots. The number of elongating shoots was always higher on the medium containing 0.25 mg/l BAP concentrations. The multiplication rate was 10-15 shoots (0.5-6 cm high) per explant after four weeks of culture in presence of 0.25 mg/l BAP. The shoots have been subcultured for two year without any loss of multiplication potential (Fig. 2).

Kinetin at 2-4 mg/l alone or in combination with NAA at 0.1 or 0.01 mg/l gave rise to only callus tissues.

On the transfer of *in vitro* produced shoots to sand beds over 35 percent developed into rooted plantlets within 10 days, under day and night temperatures of 34° C/l5°C under 70-80 percent humidity. The rooted plantlets grew to a height of 10-15 cm in four weeks (Fig. 3). Such plantlets were transferred to soil in pots and subsequently planted out in the field. All the plants grew straight, without any tendency to horizontal growth (Fig. 4).

Discussion

Cotyledons have been reported to be the most regenerative part of the plant (Murashige, 1974). Of 25 legume species that have been regenerated *in vitro* in five, *Ceratonia siliqua, Indigofera ennccaphylla, Psophocarpus tetragono-lobus, Stylosanthes hamala* and *Trifolium repens* cotyledons were used as explants (Flick et al., 1983). Chang et al. (1980) reported multiple shoot bud formation of soybean from cotyledonary node culture. Usha Mehta and Mohan Ra (1980) observed fascinated shoot bud development at the cotyledonary node culture of *Cajanus cajan.* Our results corroborate the findings of the above workers.

In vitro micropropagation of *Dalbergia sissoo* through axillary bud proliferation was easily accomplished using excised cotyledons as explants. Therefore it is reasonable to assume that the procedure developed in the present work has possibilities in the cloning of elite genotypes of *D. sissoo* from meristem or shoot tip or bud cultures.

The fact that it was possible to omit the sterile rooting stage (Karki and Rajbhandary, 1984), and the successful rooting of over 85 percent of the shoot cuttings in non sterile sand beds, suggests the commercial applicability of using this method in large scale plantations of *D. sissoo*. Mukho-Padhya and Mohan Ram (1981) reported the formation of 2 or 3 plantlets in 30-45 days from root culture of D. sissoo. Their method involved the use of aseptic cultures in both the shoot induction and rooting stages. This would therefore, limit the possibility of using this method in the large scale production of *D. sissoo* plants.

Since no loss of multiplication potential in *D. sissoo* shoot culture was observed in over two years of 8 weekly sub-cultures, production of more than a million *D. sissoo* plants from a single plant in a year is apparently feasible by use of the present method. Our observations are consistent with the suggestion by Bonga (1977), that the solution to the problem of producing large number of trues of improved qualities on shortened rotations could possibly be obtained through the use of micropropagation techniques.

However, despite the great advantages offered by tissue Culture propagation methods, to date only a few species in the genera *Populus, Eucalyptus, Betula*, *Liquidambar, Acacia, Ulmus, Castanea, Tectona, Santalum,* and *Alnus*, have been micropropagated (Perinet and Lalonde, 1983).

Acknowledgements

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In vitro propagation of *Dalbergia sissoo*

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Summary

In vitro propagation from the cotyledonary node of *Dalbergia sissoo* seedling has been successfully practiced. A field trial has shown that the resulting plants compare well with those raised by traditional methods.

Introduction

Large numbers of plants are required for reforestation, to increase the supply of fuelwood, fodder and timber. Vegetative propagation can be used to multiply trees with desirable heritable characteristics, including rapid growth and good form. Vegetative propagation from cuttings, however, requires the maintenance of mother trees in hedges or orchards, and each tree a provide at most only a few hundred cuttings a year.

Tissue culture can give a much greater rate of multiplication; a million plants can be produced in one year from a single explant. Because the plants are produced under sterile laboratory conditions they have the added vantage of being free from pests and pathogens.

Tissue culture was started in Godavari 1976, beginning with orchids, potatoes, and medicinal plants. In 1983 Mr Emrald J. Rana, then Secretary to the Ministry of Forests and Soil Conservation, suggested that tissue culture of *Dalbergia sissoo* should be tried. It is a good multipurpose species, very popular with the people of the terai for fodder, fuel, and furniture.

In vitro propagation

Although it might have appeared more rational to use mature tissue from elite trees, difficulties were encountered in the sterilization of such tissue, and so it was decided to make the first experiments with seeds as be starting material this is a common procedure when starting tissue culture experiments with a new species. The object was to find a medium that would support sustained shoot proliferation, and which might later be used with other kinds of starting material.

Seeds of *D. sissoo* were washed in running water for one hour, and then briefly dipped in detergent (two drops of Teepol in 100 ml water). The detergent was washed off with five washings of distilled water. The seeds were then sterilized in 0.1% $HgCl_2$ Solution for 20 minutes, and this was washed off with five washings of sterile distilled water. The seeds were then placed on solidified agar (0.7%

Table 1 : Murashige & Skoog medium (1962)

*Mineral salts*mg/litre	
NH_4NO_3	1650
KNO_3	1900
$CaC1_2.2H_20$	440
$MgSO_4.7H_2O$	370
Kl	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.3
Vitamins	
Inositol	100
Nicotinic acid	0.5
Pyridoxine.HCl	0.5
Thiamine.HCl	0.1
Amino acid, Glycine	2.0
Sucrose (g)	30
Supplements for shoot proliferation	
Benzylaminopurine	0.26
Casein hydrolysate	1000

bacteriological agar) in conical flasks (100 ml flasks containing 40 ml of agar), covered with aluminium foil. All these aseptic operations were carried out under a laminar flow hood (where there is only clean air).

The seeds germinated in four days. The cotyledons were excised, and cultured in Murashige and Skoog medium, with additional casein hydrolysate and benzylaminopurine. See Table 1.

Fig. 1 : Shoot proliferation at ten weeks on Murashige and Skoog medium supplemented with benzylaminopurine and casein hydrolysate

A proliferation rate of 10 15 shoots every 8 12 weeks was obtained and this has been maintained over the last four years by a sequence of subcultures. See Fig. 1.

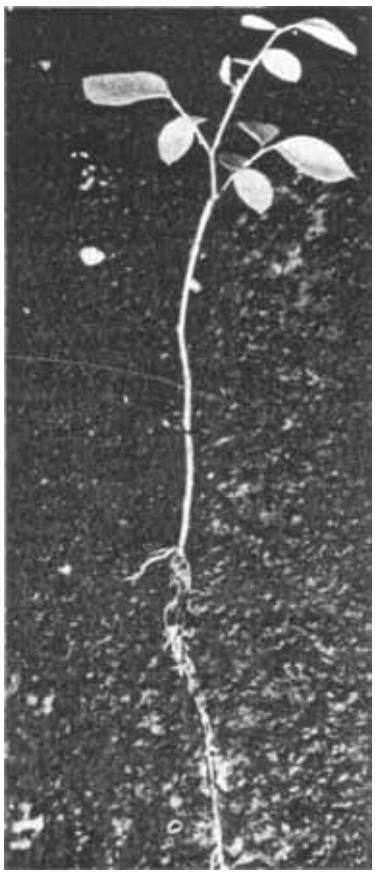

Fig. 2 : Rooted plant after four weeks in the sandbed

Table 2 : Sagarnath sissoo provenance trial (planted 9 September 1985)

	24 November 1985		24 November 1986	
	Av. ht. (cm)	Survival/50	Av. ht. (cm)	Survival/50
Plants of seedling origin: provenance				
Sunsari	24.1	46	189	37
Sagarnath	27.6	46	188	38
Kailali	22.6	45	240	40
Sagarnath	28.4	46	210	43
Banke	25.9	48	230	44
Jhapa	24.4	46	234	41
Kosi	23.5	46	214	41
Stem cuttings (from Sagarnath)				
(1)	30.7	44	212	40
(2)	31.3	47	235	44
Tissue culture	19.2	38	214	36

The shoots obtained in this way were rooted in non sterile sand beds. A high proportion was successfully rooted; it was more than 80% in the months June and July (withaverage day/night temperatures of 34°C/l5°C and 70-80% relative humidity. The roots developed within ten days. See Fig. 2.

Field Trails

In September 1985 more than 200 plants were uprooted from the sandbed, wrapped in moist paper, and transported fron Godavari (near Kathmandu) to Sagarnath in the terai, a journey of some six or seven hours. Two lines, each of fifty tissue cultured plants, were laid out along with other lines of plants raised from seed of various provenances, and some from traditional shoot cutting. Table 2 records the survival and average heights measured November 1985 and 1986. See also Fig. 3. So Far it seems that tissue-cultured plants have a similar to that of traditionally pro-pagated ones, but the plots will continue to be observed over the next few years.

Conclusions

The success so far achieved has justified continuing experiments with the propagation of *Dalbergia sissoo* from other kinds of tissue, taken from selected mature trees.

Acknowledgements

Thanks are due to Mr K.J. White for his keen interest in this work and for including the tissue cultured plants in the Sagarnath plots, and to Mr B. Roy for providing the data for Table 2 and the photograph used as Fig. 3.

Keywords: *Dalbergia sissoo;* tissue culture.

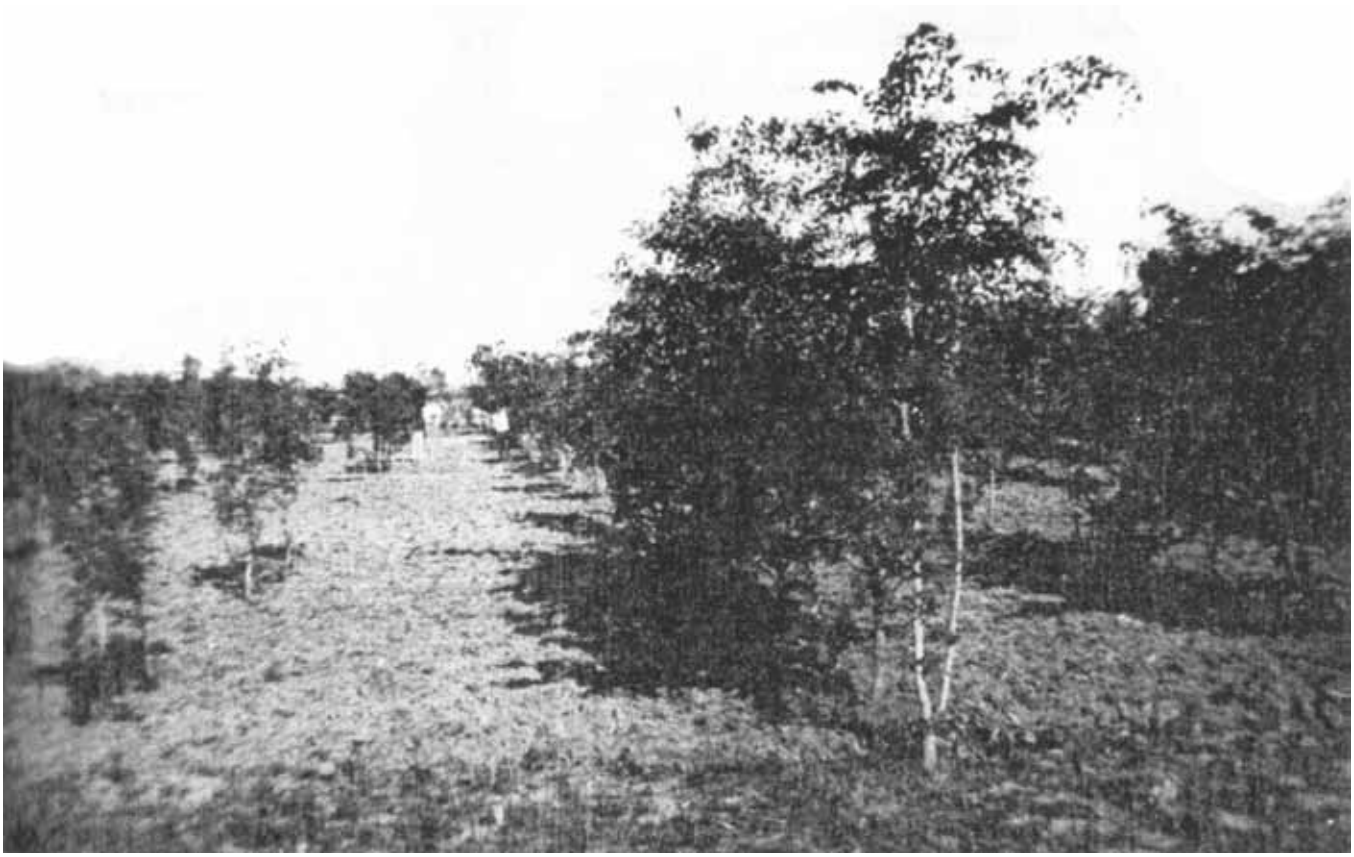

Fig. 3 : Sagarnath provenance trial at nineteen months the trees derived from tissue culture are in the centre.

Micropropagation of Potato Cultivars and Their Field Performance

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Abstract

Seven potato cultivars obtained as test tube plants were multiplied rapidly by using tissue culture method of propagation. The tissue culture derived seed tubers of these cultivars and the other 'Cardinal' cultivar were planted in the field to assess the growth yield and late blight resistance. In the spring as well as in the autumn plantations in 1986, the two CIP cultivars 1853 and MS 91.18 were found resistant to the late blight where as the other CIP cultivars MS 82.60, MS 35.22, BR 63.15, and Sangema showed various levels of late blight infection. Cardinal cultivar was found to be severely infected by the late blight.

Introduction and Review

Potato is one of the important crops of the world. In the developing countries the potato crop is vulnerable to attack by more than 268 diseases and pests (Mendoza H.A,4). Some of these are distributed worldwide, and cause extensive destruction. For example, the late blight can reduce more than 50% of the total production of the crop in Nepal (Khergoli L.P,2). Within certain geographical zones there are some localised insects which may damage the crop.

In order to improve the quality of potato crop, the disease free seed tubers of suitable cultivars and the cultivars resistant to pests and pathogens should be used.

The developing countries, however, depend upon developed countries for the supply of such certified seed tubers. This accounts for 40-60% increase in the total production cost of a potato crop (Wattemina, 7). Several countries therefore, use some sort of multiplication technique in their basic seed production program. In recent years tissue culture for germplasm maintenance in a disease free state has been a great break-through for the production of clean planting materials. In addition, the advantage of rapid clonal propagation through tissue culture has generated increasing interest among the plant breeders. In view of the potential application of tissue culture in potato crop improvement, plant regeneration via multiple shoot proliferation was carried out with seven International Potato Centre (CIP) cultivars and the Cardinal cultivar. This was followed by field evaluation of these cultivars at Godawari during spring plantation (January 1986) and autumn plantation (August 1986).

Materials and Methods

Two test tubes containing four 'in vitro' plants of each of seven potato cultivars, 1853, MS 91.18, MS 35.22, MS 82.60, MS 42.3, BR 63.15 and Sangema were received from CIP, Peru in September 1983, through National Potato Development Programme (NPDP). The plants from one set of test tubes were planted directly in pot, and from the other set of test tubes nodal cuttings were transferred in Murashige and Skoog medium (Murashige and Skoog, 5) supplemented with 1.5mg/l kinetin, 1mg/l 6 benzyl amino purine (BAP) and 1000mg/lcasein bydrolysate, This medium was used because the Cardinal cultivar was successfully micropropagated in the medium (Manandhar, A,3). The medium was solidified with 0.7% becteriological agar with pH adjusted to 5.8 before autoclaving. The cultures were incubated at 25°C±with 16 hrs. photoperiod (3000lux) provided by fluorescent tubes. New shoots

were developed from auxiliary buds. After 6 weeks of culture, the shoot tips were harvested and placed horizontally in the fresh medium. After 4-6 sub cultures, uniform shoot growth and multiplication were established. For rooting, excised shoot tips were treated with 100ppm indole acetic acid for 5 minutes and planted in non sterile potting mixture in green house under plastic cover. Rooting was observed after 10-12 days under humid tent. Thus, the rooted plantlets were transferred to soil mixture in the seed box and left to harden for two weeks in green house in open condition before field transplantation.

The well rooted plantlets were planted in the field. The spacing from one plant to another was 15 cms and from one row to anther was 70 cms. After three months period that plants produced the tubers. These tubers were cultivated in two growing seasons in 1986 in the field at Godawari.

Results and Discussion

When directly planted in potting mixture, the 'in vitro' plants of seven CIP cultivars became dead. However, one plant each of MS 42.3 and MS 35.22 survived. They produced small tubers after five months of growth.

The nodal cuttings of different cultivar proliferated uniformly after 4-6 subcultures. Such cultures produced 40-50 healthy micro cuttings from a single shoot in 8 weeks' period. The multiplied shoots lacked visible roots, a few aerial roots were, however, observed. Treatment with 100 ppm indole acetic acid (IAA) resulted in successful induction of roots after 10-12 days in the non-steriled potting mixture. The rooted plants were then transplanted in field and they produced tubers after three months. The yield was not high due to late plantation (Table I).

The seed tubers of different cultivars were planted side by side in the same area in Janaury, 1986. Tubers of eight cultivars were harvested after three months (Table II).

The tubers of Cardinal, MS 42.3, MS 35.22, MS 82.60, MS 91.18, BR 63.15 and Sangema were attacked by red ants in various levels, whereas the tubers of 1853 were found totally unaffected by red ants. Late blight appeared only in Cardinal during the harvesting period.

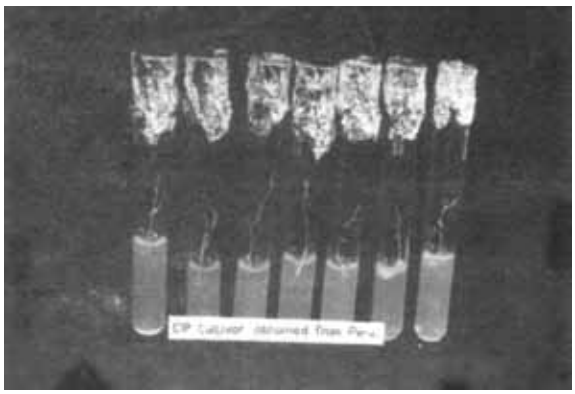

Fig. 1 : Culture Tubes from CIP.

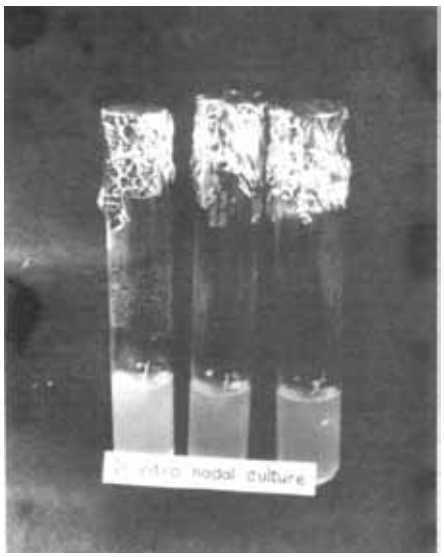

Fig. 2 : In vitro culture of nodal cutting from CIP cultuvars.

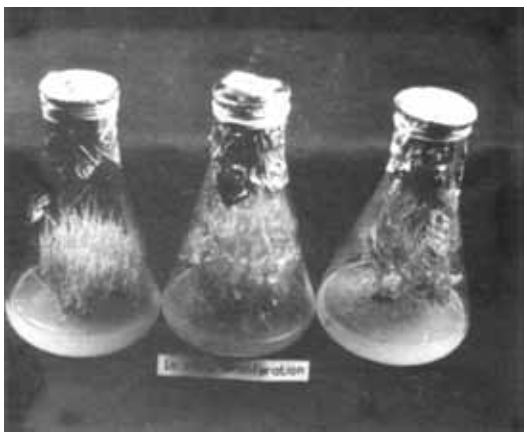

Fig. 3 : In vitro proliferation

Micropropagation of Potato Cultivars and their field performance.

Table I : The yield (fresh wt.) from Tissue culture plants of different cultivars of potato

Cultivars	Plantation	Harvesting	Average No. of potatoes per plant	Average wt. per plant (g)
I 853	27.3.1986	18.6.1986	15	151
MS 91.18	19.3.1986	15.6.1986	21	247
MS 42.3	24.3.1986	21.6.1986	30	377
MS 35.22	25.3.1986	20.6.1986	17	365
M9 82.60	10.3.1986	8.6.1986	11	160
MS 63.15	16.3.1986	15.6.1986	10	118
Sangema	31.3.1986	28.6.1986	10	85
Cardinal	31.3.1986	28.6.1986	14	390

Average of 100 plants

Table II : The yield (fresh wt.) of different cultivars of potato grown during spring (1986) at Godawari.

Cultivars	Average No. of potato es per plant	Average wt. per plant (g.)	wt of potatoes per sq. meter (Kg)	Max. yield (gm/plant)	Min yield (gm/plant)
I 853	19	543	3.75	850	175
MS 91.18	12	697	4.41	1025	300
MS 42.3	18	383	2.06	625	170
MS 35.22	12	364	2.21	750	175
MS 82.60	15	248	1.43	350	180
DR 63.15	9	317	1.90	600	200
Sangema	8	424	2.57	525	250
Cardinal	16	474	3.57	875	225

Average of 100 plants

Table III. The yield (fresh wt.) of different cultivars of potato grown during Autumn (1986) at Godawari.

Cultivars	Average No. of potatoes per pl.	Average wt. per plants (g)	wtof Potatoes per sq. meter (kg.)	max. yield (gm/plant)	Min yield (gm/plant)
I 853	21	502	3.16	1550	200
MS 91.18	7	425	2.71	975	230
MS 35.22	7	247	1.63	900	160
MS 82.60	6	296	1.78	830	150
DR 63.15	8	309	1.86	490	200
Sangema	8	267	1.53	460	175
MS 42.3	14	225	1.35	250	
Cardinal	18	91	0.56	120	

Average of 100 plants

Similarly, the seed tubers of eight cultivars were planted in the following season i.e. in August 1986. one month of plantation, the Cardinal was severely attacked by late blight. The yield was reduced drastically. In MS 42.3 disease appeared after 50 days of plantation. So, the yield was not satisfactory. Other cultivars such as BR 63.15., MS 35.22, MS 82.60 and sangema were found slightly susceptible to late

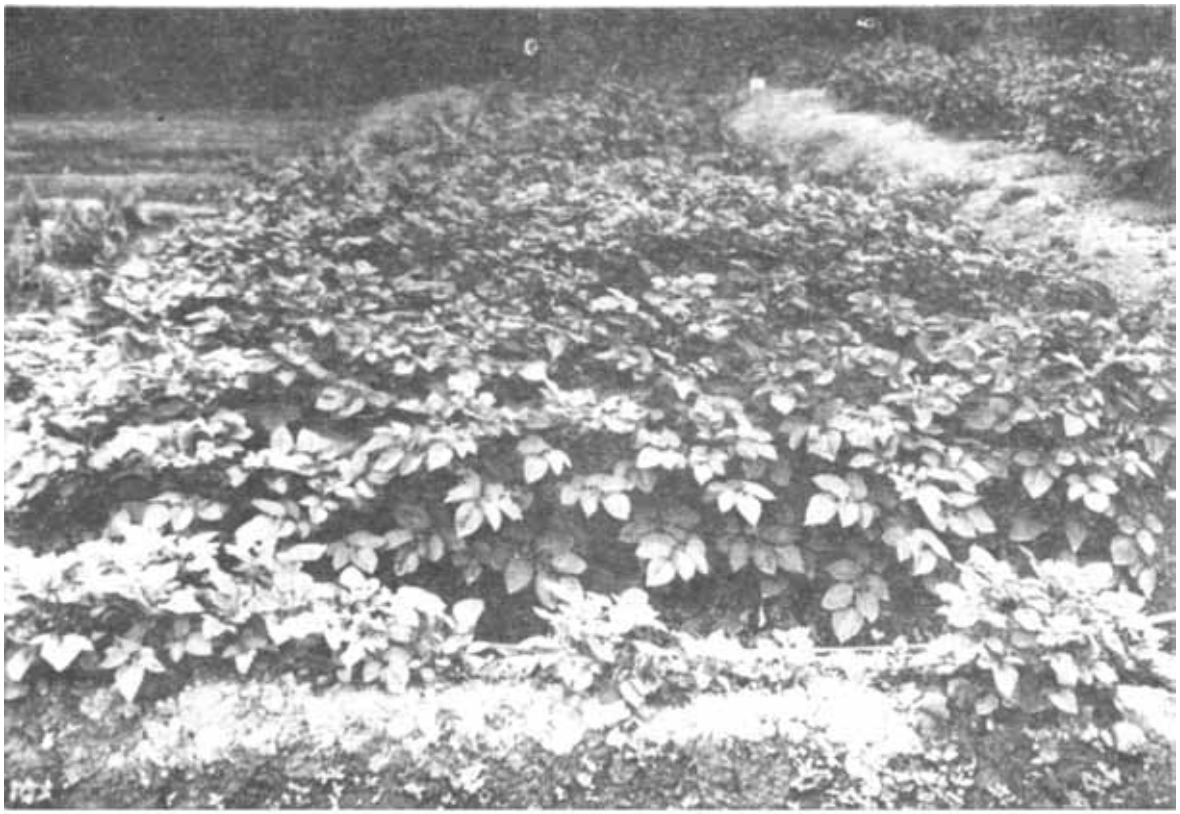

Fig. 4 : Micropropagated plants establish in field.

Fig. 5 : Plants with tubers.

blight. The two cultivars I 853 and MS 91.18 were planted along with the susceptible cultivars. The yields of tubers of I 853 and MS 91.18 were 3.61 kg and 2.71 kg per sq. meter area respectively (Table III).

Conclusion

Late blight is probably the single most serious disease of potato world wide. The occurrence of disease mostly depends upon the temperature, moisture, light intensity as well as the lost cultivar. The disease could be controlled by planting resistant cultivars in late blight affected areas. It is known that a cultivar which shows resistance to late blight in one locality may not be resistant in an other locality. Therefore, the cultivars MS 91.18 and 1 853 which have shown resistance to late blight at Godawari should be tried at different agroclimatic regions to determine their reaction against the late blight infection.

In the areas like Godawari, the local people are discouraged to grow potato because the soil is inhabited by red ants. These insects heavily damage the tubers.

The 1853 cultivar was observed to be free from red ant attack in January plantation and in August plantation. Therefore, in order to extend this cultivar in large scale plantation in the red ant problem areas, further field trials should be carried out in different locations.

Acknowledgement

The authors are grateful to Dr. S.B. Malla, Director General, Department of Medicinal Plants, Ministry of Forest and Soil Conservation, for facilities provided and Mr. B.K. Bhomi, Chief, National Potato Development Programme, Department of Agriculture, Khumaltar, Kathmandu, Nepal. for supply of invitro materials from CIP, Peru.

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In Vitro Propagation of Trifoliate Orange *(Poncirus trifoliata)*

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Abstract

In the culture of *Poncirus trifoliala,* a Citrus root stock obtained from Japan, the shoot induction was caused in the Murashige and Skoog medium (1962) (MS) in the presence of benzylaminopurine (BAP) 1.0mg/l and napthalene acetic acid (NAA) 0.1mg/l when cotyledonary node was used as explant. On subculture these shoots continued to proliferate in the basal medium supplemented with lower concentration of BAP 0. 1 mg/l and NAA 0. 1mg/l.

Roots were produced in shoots when transferred on basal medium supplemented with NAA 0.1mg/l.

Introduction

Citrus is a common fruit which is used both as a fresh or a processed product. In Nepal Citrus fruits are imported in a large scale from the neighbouring country India. Most of the Citrus orchards of local variety are destroyed by pests and viral infection. So selection of cultivars resistant to pests and diseases has become important to improve yield and quality. Breeding and selection of new scion and root stock varieties are being undertaken in many horticulture centers. In any controlled breeding programme designed to select root stocks, it is important to have a large numbers of clonal Seedlings for screening (Barlass and Skene 1982). It can take a longtime to produce a large number of seedlings (disease free) by conventional vegetative propagation. Citrus is mostly polyembryonic and the seeds are nucellar in origin. Frost and Soost 1968 stated that theoretically all nucellar trees should be genetically identical to the mother tree. The nucellar seedlings possess no problem when used as root stock, but the plant from the seed takes five years or more to flower (Button and Kochba 1977).

It is now recognised that an alternative to vegetative propagation by cutting or by seed germination is to use the tissue culture method of multiplying clones using meristems or shoot tips or buds as the explants. Advantages of in vitro method of propagation using shoot tip or bud explant include rapid multiplication (over one million plant per explant in a year), and production of true to type progeny and disease free plants.

The culture of Citrus tissues in vitro has been reported by Maheswari and Rangaswamy 1958, Rangaswamy 1958 & 1961, Sabharwal 1963. Kochba et. al 1972 and Mitra and Chaturvedi 1972 observed embryogenesis in the embryo and nucellar tissue cultures (Button and Kochba 1977).

Grinblat 1972, Chaturvedi and Mitra 1974, Raj Bhansah and Arya 1978 reported the development of normal shoots from Citrus callus cultures via formation of adventitious buds. Barlass and Skene 1982 observed regeneration of Citrus plants in vitro from vegetative tissue culture. The proliferation of shoots by culturing the cotyledonary node and stem nodal segments excised from aseptically grown seeds and rooting of these shoots are reported here.

Methodology

The virus free seeds of trifoliate orange were obtained from Japan. The seeds were washed in running water for one hour, then in teepol water for 5 minutes. The seeds were then washed with 95 percent rectified spirit for 1-2 seconds, and with 0.1 percent mercuric chloride for 25 minutes, and again washed with sterilized water 5 times. The seeds were cultured in the 100 ml culture flasks with 40ml of MS medium.

The seeds germinated after 3 weeks. The explants, cotyledonary nodes were excised from 3-6 weeks old seedling (3-5 cm. in size). The seed coat was removed from the cotyledon and were cultured in MS medium, which was supplemented with BAP 1.0mg/l NAA 0.1mg/l and casein hydrolysate 1000mg/l the pH of the medium was adjusted to 5.8 before autoclaving. Bacteriological agar 0.6 percent was added to solidify the medium. The medium was autoclaved at 151b pressure for 15 minutes. The cultures were incubated at 25±4°C under 15 hour photoperiod.

After 3 weeks of culture 8-10 shoots developed from the nodal region of the cotyledon. The nodal segments of these shoots were further sub cultured in different concentration of BAP ranging from 1-0.5mg/l either alone or in combination with NAA 0.01 0.1 mg/l. The combination of cytokinin and auxin which had the high rate of proliferation and well developed shoot was selected for further sub culture.

For rooting the shoots were excised and cultured in the culture tube containing 20 ml of MS medium with NAA 0.1 and 0.5mg/l.

Results and Discussion

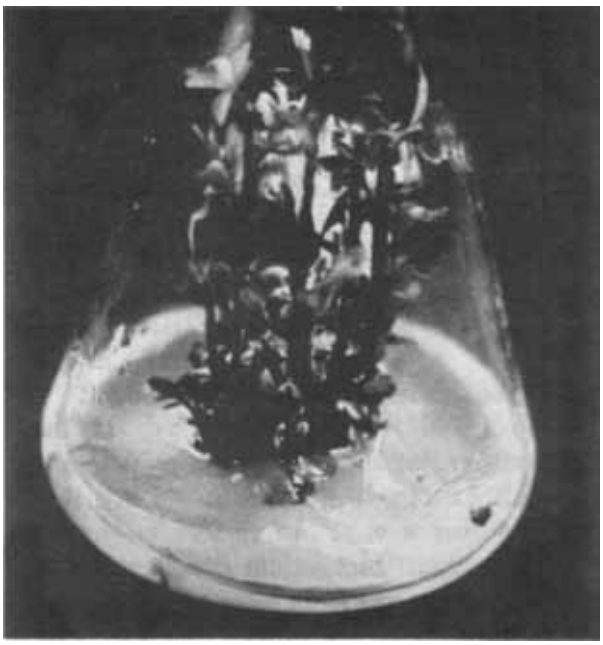
After 3 weeks of culture, 8-10 shoots developed from the cotyledonary node. If it was kept for 6-8 weeks 30-40 shots (Fig 1) would have developed from the single cotyledon.

The multiple shoots were excised and sub cultured in the media supplemented with BAP at 0.1-5.0 mg/l. In higher concentration of BAP (5.0mg/l) the explant became yellowish. 20-30 shoots buds developed in concentration of BAP 1.0mg/l with NAA 0.1mg/l, but the proliferated shoot buds had stunted growth. In lower concentration of BAP (0.1-0.5mg/l) with NAA 0.1mg/l in 6 weeks of culture the number of proliferated shoots were 15-20 with 2-4cm long shoots.

The growth of proliferated shoots was found optimal in lower concentration of BAP 0. 1mg/l with NAA 0.1 mg/l. The absence of NAA also reduced the growth of the shoots. The number of proliferated shoots were higher in BAP 0.5mg/l and 0.25mg/l, but the growth was not so good as in BAP 0. 1mg/l (Fig 2).

Fig. 1 : Multiple shoot production from cotyle-donary node. X°96

The root, of the shoots developed after 3 4 weeks of shoot transfer in MS medium supplemented with



F:g. 2 : Multiple shoot production from nodal segments in BAP 0.1mg/l and NAA 0. 1mg/l. X °94

NAA 0.1mg/l. One to two well developed roots developed from the cut end of the shoot (Fig 3).

This observation on cotyledonary node culture is consistent with the report by Barlass and Skene 1982, on multiplication of shoots from cotyledonary node culture and the nodal segments of shoots culture of Citrus.

For the culture of woody species in vitro the use of 6 benzylaminopurine is increasingly reported. It has been successfully used to stimulate shoot production from cotyledonary node of *Dalbergia* sissoo (Suwal et. al 1987). In Citrus species the use of this cytokinin has been reported by Grinblat (1972), Chaturvedi and Mitra (1974), Raj Bhamsali and Arya (1978) and Barlass and Skene (1982).

Fig. 3 Root development on an excised shoot on NAA 0.1mg/l. X °8

Conclusion

The aim of this work was to develop a method of tissue culture propagation of trifoliate orange since such a method can be used to multiply and desirable clone very rapidly.

These results with micropropagation of *Poncirus trifoliata* indicate the possibility of producing a large number of plants under controlled condition in disease free state. This would also suggest the potential of multiplying the selected scions using tissue culture method.

Transport of cultivars in aseptic conditions reduces the danger of introducing harmful pests and diseases, thereby simplifying quarantine requirements.

Acknowledgement

We are thankful to Dr. S.B. Malla, Department of Medicinal Plants, Ministry of forest and Soil Conservation, Kathmandu, Nepal for facilities provided and Mr. Kundo for providing the seeds.

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Micropropagation *of Brassica oleracea* var Capitata Through Cotyledonary Node Culture

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Abstract

Shoot proliferation of *Brassica oleracea* var capitata. (K.K. cross) was observed in Murashige and Skoog (MS) medium supplemented with Benzylarninopurine (BAP) 1.0 mg/l and naphthalene acetic acid (NAA) 0.01 mg/l, when cotyledon was used as explant. These microshoots developed root on transfer to unsterile sand under ordinary green house condition.

Introduction and Review

Brassica oleracea L. var. capitata (Cabbage, KK Cross) is a commonly cultivated vegetable crop in Kathmandu. The hybrid plant is tolerant to heavy rain and high temperature and can grow both in summer and rainy season, when other varieties are difficult to grow.

Vegetable Development Section, Ministry of Agriculture, Khumaltar obtained seeds from Japan. The seeds were of F1 generation. The farmers favour this variety more because the yield is high in comparison to other varieties. Since tissue culture technique offers the possibility of obtaining a large number of clonal plants, we have attempted to clone the hybrid plants *of Brassica oleracea* L. var. capitata via organo genetic pathway.

In vitro regeneration of Brassica. plant through cotyledonary node culture has been described by Bajaj and Neitsch (1975) and Wang and Loh (1987).

In the present paper we described multiple shoot formation from cotyledonary node culture with subsequent unsterile rooting and field establishment.

Materials and Methods

Seeds of *B.oleracea* var. capitata (K.K. cross) were obtained from vegetable Development Section, Khumaltar. The seeds were washed in running water for two hours and sterilized in 10 percent calcium hypochloride solution for 10 minutes. The seeds were washed four to five times with autoclaved distilled water and germinated aseptically on basal medium consisting of Murashige and Skoog (MS) medium. The medium was solidified with 0.65% oxoid agar. The pH of medium was adjusted to 5.8% prior to autoclaving at 15 lb pressure for 15 minutes. Ten seeds were placed in a sterilized 100 ml. conical flask containing 20 ml. of MS medium. The seeds were incubated at 25°C with 16 hours photoperiod (3000 lux) provided by fluorescent light.

The seeds germinated in 10-12 days. After two weeks, the seedlings were excised and cultured in MS medium supplemented, with 1.0 mg/l benzylaminopurine (BAP) and 0.01 mg/l naphthalene acetic acid (NAA) (Fig 1). In eight to ten weeks, the shoots were proliferated and were about 3-4 cm long. The proliferated shoots were excised for subculture.

Rooting was done by treating the excised microshoot pieces in 0.1% Indole Acetic Acid (IAA) for 5 10 minutes. The treated shoots were rooted in unsterile sand box containing sand and dried leaves (1: 1 by volume). The seed boxes were covered with polythene sheets to maintain high humidity. When roots were well developed, the plantlets were transplanted to the conventional seed boxes containing soil, sand and' compost. After 3-4 weeks the rooted plants were transplanted to the field for establishment.

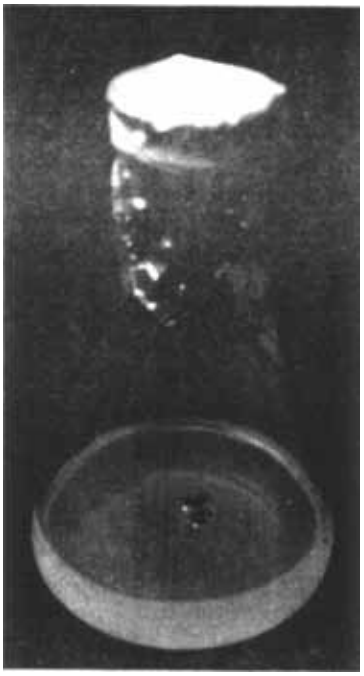

Fig. 1. Cotyledonary node culture on MS supplemented with 1.0 mg/l BAP + 0.01 mg/l NAA, and 1000 mg/l Casein hydrolysate.

Results and Discussion

After 10-12 weeks of culture shoot proliferation was observed in MS medium supplemented with BAP 1.0 mg/l and NAA 0.01 mg/l (Fig 2). In BAP 1.0 mg/l and NAA 0.1 mg/l. The number of shoot proliferation was in between 10-15, but in BAP 1.0 mg/l and NAA 0.01 mg/l, the number was generally 25 30. The number of shoots were identical in subculture also. Callus tissues (0.5-0.8 cm in diameter) were frequently formed at the basal end of the explants.

Roots appeared 10 12 days after transfer to sand. To harden these plantlets were transplanted to the seed boxes containing soil, sand and compost for 3-4 weeks (Fig 3). The rooted plants were transferred to the field and were successfully established (Fig 4).

Bajaj and Nietsch (1975) reported the cotyledonary culture of *B. oleracea* va. capitata (red cabbage) in MS medium, with IAA (1 part 10 6) and Cm (15%). Wang and Loh (1987) observed the regeneration of *B. alboglabra* var. pipple leaf plant through cotyledon culture in MS medium containing 1.0 mg/ l.0 Kn and 1.0 mg/l NAA. In this concentration they found the maximum number of 25 proliferated shoots per explant. But in our observation the higher concentration of NAA (0.1 mg/l) found to be the inhibitory factor in the number of proliferated shoots. Low concentration of NAA enhances shoot proliferation (25-30).

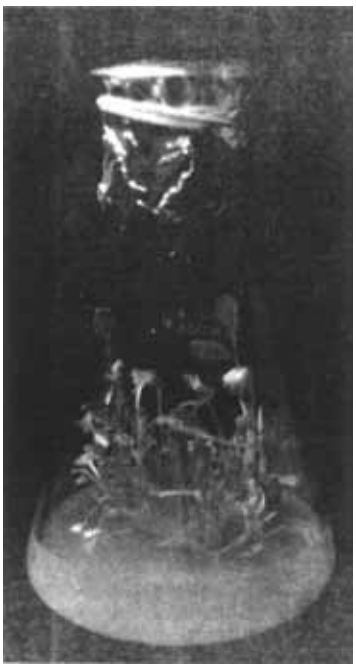

Fig. 2. Multiple shoot formation on MS supplemented with 1.0 mg/l BAP + 0.01 mg/l NAA and 1000 mg/l Casein hydrolysate. Eight weeks in culture.

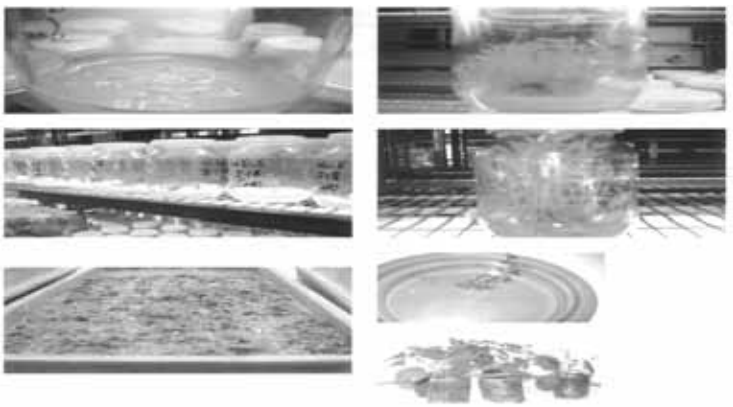

Fig. 3. Rooted plants in seed box after 6 weeks.

Conclusion

Thus, the present work has shown that the shoot proliferation can be induced in cotyledonary node culture of F1 hybrid cabbage, which leads to yield a predictable number of plantlets. The technique shows it's wide application for mass propagation of

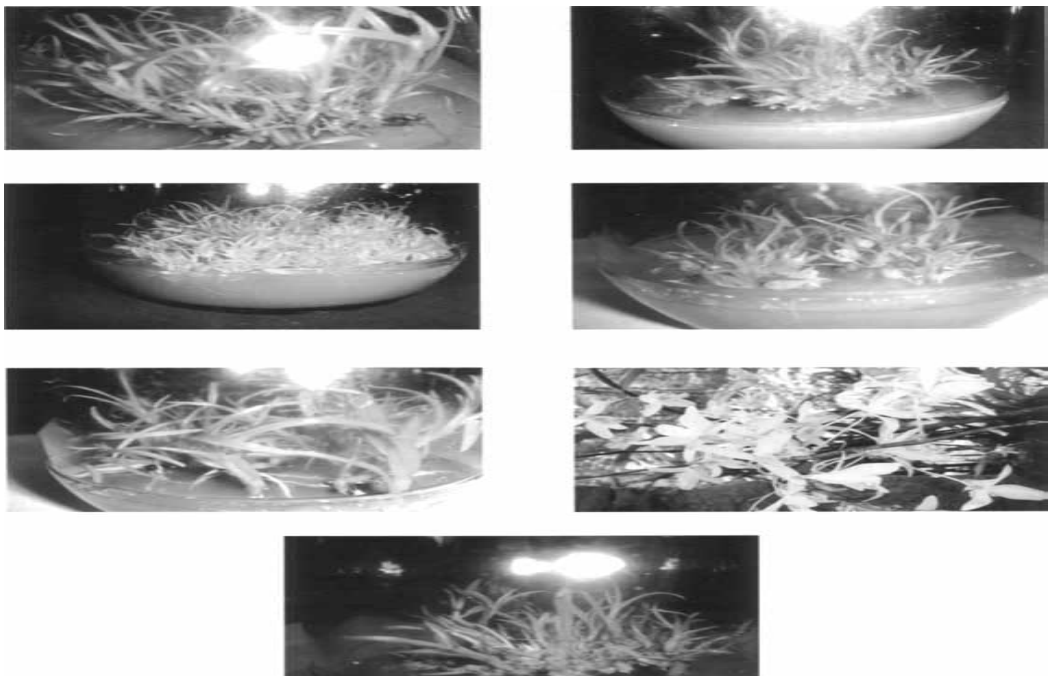

Fig. 4. Field established plant. Growth of plant in 6 weeks after the transfer.

F1 hybrid, The direct unsterile rooting of multiple shoots is an added advantage to cost effective production of tissue culture plants (Karki and Rajbhandary, 1984).

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Plant Tissue Culture Method of Propagation and its Potential

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Abstract

A brief description of tissue culture activities in the Herbarium and Botanical Laboratory building, Godawari is discussed. Achievements with regard to development of feasible methods of producing tissue culture plants are projected. As an example, the significance of using tissue culture seed potatoes in increasing the yield by more than 100% is discussed. Need for improvement of agricultural, horticultural and forestry crops through the use of tissue culture techniques is emphasized.

Introduction

A scientific thought of culturing plant cells was first put forth by a German Scientist, Haberlandt in 1902 (1). This was followed by the successful culture of root tips by Robbins and Kotte simultaneously in 1922 (2). It was, however, only after the work of George Morel in 1960s (3) that the application of tissue culture technique was widely used in commercial production of orchids. Following this, seeing the obvious advantages of tissue culture technique in rapid propagation of healthy plants a number of dicots and monocots belonging to agriculture, horticulture and forestry were experimented and successful micropropagation methods were developed in various laboratories of the world.

A brief history of tissue culture in the Department of Medicinal Plants (DMP):

In Nepal the tissue culture work began in the newly constructed Herbarium and Botanical Laboratory building, Godawari in 1976. Initially the objective of the work was to see if the alkaloids could be produced in callus or suspension culture system. A highly proliferative callus culture *of Rauwolfla serpentina* was achieved, and the culture had sustained growth rate over several years of 8 12 weekly subculture. However, no alkaloid could be detected in these cultures. So the effort to isolate alkaloid from the cultures was abandoned. And it was thought that a pragmatic approach to use the tissue culture technique would be to develop procedure that could be used for rapid propagation of plants in disease free state. Moreover, at that time the DMP wanted to have field trails of *Solanum laciniatum* but the number of seeds available then was very few. So, as a result of tissue culture approach to multiply the plants, a method of initiating shoots from leaf culture of S. laciniatum was developed (4). The plants were produced and successfully field establisbed. In this procedure the conventional method of tissue culture was followed, (a) culture establishment (b) shoot proliferation, (c) in vitro root initiation, and (d) shoot proliferation. In such procedure although the potential of shoot multiplication is at a level of over 106 shoots a year, the rooting of these shoots could hardly be worked out at 400 per person per day. A skilled man power would not be a pragmatic proposition for producing tissue culture plants. Moreover, in this procedure the cost of producing a tissue culture plant would exceed Rs. 1.50. So our further efforts were directed to bypass this rooting stage in vitro. The first skipping of this stage was achieved with Pyrethrum when the in vitro multiplied shoots were successfully rooted out but in the sand bed (5). Following this, the method was extended to other plants, as a result of which several cultivars of potato, and *Dalbergia sissoo* were successfully field established by non sterile rooting in sand. From this two step procedure a scientific person can produce 8,000 to 10,000 shoots per day which can then be easily handled by semi skilled persons for rooting them in sand beds. Hence, the production cost is drastically reduced to only 30-50 paisa per tissue culture plant.

In view of the experience so far achieved in our laboratory it is apparent that any plant can be put to micropropagation method development. Those plants that are regarded as recalcitrant to tissue culture method of propagation can be tested with various manoeuvering of the media composition and incubation conditions which may result in development of a successful micropropagation method. For instance, we can quote our work on the field establishment of tissue culture derived from bamboo plants which until now, except two reports from India, bad failed to induce shoots in other laboratories. A correct balance of media compositions, hormones, and incubation condition is perhaps critical to success and failure of micropropagation method development.

Rapid Method of Propagation:

Micropropagation now provides a very powerful tool for rapid cloning of elite individuals of horticultural plants or forest trees. Commercial production of tissue culture plants are already in operation in the developed countries. Tissue culture plants are gaining popularity not only in the developed countries but also in developing countries. Its advantage is well illustrated in Vietnam where the introduction of tissue culture potatoes has increased the yield from 2000 tons to 8000 tons per year from 450 hectares of land within a period of 1980 to 1984 (6). Here the increase in yield has been reported from 8 tons/hectare to 18 tons/hectare. A similar result can be safely projected in our context as well since the rapid and cheap method of producing tissue culture potato plants has already been developed in our laboratory. Such a scheme of producing tissue culture potato plants come into existence for distribution of seed tubers countrywide, the present yield of national average of 6.9 tons/hectare (7) may increase by 18 tons/hectare. Thus we will have 6,55,000 tons more potatoes from the same acreage, 59,000 hectares. This is indeed a very high figure.

Therefore, it is obvious that the tissue culture can play a key role in increasing the yield of plant crop by providing healthy material for plantations (8). Its importance will be still more in tree plantations because trees take several years before they can be cropped. Therefore, even a slight increase in wood volume with desirable stem form will have a significant impact on its economy.

Uniformity:

Tissue culture trees of *Dalbergia* sissoo produced by the method developed in our laboratory (9) are already under performance trials in Sagarnath Project and other forest stations. In Sagarnath the growth of tissue culture trees to height of 3 meters in a year is encouraging. Another few years of observation will perhaps be necessary before we can ascertain its advantages over seedling produced trees. However, there should be no questions unanswered as to the uniformity of the trees that will result from micropropagation. In the meantime, method development for cloning of the elite D. sissoo trees is in progress in our laboratory.

Disease Elimination:

For vegetatively propagated crops like potato etc. an ever increasing danger lies in the accumulation of invading viruses over years of successive vegetative propagations(2). This will obviously result in poor quality

and low yield of the crop. In such cases it is highly desirable to establish a production system through which the farmers will always have an access to clean planting materials. Here tissue culture can play a key role in maintaining these cultivars in disease free state, and as per need the particular cultivars can be multiplied very rapidly to meet the demand of the farmers. Moreover, the existing popular cultivars whose growth yield have lowered from virus infection could be restored to virusfree state by means of meristem culture.

Conclusion

In conclusion it is envisaged that the end of this decade the potential of tissue culture method of propagation will be so obvious to agriculture, horticulture and forestry that by that time a national effort in expanding the tissue culture activities will

perhaps be needed in order to take maximum advantages of the technology.

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In Vitro Anther Culture of *Allium fistulosum* L.

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Abstract

Callus was initiated from anthers of Allium ftstulosum L. in N 6 medium supplemented with 50g/l of sucrose, 1.0mg/l of 2,4 dichlorophenoxyacetic acid (2,4 D) and 0.25 mg/l of 6 Furfuryl amino purine (Kinetin). The callus cultured on Murashige and skoog (MS) medium plus 2% coconut milk formed green embryoids. The embryoids cultured on MS medium supplemented with 5.0mg/l of Benzyl amino purine (BAP) formed shoots.

Introduction and Review

The discovery of anther culture of *Datura innoxia* leading to plants by Guha and Maheshwari (1964, 1966, 1967) opened up a new technology to the induction of haploidy in higher plants. Since then this technique has been effectively employed in wide range of species.

Anther culture technology has been used in *Atropa belladonna* (Rashid and Street) 1973), *Brassica napus* (Dunwell, Cornish and Courcel 1985), *Capsicum annuum* (George and Narayanaswamy 1973), *Coffea arabica* (Sharp, Caldas Crocomo 1973), *Datura innoxia* (Guha and Maheshwari 1964, 1966, 1967), *Digitalis purpurea* (Corduan and Spix 1975), Hordeum (Clapham 1973), *Hyoscyamus niger* (Corduan 1975), Lilium (sharp, Raskin, and Sommer 1971), *Lycopersicum escu-lentum* (Gresshoff and Doy 1972), *Nocotiana sps.* (NItsch 1969), Oryza (Guha et al. 1970), *Solanum tuberrosum* (Dunwell and sunderland 1973), *Zea mays (Ku* Ming Kuang et al. 1978). Anther culture has been employed to obtain microspore derived callus, embryos and plants in over 247 species (Maheshwari et al 1983). However, active and sustained research in the development of anther cultured plant has been confined to a few species which include tobacco, barley, wheat, rice, maize, potato and rapeseed. Among all the plants the members of solanaceae have demostrated the greatest potential for androgenesis (BaJaj 1983).

In the present investigation, callus was initiated from the anther culture of *Allium fistulosum.* The callus formed embryoids which have been cultured to form shoots.

Methodology

Allium fistulosum inflorescences were picked fresh from the field. The unopened inflorescences were sterilized by dipping them in 70% ethanol, then immersed for 3 minutes in filtered solution of 7% calcium hypochlorite and rinsed several times with sterile distilled water in transfer room. The anthers were cultured on the N 6 medium (Chu 1978) supplemented with 50 g/l of sucrose, 1.0mg/l of 2,4 D and 0.25 mg/l of kinetin. The cultured flasks were kept in incubation room with temperature 25°C±2. The light was provided with fluorescent light, approximately 3,000 lux. Day light was maintained for 16 hours.

Following callus formation, it was cultured in MS medium (Murashige and Skoog 1962) supplement with 2% coconut milk. The embryoids were obtained in this medium which were cultured in MS medium supplemented witb 5.0 mg/l of BAP.

Results and Discussion

N6 medium supplemented with 1.0 mg/l of 2,4 D and 0.25 mg/l of kinetin was found to be most effective for the callus initiation. The callus was formed after 4 weeks (Fig. 1). It was kept in the same

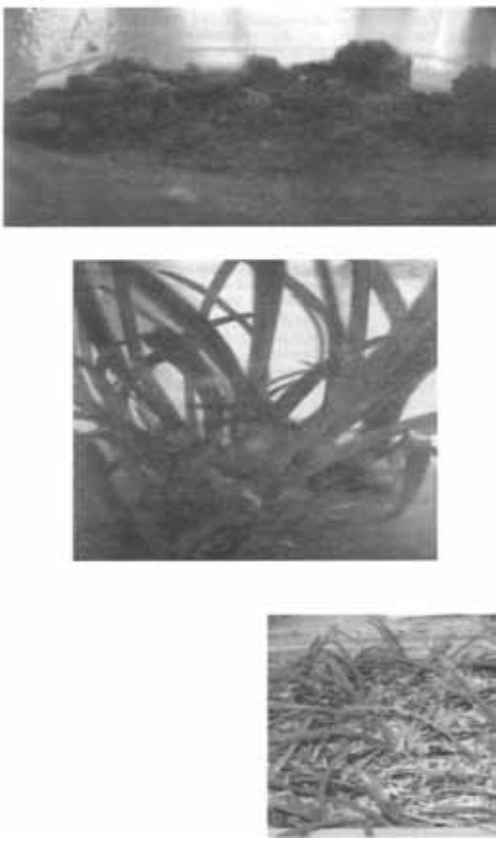

Fig. 1 : Callusing anther after 4 weeks in cuture.

Fig. 2 : Callus proliferation after 10 weeks in culture.

medium for 6 more weeks and then grew into bigger callus (Fig. 2). Then the callus was cultured in MS medium supplemented with 2% coconut milk. The callus formed green embryoids after 8 weeks. The embryoids were cultured in MS medium supplemented with 5 mg/l of BAP. Shoots sprouted from these embryoids after 10 weeks (Fig. 3).

For the anther culture of *Allium fistulosum* combination of both 2,4 D and kinetin was essential. The anthers were maintained green on the N6 medium with kinetin. Only these green anthers lead to callusing. This observation is consistent with the report by Skoog and Miller (1957) that a combination of an auxin and a cytokinin is often essential for DNA synthesis and mitosis. Embryo genesis is started in the callus which has been cultured on the MS medium supplemented with 2% coconut milk. Since watery endosperm of coconut contains the growth promoting factor at all stages of development (Steward and Caplin 1952), it is apparent that for embryogenesis from anther derived callus of A.

Fig. 3 : Shoots sprouted after 28 weeks in culture.

fistulosum such growth promoting factors are essential.

It can be concluded that culture of *Allium fistulosum* is possible in N 6 medium supplemented 2,4 D and kinetin. The embryoids are formed in MS medium added with coconut milk. These embryoids upon transfer to MS medium supplemented with 5.0mg/l of BAP lead to shoot formation.

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Meristem *Culture of Cymbidium giganteum* Wall Ex. Lindl

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Abstract

Plant regeneration through meristem. of *Cymbidium giganteum* wall ex. Lindl. is described. Shoots were initiated on Murashige and Skoog's medium (1962), MS suplemented with Benzylaminopurine (5.0mg/l), Napthalene acetic acid (1.0mg/l) and 10% Coconut milk. The proliferation continued on transfer to pots containing tree fern fiber survived in green house.

Introduction

Because of the long lasting characteristic of flowers the *Orchid cymbidum* has been widely commercialized using rapid multiplication tip culture technique. However, a fast rate of multiplication could result in a rapid spread of viruses if the stock plants from which the shoot tips are excised are not properly indexed for the presence of viruses and the size of the shoot tip should not small enough to be free from viruses. Since meristem derived Orchids have been generally reported be free from viruses it would therefore be disirable to culture the meristem of Orchids in a commercial production scheme.

This paper describes the meristem culture of *Cymbidium giganteum.*

Meterials and Methods

The plants of C. *giganteum* were grown in green house. The young shoots (8 cm) were separated from their mother plant with the help of a knife.

The shoots were washed in running tap water for half an hour followed by washing with teepol (10%) for 2 minutes shaking vigorously, and finally washed with distilled water 3-4 times.

The shoots were dipped for 5 second in ehanol the remove waxy sybstancs and then sterilized with 10% solution of calcium hypochlorite for 20 minnutes. The shoots were washed with sterilized distilled water five times. Then the outer leaves were removed one by one until the meristem was visible. the excision of meristem was done under dissecting microscope with the help of forceps and needles. The excised apical and axillary meristems (0.25-0.5mm) were cultured on Murashige and Skoog's medium (1962) supplemented with 1.0g/l Caseinacid hydrolysate, 10% coconut milk and different concentrations of BAP and NAA. pH of the medium was adjusted to 5.5 before autoclaving.

The cultures were incubated at 25°C under 16 hrs photoperiods provided by fluorescent tubes (ca. 3000 lux).

Results and Discussion

The meristems cultured on MS media with BAP (5.0 mg/l) NAA (1.0 mg/l) showed green swelling after 3 weeks. The shoots with some protocorms were differentiated after 6 weeks.

The protocorms as well as small shoots were sub cultured on MS medium for further proliferation. The sub culturing process was repeated every 2 months.

The number of plantlets were 6-10 in initial culture. Same number was observed in the first sub culture. However, after 2nd subculture, the number increased upto 10-15.

Rooting occured within two months of culture in control medium. The complete seedling developed 6-8 months after initial excision of the meristem.

The flasks which appeared ready to be transferred

into community pots were taken out from culture room, acclimatized for 1-2 weeks at room temperature, and finally transferred in pots with epiphytic medium. The plants were kept in greenhouse.

The results indicated that meristem culture can be used for rapid propagation of *Cymbidium giganteum.* The phenomenon was reported by Morel in 1964.

It was observed that small meristem piece (0.1mm) was difficult to establish in culture whereas 0.5 mm long piece survived. This shows that bigger the size of the material, the more chance of its survival. Similar results were obtained by Murashige in 1974.

The meristem cultured on media without Cytokinin and auxin was not successful to produce plants. No proliferation occured in media containing both cytokinin and auxin. This shows that for proliferation there should be proper balance of cytokinin and auxin. Skoog and Miller (1959) found that the interaction of cytokinin and auxin helps DNA synthesis and mitosis. Our observation that the explants proliferated in the presence of cytokinin and auxin corroborates the finding of the above workers.

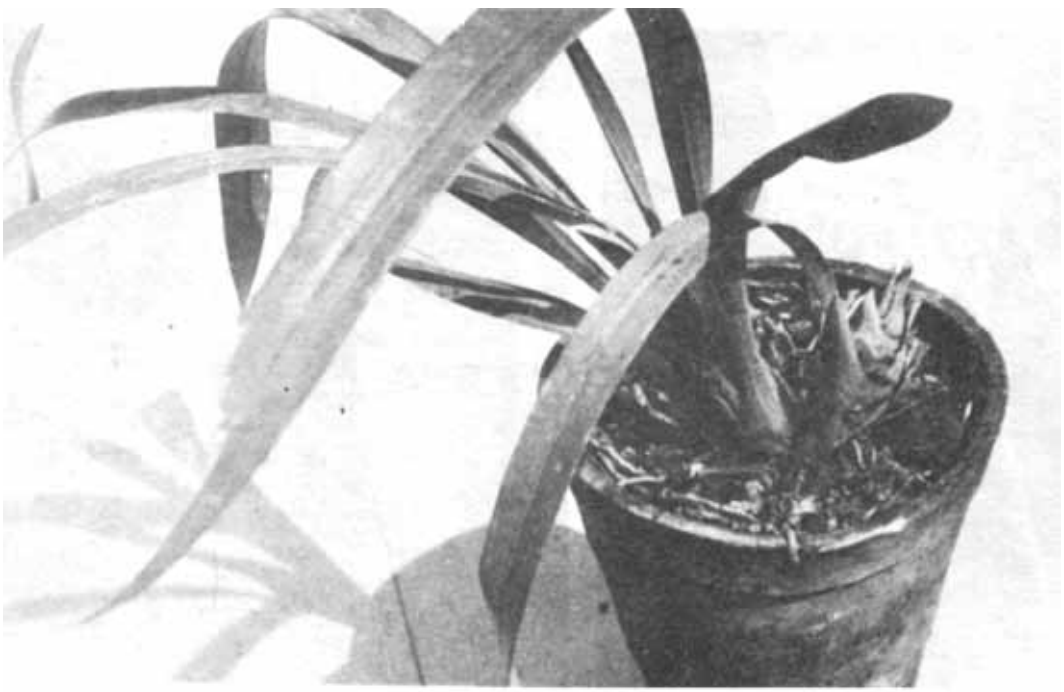
We observed that the meristem of C. giganteum cultured on media without coconut milk were slow to proliferate. But in media with coconut milk it showed faster proliferation. Our findings are consistent with the reports of the effect of coconut milk on meristem culture of orchids (Morel 1974).

Conclusion

The plants obtained in the present study were not tested for virus presence. But we assume that the plants may be virusfree because of the relatively minute size 0.25 mm 0.5 mm long of the meristem used.

However, to confirm whether the plants derived from meristem culture have been free or not from Viruses, Serological test is yet to be carried out.

The successful production of virus free *Cymbidium giganteum* may eventually lead to the development of Orchid growing on a commercial scale. Apparently the process can be applied to other valuable orchid plants also.



Plant with young shoot

Shoots proliferated
(after 12 weeks)

Shoots proliferated
(after 18 weeks)

Rooted plants
(after 2 months)

Plant ready to community pot (1 yr old)

Plant in community pot (1 yr old)

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Rooting of *in vitro* produced shoots of Agricultural, Horticultural and forestry species in non sterile sand

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Abstract

Although tissue culture mans of propagation holds potential for rapid cloning of selected genotypes, application of the technique is limited to ornamental and horticultural species only. One of the reasons that precludes the extension of the in vitro technique with other plant species including forestry trees can be attributed to the high cost of production of the tissue culture plants. The generally used in vitro cloning procedure involves 4 steps: (1) Explant establishment, (2) Shoot multiplication, (3) Rooting of shoots in culture medium, and (4) Field establishment. This paper describes the skipping of the step (3) by directly rooting the shoots into the non sterile sand. Plants obtained in our laboratory via such non sterile rooting include Pyrethrum, over twenty cultivars of potatoes, *Oxalis tuberosa, Ullucus tuberosus, Dalbergia sissoo, Eucalvotus camaldulensis, Ficus auriculata, F. lacor,F.nemralis* and *Porcirus trifoliata.*

Introduction

In vitro cloning via adventitious or axillary bud formation from shoot tip or stem node culture has now became a recognised mans of producing identical plants with selected characters. Such a procedure has become attractive to the growers because the shoot multiplication rate remains steady over several years of regular subculture passages. It is therefore apparently possible to produce over a million shoots from a single shoot in a year.

In the conventional tissue culture procedure multiple shoots produced as above are usually rooted in the auxin enriched medium followed by transfer of the rooted plants to the hardening process, and then to the field. Such a procedure limits the scope of using micropropagated clones for large scale plantations because a skilled person can handle only 90-100 shoots or shoot clumps per hour for in vitro rooting. For example, if a million plants are required skilled labour force of 2500 man days would be needed. Neither such a huge amount of skilled man power would be easily available nor the facilities to carry out the operation on such a scale. The skipping of this single step by rooting the microshoots directly from flask to non sterile sand is, therdfore, essential in order to enable production of million plants from a single explant in a year.

This paper describes the non sterile rooting of proliferated microshoots of agricultural crops (25 cultivars of potatoes, *Ullucus tuberosus,Oxalis tuberosa)*, horticultural plants (Pyrethrum a *Poncirus trifoliate*), forestry trees (*Dalbergia sissoo, Eucalyptus camaldulensis, Ficus auriculata, F.lacor* and *F.nemoralis*.

Materials and Methods

To effect organogenisis cotyledonary nodes were used as explant in *Dalbergia sissoo, Ficus auriculata, F. nemoralis,* and *Poncirus trifoliata,* tuber sprouts in potatoes*, ullucus tuberosus* and *Oxalis tuberosa*, and shoot tips from field established plants in Pyrethrum and *F. lacor.*

Murashige and Skoog medium (1962) was used in all the cultures supplemented with various concentrations of growth factors, BAP, Kinetin, NAA. The medium was autoclaved for 15 min under 15 pounds per inch. PH of the medium was adjusted to 5.6 before autoclaving. The cultures were incubated under 16 h day photoperiod with light provided by fluorescent tubes at 3000 lux.

Concentrations of growth factors that supported a steady shoot proliferation rate of 10-30 times in 3-12 weeks were selected for regular subculturing.

For rooting, elongated shoots, 2-4cm, were transferred to the ordinary non sterile sand under a high humidity polythene cover.

Results and Discussion

POTATOES

The potato plants were established in the field following the plant regeneration protocol described by Manandhar and Rajbhandary (1986). Of the 25 cultivars planted in the field, 16 cultivars were initiated from tuber sprouts and 9 from the test tube plants which were received from CIP, Peru. Only one CIP cultivar displayed no response to our culture treatment, and, died A high percentage of rooting,75 50%,of multiple shoots in the ordinary sand was observed from January to May. Rooted plants could be easily established in the field with no loss of plants during transplantation.

ULLUCUS TUBEROSUS AND OXALIS TUBEROSA

U. tuberosus and *O. tuberosa* are proteinaceous tuber crops grown from Venezuela to Argentina at elevations between 2500 and 3800m. Since their introduction in high altitude mountainous terrain of Himalaya Rey help alleviate protein deficiency (King, 1987) tubers of these crops were obtained from the New Work Botanical Garden, U.S.A.

Because the tissue culture provides a built in system of ensuring the planting stocks free from pathogens, bacteria, fungi, nematodes and possibly viruses, cultures were initiated in both species using tuber sprouts.

A sustained rated of multiplication, 15-20 shoots in 3 weekly subculture, was observed after the fourth subculture in both the species. For *U. tuberosus,* elongated shoots after three weeks of growth period were

set as individual microcuttings in the sand bed under the high humidity polythene cover to obtain rooted plants. But for *O. tuberosa* the multiplied shoot buds had to be transferred to the basic medium containing

1mg/l GAA to induce elongation. The elongated shoots, however, readily rooted in the non sterile sand. Plants transplanted in September 1988 and harvested in February 1989 have a yield of 200 g per plant with *U. tuberosus* and 70 g per plant with *O. tuberosa*.

PYRETHRUM

Pyrethrum is an insecticidal bearing plant exhibiting heterozygosity with regards to its growth form and pyrethrin content.

Cultures were initiated and plants established in the field following the protocol described by Karki and Rajbhandary (1984). Shoot multiplication rate remained same over 10 years of 8 12 weekly regular

susculture passages. Plant established from 5-6 years old cultures showed no morphological variation. Over 80% rooting of microcuttings could be achieved at day night temperature regime of 29°C 17°C.

DALBERGTA SISSOO

This tree is popular for plantation in subtropical areas in Nepal because it provides fuel wood, fodder and timber. Three year old field established trees regenerated via the protocol described by Suwal et al (1988) exhibited a uniform growth habit. Although a high rooting percentage, over 40%, could be achieved when the microshoots were set as cuttings in the ordinary sand under suitable day and night temperature regimes (Table 1), a humid atmosphere resulting from the continuous rain caused severe damage to the cuttings by the damping off effect.

PONCIRUS TRIFOLIATA

This is a citrus root stock commonly used in Nepal for grafting the scions of orange. Proliferating cultures were established by using cotyledonary nodes as explant. Shoot proliferation rate of 15-20 times in 8 10 weeks was achieved in the BAP level of 0.25mg/l. In the ordinary sand a high percentage of rooting of shoots was observed from March to September (Table 1) .

EUCALYPTUS CAMALDULENSIS

Because of its high growth yield *Eucalyptus camaldulensis* has been planted in an area of over 3000 hectares in the fuel wood plantation scheme in Nepal Terai. A marked degree of variation occurred amongst the individual trees such that the oil content varied from 0.3 to 2% and cineole content from 5 to 86%. Superior trees had wood volume three times

more compared to average. Proliferating cultures were induced from shoot tips, 5-7 mm,excised from 6 month old plant. Plants regenerated following the method described by Gurung and Rajbhandary (1987) exhibited a high growth rate in two years. Although a very encouraging result had been obtained from the field performance of the regenerated plants, at present we have been facing the problem of shoot elongation of the multiplied shoots which had undergone regular subculture possages for more than two years.

FICUS AURICULATA F.LACOR AND F. NEMORALIS

F. auricalata, F. lacor and *F. nemoralis* are important fodder trees in Nepal. Seed collection of fodder trees is difficult because unlopped trees are not easily available. More over seeds of these trees are reported to have low percentage of germination.

Shoot tips from standing tree of *F.lacor*, 2 m high, were used for culture establishment. A high shoot multiplication rate, 20-30 times, in 10-12 weeks was obtained on the MS medium suppemented with the combination of l.0 mg/l BAP and 1.5 mg/l kinetin, and 1000 mg/l casein hydrolysate. More than eighty percent rooting was observed from April to October, except in July when the percentage dropped to forty (Table 1). Saplings of plantable size could be obtained 2-3 months after transfer of the microcuttings, from flask to sand. Over 10,000 plants have been established in the soil, and they are undergoing field trials at various regions of Nepal.

Cultures of *F. auriculata* and *F. nemoralis* were initiated from the cotyledonary nodes. The growth factors BAP at 1.0 mg/l and NAA at 0.1 mg/l induced the shoots of *F. auriculata* to multiply at the rate of 20-30 times every 8-12 weeks. With *F. nemoralis,* shoot proliferation at the rate of 20-25 times in 8 weeks was observed in the MS supplemented with 1.0 mg/l BAP, 1.5 mg/l kinetin and 1000mg/l casein hydrolysate. Field established plants via sand rooting technique are being planted for their field performance.

Despite the high potential tissue culture methods of propagation hold for large scale propagation of clonal propagules of horticultural ornamental and forestry plants, the high production costs of tissue culture plants precludes their wider uses (Chu 1989). Rooting of multiple shoots in the auxin enriched medium has often been referred to as highly laborious step requiring long hours of hood operation, and occupation of large areas of shelf space.

It is however apparent from our observations with above species that for production of tissue culture plants, rooting in vitro is unnecessary. Extension of micropropagation of forestry plantations has been envisaged if the rooting in vitro is unnecessary (Hartney 1982). Ahuja (1984) reported the feasible micropropagation method for large scale forestry plantations of Aspen by setting the multiple shoots in the peat mix and obtaining rooted plants Since a high multiplication rate of shoots combined with rooting of such shoots in non sterile sand enables drastic reduction in production cost of tissue culture plants it may be presumed that they can probably compete with plants produced from traditional means (Seeds and cuttings).

Acknowledgement

I am thankful to Dr. S.B. Malla for the facilities provided and B.L. Shrestha for assistance with photography.

Table l : Effect of monthly variation of day/night tenperatures on rooting of microshoots. One hundred twenty shoots, 2-4 cm, were employed in a month, using 30 shoots each week.

Month	Max Temp.	Min Temp	Dalbergia sissoo	Eucalyptus camaldu lensis	Ficus auri culata	Ficus lacor	Ficus nemoralis	Pon1cirus trifoliata
Nov.	20	11	-	20	31	40	0	12
Dec.	24	6	-	5	10	25	0	0
Jan.	24	4	20	0	0	10	0	0
Feb.	26	6	0	0	0	15	0	36
Mar.	26	8	0	5	10	40	10	58
Apr.	28	9	40	35	35	80	15	56
May.	34	13	40	49	80	100	50	56
Jun.	34	17	65	59	80	100	50	59
Jul.	31	18	43	52	65	40	30	54
Aug.	27	18	45	43	50	95	25	-
Sep.	29	19	47	40	40	100	20	85
Oct.	29	15	34	40	38	100	5	75

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In vitro propagation of *Ficus lacor* Buch. Ham.

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Summary

Shoot tip explants from standing trees were used for micropropagation of *Ficus lacor* Buch. Ham., a fodder tree. Shoot proliferation was obtained on Murashige and Skoog medium, supplemented with benzyl aminopurine (BAP) at 1.0 mg/l, kinetin at 1.5 mg/l, and 1.0 g/l of casein hydrolysate. Shoots were multiplied by a factor of 20 30 (which remained unchanged) at each subculturing every 8-12 weeks for two years. The microshoots were rooted in sand in high humidity under a polythene cover. Rooted plants were readily established in soil, with no losses during the transfer from sand to soil.

Introduction

Fodder trees play a key role in Nepal's agriculture, providing 40% of the livestock feed (Pancley, 1982). *F. lacor* is an important species which is widely distributed at altitudes between 800 and 2000 m. Seed germination is poor, only 15% (Jackson, 1987). Although the species can be readily propagated by cuttings, this conventional vegetative propagation technique is unlikely to provide enough propagules for large scale forestry plantation programmes, as the establishment of clonal orchards would be a prerequisite for such schemes. An alternative approach is to use tissue culture techniques for rapid multiplication.

We report here the successful in vitro propagation and field establishment of *F. lacor*.

Materials and Methods

Shoot tips of *F. lacor* measuring 0.5-0.7 mm, excised from a tree 2 m high, were washed for one hour in flowing tap water. This was followed by surface disinfection with a detergent (two drops of Teepol in 100 ml of water) and then by a thorough washing in distilled water. Finally the shoot tips were sterilized in 0.1% mercuric chloride solution for fifteen minutes, and the sterilant removed with three washings of distilled water.

The medium used to induce snoot proliferation was that of Murashige and Skoog (MS) (1962), supplemented with 1.0 g/l of casein hydrolysate and 3% sugar. It was solidified with 0.7% agar, and its pH adjusted to 5.6 before being autoclaved at a pressure of 15 lb/in 2 for 15 minutes. Light was provided by white fluorescent tubes at 3000 lux. The cultures were incubated at 25 ± 4°C.

For shoot proliferation the following combina-tions of growth substances were used:

- 1.0 mg/l BAP + 1.5 mg/l kinetin
- 1.0 mg/l BAP + 1.5 mg/l kinetin + 0.01 mg/l NAA
- 1.0 mg/l BAP + 0.01 mg/l NAA
- 1.0 mg/l BAP + 0.1 mg/l NAA

Each combination was tested on five samples and each experiment was repeated at least once.

In all combinations the shoot tip explant gave rise to a number of shoots within eight weeks. In order to see the effect on shoot multiplication and shoot elongation, shoots that had been induced in each combination were subcultured to the same combination. The combination with no napthalene acetic acid (NAA) gave a high rate of multiplication, and was selected for all further subculturing. Subculturing was carried out by placing a group of 8-10 shoots in a 250 ml culture flask, at intervals of 8-12 weeks.

To induce rooting, microshoots measuring 2-4 cm were excised and immersed in a 100 p.p.m. solution of IAA for five minutes, before being planted in non sterile sand boxes. High humidity was created by covering the boxes with polythene.

To compare rooting behaviour at different seasons, fifty shoots were transferred to sand each week for a year.

to October, except in July, when the percentage dropped to 40%. A similar phenomenon has been

Rooted plants that had grown to a height of 5 – 6 cm were hardened in a glasshouse by transplanting into soil before planting them out in the field.

Results andDiscussion

Although shoot buds could be induced in all the combinations of growth hormones, a marked variation in the multiplication rate was observed in each combination (Table 1). A high rate of multiplication, 200-300 shoots in eight weeks, together with an adequate shoot elongation of 1-2.5 cm, was observed in the combination of 1.0 mg/l BAP and 1.5 mg/l kinetin. The addition of NAA at 0.01 mg/l was found to inhibit the multiplication rate to a remarkable extent, so that only 40 60 shoots were available for rooting. When kinetin was not included, the multiplication rate further decreased. An increase in NAA to 0.1 mg/l led to an even greater inhibition of multiplication.

On the medium supplemented with BAP at 1.0 mg/l and kinetin at 1.5 mg/l the high multiplication rate, 20-30 times every eight weeks, remained unchanged over a period of two years of regular subculturing (Plate 1).

Microshoots from 12 week old cultures were used for root induction, Rooting of microshoots was observed in sand throughout the year. Although the beginning of root development could be observed at seven days, the roots reached a length of 2 cm only after fifteen days (Plate 2). More than 80% rooting was observed from April

Plate 1 : Multiple shoot formation on MS media with 1.0 mg/l BAP and 1.5 mg/l kinelin

Table 1 : Effect of growth substances on shoot proliferation of *Ficus lacor* Buch. Ham.

Combination of hormones used	Concentration of hormones (mg/l)	No. of microshoots developed in each 250 ml flask	Remarks Plants healthy, microshoots 1-2.5 cm long, no callus formation
Benzyl aminopurine + kinetin	1 + 1.5	200-300	Plants healthy, microshoots 1-3 cm long.
Benzyl aminopurine + kinetin + naphthalene acetic acid	1 + 1.5+0.01	40-60	More roots are developed, no callus formation
Benzyl aminopurine + naphthalene acetic acid	1 +0.01	20-25	Plants healthy, microshoots 1-3 cm long, no callus formation
Benzyl aminopurine + naphthalene acetic acid	1 +0.1	10-15	Plants healthy, microshoots; 1-3 cm long, no callus formation

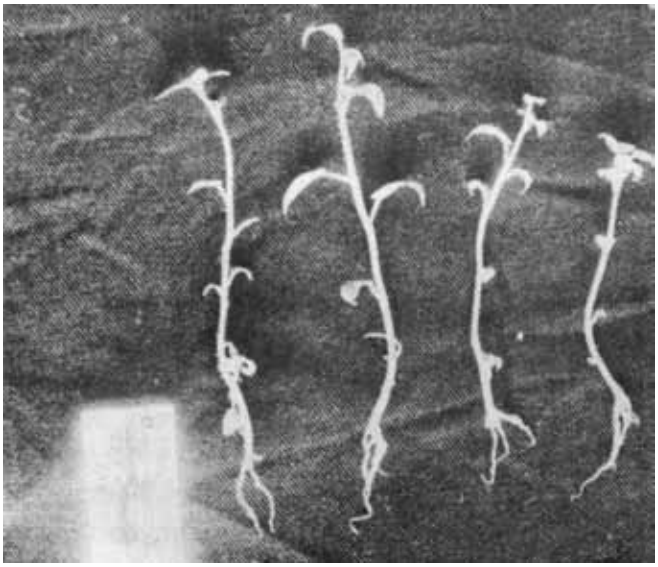

Plate 2 : Rooted plantlet 12 days after microshoots

observed in potato culture *in vitro* during the wet month of July. Manandhar and Rajbhandary (1986) suggest that a very humid atmosphere in the glasshouse under the polythene cover is perhaps not conducive to root induction. The lowest rooting percentage (10-15%) was recorded during the months of January and February.

When transplanted from sand to soil in standard polybags, three week old cuttings survived with no loss. They grew into normal nursery plants, reaching a height of 10-12 cm in eight weeks (Plate 3).

To date, 20.000 plants of *F. lacor* have been produced by the tissue culture method described above, and 12,000 of them have been distributed to different altitudinal zones of Nepal for performance trials.

A plethora of papers on tree tissue culture (Bonga, 1987) has reflected the increasing interest among tissue culturists in exploiting the technology to produce disease free, healthy planting stock. Emphasis has also been put on the development of cost effective micropropagation of forest trees (Brown and Sommer, 1982; McCown, 1985). As a result of increasing interest in the improvement of forest trees, various advances in the technique of tissue culture for such species have been reported (Bonga, 1987).

In tree breeding, a rapid increase in genetic improvement can be achieved by cloning selected individuals. Tissue culture provides the means of providing large numbers of clonal propagules for commercial forestry plantations. For maintaining genetic stability in the regenerants, it is desirable to avoid a callus phase, as it is known that plant regeneration via a callus phase generally results in chromosome changes in the progeny (D' Amoto, 1977).

Our earlier communications on pyrethrum (Karki and Rajbhandary, 1984), potatoes (Manandhar and Rajbhandary, 1986) and Ficus auriculata (Amatya and Rajbhanclary, 1989) indicate that production cost can be greatly reduced. Hartney (1982) and Ahuja (1986) have also noted that the production cost of tissue culture plants can be reduced if root for mation *in vitro* is not necessary.

Since the potential of shoot bud proliferation re-mained unchanged over two years of subcultures, and since the shoot multiplication rate was 20-30 fold every 8-12 weeks, it is reasonable to assume that the method described above offers the means of obtaining more than one million plants of *F. lacor* in a year, from a single shoot tip.

Plate 3 : Three month old soil established plants

Table 2 : Effect of day and night temperatures on the rooting of microshoots of Ficus Iscorin sand. Two hundred shoots were used each month. In all cases the roots were about 1-1.25 cm long

Months	Maximum temperature (°C)	Minimum temperature (°C)	Duration for rooting (days)	Percentage of shoots rooted
November	26	11	15	40
December	24	6	22	25
January	24	4	32	10
February	26	6	35	15
March	26	8	12	40
April	28	9	15	80
May	33	15	12	100
June	32	17	10	1100
July	31	16	7	40
August	29	19	8	95
September	29	19	7	100
October	29	15	7	100

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The authors are grateful to Dr S.B. Malla, Director General, Department of Forestry and Plant Research, Ministry of Forests and Soil Conservation, for facilities provided and to Mr Bharat L. Shrestha for photography

Keywords: *Ficus lacor;* fodder; propagation; tissue culture.

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Tissue Culture of *Lilium longiflorum* for mass production

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Abstract

Lilium longiflorum shoot tips were cultured in MS medium supplemented with NAA for the embryoid initiation. These embryoids were recultured in the medium supplemented with NAA, BAP, casein hydrolysate and coconut milk for the formation of microshoots. The microshoots were transferred in the sand for rooting. The field established plants have flowered.

Introduction

Lilium longiflorum is a big white flowered species. It is the most popular lily in Japan and the United States. Over 12 million plants of lilies have been produced through tissue culture in Europe and Japan (Chu, 1989).

Material and Methods

Lilium longiflorum plants were brought from Japan and grown in Godavari. Shoot tips were used as explant for culture. They were washed in detergent and surface sterilized in 0.1% $HgCl_2$ solution for 2 minutes. The traces of $HgC1_2$ were removed by washing with sterilized water. Murashige & Skoog, 1962 medium (MS) supplemented with 0.1 mg/l NAA, 0.65% agar and 3% sucrose was used as nutrient medium. The pH of all nutrient media was adjusted to 5.8 before steam sterilization of 15 lb p./ in.^{2} for 15 minutes. The cultured flasks were incubated in room with 25°C and light intensity of 3 kilolux (Philips flourescent lamp) was provided for 16 hours per day.

The embryoids formed in the cultured explants were subcultured in MS medium supplemented with 0.01 mg/l NAA, 0.5 mg/l BAP, 1.0 gm/l casein hydrolysate and 10% coconut milk. The microshoots formed in this medium were again cultured for more shoots proliferation.

The flasks with microshoots were brought to room temperature and kept for one week for acclamatization. Then the microshoots were transplanted in non sterile sand and covered with polyethylene hood. Once the roots developed the plantlets were transplanted in soil--bed.

Fig. 1 : Embryoids formed on MS+ 0.1 mg/l NAA.

Fig. 2 : Microshoots developed on MS

Fig. 3 : Plants in soil bed transferred from the culture

Fig. 4 : Flowering of the plants obtained from tissue cultured plants.

Results and Discussion

The sterilization of the explants with 0.1% Mercuric chloride solution for 2 minutes was found to be sufficient for tissue culture and 0.2 cm. long shoot tips established in nutrient medium.

MS medium supplemented with 0.1 mg/l NAA was effective for embryoid formation in six weeks old culture (Fig. 1). Takayama and Misawa (1982) have also used 0.1 mg/l NAA for culturing lily bulbs. The embryoids which were recultured in MS medium added with 0.01 mg/l NAA, 0.5 mg/l BAP, 1.0 gm/l Casein hydrolysate and 10% coconut milk formed microshoots in four weeks (Fig.2). Kato arid Yasulake (1977) have used a combination of NAA and BAP for the shoot proliferation in lily. Addition of casein hydrolysate and coconut milk has proliferated the number of microshoots Coconut milk contains the growth promoting factor (Steward and Caplin, 1952). It is apparent that the multiple healthy microshoots are the result of its presence in coconut milk in the nutrient medium.

The microshoots transplanted in non-sterile sand formed roots in two weeks. Rajbhandary (1989) has reported the rooting of tissue cultured plants in non-sterile sand. These rooted plants were transplanted in soil bed (Fig. 3). The field established plants did flower (Fig. 4).

Mass production of lily through tissue culture for clonal production has been successful. This technique can be used for commercial production of lily.

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Clonal propagation of *Ficus neriifolia* by Tissue Culture Technique

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Abstract

Multiple shoots were regenerated from excised shoot portions (2-3 mm) of *Ficus neriifolia* Sm. (four year old plant) in MS medium supplemented with BAP 1.0 mg/l and Kn 1.5 mg/l. The microshoots were rooted in a non sterile sand and successfully establishment in the field.

Introduction

Ficus neriifolia commonly called Dudhilo is a deciduous tree growing mainly in between 900 to 2200m and most frost tolerant other fig trees. The tree is most popular among the farmers and is highly valued for fodder. The tree is lopped for fodder from June to February and again May to June after the flush of new leaves has appeared. The seed viability is about two weeks and the germination is about 41% (Jackson, 1987). Conventional propagation though cutting is reported to be not successful (Pandey, 1982). So, for the mass propagation it is desirable to develop, a reliable method, that is the clonal propagation of *Ficus neriifolia* by tissue culture technique.

The present paper describes a method for rapid propagation of *Ficus neriifolia* plantlets from stem explants and its establishment in the field.

Materials & Methods

Shoot apices and axillary buds collected from four year old plants at Godavari, were washed thoroughly in running tap water 2-3 hours and followed by a surface wash with a few drops of teepol and finally ringsed thoroughly with distilled water for 4-5 times. Aseptically, the previously washed shoot apices and axillary buds were surface sterilized with 0.01% mercuric chloride solution for 2 minutes and washed 4-5 times with sterilized distilled water. Shoot and apices and axillary buds measuring about 2-3 mm were excised cultured on Murashige and Skoog medium supplemented with different growth hormones (Plate no. 1). The medium were solidified with 0.7% agar and were autoclaved at 15 lb pressure for 15 minutes. All cultures wee incubated at 16 hours light at 25±2°C. In all experiments 30 replicates were used and each experiment was repeated at least three times.

For rooting, the microshoots measuring about 3-4 cm. long were excised. The excise microshoots were rooted in non sterile sand boxes.

Results and Discussion

Shoot apices and axillary buds wee cultured on Murashige and Skoog media supplemented with a wide range of combination of cytokinins such as Kn, BAP and auxins including IAA and NAA. The present experiment showed that shoot formation was most consistently induced by BAP 1.0 mg/l together with Kn 1.5 mg/l. Shoot proliferation was not good in BAP in combination to NAA and IAA (Table no. 1). More than one to two shoots proliferated, callusing and browning was observed in this experiment. Transfer of shoots to the medium with BAP and Kn were revised. For routine shoot multiplication MS medium supplemented with BAP 1.0 mg/l and Kn 1.5 mg/l was used. The primary explant yields two to three shoots. These shoots inturn proliferated resulting in a dense mass of shoots after 5-6 subcultures. The number of shoots varies 30 to 40 in a 250 ml. conical flask after 6-8 week of subcultures (Plate no. 2).

Table 1: Growth responses and morphogenesis in excised shoot apex and auxiliary buds *of Ficus neriifolia* grown in various media.

Medium MS+BAP+Kn	No of shoot	Duration in week	Growth response
Control	6-8	No response	
1.0+0.25	2-3	”	shoots
1.0+0.5	5-6	”	”
1.0++0.75	8-10	”	”
1.0+1.0	12-15	”	”
1.0+1.5	30-40	”	”
MS+BAP+NAA			
1.0+0.1+0.01	1-2	”	shoots, profuse callusing and browning.
MW+BAP+NAA		”	shoots, callusing and
1.0+0.1+0.01	2-4		browning

For rooting two month old shoot buds were used. The microshoots were rooted in a sand box containing only sand (Plate no. 3). The induction of roots were observed after 8-10 days. After 15 days of rooting, the rooted plants were transferred to polybags for field establishment (Plate no. 4).

The maximum rooting (80%) was observed in June, July. August and minimum (10-12%) rooting was in January & February The result indicates that from a single shoot apex or axillary bud thousand of plants can be produced. Genetic stability *in vitro* is achieved when propagation is made directly from the explants without an intervening callus of cell suspension phase (Bonga & Durzan, 1982). Plants clones derived from shoot apices often have been observed to be uniform suggesting genetic stability as in *Asparagus, Gerbera* and *Eucalyptus citriodara* (Murashige, 1972-1974, Gupta & Mascarenhas, 1982) In the present study, plantlets produced by multiple. shoots regenerated directly from shoot apex and axillary buds are assumed to be true type. This assumption is also supported by field performances of *Brassica oleracea* var. *capitata* (Pradhan, 1991).

Thus, this technique also support in afforestation programme as well as the them paper of this conference “The regeneration of Forest”.

Table 2: Effect of temperature (day/night) on rooting of microshoots; of *Ficus neriffolia*.

Months	Minimum temperature (C°)	Maximum temperature (C°)	Humidity average %	Duration for rooting (day)	Percentage of shoots rooted
January	3	22	70-80	20-25	10
February	5	24	”	20-23	12
March	8	26	”	15	30
April	10	26	”	15	48
May	15	30	”	10	62
June	15	32	”	8	75
July	20	32	”	8	80
August	20	30	”	8	80
September	19	30	”	10	72
October	16	28	”	12	65
November	14	26	”	15	56
December	8	24	”	15	35

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Plate No. 1 : Shoot tip cultured on Murashige and Skoog's media supplemented with different growth hormones.

Plate No. 2 : Shoots proliferation in MS medium supplemented with BAP 1.0 mg/l Kn 1.50 mg/l

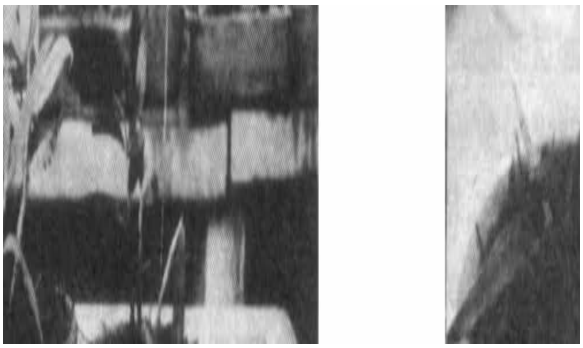

Plate No. 3 : Microshoots rooted in sand box containing only sand.

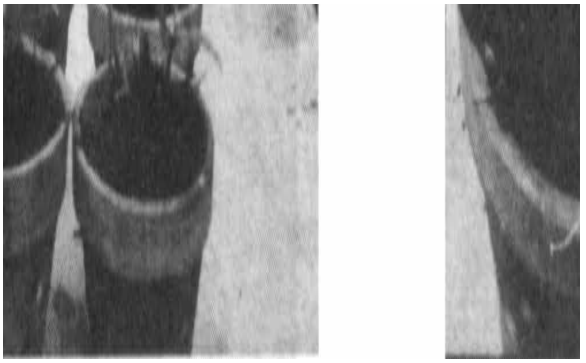

Plate No. 4 : Two month old helthy plants ready for field establishment.

Micropropagation of mulberry (*Morus alba* L.) for large scale production

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Abstract

The shoot tips from mature mulberry *(Morus alba* L.) tree were sterilized in 0.1% mercuric chloride solution for five minutes and washed thoroughly in distilled water. The innermost shoot tip was removed and cultured in Murashige and Skoog (MS) medium supplemented with 0.01mg/l NAA. 0.25mg/l BAP and 10% coconut milk. The established shoot tips were subcultured in MS medium for development of multiple micro shoots. These microshoots were rooted in non sterile sand and large scale production of mulberry plants was carried out through micropragation.

Introduction

Mulberry *(Morus alba) is* an important crop for sericulture. Its leaves can support normal cocooning and reproduction in silkworm. Why the silkworm prefers mulberry leaves is little understood, High sugars and protein content and special fragrance in mulberry might have attracted silkworm.

Mulberry is grown in temperate to tropical regions. It is commercially cultivated in China, India, Japan and Korea.

Out of thirty species of Morus found in the world, following three types are available in Nepal.

1. Morus indica 2. M. alba var. *laevigata*
3. M. alba var. *serrata*

Conventional methods of vegetative propagation of mulberry are grafting and cutting. For grafting, rootstocks are usually prepared from one year old saplings and scions are taken from branches before bud sprouting. The grafts are grown In the field for one more year. Thus two years are required for getting nursery plants. Cuttings can be taken from hardwood or softwood. They produce plants in one year. However both cuttings have some draw-backs. Softwood cuttings depend on physiological states of the cutting and environmental conditions. Hardwood cuttings have a limited rooting percentage.

These days clonal propagation of mulberry has been done with the tissue culture technique. There are two ways to obtain plants from tissue culture. One is through adventitious initiation of shoots or embryoids from callus/explants, the other is by axillary branching in bud or meristem culture.

Material and Methods

The shoot tips were excised from the field grown mature tree, cleaned with teepol solution and washed in running tap water for one hour. Then the buds were sterilized in 0.1% mercuric chloride for five minutes. The innermost shoot tip was removed and cultured for four weeks in Murashige and Skoog medium (MS) supplemented with 10% cocount milk, 0.01 mg/l NAA and BAP (0.25, 0.5, 1 and 2mg/l) for the establishment of the explant. After four weeks the established explants were subcultured horizontally in MS medium suplemented with 10% cocount milk, 0.01mg/l NAA and different concentrations of BAP (0.25, 0.5, 1 and 2.0mg/l), Subculturing was done every two months.

The microcuttings were transferred in fine non sterile sand and covered with polythene hood. Watering was done regularly in order to maintain the humidity of 80% until the development of roots.

Results

The shoot tips, washed with teepol water, sterilized with mercuric choride were established in the medium. Out of fifty shoot tips cultured in each medium supplemented with BAP (0.25, 0.5, 1.0 and

2.0mg/l) 40, 20, 10 and 0 shoot tips were established respectively after four weeks (Table 1). The shoot tip was best established in medium supplemented with 10% cocount milk, 0.01mg/l NAA and 0.25mg/l BAP in four weeks (Fig. 1). The green shoot tips subcultured every two months in MS medium supplemented with 10% coconut milk, 0.01 mg/l NAA and 0.5mg/l BAP produced upto sixteen micro shoots from an explant after 6th subculture. Two to three micro shoots were produced in the medium with 1.0 mg/l BAP, whereas one to two micro shoots were produced in the medium with 0.2 and 2.0mg/l BAP (Table 2).

The microshoots transferred in sand produced roots within two weeks. For one more week, they were left in the same box to develop more roots (Fig. 3). The sand rooted plants were well established in soil (Fig. 4). In this way large scale production of mulberry through micro propagation was carried out.

Discussion

Shoot tips cleaned in teepol water and sterilized in 0.1% mercuric chloride for five minutes established well in the medium. Oka and Ohyama (1986) used 4% calcium hypo chlorite for 20 minutes to sterilize the shoot tips. A combination of 0.5mg/l BAP, 0.01 mg/l NAA and 10% coconut milk was found to be the best mixture to produce sixteen microshoots from each explant. Whereas Oka and Ohyama (1986) found a single shoot development at 0.1 mg/l BAP and three to five shoots proliferation at 1.0 mg/l BAP.

The microshoot of mulberry produced roots in non sterile sand after two weeks. Rajbhandary and Bajaj (1991) had previously reported sand rooting of microshoots in 25 different species.

Biondi and Thorpe (1982) and Bonga (1982) stated that one of the problem in tissue culture technique is to transfer in vitro produced plants in soil. In the present experiment sand rooted mulberry plants established well in soil.

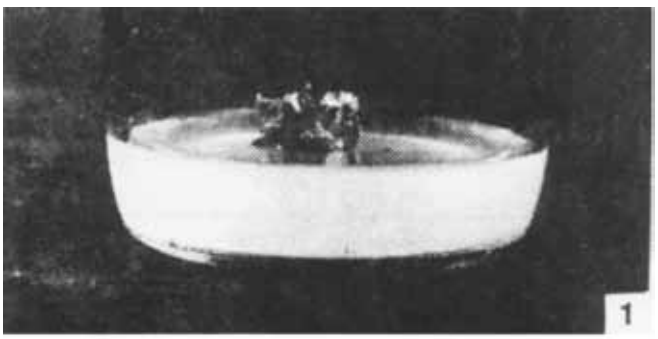

Fig. 1 : Shoot tip established after four weeks.

Table 1 : Shoot tip establishment in MS medium supplemented with coconut milk, NAA and BAP.

Coconut milk %	NAA mg/l	BAP mg/l	No. of cultured shoot tips	Response Green
10	0.01	0.25	50	40
10	0.01	0.5	50	20
10	0.01	1.0	50	10
10	0.01	2.0	50	0

Table 2 : Micro shoot multiplication in MS medium supplemented with coconut milk, NAA and BAP.

Cocount milk %	NAA mg/l	BAP mg/l	No. of microshoots produced
10	0.01	0.25	1-2
10	0.01	0.5	14-16
10	0.01	1.0	2-3
10	0.01	2.0	1-2

Fig. 2 : Multiple mocroshoots produced after sixth subculture.

Fig. 3 : Sand rooted microshoot after three weeks.

Fig. 4 : Micropropagated plants established in soil.

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Mass propagation of *Gerbera* plant through Tissue Culture

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Abstract

The shoot tips of Gerbera plants were cultured on Murashige and Skoog (1962) medium (MS) containing organic constituents of MS, BAP 1.0 mg/l and NAA 0.1mg/l. After eight weeks of culture 2-4 shoots developed from the explant. Shoots of 1-2 cm in length were sub cultured in the same medium except the NAA was reduced to 0.01mg/l, 10% coconut milk and 4% sucrose. After fourth sub culture 30-40 shoots developed. Shoots which were 3 cm or more in length were transplanted in sand bed. Roots developed in about two to three weeks. 80% of roots were found and the rooted plants survived easily.

Introduction

Gerbera plant commonly known as Transvaal Dairy belongs to the family composite and it is a dwarf perennial herb. About forty five herbaceous species of perennials, the best known among these species and the only one to be horticulturally important is *Gerbera jamsonii* and its number of cultivars. It is a beautiful cut flower for floral arrangement because of its numerous colours and shapes. Its hardy upright flower, free of leaves on the stalks are the added merits of this plants.

In *Gerbera* the conventional method of propagation by division or by rhizome cutting is quite in efficient. In recent years, tissue culture as an alternative has been used to asexual propagation. Clonal propagation has the benefit of producing plantlets with the same genetical characters or the plants are most likely to be true to type. It is faster for mass propagation producing high quality cut flowers. Thus micropropagation of gerbera has almost completely captured the cut flower market.

Pierik *et al*. (1973, 1975) showed that excised gerbera capitulum explant could be forced to form shoots in vitro. Chu and Huang (1983) used the scape as explant. Murashige *et al.* (1974), Huang and Chu used the shoot tip as explant. In this paper the technique of shoot tip culture of *Gerbera* for mass propagation and rooting in sand are presented.

Materials and Method

The initial shoot tips were obtained from plants in green house. The roots were removed and plants separated. The soil was washed off, the leaves were stripped off, each division leaving only the last unexpanded leaf. The outer layer of rhizome peeled off. The material were washed in running water for one hour, teepol water for five minutes and again with water. These shoots were sterlised with 0.1% mercuric chloride solution for ten minutes, and washed with sterlize water for five times. Then they were cut into 2-3 mm long shoot tip explants and cultured in the MS (1962) medium with 1000 mg/l casein hydrolysate, benzylaminopurine (BAP) 1.0mg/l and nepthylacetic acid (NAA) 0.1 mg/l (Niroula and Rajbhandari, 1989) pH 5-8 before autoclaving. The culture was incubated at 25°C under 3000 lux for 16 hours photoperiod.

2-4 shoots regenerated after 8-10 weeks of culture. These shoots were further subcultured in the same medium or in different concentration of auxin and cytokinin. Rooting of microshoots were in sterile sand. 2-3 cm or more in length of the shoot were used for rooting.

Result and Discussion

Establishment of aseptic shoot tips were very low (5-10%) due to high contamination because the plant material is in the contact with the soil and there is no any leaf sheath to remove. The established shoot

regenerate 2-4 shoots aftor 8-10 weeks of culture. The regenerated shoots when sub cultured in the same medium (initial medium), the shoots were proliferated and the formation of callus (hardy) was observed and the plants were yellow. The concentration of auxin was reduced from 0.1mg/l to 0.01mg/l to avoid callus formation and 10% coconut milk and 10g/l of sucrose was added. After 4-5 sub culure the regeneration was optimum, 30-40 shoots per flask were found (plate I) Root was found 70-80% in non sterile sand (plate II) at temperature of maximum 35°C and minimurn 15°C, humidity 70-80° under the light intensity of 4000-5000 lux after two to three weeks (plate III). The rooted plants survived easily in soil (plate IV). The plant flowered after one year of initial culture (plate V).

For the shoot tip culture of gerbera, the salt mixture of MS (Murashige and Skoog, 1962) has proved satisfactory. Pierik *et. al.* (1975), and Chu and Huang (1983) showed that the medium of Murashige *et. al.* (1974) was less effective than. 1/2 MS medium and micro of Hellers and high concentration of BAP (5-10 mg/l). For the initial culture MS with AP 1.0 mg/l and NAA 0.1 mg/l was beneficial. The best shoot multiplication or the optimum shoot growth occurred on the MS medium containing BAP 1.0mg/l, NAA 0.01mg/l, 10% coconut milk and 4% sucrose where other used the BAP 5-10 mg/l and kinetin (Murashige, 1974).

Plate I : Multiplication of Shoots

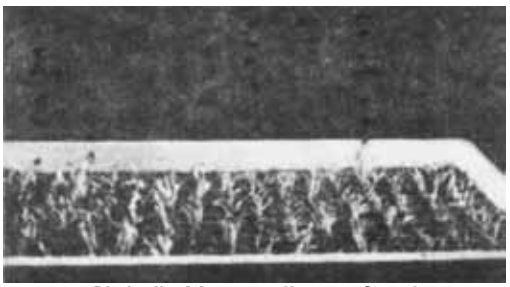

Plate II : Micro-cutting in Sand

Plate III : Rooted Plants after 3 weeks

Plate IV : Established Plant in Soil

Plate V : Flowering Plant

Establishment of gerbera plantlets through shoot tip culture is rapid but the initial number of shoot tips required is very high due to the higher infection rate. Where explants as capitulum, scape or pith of rhizome are cultured, the number of shoots per explant is small and success depends upon the individual cultivars (Chu and Huang, 1983). The method of axillary shoot multiplication has virtually eliminated genetically deviant plants (Murasheige, 1974). Therefore the shoot tip system is more efficient for commercial propagation than other methods.

Thus a combination of tissue culture with micro stock plant and sand rooting of micro cutting might be desirable for commercial production of plantlets of *Gerbera.*

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Large scale propagation of Strawberry through Tissue Culture

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Abstract

Two test tubes containing in vitro plants of Strawberry *(Nara* variety) was received from Japan. In vitro explants were cultured in Murashige and Skoog's medium (1962) supplemented with 6 Benzylaminopurine 1 mg/l and Kinetin 1.5 mg/l with 10% cocount milk. 60-80 multiple shoots were formed after 10-12 weeks. These shoots were subcultured every 4 weeks interval in same medium. Rooting of microshoots were observed after 8-10 days in sand. Rooted plantlets were established in the field.

Introduction

Strawberry, botanically known as *Fragaria sp.* is a prominent herb which belongs to the Rosaceae family. The fruit or the berries are red in colour, juicy and delicious in taste, They are distributed throughout the temperate region of the northern Hemisphere as well as the South America.

The fruit as well as the leaves of the plant contain ascorbic acid which is an essential element for preserving vegetable and fruit.

Jam and jelly of good taste can be prepared from the strawberries. The juice can be used as soft drink and the slice of the well rippened red berries are used as a decorative agent over ice creams.

A good quality of wine can be produced by adding a small quantity of yeast in the syrup of strawberries.

Traditionally the berries are propagated from seeds, splits and runners. Propagation by split is adopted for the multiplication of strains of proved merit. Under field conditions the seedlings produced by this method were found susceptible to virus, mycoplasma disease, nematodes and soil fungi which are often directly or indirectly transmitted to the descendants. The seedlings produced by seeds take a long time to propagate it is therefore preferred to propagate seedling from runners for commercial purposes. Propagation by runners under field condition however faces similar problem as that of propagation by splits with regards to disease contamination. Nishi and Ogasawa (1973), and Boxus (1974) have reported that the mass production of virus free seedlings can be done by tissue culture using *in vitro* rooting procedure.

Since *in vitro* rooting is an expensive and slow process attempts have been made in the present study to produce seedlings of strawberry using simple, inexpensive and efficient sand rooting procedure.

Materials and Methods

In vitro strawberry plants were received from Japan. The shoots of the plants were then excised from test tubes aseptically and cultured in Murashige and Skoog medium (1962) in sterile jam bottles containing 70 ml solidified medium. To induce shoot proliferation, the medium used was that of Murashige and Skoog medium supplemented with 1.0 mg/l Benzylaminopurine (BAP) and 1.5 mg/l Kinetin with 10% coconut milk. The medium was solidified with 0.7% Agar and the pH was adjusted to 5.8 before sterilization by 18 minute of autoclaving. The cultures were then incubated at 25±2°C with a 16 hour photo period (3000 lux) provided by fluorescent lights.

Under these conditions, after 12 weeks of culture the shoots proliferated and grew to a height of 2-3 cm. Cultures were then routinely transferred every 4 weeks. For rooting, multiple shoots were seperated out and transferred to non sterile sand box in a greenhouse under high humidity of about 70-80%. After 10-15 days, visibly strong roots began to appear and these plantlets were then transplanted in

polythene bags containing soil, sand and compost and were kept in greenhouse. After 8-10 weeks, they were planted out in the field.

Results and Discussion

Shoot proliferation was observed in all concentration of 6 Benzylaminopurine and Kinetin (Table 1). The best multiplication was observed when shoot tips were transferred to the Murashige and Skoog medium supplemented with BAP 1.0 mg/l and Kinetin 1.5 mg/l with 10% cocount milk, the multiplicaiton rate was 60-80 shoots per explant after 12 weeks. No loss of multiplication potential was observed after 4 years of regular subculture.

Boxus (1974) reported that the multiplication of shoots of strawberry can be achieved in MS medium supplemented with 1.0 mg/l BAP without coconut milk. We observed that the combination of two Cytokinins with 10% coconut milk gave good result for multiplication. Regeneration of plants from in vitro culture has been applied in many species (Murashige, 1971).

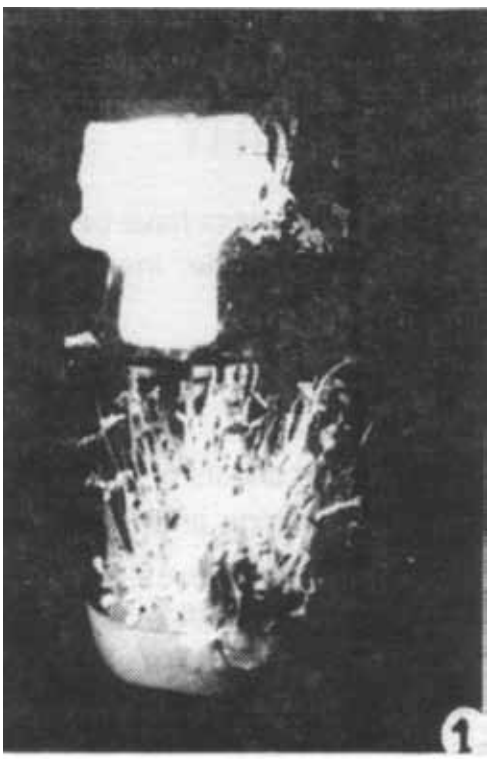

Fig. 1 : Multiple shoot formation in MS medium with BAP 1.0 mg/l and Kinetin and Kinetin 1.5 mg/l after 12 weeks.

Fig. 2 : Rooted plantlets after 4 weeks.

Fig. 3 : Mature plant in pots after 3 months.

Fig 4 : Flowering of plants in the field after 5-6 months.

Table 1 : Response of Shoot tips after 12 weeks culture in different combinations of Cytokinin

Explant	Medium (Murashige and Skoog) BAP + Kinetin (mg/l)	Response Shoots
Shoot tip	1 0+0.5	4-6
	1.0+1.0	20-25
	1.0+1.5	60-80
	1.0+2.5	20-40
	1.0+5.0	20-25
	Control	No response

While transferring the in vitro produced shoots to sand beds 60-80 percent developed into rooted plantlets within 10-15 days (Fig. 2), under day and night condition of temperature (34/l5°C) and 70-80 percent humidity. These plantlets were then trasterred to soil in pots (Fig. 3) and subsquently planted out in the field (Fig. 4).

The result of this findings can be useful for large scale commercial production of strawberry for plants.

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In Vitro Propagation of Rose for Mass Production

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Abstract

The shoot tips (2-3 mm) of rose were cultural in Murashige and Skoog medium supplemented with Benzylaminopurina (BAP) 1.0mg/l and Naehthalene Acetic Acid (NAA) 0.01 mg/l. The microshoots were recultured in the medium supplemented with BAP (1.0 mg/l, Kinetin (1.5 mg/l) and coconut milk (10%). The microshoots were transferred in a non-sterile sand for rooting and successfully established in the field.

Introduction

Rose in considered as one of the most important commercial cut flowers. The demand for flowers is increasing substantially in the local market too. The data available showed that rose could be a big business in local market in the future.

A number of reports are available on the callus culture of rose (Jacobs et.al., 1968, 1969, 1970, Wuestev and Sacalis, 1980). Hill (1967) reported the formation of shoot primordia' in long term callus cultures of hybride rose. He tried various experiments to stimulate the growth and proliferation of shoot like structure. Tweddle et. al., (1984) and Lloyd et. al., (1988) reported the adventitious shoot formation from callus culture of *Rosa persica* and *R. xanthina* on MS media.

The first report of rose shoot proliferation and rooting was made of *R. multiflora* by Ellioti (1970). During the same period, some others reported the callus and shoot proliferation by using various combinations of growth regulators. As is common with research in any field of science, there studies were independently conducted in different parts of the world. Clasical methods to propagate rose plants is tedious. Moreover, it is the demand of time to develop methods which are commercially feasible to produce rose plants on a large scale. Realising this, we have attempted to develop micropropagation technique for some popular rose species in Nepal.

Methodology

Shoot tips (2-3 cm) were collected from healthy rose plant. The explants were washed in running tap water for about 2-3 hours and followed by a surface wash with a few drops of Teepol and finally rinsed thoroughly with autoclaved distilled water for 4-5 times. The shoot tips were sterilized with 0.1 percent mercuric chloride solution for 13 to 15 minutes followed by through washing with autoclaved distilled water. Shoot tips (2-3 mm) were excised and cultured on solidified Murashige and Skoog's (MS) medium supplemented with BAP 1.0mg/l and NAA 0.01 mg/l. The medium was solidified with 0.7 percent agar and pH was adjusted to 5.8 before autoclaving at 15 lb/in^2 pressure for 15 minutes. The cultures were incubated at 25°C under fluorescent tubes at 3000 lux.

After 2-3 days of culture browning was seen at the base of the excised shoots. In our laboratory, we observed that browning is particular problem with fresh explants. Browning is believed to be the result of polyphenol substances which exclude from the cut surface of the explants. To overcome the problem, the explants were transferred to fresh medium every 2-3 days for several weeks.

The incidence of browning reduced gradually. Shoot initiation developed after 8-10 weeks. The multiple shoots were excised and transferred to MS medium supplemented with different concentrations of benzylaminoruine (0.5-2.0mg/l) kinetin (1.5 mg/l) and naphthalene acetic acid (0.01-0.1 mg/l).

Result and Discussion

After 8-10 weeks of culture, shoot proliferation was observed in all concentrations of BAP, Kin and NAA but the number of shoot proliferation was varied (Table 1). It was observed that at higher concentration of BAP (1.5 and 2.0 mg/l), shoots proliferated with callusing and browning. The number of shoot proliferation was less in BAP (1 mg/l) and NAA (0.1 mg/l). A lesser number of multiple shoots was observed at BAP (1 mg/l) and NAA (0.1 mg/l). A combination of BAP (1 mg/l), Kn (1.5 mg/l) and 10 perent coconut milk was found more effective. This medium produced 6-8 shoots per explants within 8-10 weeks. This means that different sources of cytokinin such as benzyl adenine, kinetin and even coconut milk have together exerted a favourable effect on shoot proliferation on *Rosa*. When auxin is added to the medium, the effect on shoot proliferation was inhibited. At BAP (1.0mg/l) and NAA (0.01 mg/l), only 3-4 multiple shoots were observed. After fifth to sixth subculture, the number of multiple shoots were 40-50 in MS medium supplemented with BAP (1.0 mg/l), Kn (1.5 mg/l) and 10 percent coconut milk after 10 weeks of culture. Then the shoots were cut off and planted in non-steriles sand boxes in the green house for rooting. They regenerated roots within 4-5 weeks. Rooted plants (3-4 cm long) were hardened in the greenhouse by transplanting in the soil and planting out in the field.

The maximum rooting (75%) were observed in August, September and October followed by only 60% rooting in March and April. However, rainy days were found to be deleterious for the rooting of microshoots when the rooting frequency became less than 20%.

Table 1 : Effect of BAP, NAA and Kn for multiplication of shoot tip explants of rose - cultivar (Hybrid Rose)

Cultivar	Medium MS + BAP +KN + NAA	No of shoots	Growth response
Pink Peace, Fragrant Cloud King's Ransom and Oklohoma	Control	-	No response
	2.0 + x + 0.1 mg/1	-	Brown callus
	1.5 + x + 0.1 mg/1	-	Brown callus
	1.0 + x + 0.1 mg/1	2-3	shoots
	0.5 + x + 0.1 mg/1	1-2	Shoots
	1.0 + 1.5 mg/1	6-8	Shoots
	coconut milk		Shoots
	1.0 + x + 0.01	3-4	Shoots

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Clonal Multiplication of *Cymbidium longifolium* D Don by Shoot Tip Culture

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Abstract

Clonal multiplication of *Cymbidium longifolium* D.Don was achieved with shoot apical meristerns cultured in vitro. Protocorm like bodies (PLB) were initiated in (Murashige and Skoog) 1962 medium supplemented with Benzylamino purine (2.0 mg/l), 1.0 mg/l Nepthalene acetic acid (NAA), 10 percent Coconut milk and 3 percent Sucrose. Rapid multiplication of shoots was found in MS containing 1.0 mg/l BAP, 1.5 mg/l kinetine and 10 mg/l Adenine sulphate. The proliferation continued on sub culturing on same medium. Rooting occurred in MS basal medium without growth hormones and coconut milk. Establishment of the mericloned plants in community pots was 80 percent successful.

Introduction

Orchids are of high commercial value for cut flowers in European countries. It comprises 900 genera and 3500 spp. out of which 86 genera and 350 spp. have been recorded in Nepal. There are 70 species of *Cymbidium* in the world, and in Nepal, there are nine species only. The genus *Cymbidium* was first found by Swedish Botanist Olob Swartz in 1800. Its name was derived from the Greek Word and refers to the boat like appearance of the lip of flowers. The flowers are long lasting in character, attractive in shape and size, hence there is high demand of this genus in comparision to other orchids.

Cymbidium longifolium, a native Himalayan orchid from Nepal has been enlisted as an endangered species and it is dwindling rapidly (Muralidhar and Mehta, 1982). They are found at an altitude of 2000 to 2400 in above sea level in Nepal.

Leaves of *Cymbidium longifolium* are linear and accurninate. Inflonescences, are as long as leaves and has densely flowered racemes having dropping in nature. Sepals and patals are generally pale green with purplish brown strips. Lalullume is white stripped and spotted with purplish color. Flowers last for more than one month under Nepalese climatic conditions. Its flowering time is September to October.

The fast rate of deforestation in Nepal has resulted in the rapid disappearance of orchid population in Nepal. Hence it is of utmost necessary to conserve the orchid spp., by developing rapid method of propagation of *Cymbidium longifolium.* Meristem or shoot tip culture is one of the quick method for producing large number of plantlets in a short time.

Orchid is the first horticultural crop in which tissue culture technique was applied commercially. The early work of many orchid genera have been successfully cloned by tissue culture technique. Murashige (1974) enumurated 22 genera and Anditti (1977) listed 35, including some inter generic hybrids (Soh, 1990)

Materials and Method

The young shoot 8 cm long was separated from plant with the help sharp knife. The shoot was washed in running tap water for half an hour followed by washing with teepol (10 percent) for 2 minutes shaking vigorously and finally washed with distilled water 4 times.

The material was dipped for 5 seconds in ethanol to remove waxy substances and then sterilized with 0.1percent solution of mercuric chloride for 5 minutes. The shoot was washed with sterilized distilled water 5-6 times. Then the outer leaves were removed one by one with the help of forceps until meristem was visible. The meristem was excised

under dissecting microscope with the help of forceps and needles.

The excised apical. meristem was cultured on MS media supplemented with 1.0 g/l casein acid hydrolysate, 10 percent coconut water and different concentration of cytokinin and auxin ranging from BAP 0.1-5.0mg/l NAA.0.01-2.0 mg/l.

The pH of the medium was adjusted to 5.5 before autoclaving. The cultures were incubated at 25°C ± 4°C under 16 hrs photo periods provided by 3000 Lux of florescent light.

Result and Discussion

The shoot tips cultured in MS with BAP (2.2 mg/l) and NAA (1.8 mg/l) showed green swelling after 4 weeks. New protocorms 6-8 in number from a piece of protocom were produced in 6.weeks. These protocorms were cultured as such in the same media for further proliferation of protocorms and shoots.

The whole protocorms produced shoots but cut pieces of protocorms when sub cultured produced more protocorms.

Rapid multiplication of shoots occurred in MS media containing 1.0mg/l BAP, 1.5mg/l kinetine, 10 mg/l adenine sulphate. The proliferation continued on sub culturing in the same medium. The sub culturing process was repeated at intetvals of 8 weeks.

The number of shoots were 6-8 on initial culture, after 3rd subculture, the number increased upto 20.

2-4 cm long shoots were cultured in control media without growth hormones. Roots developed within two month of culture. Normal plantlets with well formed leaves and roots were obtained within 6-8 months after initial excision of the meristem.

The culture flasks were acclimatized for 1-2 weeks at room temperature and finally transferred in pots containing epiphytic medium. The plants were kept in green house.

The results indicated that shoot tip or meristem culture technique can be used for rapid propagation *of Cymbidium longifolium.* This phenomenon had been reported by Morel in 1964.

The bigger the size of the material, more the chance of survival. Similar results were obtained by Murashige in 1974.

The above findings show that there should be proper balance of cytokinin and auxin for proliferation. Skoog apd Miller (1959) found that the interaction of cytokinin and auxin helps DNA synthesis and mitosis. Tradition of using coconut milk and adenine sulphate enhanced rapid proliferation and good growth. Without coconut milk growth was slow. Our findings are consistent with the reports of the effects of coconut milk on meristem culture of orchids (Morel, 1960).

Conclusion

It is clear that tissue culture of Orchids has opened a new frontier for the orchid world. Tissue culture of *Cymbidium longifolium* has been successfully carried out. Development of such procedure opens up the possibility of mass producing, native as well as exotic orchids for both local and international markets.

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Protocorms (after 6Weeks)

Shoots pooliferatal

Plants ready to community

Individul Plant (3years old)

Plant with flower

372

Tissue Culture of Sugarane (Saccharum Sps) for Mass production

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Abstract

Meristem and meristematic tissue of sugarcane were exised under aseptic condition and cultured in MS media (Murashige & Skoog 1962) supplemented with 2.0 mg/l 2,4-D and 1.5 percent coconut milk for callus initiation. These callus were recultured in the medium supplemented with 1.5 mg/l kinetin and 2.0 mg/l IBA for the formation of plantlets (shoots). The plantlets were transferred to non-sterile sand for rooting. The rooted plantlets were ready to transplant in soil within 4-6 weeks.

Introduction

Sugarcane is one of the major agricultural crop in tropical and subtropical regions. From sugarcane, sugar and other biproducts are derived, such as, canefibre for fuel, paper board, molasses for animal feed and alcohol.

Sugar is an important cash crop of Nepal. In 1992/93 sugarcane was cultivated in 38330 hectare with average yield of 35-60 ml/ha and 1365870 tons of sugarcane was produced (Agri, stat 1992/l993).

Sugarcane is readily propagated by stem cuttings but this method of propagation does not ensure the production of disease free planning stock. Thus research on sugarcane tissue and cell culture was started in Hawaii in 1961 by Nickell (1964). Barbara, Nickell & M. Krishnamurti reported the production of disease resistant and improve varieties of sugarcane using tissue culture technique.

In the present case sugarcane plants of the following varieties were attempted to be produced.

Cos :	767,	8001,	8407,	86312,	1158	&	pusa
BO :	99,	91	901,	110,	113		120

Materials and Methods

Explants were taken out and kept in running water with a few drops of teepol, a liquid detergent for four hours. Explants were sterilized with 0.1 percent $HgC1_2$ for 15 minutes and thoroughly washed with sterilized distilled water.

Isolated explants were cultured on MS medium with 2,4-D (2.0 mg/l) and coconut milk. The medium was solidified with 0.75 percent agar and pH adjusted to 6 before autoclaving. The cultured flasks were incubated at $25^0c + 2^0c$ under fluourescent light of 3000 lux. (Fig 1)

Callus initiation was obtained within four days. Well developed calli were recultured on MS medium with kinetin (15.0 mg/l) and IBA (2.0 mg/l) for shoot regeneration (Fig. 2). In 4-8 weeks the calli regenerated into shoots attaining a height of 8 to 10 cm. (Fig. 3). Shoots were transferred to non-sterile sand tray and kept in greenhouse under high humidity with 30^0 c to 35^0c temperature. The root initiation was taken place in two weeks (Fig 4). The rooted plantlets were ready to be transplanted in soil within four to six weeks (Fig 5).

Results and Discussion

Explants were cultured on MS medium with 2,4-D (0.5 mg/l to 5.0 mg/l). Calli development were observed in all concentration. Calli in 5.0 mg/l and 0.5 mg/l were found black in color and so those were discarded. It was found optimum regeneration of shoots in 2.00 mg/l 2,4-D.

Combination of kinetin (15.0 mg/l & IBA (2.0 mg/l) was found best for shoot regeneration. M. Krishnamurti and J. Tlaskal (1974) have also used kinetin (15.0 mg/l) & IBA (2.0 mg/l) for shoot regeneration M. Krishnamurti (1981) used kinetin (15.0 mg/l), IBA (2.0 mg/l) & IAA (2.0 mg/l) for

Table 1 : Effect of different concentrations of kinetin and IBA on shoots productions

Explant	Kinetin + IBA	No. of shoot multiplication
Explant (callus)	2.0 + 2.0 (mg/1)	2-4
	2.5 + 2.0	2-4
	5.0 + 2.0	5-20
	10.0 + 2.0	10-40
	15.0 + 2.0	Whole calli regenerated into shoots.

shoots regeneration . The present experiment results are consistant with that of M. Krishnamurti & J. Tlaskar (1974).

Shoot regeneration occurred when callus was creamy and green. Some albino plantlets were also observed in cultured medium but such plants did not survive when transferred in the sand. When the shoots were transplanted, the rooting percentage was 70-80 in February to April and 80 to 90 from May to August.

The rooted plantlets were transplanted again in field for further growth and found at least 10-14 tillers, compared to conventional propagation method which give only 4-6 tillers.

Thus in vitro procedure of sugarcane tissue culture can be employed for rapid production of large in number of improved plants with desirable characters within a short period of time. It also helps to have disease-free sugarcane plantlets leading to increase in productivity per unit area. A further field trial is necessary to confirm the productivity of the crop over time.

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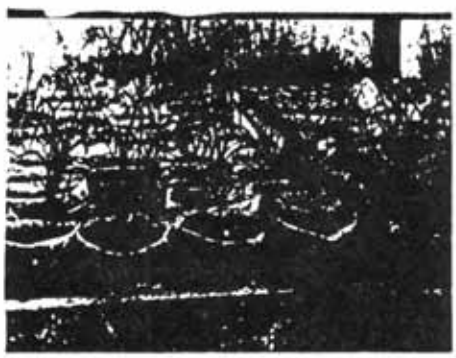
Fig 1

Fig 2

Fig 3

Fig 4



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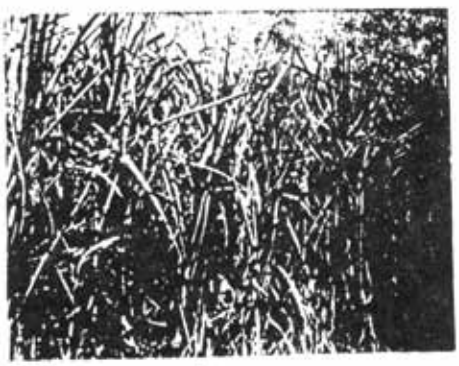

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In Vitro Propagation of Citrus Sinensis (Junar)

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Abstract

The shoots were regenerated from the cotyledonary node and stem segment of *citrus sinensis* when cultured in Murashige and Skog medium (MS) in the presence of benzylaminopurine (BAP) 1mg/l and napthyl - acetic acid (NAA) 0.1 mg/l. The shoots increased in number when the microshoots were sub-cultured in the same medium supplemented with lower concentration of BAP and NAA and addition of coconut milk. The microshoots produced roots when transferred to sand and the rooted plants were established in the field.

Introduction

Citrus sinensis (Junar) constitute an important group among citrus fruits and one of the most valued commercial citrus in the world. It is a native of China and is now cultivated widely in the sub-tropical regions. The major Junar growing areas in Nepal are Dhankuta, Sindhuli and Ramechhp districts. The Junar of Sindhuli is excellent and the best among the many foreign varieties grown in Nepal (NCDP 1980/81 - 1984.85). The fruit is nutritious, sweet, and juicy. It is highly esteemed as a dessert fruit.

Citrus orchard in Nepal were entirely of seedling origin before 1980. Recently grafted plants are getting popularity. Very little research has been done on citrus species indigenous to Nepal. In vitro propagation method has proved to be a useful method for crop improvement and to overcome the limitation of the conventional method of propagation.

Chaturvedi and Mitra (1974), Raj bhansali and Arya (1978), Spiegel Roy and Kochha (1980), Barlass and Skene (1982) reported the formation of adventitious buds from callus. The present paper reports on in vitro regeneration potential of cotyledonary node and stem nodal segments of *Citrus sinensis*.

Material and Method

The seeds were obtained from fresh fruit available in local market. The seeds were washed in running water for one hour, then surface sterlised with 0.1 percent mercuric chloride solution for twenty minutes. All the seeds were then rinsed five to six times with sterlised water. The seed were cultured in basal Murashige and Skoog (1962) medium.

The cotyledonary node and nodal segments of the germinated seedlings from 6-8 weeks old seedlings and were cultured in MS medium supplemented with BAP 1.0 mg/l, NAA 0.1 mg/l and casein hydrolysate 1000 mg/l. (Niroula and Rajbhandari 1989).

The pH of the medium was adjusted to 5.8 before autoclaving and 0.65 percent agar was added to solidify the medium. The medium was sterilized at 15lb/sq inches pressure for 15 minutes. The cluture was incubated at $25^0c \pm 4^0c$ under 3000 lux for 16 hour photoperiod.

After 6-8 weeks of culture, (2-4 shoots developed from the nodal region of the stem segment and the cotyledon), the developed shoots were excised at the basal region and were further sub-cultured in different concentration of BAP ranging from 1-5 mg/l either alone or in combination with NAA 0.01 - 0.1 mg/l. The combination of cytokinin and auxin which gave high rate of proliferation and well developed shoots was selected for further sub-culture. For rooting the multiple shoots were excised and transferred to non sterile sand.

Results and Discussion

After 6-8 weeks of culture 4-6 shoots were developed from the explants in BAP 1.0mg/l and NAA 0.1 mg/l. The multiple shoots were further sub-cultured in the MS medium with different concentration of BAP and NAA. The higher concentration of BAP (5.0mg/l) inhibited shoot proliferation. The shoots buds were

Table No. 1 : Growth Response of *Citrus Sinensis* Shoots in MS Medium with Different Conc. of BAP + NAA.

S.N.	BAP + NAA mg/1	No. of shoots	Growth response
1.	5 + 0.1		Explant became yellow
2.	1 + 0.1	20-25	Shoots were stunted
3.	5 + 0.1	20-25	Shoots were well developed
4.	25 + 0.1	15-25	Not well developed
5.	1 + 0.1	15-25	Not well developed

developed in BAP 1.0mg/l and NAA 0.1 mg/l but they had stunted growth. The shootbuds were developed after 6-8 weeks of culture in lower concentration of BAP but the growth was less as compared to that of BAP 0.5 mg/l. The growth of proliferated shoots found optimal in BAP 0.5 mg/l, NAA 0.01 mg/l with the addition of 15% coconut milk. In NAA 0.1 mg/l callus developed at the base. The absence of NAA also reduced the growth of the shoots. The growth response of shoot in different combination of BAP and NAA is shown in Table No. 1.

The addition of 15% coconut milk was beneficial for shoot regeneration. After regular sub-culture for three years, cultures had maintained their ability to proliferate.

Shoot developed roots after 4-6 weeks of transfer in non-sterile sand. Forty to fifty percent of shoots were rooted under day and night temperature of 35^0c/15^0c under 60-80 percent humidity and light intensity of 4000-5000 lux. The rooted plants were transplanted in soil.

In general maximum shoot production in citrus culture has been achieved through the use of BAP and other cytokinins are less effective (Grinblat, 1972; Rajbhansali and Arya. 1978; Barlass and Skene, 1982) Taking into consideration this fact BAP is the only cytokinin tested in this research. A general finding from other work is that malt extract was beneficial for shoot regeneration. In our culture 15% coconut milk is sufficient for shoot development.

Conclusion

Tissue culture has proved to be useful for citrus crop improvement and propagation. Our results indicate the potential of multiplying the selected scion under controlled condition. The transport cultivars in aseptic condition may simplify the quarantine requirements. The seeds do not carry any bacterial or viral diseases, so the plants are disease free. The successful rooting of the shoot in non sterile sand suggests the applicability of using this method in large scale production of citrus plants.

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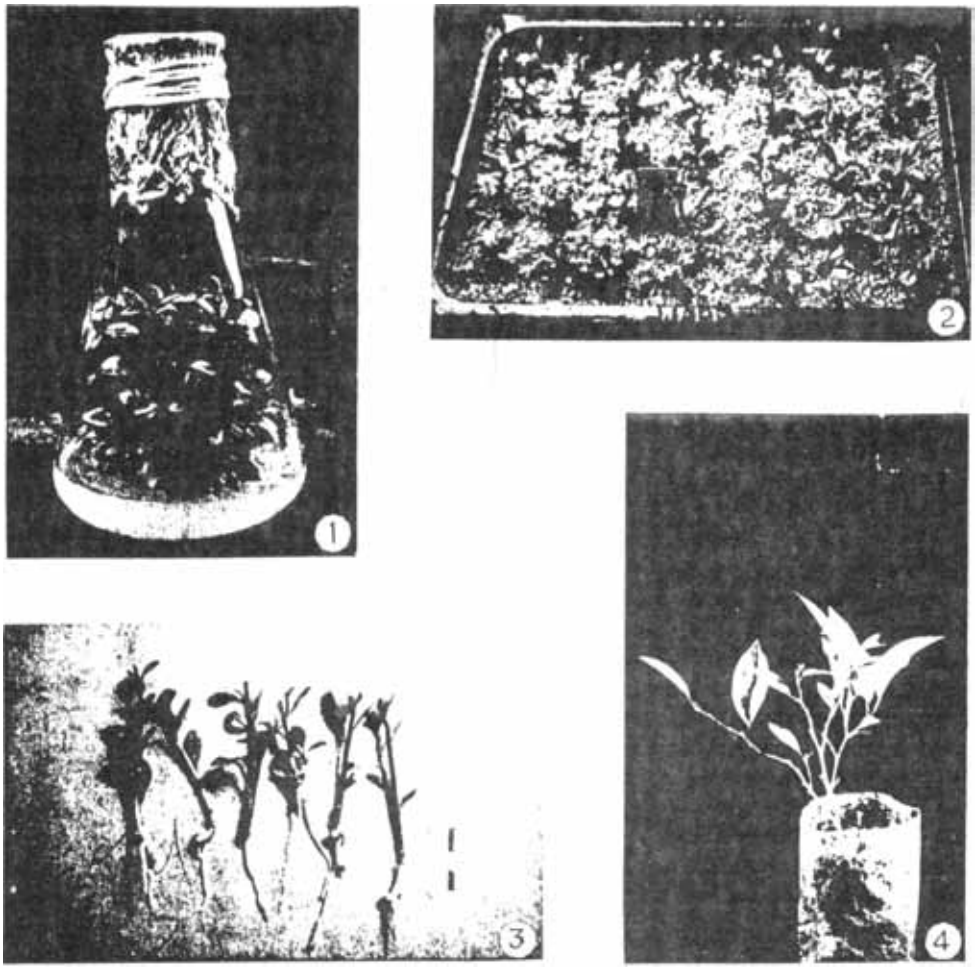

1. **Multiple shoot formation after 8-12 weeks.**
2. **Shoots transferred in sand.**
3. **Rooted plants after 4 weeks.**
4. **Established plant in soil, one year old.**

In vitro culture of Gladiolus

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Abstract

The present paper describes the initiation of shoot buds from apical and axillary corm buds of Gladiolus in Murashige and Skoog's medium (1962) supplemented with 1mg/lit of BAP (Benzyl amino purine) and 0.02 mg/lit of NAA (Napthalene acetic acid). The initiated buds produced microshoots when cultured in MS medium supplemented with 1.0 mg/lit of kinetin (KN). The micro-shoots were rooted in non-sterile sand. The rooted shoots produced in soil within 2 to 3 months.

Introduction

Genus Gladiolus belong to family Iridaceas and sub-family lxioideae. It includes over 180 spp. with more than 10,000 cultivars (Wilfret, 1980). It originated in the Mediterranean area and South Africa (Puch 1972, Wifret 1980). The present Gladioli are successive hybrids of these wild grown species.

Gladioli are herbaceous and annually flowering plants. The leaves are mostly linear 2.5 to 5 cm broad, 30 cm to 120 cm long with many nerves, arising from the swollen corm. Infloresence is spiked, flower bisexual, funnel shaped, epipetalous, perianth of various color red, white, blue, pink, yellow, maroon etc.

Gladioli have beautiful flowers and extensively used as cut-flowers. These are propagated vegetatively by corms and cormlets. The rate of its propagation is very slow. Hence tissue culture techniques have been attempted for clonal propagation of new selected hybrid, and for disease free plants.

Materials and Methods

Five cultivars of Gladiolus were used namely (i) shell pink (ii) Thombolina (iii) Yellow frilled (iv) Maroon and (v) American beauty for the present study.

The corms were washed for 2-3 hrs in running tap water, surface sterilized 5 min with teepol and thoroughly washed with distilled water. The dried scaly leaf bases covering apical bud and axillary buds were removed from corm. These corms were sterilized with 0.1% mercuric chloride solution for 15 to 20 minutes and rinsed thoroughly with sterile distilled water. The apical and axillary buds were excised with the minimum amount of corm tissue. These excised buds were kept in Murashige and Skoog's (1962) medium with different concentration of BAP and NAA.

The medium was supplemented with 3% sucrose, and 0.7% agar and pH adjusted to 5.6 - 5.8 before autoclaving. The media were autoclaved at a pressure of 15 1b/in^2 for 15 minutes. The cultures were incubated at $25^0 \pm 2^0$C temperature and 16 hours photoperiod.

The proliferating shoot buds obtained from Murashige and skoog medium with BAP and NAA were subcultured on MS medium with 1 mg/lit of kinetin for shoot elongation. The elongated shoots were transferred to non-sterile sand for rooting under glasshouse condition . The rooted plantlets were transferred to polybags with soil for field establishment.

Results andDiscussion

Explants began to swell within 1 to 2 weeks after inoculation in all concentrations. The shoot buds slowly proliferated from base of the explants within 4 to 6 weeks. The shoot buds formation in BAP 1.0mg/lit + NAA 0.02 mg/lit and BAP 0.5 mg/lit + NAA 0.02 were loosely arranged, but in other concentrations, the shoot buds were disorganized mass and compactly arranged.

Table No. 1 : Growth responses of explants in different concentrations of BAP and NAA

Medium	In 1 to 2 weeks explants response	Growth response in 4 to 6 weeks
BAP + NAA (mg/lit)	Swelling	Compact shoot bud
MS .5 + /02	"	Loosely arranged shoot but
MS 1 + .02	"	More "
MS .5 + .02	"	Compact shoot buds
MS .5 + .1	"	"
MS .1 + .1	"	"

MS medium with BAP 1.0mg/lit + NAA 0.02 mg/lit was found to be best in comparision to the concentrations for production of shoot buds. Ziv (1979) also used MS medium with BAP 0.2 mg/lit and NAA 0.5 mg/lit for culturing axillary buds in cultivar Euro-vision. Hussey (1977) used ½ MS with BAP 1.0mg/lit in cultivar Forest fire, Elvira, Kiprus and Kochba (1987) also used MS medium with BAP and NAA for shoot proliferation from axillary buds.

The micro-shoots were elongated within 8 to 10 weeks on MS medium with 1.0mg/lit kinetin.

The microshoots transplanted in non-sterile sand developed roots in one to two weeks. Three to five months old plants in soil developed cormlets of various sizes. Ziv et. al (1979) used MS medium containing only auxin to develop roots. Hussey (1977) obtained cormlets from cultures when incubated for a longer period of time.

The present result are consistent with that of Hussey and Ziv. In the Hussey and Ziv experiments the formation of root and cormlets were in media, but in this experiment, the development of roots & cormlets were in non-sterile sand and soil.

In the first year of experiment, plantlets transferred to polybags did not flower and cormlets of sizes 5mm to 1.0 cm diameter were obtained. In 2nd generation, the cormlets obtained from 1st year did not flower, but the sizes of cormlets increases to 2 cm to 3 cm in diameter. Further studies are being carried out to obtain flowers from these enlarged corms.

It is therefore apparent that mass production of improved planting stocks of different cultivars of Gladiolus is possible, through tissue culture method.

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Figure No. 1

Figure No. 2

Figure No. 3

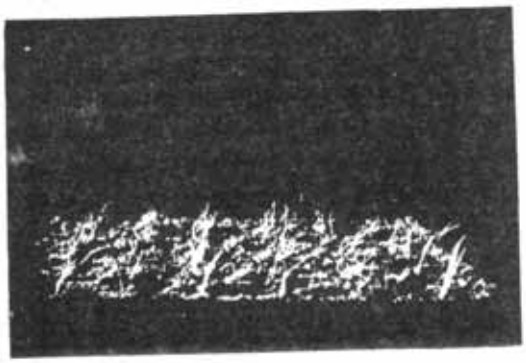

Figure No. 4

Figure No. 5

Nicotiana Tabacum L. Plants from Anther Culture

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Abstract

Nicotiana tabacum L. anthers were cultured In n Murashige and Skoog medium supplemented with 0.5 mg/l Napthaleneacetic acid and 1.0 mg/l Benzylamino purine. Some anthers formed shoots directly whereas others formed callus only. The callus formed shoots after transfer to medium supplemented with 1.0 mg/l Benzylamino purine. The microshoots are transplanted to non-sterile sand for the formation of roots. The rooted plants were transferred to soil. The plants derived directly from anthers were haploid whereas callus derived plants were diploid.

Introduction

The discovery of anther culture of *Datura innoxia* leading to plants by Guha and Maheshwri (1964, 1966, 1967) opened up a new technique of inducting of haploidy in higher plants. Anther culture technique has been employed to obtain microspore derived callus, embryos and plants in over 247 plant species (Maheshwari et al., 1983) The member of solanaceae have the greatest potential for androgenesis (Bajaj, 1983).

The production of haploid plants from the pollen of *Nicotiana tabacum* has been reported by Bourgin and Nithsch, 1967; Nakata and Tanaka, 1968; Nitsch and Nitsch, 1969; Sunderland and wocks, 1969, 1971; Sunderland, 1984; Aruga et al., 1985. Non-haploid production has also been reported in tobacco (Tanaka and Nakata, 1969).

Methodology

Nicotiana tabacum L. flower buds were picked fresh from the field. The young flower buds, about 0.5 cm long, were washed three times with sterilized distilled water in the culture room. The anthers were taken out from the bud and transferred to the culture flasks. One thousand five hundred anthers were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/l Napthylaceti acid (NAA) an 1.0 mg/l Benzylamino purine (BAP). The medium was adjusted to 5.8 pH before autoclaving. The anther cultured flasks were incubated at 25^0C temperature. Light was provided with fluorescent tube-lights approximately 3,000 lux. Light was maintained for 16 hours.

Results andDiscussion

Sterilization of *Nicotiana tabacum* flower buds only sterilized distilled water was found to be sufficient for cleaning the buds before anther culture.

Out of 1500 cultured anthers in MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/lBAP, 100 anthers (6.6%) responded to direct shoots regeneration, whereas 500 anthers (33.3%) responded to form callus, and 900 anthers (60%) did not respond to this medium (Table 1). It is probable that only a few anthers in a bud tend to have the capacity to form shoots directly, others have the tendency to callusing whereas others do not have either of these tendencies. This result is similar to the reports given by suderland and Wicks (1971).

The callus formed through anther culture upon transfer to MS medium supplemented with 1 mg/l BAP formed shoots after eight weeks. Once the shoots formed, they were transferred to non-sterile sand and covered with polythene sheet hood to keep 79-80% humidity with 30^0C temperature. The roots were formed after four weeks. The rooted plants were transplanted in field. Manandhar and Rajbhandary (1986) have reported this technique of rooting of tissue cultured plants. The present study showed that rooting of shoots derived from anther culture is also possible in sand under glass house condition.

Table no. 1 : Response of in vitro cultured anthers in MS medium supplemented with NAA (0.5 mg/l and BAP (1.0 mg/l).

No. of in vitro cultured anthers	Response		
1,5000	No. of anthers	Result	%
	100	Direct shoot formation	6.6%
	500	Callusing	33.3%
	900	No response	60%

The majority of the anther derived plants were haploid. But diploid and polyploidy plants have also been regenerated. In *Nicotiana tabacum* L. haploid (Sunderland, 1974), and completely diploid (Kasperbauer and Collins, 1972) plants have been obtained. Diploid strains of Nicotiana have been obtained by endomitotic phenomenon which commonly occurs in callus cultures (Nitsch, 1969, 1969). In the present investigation also haploid plants might have been the product of embryogencsis in the pollens and diploid plants might have been the product of organogensis in anther derived callus. A further research on callus culture is necessary to produce shoots as has been obtained 33.3% from 500 anthers cultured.

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Anther culture *of Allium fistulosum* L.[1]

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Abstract

Anthers of *Allium fistulosum* were cultured on N_6 medium supplemented with 2,4 Dichlorophenoxyacetic acid (2,4 D) and 6 furfuryl amino purine (kinetin). The callus developed from cultured anthers. The callus was then subcultured on Murashige & Skoog's (MS) medium with coconut milk for the formation of green embryo like structures. These developed shoots after transfer to MS medium supplemented with Benzyl amino purine (BAP) formed roots after transferring to non sterile sand.

Key Words : *Allium fistulosum, anther*, callus.

The discovery of anther culture of *Datura innoxia* leading to plant formation by Guha & Maheshwari (1964, 1966, 1967) opened up a new technique of inducing haploidy in higher plants. Technique of anther culture has been employed to obtain microspore derived callii, embryos, and plants in over 247 plant species (Maheshwari et al. 1983). The members of Solanaceae possess the greatest potential for androgenesis (Bajaj 1983). However, active research in the development of anther cultured plant has been confined to a few crop species like barley, maize, rapeseed, rice, potato tobacco and wheat.

Allium fistulosum is a member of Liliaceae. Its leaves are used as vegetable. Due to its cross pollination nature it is highly heterozygous.

In this paper steps involved in anther culture of *Allium fistulosum* leading to plants have been described.

Materials and Methods

Inflorescences of *Allium fistulosum* were picked fresh from the glass house. The inflorescences were sterilized by dipping in 70% ethanol, then immersed for 3 minutes in filtered solution of 7% calcium hypochlorite and rinsed three times with sterile distilled water in the clean air bench. The anthers were cultured on the N, medium (Chu 1978) supplemented with 25g/l sucrose, 1.0mg/l of 2,4 D and 0.25 mg/l of kinetin. The flasks with explants were incubated at temperature 25°C±4 under fluorescence light intensity of approximately 3,000 lux. Day light was maintained for 16 hours. After callus formation, it was cultured in MS medium (Murashige & Skoog 1962) supplemented with 2% coconut milk. The green embryo-like structures were subcultured in MS medium supplemented with 2% coconut milk and 5 mg/l of BAP to induce shoots. Once the shoots are formed they are transferred to non-sterile sand for rooting.

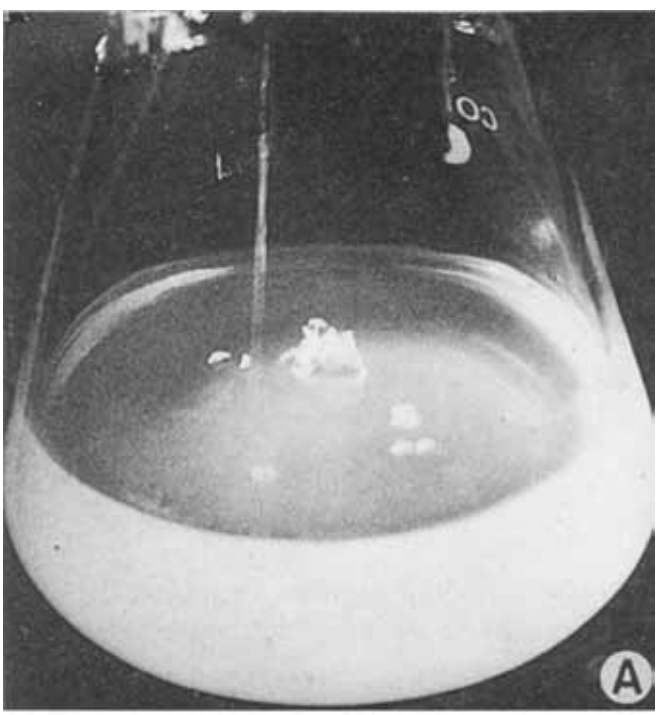

Fig. : A. Callusing anther

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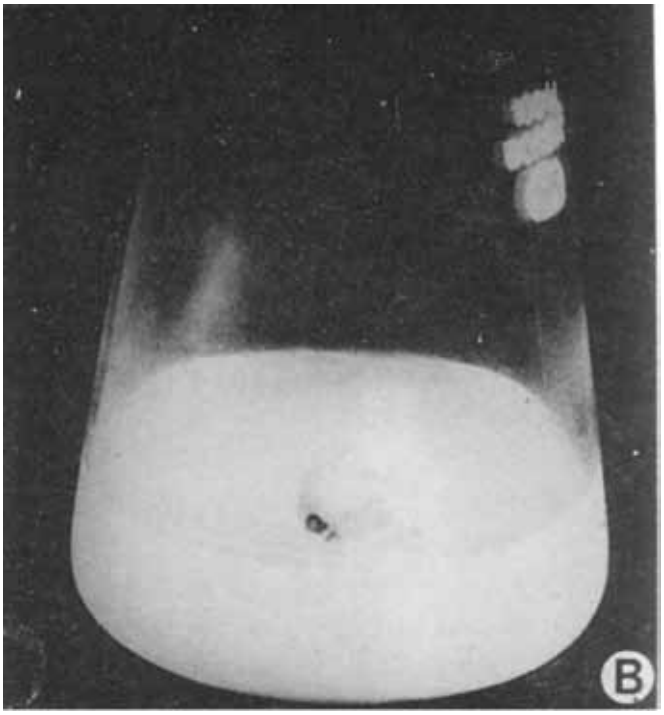
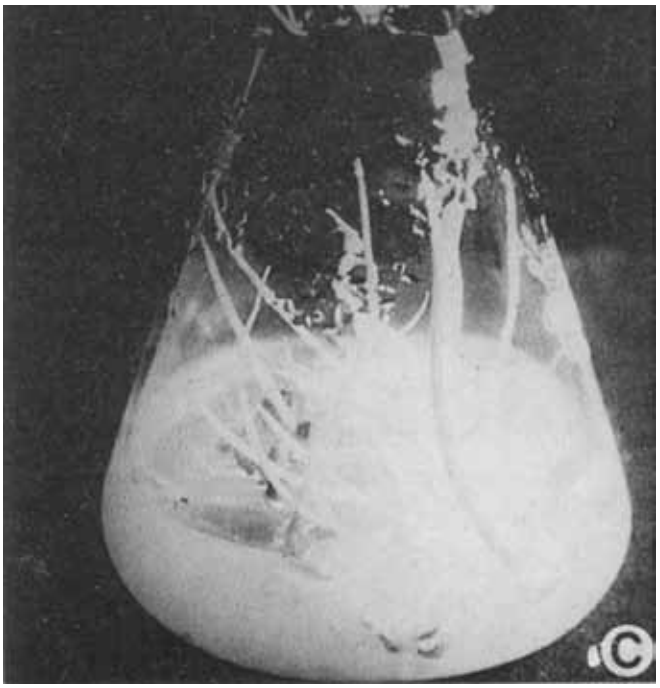
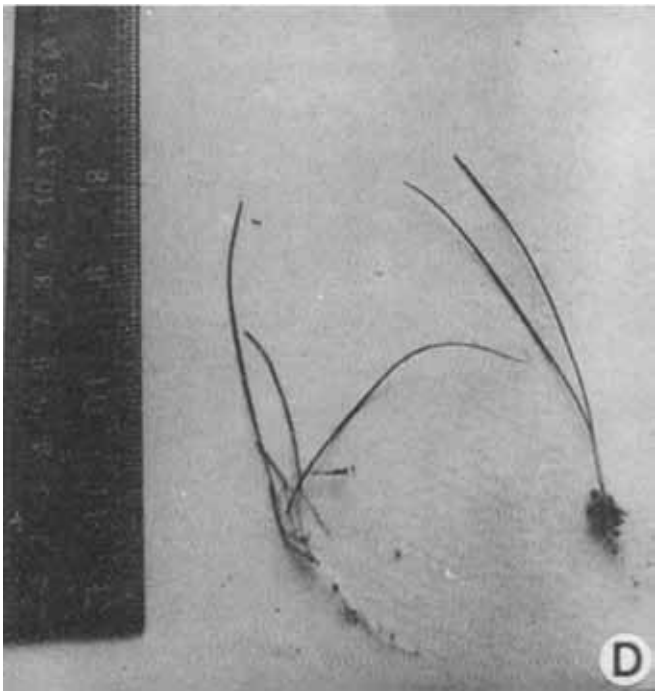


B. Callus with embryoids



C. Shoots formation from embryoids



D. Root formation in non-sterile sand.

Observations and Discussion

Callus was formed within 4 weeks (Fig. 1A) of incubation in N_6 medium supplemented with sucrose and growth hormones. It was kept in the same medium for 6 more weeks to allow more callus growth. Subsequently, the callus was cultured on MS medium supplemented with coconut milk. Few green embryo like structures were formed in callus mass (Fig. III). The embryoid bearing callus mass was subcultured on MS medium supplemented with coconut milk and cytokinin. Leaves appeared from the green embryoids after 10 weeks of incubation (Fig. 1C). The shoots with 7 cm long leaves were transplanted in non sterile sand in the wooden box and covered with polythene sheet to keep humid. The roots were formed in the sand box after 5 weeks (Fig. 1D). Once the roots were formed, they were transplanted in the soil. It was interesting to note that the plants obtained, from anther culture have non functional pollen.

A combination of both 2,4 D and kinetin was found to be essential for the anther culture of *Allium fistulosum*. The anthers maintained green on the N, medium with kinetin. These green anthers only led to callusing. This observation is related with the report by Skoog & Miller (1957) that a combination of an auxin and a cytokinin is often essential for DNA synthesis and mitosis in cultured material.

Embryogenesis is induced in the callus which has been cultured on the MS medium supplemented with coconut milk. Since watery endosperm of coconut contains the growth promoting factor at all stages

of development (Steward & Caplin 1952), it is apparent that for embryogenesis in callus of *Allium fistulosum* such growth promoting factors play an important role. Cytokinins in high concentration was found to induce shoots in monocots (Woo & Chen 1982); in *Allium fistulosum* also, high concentration of BAP led to shoot proliferation. Once the shoots are formed in in vitro culture it can be rooted in non sterile sand (Karki & Rajbhandary 1984, Manandhar & Rajbhandary 1986). The rooting of shoots of *Allium fistulosum* obtained from anther culture was also possible in non sterile sand kept in box and covered with polythene sheets for maintaining humid and warm condition.

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Plant Tissue Culture in Nepal

H.K. Saiju

Introduction

Plant tissue culture is an important aspect of biotechnology. It has great potential for rapid, large scale and true to type multiplication. The plants from tissue culture are generally clean and healthy. This technique has been applied to several crop species. This technique is of particular interest in case of highly heterozygous species which are virus infected and are generally vegetatively propagated.

Since 1963, in vitro culture has been growing rapidly throughout the world. Mass propagation of some ornamentals is one of the most successful examples of commercialization of tissue culture technology. Multimillion dollar industries have been set up in the world to meet the demand for quality plants. Realizing the potential, tissue culture techniques protocol development for different species are being carried out in different laboratories.

A brief review of the activities on plant tissue culture carried out by different institutes in Nepal is summarized as follows.

Tissue Culture Laboratory (TCL)

Tissue Culture Laboratory was established in 1976 in National Herbarium & Plant Laboratories. This laboratory has developed the techniques for micropropagation of economically important plants and elite trees to produce best quality clone plants. Micropropagation protocols have been developed for 75 plant species and varieties including trees, horticultural plants, medicinal plants, ornamentals and orchids. Anther culture technique has been developed for 3 species. The most significant achievement of this laboratory has been the development of the "Technique of micro shoots rooting in non sterile sand". This method of rooting has been successfully done in all micropropagated shoots. This technique has significantly reduced the cost of production of tissue cultured plants. Rooted plants have easily survived in field plantations.

Training on tissue culture laboratory technique and sand rooting of micropropagated shoots has been a regular program of this labdratory, Three private tissue culture factories in Nepal have been using the micropropagation protocols and sand rooting techniques developed in this laboratory for commercial production of economically important plants.

Tissue Culture Protocols Developed at TCL, Godavari

Protocols developed at the Tissue Culture Laboratory, Godavari are listed below:

Trees:

Artocarpus lakoocha, A. heterophyllus, DaLbergia sissoo, Eucalyptus camaldulensis, E. citriodora, Ficus auriculata, F. carica, F. elastica, F. lacor, F. nemorallis, F. semicordata, Morus sp., M. alba, Populus ciliata.

Horticultural Plants:

Banana, Cabbage, Cardamom, Cauliflower, Cherry, Potatoes, Strawberry, Sweet potato, Zinger, *Citrus lemon, C. sinensis, Curcuma sp., Fortunella sp., Poncirus trifoliata, Ullucus tuberosus.*

Medicinal Plants:

Atropa belladonna, Chrysanthemum cinerafifolium, Solanum laciniatum.

Ornamental Plants:

African violet, Carnation, Chrysanthemum, Gladiolus, Lily, Rose

Orchids:

Over 37 varieties of orchids belonging to genera *Coelogyne, Cymbidium, Dendrobium, Epidendrum, Poniorchis, Rhynchostylis and Vanda.*

Anther Culture

Allium fistulosum, Nicotiana tabaccum, Oryza sativa.

Potato Research Programme, National Agriculture Research Council

Potato Research Programme was initiated in Nepal for promoting potato development in Nepal. Under this programme, a modern plant tissue culture laboratory was established in collaboration with Swiss Development Cooperation. The main aim of this laboratory is to produce and supply virus tested, disease free minitubers, the pre basic seed potato production. Virus free and pathogen free *in vitro* mother plants of potatoes are obtained from International Potato Center. Virus free plants are tissue cultured. The plantlets are tested for virus using double anti body sandwich enzyme linked immunosorbent assay technique (DAS-ELISA). Presently prebasic seeds of Kufrijyoti, Desire, Achirana Inta, CFJ, CFM, Sarkari seto, Kufri Sindhuri are produced. These prebasic seeds were distributed for plantation in government farms and seed growers association in the country.

In vitro germplasm conservation of 94 potato cultivars has been taken up. Every year local potato varieties are cleaned from six potato viruses viz. PVA, PVM, PVS, PVX, PVY, and PLRV by using thermotherapy and apical meristem culture.

Central Department of Botany (CDB), Tribhuvan University

The main objective of tissue culture laboratory at CDB is to provide training to M.Sc. students on plant tissue culture and conduct academic research. Dissertation researches are the main activities of post graduate students.

More than a dozen students have been trained in plant tissue culture at CDB. Most of them are working in different tissue culture laboratories. Research on *in vitro* morphogenesis and micropropagation of ferns, oilseeds, legumes, pseudocereals, medicinal plants was carried out in the past and it is still continuing. Similarly, studies on effect of different plant growth regulators on morphogenesis, embryogenesis, totipotency of cells, induction of haploidy, genetic variation and micropropagation, somaclonal variation is being carried out.

Research Laboratory for Agricultural Biotechnology and Biochemistry (RLABB)

RLABB is a private research institute established in 1986 with the objective of basic and applied research in the field of agricultural biotechnology. This laboratory has facilities for plant tissue culture research, It has developed a new technique, inverted embryo technique for micropropagation of Pinus roxburghii and P. wallichiana. In this technique, embryos are implanted in inverted position in medium. Micropropagation of Artocarpus lakoocha from nodal segments and Brassica campestris from leaf segments have been done in RLABB. This laboratory has carried out anther culture of 70 parental and hybrid rice varieties. Studies on albinism in rice anther culture has been taken up.

RLABB, also provides training in plant cell, tissue culture and gene technology.

Biotechnology Research Laboratory, (BRL), RONAST

BRL is currently engaged in shoot tip micro grafting to eliminate virus and virus like diseases of citrus. Some disease free plants have been produced by this technique. Protocols of modified shoot tip grafting has been developed. This technique has been used to eliminate greening, citrus tristeza virus (CTV), xyloporosis and other virus and virus like diseases of citrus, specially of oranges/mandarins.

Tissue Culture Factory, Botanical Enterprises Private Ltd. (BE)

BE is a private enterprise. It is engaged in large scale production of tissue cultured potatoes, orchids, *Miscanthus* spp.

Nepal Biotech Nursery (NBN)

NBN, is a private tissue culture laboratory engaged in tissue culture of banana, potato, ginger, fodder trees, orchids and ornamental plants.

Himalayan Floratech, Private Ltd. (HF)

HF is a private company established in 1992. It is engaged in tissue culture of banana and strawberry for large scale production. This company has a programme to encourage small farmers for the plantation of tissue cultured plants. This company plans to expand its activities through joint ventures.

The review of major ongoing activities of plant tissue culture in Nepal shows that the activities are geared towards:

a. Micropropagation of selected economic plants.
b. Production of disease free pre basic seed potato.
c. Training of manpower in tissue culture technique.
d. Sand rooting of micro shoots.
e. Commercialization of tissue cultured plants.

Conclusions

In Nepal, tissue culture scientists have developed an innovative technique of sand rooting which has been successfully applied by private laboratories in the country for the large scale production of economically important plants. In addition, Nepal has developed expertise in the production of virus tested and disease free pre basic seed potato. Nepal is ready to share its experiences with other developing countries.

Rooting of microshoots of *Artocarpus heterophyllus* Lam. on non sterile sand as a potentially cost effective means of mass propagation

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Abstract

Cotyledonary nodes of *Artocarpus heterophyllus* Lam. when cultured in Mura-shige and Skoog medium (MS) supplemented with 5.0 mgl^{-1} benzylaminopurine (BAP) initiated shoots. These shoots were subcultured in the same medium supplemented with 1.0 mgl^{-1} BAP and 0.01 mg 1^{-1} napthalene acetic acid (NAA) for multiplication. The roots initiated on non sterile sand after five weeks but did not survive well on transplantation to the field.

Keywords: *Artocarpus heterophyllus,* Benzylaminopurine, micro shoots, MS medium, Napthalene aceti acid, propagation, rooting.

Introduction

Artocarpus heterophyllus Lam. is commonly known as jackfruit. The tree is mainly cultivated in tropical and subtropical region of Nepal for its fruits, though its timber is of high quality. The plant is also used for dye, fuel, fodder, building materials and for making musical instruments. It is regarded as the second most important multipurpose tree (MPTS) (Raintree, 1991). Locally, the plant is mainly propagated through seed but as it loses viability within a few days (Jackson, 1994), tissue culture may be a better option for mass propagation.

The production of microshoots and root initiation *in vitro* has been described by Rahman (1988), and Rahman and Blake (1988) but is costly. An alternative method has therefore been applied to reduce the production cost of tissue cultured plants through a new rooting technique in non sterile sand.

The present paper describes the proliferation of shoots *in vitro* for mass propagation, and rooting of these shoots in non sterile sand for field establishment.

Materials and Methods

Seeds were recovered from the fresh fruit four hours before culture. They were washed in running water for two hours and then in teepol for a few minutes. Finally they were sterilized in 0.1% mercuric chloride for 15 minutes and washed two to three times with distilled water. The seeds were then left to germinate in MS medium. (Murashige and Skoog, 1962).

After germination, the cotyledonary nodes were excised from two to three weeks old seedlings and cultured in MS medium supplemented with 5.0 mgl^{-1} BAP and 1.0 gl^{-1} casein hydrolysate. The medium was solidified with 6.5 gl^{-1} agar and the pH was adjusted to 5.8 before autoclaving at a pressure of 15 lb. The cultures were incubated at 16 hour photoperiod at 25°C + 4°C.

After the development of shoots from the nodal segment, shoots were subcultured in MS medium supplemented with different combinations of growth hormones for better proliferation. Micro-shoots of 2-4 cm were excised and dipped in indole acetic acid for 15 mins. They were then rooted in non sterile sand. The rooted plants were transferred to soil for field establishment.

Results and Discussion

Seed germinated within three to four weeks. After eight weeks, 8-10 microshoots from the cotyledon-ary node were proliferated in MS medium

Table 1 : Effects of growth substances on shoot proliferation of *Artocarpus heterophyllus* Lam.

Concentration of growth harmones used	No. of shoot developed	Remarks
1 mgl^{-1} BAP	5-10	Plants healthy, 1-3 cm long, no callus formation
1 mgl^{-1} BAP+0.01 mgl^{-1} NAA	30-40	Plants healthy, 1-3 cm long, no callus formation
1 mgl^{-1} BAP+0.01 mgl^{-1} NAA+10% coconut oil	10-15	Plants healthy, 0.5-1 cm long, a large amount of callus formation.

supplemented with 5.0 mgl^{-1} BAP. A large amount of callus also developed from the nodal segment and the margin of the cotyledon when cultures were kept for more than 10 weeks.

The shoots from cotyledonary nodes were subcultured on MS medium supplemented with different combinations of auxin and cytokinine (Table 1). Experiments showed that shoot formation was most consistently induced by 1 mgl^{-1} BAP and 0.01 mgl^{-1} NAA. Hence, 1 mgl^{-1} BAP and 0.01 mgl^{-1} was used for shoot multiplication. The number of shoots increased to 40 after repeated subcultures (7-8 times) in the same medium (Fig. 1).

The proliferation continued even after 2 years. Microshoots of 12-14 weeks old were used for rooting. Root initiation was observed 4-5 weeks after planting them on non sterile sand (Fig 2). Rahman (1988) reported that *in vitro* rooted plants do not require any nutrient for transplanting to *en vitro* condition in the glass house. In the present experiment also 90% of the rooted plants survived only by water spraying, and continued to grow well in the greenhouse.

However, only a few of our rooted plants survived when transferred to the field (Fig 3). The growth of these plants was also very slow as compared to other tissue cultured plants at Godawari.

This suggests that climatic conditions at Godawari may be unsuitable for field establishment of this species. A growth study of such plants should be carried out in suitable climatic zones to derive further results.

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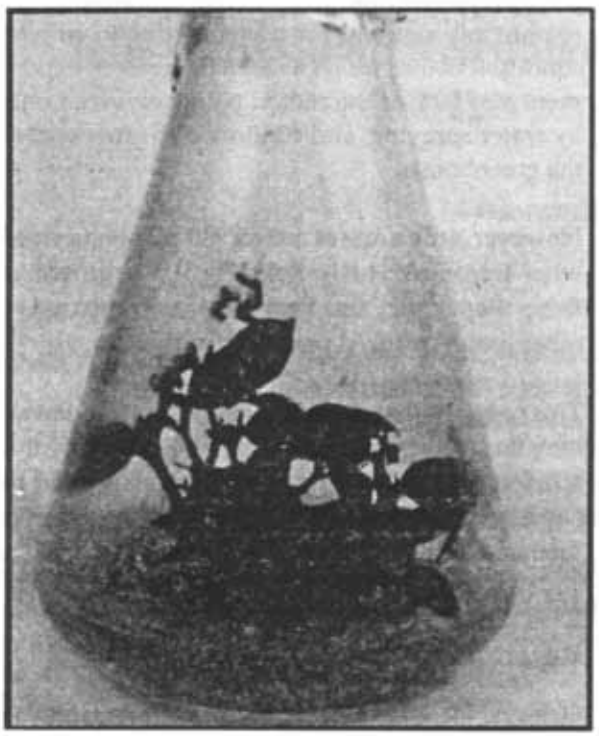

Fig.1 : Multiplication of shoots 8-9 weeks after subculture

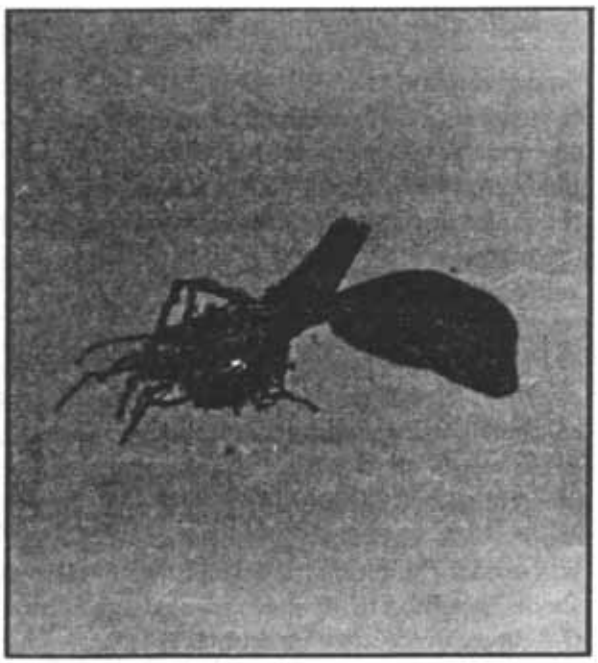

Fig. 2 : Roots appear after transplantation to non-sterile sand

Fig. 3 : A one year old plant

Rooting of *Populus ciliata* explants, on non sterile sand as a potentially cost effective means of mass propagation

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Abstract

Shoot tipexplantsfrom the cuttings of mature *Populus ciliata*, were cultured over a filter paper bridge in Murasige and Skoog (MS) liquid medium and supplemented with 5 mg/l benzylaminopurine (BAP) and 1 mg/l napthelene acetic acid (NAA). This avoids browning of explants. The latter were subcultured in MS solid medium with 0.5 mg/l BAP and 0.2 mg/l NAA and 10% coconut milk. After 10 to 12 weeks the shoots were rooted in non sterile sand. This obviates the need for transplantation in the laboratory and the associated costs that would be incurred.

Keywords: *Populus ciliata,* afforestation, benzylaminopurine, MS medium, napthalene acetic acid, sand rooting.

Populus ciliata is a large, fast growing, decidious, drought resistant tree. It is generally found in the Himalayan region at above 2000 m. It is mainly used as pulp for making papers, match sticks, plywood and, packing boxes. The trunk is used in building construction and the leaf and bark for animal feed.

Winton (1968) produced the first tissue culture plant of the species in quaking aspen (*Populus tremuloides)* from callus culture. Winton and Wolter were successful in regenerating several species of Populus from callus culture.

Mass scale propagation of selected aspen clones has been carried out by Ahuja and Muhs, (1982). This paper describes similar work in *Populus ciliata*, in propagating high yielding clones for mass scale production. The present study also attempts to avoid browning of shoot explants during culture by use of a filter paper bridge.

Method

Cuttings of mature trees, 8 10 years old, of *P. ciliata* were taken in their dormant stage. The cuttings were kept in a sand box for sprouting of shoot buds in the greenhouse. Shoot buds were observed after 20-25 days. Buds of 1-2 mm were sterilised with 0.1% $HgC1_2$ for 8-10 minutes and washed six times with sterilised, distilled water. Shoots of 2-4 mm were excised from the bud sprout and cultured in MS liquid medium supplemented with BAP 5.0 mg/l and NAA 0.1 mg/l over a filter paper bridge. After 10-15 days, green shoot buds were taken out from the filter paper bridge and cultured in MS solid medium (1963) supplemented with BAP 0.5 mg/l, NAA mg/l and adenine sulphate 20 mg/l with 10% coconut milk.

The pH of the medium was adjusted to 5.8 before adding agar and autoclaving. The medium was sterilised at 15 lbs pressure for 20 minutes. The cultures were incubated at 25±2°C in light for 16 hours and 8 hours in darkness. The light intensity was 1500 lux at the level of culture flasks.

All experiments were repeated thrice with four replicates in each experiment. For rooting, microshoots; of 3-4 cm were excised and were allowed to root in non sterilised sandboxes.

Results and Discussion

In the various culture media used (Table 1), shoot proliferation was consistently induced by in the presence of 0.5 mg/l BAP, 20 mg/l adenine sulphate and 0.2 mg/l NAA with 10% coconut milk. Shoot proliferation was not satisfactory in BAP alone. After

four weeks of culture the multiplication rate was 30-35 shoots (24 cm high) per explant in the presence of 0.5 mg/l BAP, 20 mg/l adenine sulphate and 40.2 mg/l NAA. Liquid medium was observed to cause browning of shoot explants but this problem was overcome by culturing the explants over a filter paper bridge. The latter absorbed the phenolic compounds present in the explants which are responsible for browning. The shoots were subcultured and transferred at 4-5 weeks intervals. Micro shoots rooted 15-20 days after they were transferred to the sand. A light intensity of 3000-5000 lux was found favourable for rooting. The rooted plants grew to a height of 10-15 cm in 8 weeks. These plantlets were then transferred to soil in pots.

In vitro propagation of Populus *ciliata* through shoot buds was accomplished successfully by using cuttings of shoot bud as explants.

Suwal *et. al.* (1987) reported the successful rooting of *Dalbergia sissoo* in non sterile sand beds. The commercial application of using this large scale method of planting *D. sissoo* is recommended. The present study also showed that rooting by direct transfer of multiple shoots of *Populus ciliata* into non sterile sand was possible. The method thus developed may be cost effective for large scale afforestation programmes at higher altitudes.

Acknowledgernents

The author is grateful to Dr. S. B. Rajbhandari, Ex Director General of Department of Forestry and Plant Research for encouragement and facilities.

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Table 1 : Response of shoot buds after 8-12 weeks culture in different combination of cytokinins and auxin

Explant	Medium (MS)	Results
1. Shoot buds (2-4 mm)	1.0 mg/l BAP	Turned brown
	1.0 mg/l BAP + 1.5 mgA kinetin	5-6 shoots
	20 mg/l adenine sulphate + 0.2 mgA NAA	8-10 shoots
	0.5 mg/l BAP + 20 mgA adenine sulphate	8-10 shoots
	0.5 mg/l BAP + 20 mgA adenine sulphate + 0.2 mg/l NAA	30-35 shoots
	Control	Turned brown and died

Tissue Culture of Mulberry *Morus alba* and its Microshoots Rooting in Sand

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Abstract

Mulberry (*Morus alba* L.) is an important tree for sericulture. Clonal propagation of this species is possible through tissue culture. The tissue derived from microshoots was rooted *ex vitro* in sand. This technique is convenient and easy to transfer microplants to the field.

Key words: clonal, explant, *ex vitro,* microshoot, mulberry

Introduction

Mulberry (Morus *alba* L.) is the most important tree species for sericulture. High sugar and protein content and special fragrance in its leaves attract silkworms. Its leaves support normal cocoon formation and reproduction of silkworm. Mulberry is grown in temperate and tropical regions of the world. It is commercially cultivated in China, India, Japan and Korea.

Traditionally, mulberry trees are propagated by grafting and cutting. For grafting, rootstocks are usually prepared from one year old saplings and scions are taken from branches before bud sprouting. The grafts are grown in the field for one more year. Two years are required for producing grafted plants. Cuttings are taken from hardwood or softwood. They produce plants in one year. The success of softwood cuttings depends on the physiological state of the cuttings and the environmental conditions. Hardwood cuttings have limited rooting percentage. Clonal plants propagated through tissue culture, are either produced through adventitious initiation of shoots or embryoids from callus or axillary branching in buds or meristems.

The aim of this research was to produce microshoots from tissue culture and ex vitro rooting in sand for field plantation.

Materials and Methods

The shoot buds were excised from the mature plus tree, cleaned with soap and washed in running tap water for an hour. The buds were sterilized in 0.1% mercuric chloride solution for 5min. The innermost shoot tip (explant) was cultured in Murashige and Skoog (1962) medium (MS) supplemented with 10% coconut milk (CM), 0.01mg/l^{-1} napthylene aceticacid (NAA) and different concentrations (0.25, 0.5, 1.0, and 2.0mg/l^{-1}) of benzylaminopurine (BAP) for explant establishment. After 4 weeks the established explant was subcultured in MS medium supplemented with 10% CM, 0.01 mg/l^{-1} NAA and different concentrations (0.25, 0.5, 1.0 and 2.0 mg/l of BAP The culture flasks were incubated at 25°C with an 18h light period. Subculturing was carried out every 2 months.

The flasks with microshoots were brought to room temperature for 2 weeks before rooting was done in non sterile sand. The sand was washed with water and sun dried for 2 days, sieved through 2 *x* 2 mm wash, added to a box and wetted evenly with water 10% w/wt. The microshoots were cut into 10 cm long pieces and dipped in 1 mg/l^{-1} NAA for 5 minutes, planted in sand and covered with a polythene sheet. The temperature of the sand box was maintained at 30°C. The sunlight intensity varied from 8 25 kilo lux. Watering was done with a sprayer to maintain 80% humidity.

Results

The shoot tips sterilized with 0.1% mercuric chloride solution for 5 minutes established in MS medium. Out of 100 shoot tips cultured in each medium supplemented with (0.25, 0.5, 1.0 and 2.0 mg/l^{-1}) BAP 80, 40, 20, and 0 shoot tips were established after 4 weeks (Table 1). The explant was best established in medium supplemented with 10% CM, 0.01 mg/l^{-1} NAA and 0.25 mg/l^{-1} BAP.

The green shoot tips subcultured every 2 months in medium supplemented with 0.5 mg/l^{-1} BAP produced 16 microshoots after the 6th subculture (Table 2). Three microshoots were produced in the medium with 1.0 mg/l^{-1}BAP, 2 microshoots in 2.0 mg/l^{-1} BAP and 1 microshoot in 0.25mg/l^{-1} BAP. The microshoots transferred to sand produced healthy roots within 2 weeks. The sand rooted plants established successfully in the field.

Table 1 : Shoot tip establishment in MS medium supplemented with coconut milk, NAA and BAP in mulberry.

Coconut Milk %	NAA mg/l^{-1}	BAP mg/l^{-1}	No. of cultured shoot tips	No. of green tips formed
10	0.01	0.25	100	80
10	0.01	0.5	100	40
10	0.01	1.0	100	20
10	0.01	2.0	100	0

Table 2 : Microshoot multiplication in MS medium with coconut milk, NAA and BAP in mulberry.

Coconut Milk %	NAAmg/l^{-1}	BAP mg/l^{-1}	No. of microshoots produced
10	0.01	0.25	1
10	0.01	0.5	16
10	0.01	1.0	3
10	0.01	2.0	2

DISCUSSION

Shoot tips sterilized in 0.1% mercuric chloride solution for 5 minutes established well in the medium. Oka and Ohyama (1986) sterilized tips in 4% calcium hypochlorite. A combination of 0.01 mg/l^{-1} NAA, 0.5 mg/l^{-1} BAP and 10% CM was found to be the best medium to produce multiple microshoots. Oka and Ohyama (1986) found a single shoot developed with 0.1 mg/l^{-1} BAP and 2-5 shoots with 1.0 mg/l^{-1} BAP.

Holding the flasks with microshoots kept at room temperature for 2 weeks before rooting for acclimatisation, hardening and change to an autotrophic mode of nutrition, benefited establishment. Temperature, light and moisture levels under natural or green house condition will have a positive effect on future growth and development of plants (Kozai, 1991). In vitro produced microshoots of 20 forestry species were successfully transplanted in sand for ex vitro root induction (Saiju, 1997). The non sterile sand rooting method has the potential for mass micropropagation of plants (Rajbhandary & Bajaj, 1991). The tissue culture method combined with ex *vitro* rooting in sand can be an efficient method for clonal plant propagation of forestry and commercial plant species.

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Biotechnology Action Plan of Department of Plant Resources, Nepal

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Abstract

The present status of biotechnology in Nepal is limited to tissue culture activities only, which are carried out in public and private sectors. Department of Plant Resources (DPR), Nepal, has been the pioneer to establish Tissue Culture Lab in 1976. This Lab has successfully developed tissue culture protocols for more than 90 plant species. Other public sectors involved in tissue culture works are Nepal Agriculture Research Council, Tribhuvan University and Royal Nepal Academy of Science & Technology. Few private sectors Labs have been established for commercial micro propagation of plants. Future prospects are in areas of soma clone variation, diseases free plants, germ plasm conservation and exchange, anther culture and genetic engineering. Biotech action plans of DPR incorporate the organization and operation plan. Biotech National Coordination Committee has been proposed. To implement the plan operational plan has been divided into two phases to formulate Biotech policy, use of technology, interdisciplinary linkages and seek internal and external assistance.

Introduction

Nepal is situated on the Himalayan range from 80°04' to 88°12'E longitude, and 26°22'N to 30°27'N latitude. It has area of 147,181 square km with 885 km length east to west and width of about 193 km north to south. The altitude range varies from 60 m in the south to 8848 m in the north. It has a rich bio diversity. Having diversified physiographic zones, it has 10 bio climatic zones ranging from tropical to the alpine and zones making it rich with 35 forest types and 75 vegetation types, Over 2% of the world's flowering plants are found here though it occupies only 0.1% of the world land mass. Master plan forestry sector (1988) has indicated that 15% of the GDP in Nepal is contributed by the forestry sector. More than 5000 species of vascular plants have been collected from Nepal. 246 species are endemic. 21 species (13 species CITES Appendix I and 8 species HMG protected) are endangered. 14% of the land is protected as national parks, wild fife reserves and conservation area in Nepal.

Biotechnology is broadly defined as the use of living organism for the production of consumable goods and ser-vices. Modern biotechnology consists of tissue culture and recombinant DNA technology. The technology is expected to bring a major impact in the near future. In a forest based agriculture country like Nepal this technology can play a vital role in upgrading the economy through conservation and use of its plant resources.

Plant tissue culture is one of the important aspects of biotechnology. Its products could make significant impact on agriculture and forestry development. Besides the commercial production of ornamental and horticulture plants, cloning of trees for afforestation will be of major importance.

CURRENT STATUS OF BIOTECHNOLOGY IN NEPAL

Biotechnology in Nepal is confined to tissue culture activities in government and private sectors. Protocols for micro-propagation have been developed in government laboratories, Tissue Culture Laboratory of Department of Plant Resources. The laboratory has developed tissue culture protocol for ninety plant species up to now. Potato Research Division of Nepal Agriculture Research Council has started tissue culture efforts to produce virus free potato. Central Department of Botany of Tribhuvan University has been providing

training to post graduate students of Botany. Royal Nepal Academy of Science and Technology is involved in micro grafting in vitro for eliminating virus and citrus greening disease.

The private sector tissue culture laboratories, Nepal Biotech Nursery, Botanical 'Enterprises, Himalayan Flora Tech, Micro plant Nepal has been engaged in commercial production of elite ornamental and horticulture plants for commercial purposes.

FUTURE EXPECTANCE OF BIOTECHNOLOGY IN NEPAL

At present, biotechnology in Nepal is confined to tissue culture method for the propagation of wide varieties of species. Other areas of biotechnology having prospects are somaclonal variation, production of virus free plants. Germ plasm conservation and exchange, wide hybridization and embryo rescue, anther culture, secondary metabolites, cell culture and genetic engineering.

BIOTECHNOLOGY ACTION PLAN

Department of Plant Resources of His Majesty's Government of Nepal established the first tissue culture laboratory in Nepal in 1976. This laboratory has developed micro propagation and anther culture technique of different economic plants. It has encouraged the establishment of tissue culture laboratories in the public and private sectors. The Department is taking initiatives to formulate biotechnology action plan to systematize biotechnological activities and provide a clear guidance for their implementation. The action plan has planned three levels namely strategic, commodity and tactical level.

1. Strategic or National Level

Strategic policies for the development of biotechnology are formulated at the national level to ensure policy commitment from the government.

2. Commodity or Regional Level

Biotechnological researches are prioritized based on importance of the commodity and agro ecological zones of the country.

3. Tactical of Researcher's Level

Researches are prioritized based on their importance. The available resources are allocated accordingly.

NATIONAL COMMITTEE ON BIOTECHNOLOGY

A national committee on biotechnology will be set up to coordinate activities of all three levels. The main function of this committee is to examine critically all project proposal submitted to it and sanction final approval. The committee has to coordinate all the agencies involved in biotechnology, explore funding sources. It will advise on allocation of resources on priorities basis and provide research and market information.

The Biotechnology Action Plan has been divided into two sections.
1. Organizational plan
2. Operational plan

1. Organizational Plan

The Department of Plant Resources is guided by the national committee on biotechnology for policy formulation and is advised by two committees namely Research Committee and Training and Outreach committee for program implementa-tion.

2. Operational Plan

The Department of Plant Resources should execute the following operational plan for the implementation of the Action Plan. The first phase has to carry out the following activities.

a. Plant species selection and justification
b. Establishment of interdisciplinary linkage
c. Transfer of in vitro technology to the field
d. Development of feedback mechanism
e. Establishment of institutional linkage
f. Establishment of international linkage
g. Explore external assistance

The second phase of the operational plan has to focus the following activities.

a. Recruitment of additional manpower
b. Procurement of additional equipment
c. Training of additional manpower

d. Initiation of research and technical work

Prioritization

Priority has been set for the following activities.

a. Micro propagation
b. Cell and tissue culture, anther culture and somaclonal variation
c. In vitro conservation of rare and endangered species
d. Plant improvement
e. Genetic engineering

COLLABORATION

Department of Plant Resources is seeking collaboration with national and international organizations for implementing and executing the Biotechnology Action Plan. A national coordination committee is to be framed at the national level consisting of Department of Plant Resources, Department of Forest, Department of Agriculture, Nepal Agriculture Research Council, Royal Nepal Academy of Science and Technology, Tribhuvan University and Katmandu University. Nepal has to be a member of International Center for Genetic Engineering and Biotechnology (ICGEB). International agencies like World Bank, Asian Development Bank, UNDP, FAO, UNESCO, UNIDO, GTZ, ODA (U. K.), USAID, SDC, CIDA, SNV, DANIDA, JICA, ODA (Japan) and other friendly donor agencies is requested for technical and other supports.

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Root Development *of Lilium longiflorum* Micro Cuttings *Ex Vitro* in Sand

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Abstract

The micro cuttings of *Lilium longiflorum* develop root system *ex vitro* in sand. Sand was cleaned with water and sun dried for two days. It was sieved through 2x2 mm pore. Sand was kept in a box and mixed with 10% water. The flasks with micro shoots were transferred to glass house for changing it to autotrophic nutrition. After two weeks, the micro cuttings were transplanted in sand and kept in the glass house. Forty days after the transplantation, the micro plants were evaluated for their development. 700 micro plants were produced out of 1,360 micro cuttings (i.e., 51.47%). These micro plants established well in field and produced normal flowers.

Key words: autotrophic, in vitro, ex vitro, *Lilium longiflorum,* micro cuttings, micro plants, micro-shoots.

Introduction

Conventionally micro shoots are rooted in vitro in the auxin rich medium. It is followed by transfer to hardening process and finally to the field. The conventional method of in vitro rooting develops incomplete roots which survive in nature with low survival percentage and growth rates (Kazai, 1991b). The crucial problem to be solved for mass scale production of plants through micropropagation is the induction of rooting in *in vitro* produced shoots (Nemeth, 1986). In vitro proliferated shoots were rooted in non sterile sand and dried leaves mixed in equal proportions by volume (Manandhar and Rajbhandary, 1986). In vitro produced plant species have rooted in non sterile sand (Rajbhandary and Bajaj, 1991). The objective of the present research is to study the development process of micro cuttings ex vitro in sand.

Materials and Methods

Lilium longiflorum plants were brought from Japan and grown in Godavari, Nepal. Shoot tip was used as explant for culture in MS medium (Murashige & Skoog, 1962) supplemented with 0.01 mg/l NAA, 0.65% agar and 3% commercial cane sugar. The embryoids formed from the cultured explants were subcultured in MS medium supplemented with 0.01mg/l NAA, 0.5mg/l BAP, 1.0gm/l casein hydrolysate and 10% coconut milk. The micro shoots formed in this medium were recultured for proliferation of more shoots (Saiju et al., 1992). Sand was cleaned with water and sundried for two, days. They were sieved through 2x2 mm pore. Sand was kept in a box and wetted evenly with 10% water. The flasks with micro shoots were transferred to glass house for changing it to autotrophic nutrition. After two weeks, leafy single node, 2 cm long, micro cuttings were transplanted in sand, One hundred and thirty six micro cuttings were transplanted in each box. In total, 1360 micro cuttings were transplanted in ten boxes. The boxes were covered with polythene sheet. The maximum and minimum temperature were 30°C and 20°C respectively in a glass house. The sunlight intensity varied from 4 to 15 kilolux. Watering was done regularly to maintain eighty percent humidity.

Results

Fifteen to twenty micro shoots were formed in each flask (Fig. 1). The micro cuttings started to produce roots in twenty days. They were left in the same box for growth and development of more healthy roots. In each micro cutting two to four roots were formed (Fig. 2). The roots were 2-4 cm long. After forty days of transplantation in sand the micro plants were evaluated for their development.

Fig. 1: Micro shoots in NIS medium

Fig. 2: Micro plants rooted in sand

The root development was monitored and evaluated through the change in weight of micro-plants. The micro cuttings with weight range 0. 013 to 0.100g produced four hundred forty plants. The micro cuttings with weight range 0.109 to 0.192g produced one hundred eighty plants. The micro-cuttings with 0.229 to 0.093g produced only eighty plants. In this way, out of 1360 micro cuttings 700 micro plants (i.e. 51.47%) developed roots ex vitro in sand (Table 1).

Table 1: The range of micro cuttings weight and no. of micro plants production ex vitro in sand.

Micro cutting weight range (g)	No. of micro plants production
0.0 13 to 0. 100	440
0.109 to 0. 192	180
0.229 to 1.093	80
Total no. of micro plants formed	700

1.42% micro plants lost weight 8.38%. 7.14% micro plants gained 0-25% weight, 8.57% got 26-50%, 11.42% micro plants added 51-75% weight., 11.42% micro plants gained 76-100%, 24.28% gained weight by 101-125%, 10% micro plants gained by 126-150%. 7.14% micro plants gained 151-175%, 7.14% micro plants gained 176 200% weight, 5.71% gained 201-225%, 1.42% gained 226-250%. 1.42% added 251-276% weight, 2.85% micro plants gained 276 300% within forty days of root development in sand (Table 2). These micro plants rooted in sand easily established in field. They produced normal flowers.

Table 2 : The percentage of micro plants and weight difference (decrease or increase) after forty days in sand box.

S. No.	Micro plants %	Difference in Weight %
1.	1.42	Decrease -8.38%
2.	7.14	Increase 0-25%
3.	8.57	Increase 26-50%
4.	11.42	Increase 51-75%
5.	11.42	Increase 76-100%
6.	24.28	Increase 101-125%
7.	10.00	Increase 126-150%
8.	7.14	Increase 151-175%
9.	7.14	Increase 176-200%
10.	5.71	Increase 2 01-225%
11.	1.42	Increase 226-250%
12.	1.42	Increase 251-275%
13.	2.85	Increase 276-300%

Discussion

In *Lilium longiflorum* the results indicated that the micro cuttings for ex vitro rooting in sand should

be of weight range 0.013 to 0.100 g for the production of maximum number of micro plants. If micro cutting is bigger than this size the rooting percentage decreases. It is most probable that the smaller micro cuttings have more vigour than bigger ones for rooting in sand. Early works also showed that the best rooting response was obtained with young plant material (Moncousin, 1991). Natural glass house condition with maximum temperature 30°C and minimum temperature 20°C, the sunlight intensity of 4 15 kilolux and 80% humidity was good for ex vitro root development. Temperature, light and hydricity under natural or green house condition will have a positive effect on future growth (Kozai, 1991a). The loss of weight in some micro plant during ex vitro rooting indicates that the physiology of micropropagated cuttings have changed during autotrophic mode of nutrition in glass house condition. The plantlets during acclimatisation are forced to autotrophy (Kozai, 1991b). In the future research, the role of temperature, humidity and light for increasing ex vitro rooting percentage in sand has to be studied in detail. The mechanisms involved in root formation during ex vitro condition have to be studied. For the commercialisation of micro propagation, low production cost is necessary. In the present investigation, cheap commercial cane sugar has been used in the medium instead of expensive sucrose. Ex vitro rooting of micro cuttings in sand is also an efficient and inexpensive method of propagation. This method can help for the lab to land programme of micropropagation.

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Micropropagation of Syngonium 'cv' White Butterfly by Culturing Axillary Buds of Foliage Plants

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Abstract

Shoot calli induced on MS medium containing low concentration of NAA 0.01 mg/l is combination with 1 mg/l BAP were found to be competent for shoot regeneration. upon transfer of regenerated shoots on 1.0 mg/l BAP and 0.5 mg/l with Kinetin liquid medium an increase in multiple shoots was observed. Those shoots increased in multiple frequency ratio. Such multiple shoots were readily rooted in non sterile sand and subsequently established successfully in the field. These results suggest that the methodology can be used on a commercial scale for micropropagation of *Synogonium* from axillary buds.

Introduction

Syngonium belongs to Araceae family which consists of twenty different varieties. A native of Central America and West Indies, *Syngonium* is a green foliage potted plant and is tolerant towards low light and low humidity. Because of such inherent characteristics, these plants are popularly used as indoor plants for decoration purposes. George and Sherington (1984) reported that 16 million tissue cultured plants were produced in the year 1984 to meet the commercial market. Similarly, Jones (1986) stated that The United States of America produced 15 million plants of *Synogonium* podophyllum. It was only towards the end of 1985 that tissue cultured *Synogonium* flooded the market.

Miller and Murashige (1976) reported the *in vitro* propagation of *Syngonium* from lateral buds. The vegetative propagation of *Syngonium* which is done through the cuttings, is found to be slow for mass propagation. In vitro propagation of *Syngonium,* however, was found be useful for commercial production to overcome the limitations of the conventional method of propagation.

The present paper attempts to explain the *in vitro* propagation from axillary buds of *Syngonium* to produce for commercial production.

Methodology

Green house grown plants were used as explants (Figure 1). Axillary buds bearing leaves were removed from the plants and its surface was disinfected on dipping in 0.1% Mercuric Chloride for 4-6 minutes and washed thoroughly for 4-6 times with sterilized distilled water. Isolated axillary buds (2-4 mm in size) were placed in Murashige and Skoog medium (1962) containing 3% Surose, 0.7% Agar and pH were adjusted to 5.8 prior to autoclaving at 15 Ibs/sq in for 20 min. Cultures were incubated at 25°C + under 24 hours continuous florescent light.

Figure 1

Calli were obtained in MS medium supplemented with BAP 1.0 $mg.1^{-1}$ and NAA 0.01 $mg.1^{-1}$. Multiple shoots were observed when these calli were transferred to liquid medium containing BAP 1.0 $mg.\ 1^{-1}$ and Kinetin 0.5 $mg.1^{-1}$. The liquid medium contained 1.5% Sucrose and pH adjusted to 5.5 was then autoclaved. The multiple shoots having 5.5 cm to 6.5 cm long were excised for rooting study.

The excised multiple shoots were rooted in non sterile sand boxes. The boxes were covered in polythene to create high humidity and temperature and kept in poly house. In this condition, 90% of successful rooting was observed. The rooted plants were ready to be transplanted to soil within 4-5 weeks.

Results and Discussion

Axillary buds 2-4 mm in size were cultured on Murashige and Skoog's medium containing BAP 1.0 mg.1^{-1} combined with NAA 0.01 mg. 1^{-1}. After 4 weeks, compact light greenish white calli were produced.

Multiple shoots were observed in Murashige and Skoog liquid medium containing BAP 1.0 mg. 1^{-1} and kinetin 0.5 mg. 1^{-1}. After 6 weeks 10-12 shoots were observed in this combination (Figure 2).

Figure 2

For this experiment, different concentration of BAP and NAA were used. It shows that higher concentration of BAP 2.5 mg.1^{-1} and Kinetin 0.5 mg.1^{-1} produced less shoots. The growth of proliferated shoots were found optimal in BAP 1.0 mg.1^{-1} and Kinetin 0.5 mg.1^{-1} as shown in the Table below.

Healthy roots were observed in non sterile sand within 4-5 weeks (Figure 3). Ninty percent of shoots were rooted under day and night temperature of 30˚C and 10˚C under 70% humidity. The rooted plants were transplanted in soil (Figure 4).

Figure 3

Figure 4

Table 1 : Response of *Syngonium* in MS medium with different concentration of BAP and KN

Cultivar	6 Benzyl Ainopurine + Kinetin (mg.1^{-1})	No. of Shoots	Growth Response
Syngonium 'cv' White Butterfly	2.5 + 0.5	2 - 4	less
	1.5 + 0.5	4 - 6	less
	1.0 + 0.5	10 - 12	well developed shoots
	0.5 + 0.5	2 - 4	less
	control	-	No. response

Figure 5

After the transplantation in soil, micropropagated *syngoniums* were characterized by a greater degree of basal branching than that produced by conventational stem tip cutting (Figure 3). It was generally thought that the increased branching was due to a "hormone carry over effect" typical of Cytokinin which can cause increased branching when applied to the whole plant.

Thus, the protocol of transferring the multiple shoots in the green house for its rooting can minimize the cost of production of tissue culture plants and can be used on a commercial production of syngonum.

Micropropagation is effective for reducing production cost and increasing production plays an important role in the successful industrialization of plant production.

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Application of Plant Tissue Culture in Nepal

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Abstract

Plant tissue culture was initiated in the National Herbarium and Plant Laboratories, Godawari, Department of Plant Resources in 1976. In the beginning callus initiation and its subsequent development was achieved from the culture of *Rauwolfia surpentina* seedlings. Since no alkaloid was detected in the *R. serpentine* calli, this work was discontinued. Attempts were then made to generate orchid plants from seed culture and meristem culture. A brief note on orhid culture will be presented. From the early 1980s efforts were directed to address the problem of high production cost of tissue culture plants. In this regard sand rooting of microshoots of various floricultural, horticultural, forestry and medicinal plants were developed. A substantial reduction in production cost of tissue culture plants using sand rooting technique will be presented.

Introduction

Plant tissue culture was initiated in the newly constructed Herbarium Building, Godawari in 1976. In order to see if the undifferentiated mass of cells would produce alkaloids, calli were developed from the seedlings of *Rauwolfia serpentine* in the MS medium (Murashige and Skoog, 1962) using 2,4-D. A substantial amount of calli were produced from *R. Serpentina* then shifted our emphasis to seed and meristem culture of orchid. Both from seed and meristem culture over 50 kinds of orchids were regenerated and placed in the pots. An efficient method of rooting of microshoots in clean sand was developed replacing the in vitro rooting of tissue culture plants.

Pyrethrum

In the late 1970s Pyrethrum *(Chrysanthemum cinerariaefolium)* was chosen to establish shoot culture since it was reported that individual plants grown from seeds exhibit heterozygosity with regards to plant form and pyrethrine content. In average pyrethrine content was found to be 1%, but in the selected clones pyrethrine content was reported to be 2%.

Shoot proliferation was obtained in MS supplemented with kinetine and BAP, and we were assured of a high rate of multiplication because the multiplication rate of shoots remained high through several 6-8 weekly subculture passages. The prevalent method of rooting of microshoots in medium was reported to be cumbersome and costly. We addressed the problem by transferring the microshoots to the clean sand under high humidity to induce root development. To our great encouragement a few out of hundreds of transferred shoots developed roots, and these rooted plants were grown to blooming stage. Later we came to know that under certain day/night temperature regime these microshoots exhibit a high percentage of rooting (Karki and Rajbhandary, 1984)

Potato

Potato is an important crop in Nepal especially in the hilly region where it becomes a staple food. Although potatoes are grown extensively all over Nepal, an improvement in the quality of seed tubers is presumed to enhance both quality an quantity of the yield. In collaboration with the National Potato Development Program (NPDP) various cultivars including those introduction from CIP were established in cultures. And it was found that a scheme of producing clean tubers of 1st generation could be worked out following the sand rooting technique (Manandhar and Rajbhandary, 1986). Such a scheme is projected to increase the potato production substantially in Nepal. However such a scheme is yet to be worked out in Nepal. We believe such a procedure is applicable to produce clean tubers in the developed countries as well.

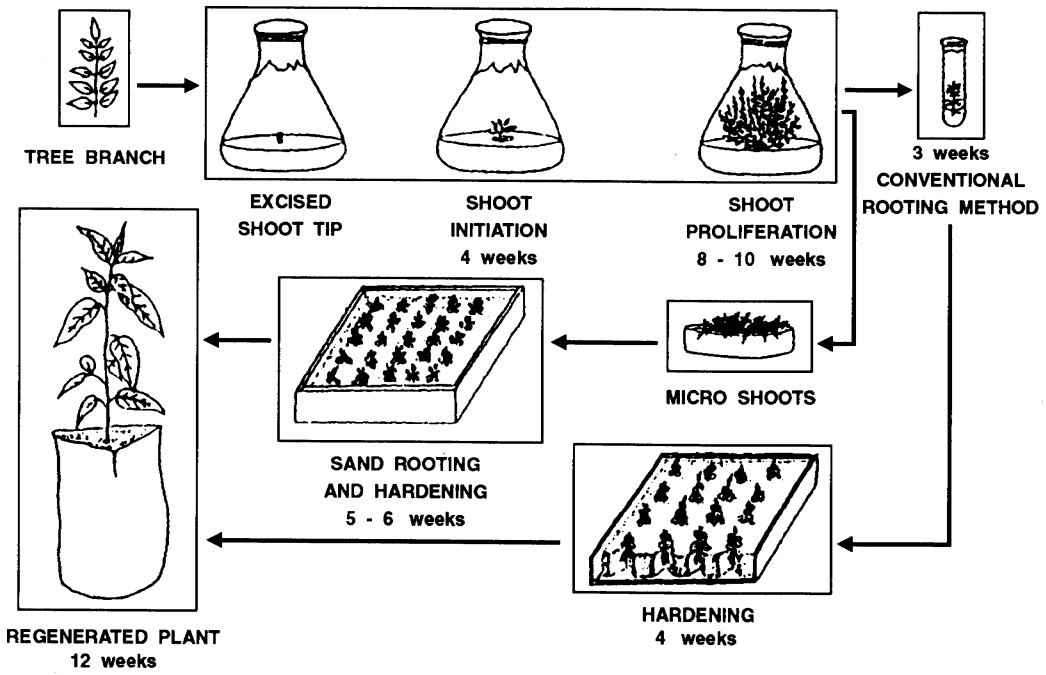

Fig. 1 : Tree Tissue Culture

Tree Tissue Culture

In the early 1980s tree tissue culture received a little attention. In view of the potential application of sand rooting for mass cloning we started working on tree tissue culture. The advantage of sand rooting over in vitro is that both hardening and rooting can be done in one step (Fig. 1).

Critical factors that apparently influence sand rooting of microshoots are humidity, temperature and light intensity. Generally a high relative humidity, over 80%, day/night temperature, 25˚C/l5˚C, and light intensity between 10-15 Kilolux seems to be adequate for the microshoots to develop root.

Eucalyptus camaldulensis

Eucalyptus camaldulensis was planted in thousands of hectares in Terai with an objective to provide fuelwood. Besides being a fast growing tree *E. camaldulensis* contains an essential oil, and cineole is an important component of the oil. On analysis a substantial variation was noted in both oil and cineole content in trees grown from seeds. Tissue culture protocol was developed to clone the selected individuals with desirable traits (Gurung and Rajbhandary, 1989). They were grown in trial plots.

Dalbergia sissoo

Dalbergia sissoo is an important tree popular in plantation all over Nepal Terai. Although plants could be regenerated in large numbers through shoot multiplication and sand rooting using cotyledonary node culture, culture establishment from mature trees is yet to be achieved (Suwal *et al*, 1988). However it is interesting to note that the preliminary survey indicates that 14- year-old tissue culture sissoo trees have not been affected by the top-dying disease of *D. sissoo* plantations. Further investigation is needed to find out why tissue culture trees resist such disease. Moreover a systematic trial of tissue culture trees should be carried out to assess the repeatability of such observation.

Fodder Trees

Fodder trees provide a significant amount of animal feed in Nepal. Most of the fodder trees were reported to have a low percentage of seed germination. To overcome this problem and also to clone the selected individual trees protocols were developed for a number of fodder trees such as *Ficus auriculata, F. lacor, F. nemoralis, F. semicordata* (Rajbhandary, 1993). All these these have been planted out and started bearing fruits and seeds.

Populus ciliate is seen widely growing in high mountains. Tissue culture protocol was developed to enable plantation of the tree.

Tissue culturists are frequently faced with the question - if tissue culture trees would ever receive commercial application. In this regard I understand that tissue culture trees are being used in commercial scale by Plumber Companies in USA. Each week two plane loads of tissue culture trees produced in a South American Tissue Culture Company are being flown to USA for plantations (Walden, 1998).

Horticultural Crops

Banana has been commercialized. Private tissue culture labs have been supplying tissue culture plants to farmers. It has received an increasing demand.

Another horticultural crop which has received interest by farmers is strawberry. it seems because of the introduction of strawberry tissue culture plants, a small scale strawberry farming has been established in Nepal.

Other floricultural crops we have worked with include carnation, chrysanthemum, lily, African violet, gerbera etc.

Ex vitro rooted plants have been packed in plastic containers and sent to different countries such as USA, UK, Japan, Germany, France and India. These plants were reported to have established in nurseries. In this regard I would like to emphasize that there exists an immense potential of supplying ex-vitro rooted plants to developed countries. A comparative production cost of ex vitro and in vitro rooted plants (Table 1) will illustrate how efficient and cheap the sand rooting technique is.

Conclusion

I remember one article by I. Roberston in a UNESCO publication (1991) that in this world facing food and fuelwood scarcity tissue culture is such a tool which has the potential of greatly alleviating the hunger of the world by substantially increasing the productivity. I quote his words-A bus load of dedicated, committed, funded scientists, plus a container load of equipment could soon cope with the pressing problems-the crushing poverty and hunger and the intellectual loneliness of the third world.

I would like to express my gratitude to my over two down colleagues of Department of Plant Resources with whom I had the opportunity to work in tissue culture.

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Table 1 : Cost of Producing 1000 Tissue Culture Plants, 1999

Expenditure in Tissue Culture Laboratory				
	For Sand rooting		For in vitro rooting	
	Rs.	%	Rs.	%
Labour	700	25	1680	20
Electricity	840	30	3360	40
Medium & Chemicals	280	10	840	10
Other Materials	140	5	420	5
Overheads	840	30	2100	25
Expenditure in Transplanting				
Sand Rooting and Acclimatization	1400			
Acclimatization			2800	
Total	4200		11200	
Note: US$ = Rs. 70				

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In Vitro Propagation of *Acacia auriculiformis* A. Cunn. ex Benth.

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Abstract

Acacia auriculiformis, an Australian species is an important agro-forestry tree. It thrives in harsh sites on poor soil. This species is valued for good fuel woods and makes good charcoal production. It is an important species for avenue plantation and reforestation programme. In Nepal, the speies was introduced in 1950 and has been planted in different parts of the country. It is suitable for Terai region and inner Terai region. In the present paper devised a method for large scale production of plantlets all the year round by in vitro culture method. The in vitro propagation of *A. auriculiformis* seedlings were investigated. The shoot tips from 12-16 days old seedlings were excised and cultured in Murashige and Skoog medium (1962) Supplemented with different concentration of 6 Benzylamino purine and Napthylene acetic acid 25-30 multiple shoots were proliferated in MS medium supplemented with 6 Benzaylamino purine 0.5 mg/l and Napthylene acetic acid 0.1 mg/l after fourth sub-culture. The micro shoots 3-4 cm long were excised and rooted in non sterile sand. The roots were initiated after 15-21 days. The rooted plants were successfully established in soil.

Introduction

Acacia is a large genus comprising over 500 species found in the warmer and drier parts of the world, mainly in Australia and Africa. Acacia species were introduced into Peninsular Malaysia since the early 1930. *Acacia auriculiformis* is a native of Saranas in Papua New Guinea Island of Torres strait and Northern Australia. It is a popular species on soil improvement trails and good fuel wood and makes good charcoal. (Chaturvedi *et al*. 1986). It is also an important agro-forestry tree. In India it is widely used in social forestry project (Hawkins 1987). It is good species for plantation where grazing is a problems because its leaves are not eaten by cattle. Beside being used in plantation, it is also an ornamental plant used to provide shade in parks and road sides.

In Nepal species was introduced before 1950 in Jhapa on the east-west highway (Jackson, J.K. 1987) and found suitable for Terai and inner Terai region. In recent years the potential use of Australian *Acacia* as exotic have been realized. Vegetative propagation of selected clones through tissue culture is a valuable tool for tree improvement programme. Keeping in view with the present demand of this species it was felt necessary to investigate and devise methods by which large scale population of selected fast growing and disease resistant elites can be raised for plantation.

In vitro development of plantlets from axilliary buds of *Acacia auriculiformis* and *Acacia nilotica* have been Gupta and Agrawal reported by Mittal, et al (1989) and Gupta and Agrawal (1992). Tissue culture of *Acacia mangium* and *Acacia stenophylla* have been reported by Crowford and Hartney (Anonymous 1987) using cotyledons. Complete plantlets of *Acacia koa* were successfully obtained on solid MS medium by Skolmen and Mapes in 1976.

The present paper highlights to develop rapid multiplication of woody species *Acacia auriculiformis* followed by non sterile sand rooting of the shoots for large scale production and its propagation all the year round.

Materials and Methods

Seeds of *Acacia auriculiformis* were obtained from Department of Forest Research and Survey. Seeds without any obvious damage were selected and washed under running tap water for an hour and sterilized with 0.1% mercury chloride for 8-10 minutes and again washed with sterilized water for

five times. Seeds were then cultured in MS basal medium (Murashige and Skoog 1962). The pH of the medium was adjusted to 5.8 before autoclaving and 0.7% agar was added to solidify the medium. The cultures were incubated at 25 ± 2°C under 16 hours photo-period at light intensity of 3000 lux.

The seeds germinated after 6-8 days of culture. After 12-16 days the shoots were excised from the seedling and cultured in MS medium supplemented with different concentration of Benzylamino Purine (BAP) and Naphthalene Acetic Acid (NAA). The cultures were incubated under the same condition of seed culture.

For rooting the mirco-shoots about 3-4 cm were excised and rooted in non sterile sand (Rajbhandari and Bajaj, 1991).

Results and Discussion

The shoot tip explants excised from the in vitro grown seedlings were cultured in MS medium with different concentrating of BAP ranging from 0.1 to 5.0 mg/l in combination with NAA 0.1 mg/l. After 4-6 weeks of culture 2-4 axillary shoots regenerated from the explants in BAP 0.5 and 1.0 mg/l combination. In lower concentration of BAP (0.1 mg/l) there was no regeneration and higher concentration of BAP (5.0 mg/l) produced stunted shoot buds. The multiple shoots were further sub-cultured in BAP 0.5 and 1.0 mg/l combination 25-30 shoots were developed after fourth and fifth sub-culture in 6-8 weeks. (Fig. 1). The formation of callus at the base was observed In BAP 1.0 mg/l and NAA 0.1 mg/l combination, whereas in BAP 0.5 mg/l nd NAA 0.1 mg/l the shoots were well developed and no callus mg/l the shoots were well developed and no callus formation occurred. The growth response of shoot in different concentration of BAP and NAA

Fig. 1 : Multiple shoots formation in MS Medium

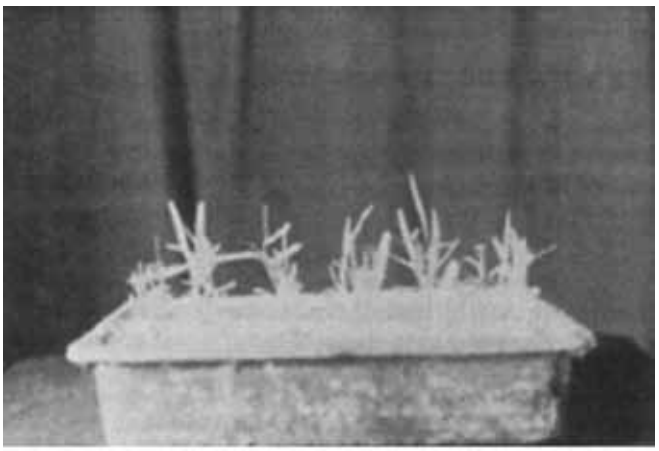

Fig. 2 : Microshoots for rooting in non-sterile sand

Table 1: Effects of Cytokinin and Auxin for regeneration of shoot tip Explants of *Acacia auriculiforis*

Species	Medium MS + BAP + NAA	Growth response
Acacia auriculformis	Control	No response
	0.1 mg/1 + 0.1 mg/1	No response
	0.5 mg/1 + 0.1 mg/1	Shoots regeneration
	1.0 mg/1 + 0.1 mg/1	Shoots with Callus
	5.0 mg/1 + 0.1 mg/1	Stunted shoots with callus

Fig. 3 : Micropropagated plant in pot

is shown in Table 1. The shoots were sub-cultured in every 4-6 weeks interval.

For rooting, 3-4 cm microshoots were transferred in non sterile sand. 70 to 80% shoots developed roots after 2-3 weeks (Fig. 2) under day and night temperature of 35^0 C/$l5^0$ C and 80% humidity. The light intensity was measured, to be 3000-5000 lux. The rooted plantlets grew to a height of 6-8 cm in 8 weeks. The plantlets are well established in soil (Fig.3).

Mittal et al. (1998) and Gupta et al (1992) used Gamborg medium with 10% coconut milk for *A. auriculiforis* and *A. nilotica*, but in our experiment MS medium without coconut milk was found suitable for shoot multiplication. in most of the woody taxa investigated so for, cytokinins have played a major role in shoot differentiation and BAP was necessary for development of shoots.

The in vitro propagation of *A. auriculiformis* using juvenile explants was developed successfully. Thus it can be concluded that the method developed by axillary shoot ultiplication and sand rooting of micro shoots would help to strengthen the microprpagation in mass production and tree improvement and also useful for conservation of superior genotypes.

Acknowledgements

We are very thankful to Mr. Madhusudan Bista, Director General, Department of Plant Resources, Ministry of Forest and Soil Conservation, HMG, Nepal for providing facilities for the study.

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Propagation of *Santalum album* L. Through Tissue Culture

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Abstract

Regeneration of microplants were observed from the excised shoot tips (2-3 mm) from five-years-old plant of *Santalum album* L. Green callus were initiated in Murashige and Skoog (MS) medium supplemented with Benzyl amino purine (BAP), 1.0 mg/l and Naphtylene acitic acid (NAA), 0.1 mg/l. Microplants were regenerated in MS medium supplemented with BAP, 0.1 mg/l and Kinetin, 1.5 mg/l and coconut milk 10 percent. Roots were observed in non-sterile sand. The rooted plants were successfully planted in the field.

Key words: Sandalwood, propagation, microshoots, sand rooting, plantation

Introduction

There is an increasing need to select tree species and cultivate them to fulfill the basic requirements of people such as fuel wood, fodder, medicine, biomass and other industrial processed products. One of such remarkable tree is sandalwood which is highly prized for its wood, essential oil and also for medicinal purpose. The plant is acknowledged as one of the most percious perfumery items from antiquity to modern time. The plant is highly demanded in Nepal, India and abroad for its economic and religious values. The seed raised plants may not produce elite clones due to genetic variation and seeds need to be imported from other countries as the plant is exotic in Nepal. So to overcome this problem, a tissue culture technique is developed for clonal multiplication of elite species of sandalwood in Nepal for plantation.

Conventional propagation of the sandalwood is not carried out yet in Nepal. Only a report on possible conventional technique for raising and planting of this species in Nepal was published by Neil (1990). In India extensive work on tissue culture of sandalwood has been reported by Rao and Raghav Ram (1983), Rao *et al.* (1984) and Laxmi Sita (1986). They have regenerated the plant from stem segment and shoot tips from a mature plant. The present paper deals with the regeneration of plantlets from shoot explants of five year old tree of sandalwood and subsequent establishment in the soil.

Materials and Methods

Stem segment and shoot tip explants were collected from five years old tree of sandalwood growing at tropical house of Royle Botanical Garden, Godavari. The materials were dipped in water 12-24 hour for leaching out of phenolic compound. Then the explants were washed in running water for 1-2 hour followed by surface wash with a drop of teepol and again washed thoroughly in distilled water. Aseptically the materials were surface sterilized with 0.01 percent mercuric chloride solution for five minutes and washed for 4-5 times with sterilized distilled water.

The MS medium was solidified with 0.65 percent agar and pH of the medium was adjusted to 5.5 before autoclaving. The cultures were incubated at 25˚C. The sterilized tips (2-3 mm in size) were excised and transferred to MS medium supplemented with BAP 1.0 mg/l and NAA 0.1 mg/l for shoot initiation. Rooting was done by treating the excised shoots in 1 percent IBA for 20 minutes on sand box containing only sand.

Result

The explants developed into green embryoides in two months in MS media supplemented with BAP 1.0 mg/l and NAA 0.1 mg/l (Fig. 1). These embryoides, when subcultures in MS medium supplemented with BAP 1.0 mg/l and Kinetin 1.5 mg/l and coconut milk % developed into plantlets in two months (Fig. 2). For routine subcultured, MS media supplemented with BAP 1.0 mg/l and Kn 1.5 mg/l and 10 percent coconut milk were used. For elongation of microplants gibberlin (GA3) 1.0 mg/l was used, which showed elongation (4-5 cm) of the plantlets. The number of plants varies from 15-20 in 250 ml conical flask. The plantlets were subcultured for mass propagation.

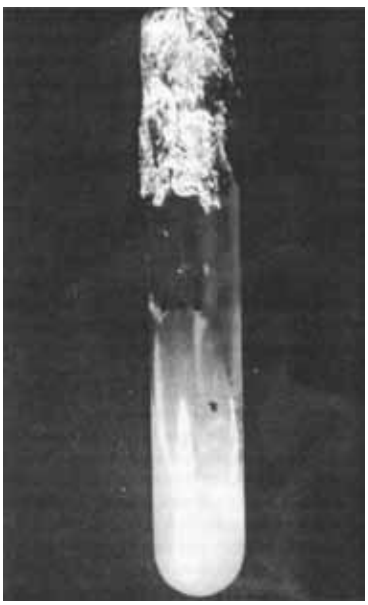

Fig. 1 : MS medium supplemented with BAP, 1 mg/l and NAA 0.1 mg/l

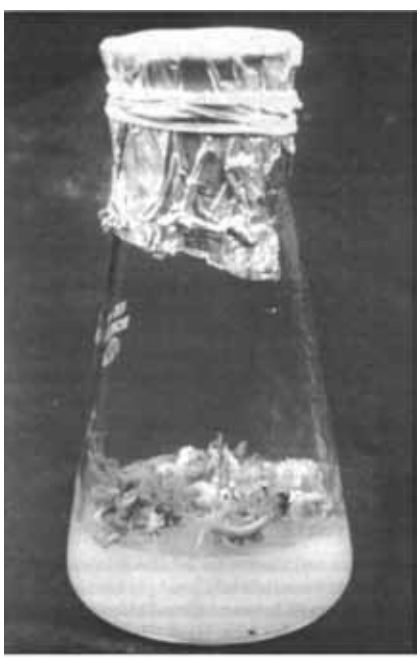

Fig. 2 : Plantlets formation in MS medium

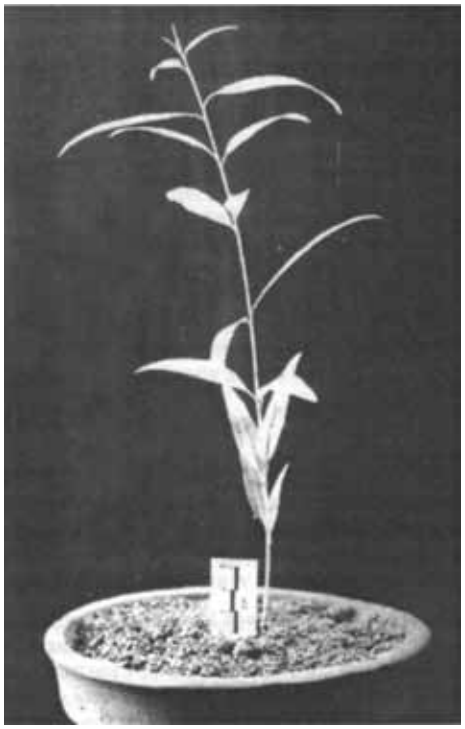

Fig. 3 : Sand rooted plant in pot

For rooting, plantlets were washed and were dipped in 1 percent indolebutyric acid (IBA) for 20 minutes before rooting in sand. Roots were visible after 1-2 months of transferring of plants to sand. Only 15 percent rooting were observed and rooted plants were transferred to the pots (Fig. 3). The plants were transferred to the field with *Duranta plumerie* as a host plant. Although the plant is obligate root parasite, rooting in sand was observed. Rooting percentage was not so encouraging, i.e. 15 percent in comparison to other tissue cultured tree species such as *Eucalyptus cameldulensis* (Pradhan, 1993). *E. citriodora* (Pradhan, 1997) and *Ficus neriifolia* (Pradhan. 1993), where rooting percentage was 70-90. Some microshoots remained green without developing root upto six months in sand. This may be due to slow growth nature of the plants as well as the climatic condition of research area (temperate) as the plant is tropical species.

Acknowledgements

We want to thank M.S. Bista, Director General, Department of Plant Resources for facilitating the research work.

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Micro-Propagation of *Rheum Emodi* wall

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Abstract

Rheum emodi wall, a widely used medicinal plant is in the verge of extinction because of its wide collection. In order to conserve its germplasm and to cultivate it in mass scale, attempts have been made to micro-propagation of *R. emodi* wall as an ex-plant cultured in Murashige and Skoog medium supplemented with benzyl-amino-purine (BAP) and napthyl-acetic acid (NAA) 0.1 mg/l. The roots were initiated when the micro-shoots were transferred in non-sterile sand.

Introduction

Rheum emodi wall (Padam Chal) belongs to polygonaceae family and its trade name is known as Rhubarb. In Nepal, the distribution of the plant is through the alpine and sub-alpine zones of Himalayas at altitude between 3300-3600m (Anonymous 1993). This is the perennial herb and therapeutic use of its root and rhizome is purgative, astringent tonic and diarrhea (Anonymous 1993). Leaves and flowers are also edible. The petioles are pickled and locally known as "Chulthi amilo" (Manandhar 1980). The pharmaceutical industry and the local people are largely dependent upon wild plants for supply of the plant material. Due to over exploitation of these natural resources to meet the ever-increasing demand, the wild sources are progressively declining and is expected to be endangered in the near future. To prevent further depletion, it plant by means of tissue culture technique could offer a convenient way to provide sufficient drug material as well as to avoid eradication of the natural stock. Lal and Ahuja (1989) have reported *in vitro* regeneration of *R. emodi* through shoot tip and leaf explants culture. The present study was undertaken to develop the technique to develop the technique of in vitro propagation and mass production procedure of this plant.

Methodology

Seeds of R. emodi were collected from Langtang of Rasuwa district. First the seeds were washed under running tap water for an hour and sterilized with 0.1% mercuric chloride for twelve minutes and again washed with sterilized water for five times. Seeds were cultured in M.S. basal medium (Murashige and Skoog 1962). The cultures were incubated at 25° ± 2°C under 16 hours photo-period at light intensity of 3000 lux.

The seeds germinated after two weeks of culture. The cotyledonary shoots was exicised from the seedling. The leaf lamina were removed and cultured in MS medium supplemented with different concentration of benzyl-amino purine (BAP) and napthyl-acetic acid (NAA) (Niroula and Rajbhandari 1988). In 6 to 8 weeks the shoots were proliferated and were shout 2-4 cm long. The proliferated shoots were excised for sub-culture.

For tooting, the sand rooting procedure (Rajbhandari and Bajaj 1991) was applied for the micro-shoots.

Results and Discussion

After 6 to 8 weeks of culture shoot proliferation was observed in MS medium supplemented wit BAP 1 mg/l and NAA 0.1 mg/l. The number of axillary shoot proliferation was 2-4 from the initial explants. The number of shoots proliferation was increased on sub-culture, and optimum number of shoots proliferation was increased on sub-culture, and optimum number was 15-20 (figure 1) after 4 to 6 sub-culture. Roggemans and Boxus (1988) in *R. rhaponticum* and Lal and Ahuja (1989) in *R. emodi* had reported meristem and shoot tip culture in MS medium supplemented with BAP and IBA (Indole butalic acid). But in our observation NAA 0.1 mg/l

in combination with BAP 1 mg/l showed better result than IBA 0.1 mg/l.

The micro-shoot transferred in sand (Figure II) produced roots within there in four week. The sand rooted plantlets were well established in soil. The result of present work demonstrate a procedure for the propagation of a medicinal herb *R. emodi*. Stable rates of regeneration have been observed for a period of over four years during repeated sub-culturing. The advantages of this procedure have direct implication in mass propagation of this species. In addition, the present work shows the importance of *in vitro* conservation for the maintenance of its germplasm.

Figure I : Multiple shoot formation after sixth subculture

Figure II : Micro-shoots frooting in sand

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In vitro Propagation of Cardamom (Amonum subulatum Roxb.)

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Abstract

Cardamom was introduced and has been in use since time immemorial in Nepal. However people were ignorant in the early days about its commercial value which off late has slowly begun to attract people's attention. More and more people are becoming increasingly aware of its commercial value and are being drawn into its cultivation. This has contributed tremendously in increasing the social, economic status of the farmers. The crop being environment friendly as well as exportable commodity can leave a very fruitful result for the people and the nation as a whole. The Cardamom is very useful in agro forestry inter cropping and also helps in nature conservation as well as in medicine formulation.

In the present paper the protocol for the two cultivars of Ramsai and Golsai were developed for large scale production of plantlets round the year by *in vitro* culture. The shoot tips (1 2mm) were excised from the mother plants and cultured in Murashige and Skoog solid media (1962) supplemented with 1.0mg/l 6 Benzyl Amino Purine and Alfa Naphthelene Acetic Acid 0.1mg/l. Multiple shoots with roots were produced by repeated sub cultures in same concentration of liquid media. Rooted plants were successfully planted in the field.

Introduction

Cardamom of Zingiberaceae family is a native of south India. It prefer shady, high humid area and an altitude of 500 to 1800m for its favorable growth. It is underground rhizome of the perennial herb with leafy stems. It is dark red brown capsule (fruit) and measures about 2-3mm length. Mostly it is used as a spice/condiment which carries high medicinal value.

Cardamom is one of the major cash crop of Nepalese farmers of eastern hilly region of Nepal, may be because of the favorable climate. The farmers are making a good profit out of this with a little investment and little labour.

Uses

- Domestically used as a flavoring agent in the preparation of spices.
- Extract from Cardamom, use in the formulation and production of the medicine.
- In Great Britain, U.S.A., used as an ingredient for curry powder.
- In Russia, Sweden and Germany, largely used for flavoring cakes and in preparation of liquiers.
- In Egypt they used in coffee.
- In East Indies, used both as a condiment and for chewing a betel.
- In France, oil is used in perfumes.

The seeds are aromatic, and pungent with a sharp, pleasant taste. Medically, it is prescribed for the treatment of indigestion, vomiting, abdominal pain, and rectal diseases. A decoction of the seed is used as gargle in affection of the teeth and gums. The aromatic oil extracted from the seed is applied to the eyes to allay inflammation.

Nepal is playing an important role in producing and exporting Cardamom. In fiscal year 1996/97 the total production was 4400 metric tones, where as in 1997/98 the maximum production was 5200 metric tones and gradually declined in 1998/99. It was only 4300m.t. The production declined due to non favorable weather conditions (source Agro Enterprise Centre).

In fiscal year 1998/99 Cardamom was exported to 6509m.t. to India from where it is destined to other countries (source Agro Enterprise Center).

So, far in Nepal Cardamom is being cultivated with conventional method. In this method Cardamom is

propagated vegetatively which is very slow process and allows pathogens.

The first report on the successful regeneration of Cardamom plants *in vitro* (Rao *et al.,* 1982; Nadgauda is *et al.,* 1981; Nadgauda *et al.,* 1983) have been developed a successful, cost effective commercial micropropagation to multiply elite Cardamom.

In vitro propagation of Cardamom, however was found to be useful for commercial production to overcome the conventional method.

The present paper attempts to develop a *in vitro* propagation of Cardamom through shoot tip culture to produce pathogen free plantlets for commercial production.

Materials and Methods

Two cultivars of Cardamom Ramsai and Golsai plants were obtained from Cardamom Development Centre, Phikkal, Ilam. The explant (2-3 cm) shoot tips of Cardamom was taken out by removing the scale and kept in running tapwater for 2 hours with 4 dorps of teepole liquid detergent. The shoot tips were then sterilized with 0.1% mercuric chloride for 20 minutes and rinsed 6 times with sterilized distilled water. Trimmed shoot tips (1-2mm) in size were cultured in sterilized Jams bottles 250ml containing 70ml of solidified MS medium. The culture medium was composed of Murashige & Skoog's (1962) supplemented with 3% Sucrose and 10% coconut milk. The medium was solidified with 0.7% Agar and pH was adjusted to 5.8 prior to autoclaving at 15 lb/sq for 20 minutes. Cultures were incubated at 25°C±2 under fluorescent light of 3000 lux for 16 hrs. Observation for all experiments were made 4-6 weeks after culture.

For rooting the microshoots about 3-4cm were excised and rooted in non sterile sand (Rajbhandary and Bajaj, 1991).

Results andDiscussion

Shoot tips (1 2 mm) of Ramsai and Golsai cultivars of Cardamom were cultured on Murashige and Skoog's medium (1962) containing 6 Benzyl Aminopurine and Alfa Napthalene Acetic Acid. In this experiment the colour of survival explant change reddish creamy to green within 20 days. The green shoot was grown and produced single shoot only. After 4 weeks the shoot was transferred to the fresh medium of same concentration (Fig. 1). 8-12 shoots were proliferated after 12 weeks in BAP 1.0 mg/l and NAA 0.5 mg/l solid media to transferred in liquid media after 4 weeks (Table 1). It has been found that solid to liquid medium is good in proliferation rather than solid media only.

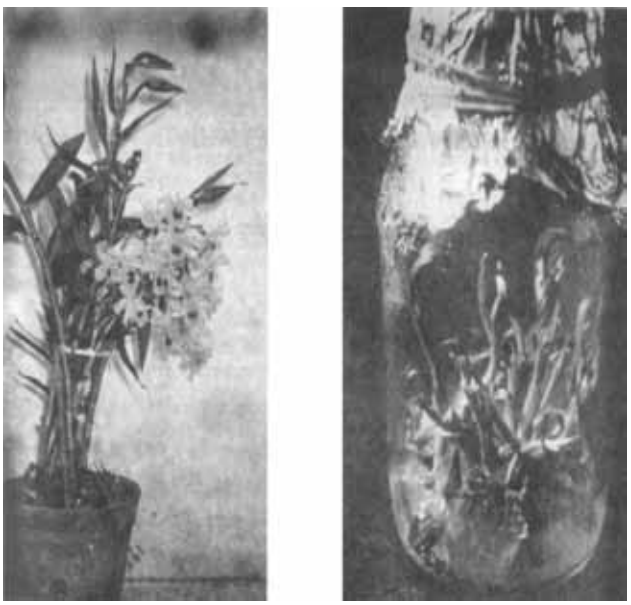

Fig. 1. Shoot multiplication after four weeks

No significant difference was observed in multiplication of shoots while comparing with or without coconut milk. For this experiment, different concentration of BAP and NAA were used.

In comparison of two cultivars, the multiplication rate of Ramsai was higher than Golsai. It seems that growth of proliferated shoots were found optimal in BAP 1.0 mg/l and NAA 0.5 mg./l in both cultivars.

Shoots developed roots after 6-8 weeks of transfer in non sterile sand (Fig. 2) 90 percent of shoots were developed root under day and night temperature 35°C.15°C under 70 percent humidity and light intensity 3000-4000 Lux. The rooted plants were transplanted in pots (Fig. 3). After one year the mature plants were transplanted in the field (Fig. 4).

Table 1 : Response of Cardamom cultivars Ramsai and Golsai in MS medium with different concentration of BAP and NAA

Cultivars	6 Benzyl Amino Purime + NAA	No. of Shoots	Growth Description
Ramsai	2.5+0.5	2 6	Less
	1.5+0.5	4-6	Less
	1.0+0.5	20-25	Well developed shoots with root
	0.5+0.5	2-4	Less shoots
Golsai	2.5+0.5	-	No growth
	1.5+0.5	2-4	Less
	1.0+0.5	8-10	Well developed shoots with root
	0.5+0.5	2-4	Less
	Control	No response	

(a) Ramsai (b) Golsai
Fig. 2 : Shoot multiplication after 8 12 weeks

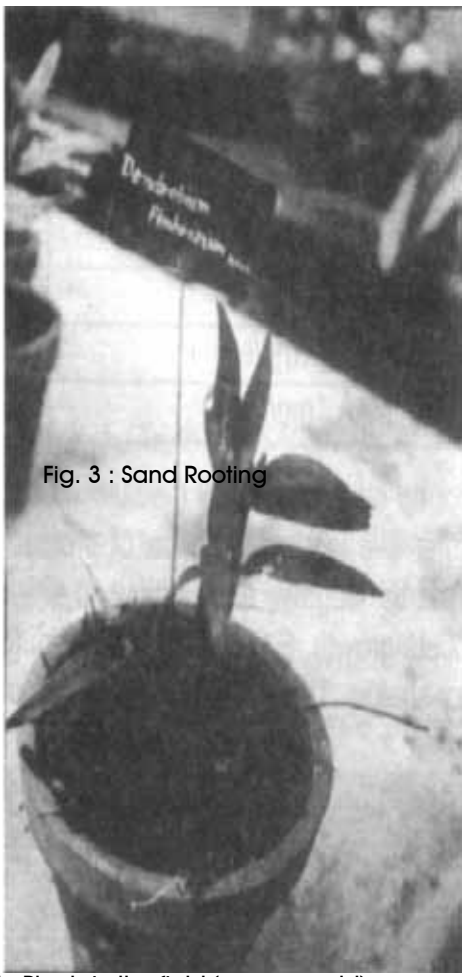

Fig. 3 : Sand Rooting

Fig. 4 : Established plants

Fig. 5 : Plants in the field (one year old)

Nadgauda *et al.,* 1981 have cultured three cultivars of Cardamom such as Vazhukkai, Malbar and Mysore and compared with seed raised plants and observed that tissue culture plants shows significantly higher value than seed raised plants.

Nadgauda *et al.,* 1983 have reported the clonal multiplication of Cardamom *(Elettaria cardamom* maton) by using shoot tip culture for plant regeneration.

Priyadarshan *et al.,* 1992 reported that Schenk and Hilderbrand (SH medium) showed better results in comparision to Murshige and Skoog medium. Roa *et al.,* 1982 reported the regeneration of plantlets from callus of *Elettaria cardamom.*

Karki and Rajbhandary 1984 regenerated multiple shoots from shoot tips of *Chrysanthemun cinerarifolium* coupled with successful rooting in non sterilized sand bed.

In our experiment there is no callus formation from explant. They produce plantlets directly from shoot tip culture. Thus it can be concluded that plant regeneration from shoot tip culture and sand rooting in mass scale would be useful for conservation of superior cultivars.

Acknowledgments

We are thankful to Mr. M.S. Bista, Director General, Department of Plant Resources for facilitating the research work and Devendra Saraf Chief of Cardamom Development Center Khumaltar for providing the mother plants of Cardamom cultivars.

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In vitro Propagation of *Elaeocarpus sphaericus* (Gaertn) K. Schum.

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Abstract

Rudraksha *[Elaeocarpus sphaericus* (Gaertn) K. Schum.] belonging to the family Elaeocarpaceae is the important religious and medicinal plant. Because of its high valued fruit there is a urgent need to produce it in mass scale by *in vitro* propagation technique.

The choice of explant as nodal cuttings (from 2 to 5 yrs. plant) in liquid MS medium was observed appropriate. Otherwise there was a great possibility of necrotic exudation of phenolic compound. The established green explants were then inoculated in MS medium with the combination of different concentration of BAP and NAA as well as in MS medium with different concentration of BAP alone. It was also tried in MS medium with less ammonium nitrate (300mg/l). The MS medium supplemented with BAP 0.5gm/l and NAA 0.01 mg/l as well as MS medium with BAP 0.25mg/l were observed good for proliferation of microshoots. The proliferated microshoots when subcultured in MS medium with less ammonium nitrate (300mg/l) supplemented with BAP 0.25mg/l at the interval of 3 to 4 months, it showed good result of proliferation with no browning of microshoots. The microshoots develop roots successfully in non sterile sand within 15 to 20 days in house condition.

Introduction

The *Elaeocarpus sphaericus* (Gaertn) K. Schum., utrasum bead tree belongs to family Elaeocarpaceae. The plant is a medium sized tree occuring from south and east Asia through Malaysia to Australia and Pacific islands. The status of tree is vulnerable in context to country Nepal. It is cultivated in many parts of eastern Nepal as religious tree as well as for the harvest of religious seeds. However, few examples are seen in the wild as indigenous forest tree; in central and eastern Nepal 650 to 1700m altitude. The leaves of the plant are elliptic to 15cm long, acute, sub entire, nearly glabsous, flowers white 1.25cm across in drooping raceme, stamens 35 to 40 with bearded anthers; fruits globose, 1 seeded stone tubercled, grooved, 5 celled. Flowering and fruiting time is May to October. The important part of the plant is the fruit. The fruit is used in the cure of diseases of head and epileptic fits as well as the important religious beads for Hindus.

Materials and Methods

Different explants of plant parts were tried from trees of 2-5 years plants. The explants were first taken in 4 to 5 cm sizes and washed for half an hour on flowing tap water. Then they were again washed with tween 20 and rinsed with distilled water. Surface sterilization was achieved by treating the explants with $HgC1_2$ (0.1%) solution for 3 to 4 minutes.

Murashige & Skoog medium either liquid or solid were tried to establish the explant of appropriate size 0.5 to 1.0 cm. After solving the browning problem (due to exudation of phenolic compound), the explants were tried on solid MS medium supplemented with different concentration of BAP alone as well as BAP in combination with NAA. The medium was solidified with 0.8% agar with sucrose 3%, 1.0 g/l casein hydrosylate; pH was 5.8. It was also tried on MS medium with less ammonium nitrate (330 mg/l). The cultures were incubated at 25±2°C and 16 hr. of photo period for 4 weeks. Then they were again subcultured to find out the appropriate proliferation medium in every 4 weeks period.

The established microshoots were allowed to root on non sterile sand under green house condition in Godawari.

Results andDiscussion

The best explant for this job is the nodel explant which when established on liquid MS medium on filter paper bridge; the problem of phenolic compound is minimized. It takes 2 to 3 weeks culture time. The established explants when tried on solid MS medium supplemented with different concentration of BAP and NAA as shown in table 1. It shows that it does not need NAA and the appropriate composition is MS medium supplemented with BAP 0.25 mg/l.

As shows in table 2, the microshoots when subcultured for 4 to 5 times; the best medium seems to be MS medium supplemented with BAP 0.25 mg/l.

As shown in table 3, when they were subcultured on MS medium with less ammonium nitrate, supplemented with BAP; the result was very good, without browning. Because in former cases, little browning began after long period.

It was also observed that when they were subcultured from MS + BAP 0.5 mg/l to MS + BAP 0.25 mg/l; the production was very good.

Conclusion

The best way to mass production of the plant, Elaeocarpus sphaericus is to first culture on liquid MS control medium and then transferred to MS + BAP 0.5 mg1l then again subcultured from high BAP that is MS + BAP 0.5 mg/l to MS + BAP 0.25 mg/l and then again continuos subcluture to MS with less ammonium nitrate + BAP 0.25 mg/l.

Acknowledgments

We thank to Mr. M.S. Bista, Director General, Department of Plant Resources for providing the necessary facilities.

Table 1 : Record of prolferation of microshoots of *Elaeocarpus sphaericus* with different concentration of BAP and NAA (BAP alone after 4 weeks of culture)

Composition of Media	No. of microshoots in the culture flask	Remarks
MS + BAP 1.0 mg/l + NAA 0.01 mg/l		Survival of explants for few days and then browning to death; during their survival the plants were in undistinguished form
MS + BAP 0.5 mg/l + NAA 0.01 mg/l	2 to 3	There was proliferation and elongation; but later changing to brown
MS control	2 to 4	Good elongation
MS + BAP 05 mg/l	4 to 8	Good elongation; no browning
MS + BAP 0.25 mg/l	4 to 10	Very good elongation; no browning

Table 2 : Record showing the proliferation of microshoots of *Elaeocarpus sphaericus* while sub-culturing on proliferation media

Composition of Media	No. of microshoots in the culture flask	Remarks
MS + BAP 0.5 mg/l	6 to 10	The elongation was good
MS + BAP 0.25 mg/l	10 to 14	The elongation was good; appropriate for rooting

Table 3 : Showing the record of subcultured on MS medium supplemented with less ammonium nitrate

Composition of Media	No. of microshoots in the culture flask	Remarks
MS + BAP 0.5 mg/l	8 to 10	Good elongation
MS + BAP 0.25 mg/l	12 to 15	Good elongation; appropriate size for rooting

Fig. 1 : Explant in MS liquid medium

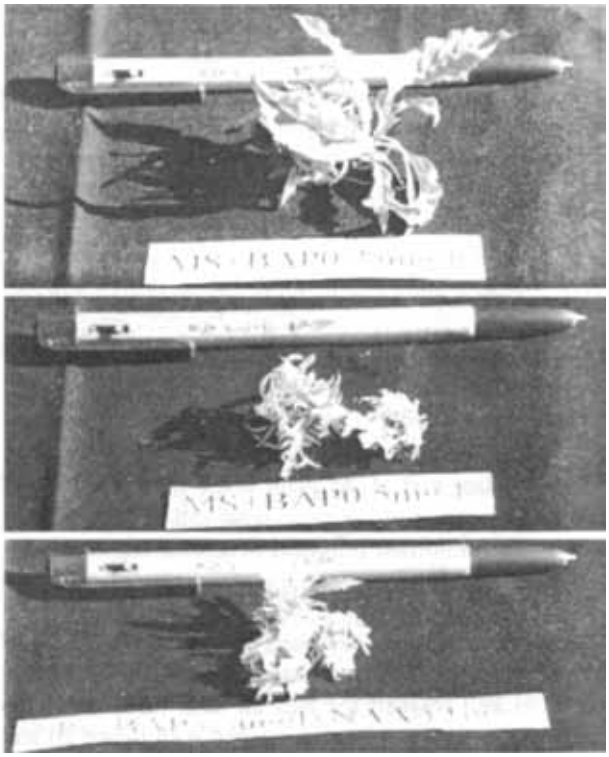

Fig. 2 : Proliferation of explant in different concentration of BAP and NAA

Fig. 3 : Multiple shoots formation in MS medium with 0.25 mg/lit of BAP

Fig. 4 : Micropropagated plant in pot

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Micropropagation of the Nepalese Medicinal Plant *Swertia chirata* (Wall.) C.B. Clarke

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Abstract

Swertia chirata, the Nepalese medicinally valuable plant that is used in the treatment of different ailments by different ethnic groups, is being tested for its mass propagation through tissue culture technique.

The shoot tips of two months old plants were cultured in Murashige & Skoog (MS) medium supplemented with 1.0 mg/l of 6 Benzyl Amino Purine (BAP) and 0.01mg/l of Naphthalene Acetic Acid (NAA) for multiplication. Ten-fifteen microshoots were developed after 4th subculture. These microshoots were transferred in non sterile sand for rooting. The roots were developed within two to three weeks. The rooted plants have been established successfully in the field.

Introduction

Swertia chirata locally called as Chiraeto is one of the important medicinal plants found in Himalayan region of Nepal between 1500-2500m altitude (Shrestha & Joshi, 1996). This plant is used to cure leucoderma, inflammation, burning sensation, pain in body and joints, ulcers, asthma, bronchitis, piles, chronic fever, liver cirrhosis and other many diseases (Kirtikar & Basu, 1980). It is also used in the preparation of other Ayurvedic medicine for the treatment of hepatitis, fever, malaria, pulmonary tuberculosis and typhoid fever (Bajracharya, 1979). Due to its great medicinal value people collected this plant from natural sites for their uses and for export which makes the plant endangered in Nepal in near future. It is therefore, an attempt has been under taken for mass propagation of the plant through tissue culture technique.

Kitamura *et al.* (1986, 1988) reported the regeneration of multiple shoots from callus cultures and root cultures of *S. pseudochinensis.* No reports on micropropagation of *S. chirala* was found. The present paper deals with proliferation of microshoots from the shoot tip of two months old plants and their rooting in non sterile sand for large scale production.

Materials and Methods

The shoot tips of two month old plants were collected from the green house of Conservation section of Royal Botanical Garden, Godawari. They were washed in running water for one hour and with teepol for few minutes. After this, the shoot tips were rinsed two-three times with distilled water. Finally they were surface sterilized with 0.01% Mercuric Chloride for ten minutes and washed for 2-3 times with sterilized water.

After surface sterilization, the explants were cultured in Murashige & Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 1.0mg/l BAP and 0.01mg/l NAA, pH of the medium was adjusted to 5.8 before autoclaving. 0.8% agar was added to solidify the medium. The cultures were incubated at 25°C±4°C under 16 hour photoperiod at light intensity of 3000 lux.

After the development of microshoots from the explant, these shoots were subcultured in the same medium supplemented with different concentration of BAP and NAA. The best proliferation of microshoots was observed in the medium supplemented with 1.0mg/l BAP and 0.01mg/l NAA. 2-4 long microshoots were excised and transferred in non sterile sand for rooting (Rajbhandari & Bajaj, 1991). The rooted plants were transferred to polybags with soil for field establishment.

Results andDiscussion

When the explant were cultured in the medium supplemented with 1.0mg/l BAP and 0.01 mg/l NAA

a mass of embroids were observed around the cutting end of explant after 2 weeks. But when these embroids were subcultured in the same medium 2-4 microshoots were developed after 4 weeks. 10-15 microshoots were developed after 4th subculture. The shoots were subcultured in every 4-6 weeks of interval.

The formation of callus and development of roots were observed when the microshoots were subcultured in MS medium supplemented with BAP and 0.1 mg/l NAA. The growth response of shoot in different concentration of BAP and NAA is shown in the table 1.

Table 1 : Effects of BAP and NAA for proliferation of shoot tip explant of *Swertia chirata*

Medium MS+ BAP+NAA(mg/l)	No. of Shoots	Growth Response
1.0+0.01	10-15 shoots	Well developed
0.5+0.01	10-15 shoots	Not well developed
1+0.1	4-5 shoots	Callus + Roots
0.5+0.1	4-5 shoots	Callus + Roots

In this experiment, when 0.1 mg/l NAA and cytokinine was used initation of callus and root was observed after 4 weeks. But when the medium is supplemented with 0.5 mg/l of BAP and 0.01 mg/l NAA the microshoots and few roots were developed but the microshoots were not well developed. The microplants were well developed when they were subcultured in the medium supplemented with 1.0 mg/l BAP and 0.01 mg/l NAA. Therefore, the medium supplemented with 1.0 mg/l BAP and 0.01 mg/l NAA was used for the subculture of the plants. The proliferation of microshoots increased in number as they were subcultured in the same medium (Fig. 1).

For rooting, 2-4 cm microshoots were transferred in non sterile sand, 80-85% shoots developed roots after 2-3 weeks (Fig. 2). The rooted plants were well established in soil (Fig. 3). The cultures had maintained their ability to proliferate after regular subculture for 4 years.

Kitamura *et al.* (1988) reported that high level of NAA is needed for the initiation of callus in *S. pseudochinensis.* In this experiment also, high level of NAA initiated callus in *S. chirata.* BAP plays important role in shoot proliferation.

The *in vitro* propagation of S. *chirata* from shoot tip was developed successfully. Thus it can bc concluded that the tissue culture technique is useful for mass propagation of *S. chirata* from which the plant can be conserved.

Acknowledgments

I am very grateful to Mr. M.S. Bista, Director General, Department of Plant Resources and Ministry of Forest and Soil Conservation, HMG Nepal for providing facilities for this research.

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Fig. 1: Microshoots of *Swertia chirata* after 4 weeks

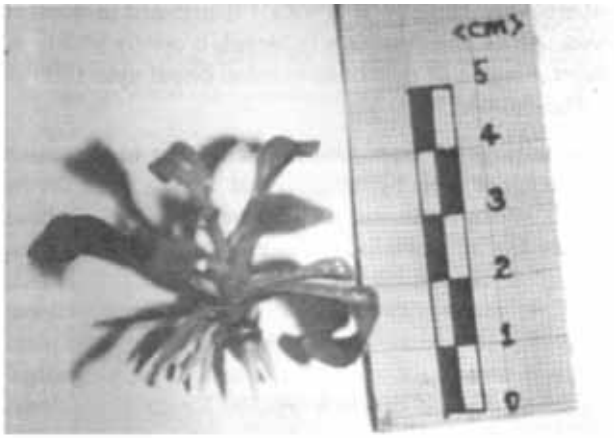

Fig. 2 : Rooted plant

Fig. 3 : Established plant

In vitro Culture of *Rauvolfia serpentina* L. Benth. ex Kurz.

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Introduction

Rauvolfia serpentina is most important medicinal plant. Its dried roots of 3-4 years old plant has been used in the Ayurvedic system of medicines for the treatment of hypertension or as a sedative and tranquillizing agent. The major alkaloids in roots are reserpine, deserpine, ajmaline, ajrnalicine, serpentine.

Rauvolfia serpentina belongs to family Apocynaceae. It is found on moist and shady place in forest. It is perennial under shrub usually to 45cm tall with white bark. It is characterized by long elliptic lanceolate leaves occurring in whorls of three to five at nodes of short terete stem, a many flowered corymbose inflorescence with white or pink flower. The root system consists of a prominent tuberous soft taproot. It is widely distributed in tropical Asia, Africa and America. In Nepal, it occurs widely in tropical region of eastern and central Nepal upto 1000 to 1150m altitude.

It is endangered from natural habitat due to excessive collection of roots by villagers and sold by them for its multipurpose medicinal uses as well as due to habitat destruction from human encroachment. The plant is band for export outside the country, under Forest Act 1993, the Government of Nepal. Its propagation by conventional method is not satisfactory. For such an endangered plant species, tissue culture method is an alternative method for its germplasm conservation and for its rapid clonal multiplication. Hence its tissue culture technique has been attemped.

Materials and Methods

The explants were taken from field established plant in Royal Botanical Garden. The shoot tips were washed for one hour. in running water, surface sterilized 5 minute with teepol and throughly washed with distilled water. These shoots were sterilized with 0.1% mercuric chloride solution for 3-4 minutes. These sterilized shoots were rinsed thoroughly with sterilized distilled water. Then the shoot explants, 2 to 3mm were cultured in Murashige and Skoog's medium (1962) with different concentration of Benzyl amino purine (BAP) and Napthalene acetic acid (NAA).

The MS medium supplemented with 3% sucrose, 0.1% casein hydrolysate, 0.8% agar and its pH range 5.6 to 5.8 before autoclaving. They were autoclaved at a pressure (15 lb/in^2 for 15 minutes. The culture were incubated at 25±2°C temperature and 16 hrs photoperiod. The established shoots were again subcultured in different concentration of BAP and NAA. Subcultured of these proliferated micro shoots was done in regular interval of 5 to 6 weeks in the medium.

The regenerated shoots were acclimatized for a week in green house. Then these shoots were transferred to non sterile sand for root induction. The rooted plants were transferred to seed box and then in clay pots before field establishment.

Result

The explants response in all concentration of auxin and cytokinin initially (upto 1-2 week). After 3 to 4 weeks, the MS medium with BAP 1.0 mg/lit, 2.0 mg/lit, 3.0 mg/lit along with 0.1 mg/lit NAA showed micro shoots sprouting from the base of explant, but the medium with BAP 3 mg/lit + 0.1 mg/lit NAA was seem to be best for micro shooh sprouting little bit sooner than other medium (Table 1).

The sprouting micro shoots again subcultured in

various conc. of hormone, as previous one (Table 2). It shows not so difference in BAP 1.0 mg/lit and 2.0 mg/lit. In higher concentration of BAP and NAA, callusing occurred at the base of explants. The micro shoots were regularly subcultured in the best medium i.e. BAP 1.0 mg/lit and NAA 0.1 mg/lit at interval of 5 to 6 weeks. After 7 subculture, the multiplication rate of micro shoots were increased upto 12-16 from individual shoot (Fig. 1-2).

Discussion

In shoot tip culture of *Rauvolfia serpentina,* exudatioii of Phenolics did not occur, but V.M. Patil and M. Jayanilu (1997) reported that establishment of explants *in vitro* was difficult due to exudation of Phenolics. They also found that BAP 2.0 mg/lit alone produced better axillary sprouting and lower concentraction of BAP with 0.005 mg/lit adenine sulphate was best for multiplication of shoots but in our experiment BAP 1.0 mg/lit and NAA 0.1 mg/lit was seem to be the best for multiplication of shoot Mathur *et al.* (1987) reported that the addition of IAA along with BAP helped in higher multiplication of *Rauvolfia serpentina.* M. Jayanthi and V.P. Patil also observed better result in 1.0 mg/lit BAP and 0.1 mg/lit IBA in case of *R. densiflora* and *R. tetraphylla.*

After acclimatization, the micro shoots transferred in non-sterile sand and Covered with plastic hood (Fig. 3) 90% of micro shoots developed roots in 1-2 weeks (Fig. 4). V.M. Patil and M. Jayanthi rooted individual micro shoot in auxin free media and media with 2.0 mg/lit IBA. They also found that rooting percentage is increased by 26% in *R. tetraphylla* and 20% in *R. micrantha* by doubling the sugar concentration in media. In *ex vitro* rooting, they observed 43% mortality. The rooted micro shoot were transferred to the clay pots (Fig. 5). The micro-propagated plant flowered after one year in pot (Fig. 6).

Table 1 : Growth responses by explants on MS medium with different concentration of BAP and NAA

MS Medium		Explant responses	Growth responses (After 3-4 weeks)	
BAP mg/lit	NAA mg/lit	(After 1-2 week)	Induction of shoots number in average	Shoot growth condition
0.1	0.1		only elongate	worse
1	0.1	explant	2-4	not so good
2	0.1	responses	3-5	not so good
3	0.1	in all concentration	6-9	best
4	0.1		8-10	very stunted growth
0.1	0.1			worse
1	0.1	explant	2-3	not so good
2	0.1	responses	2-3	not so good
3	0.1	in all concentration	5-7	not so good
4	0.1		6-8	stunted growth

Table 2 : Subculture of regenerated micro shoots

MS Medium		Growth responses	
BAP mg/lit	NAA mg/lit	Ave. no. shoots	Growth response
0.1	0.1	elongate	worse
1	0.1	8-12	best
2	0.1	8-11	best
3	0.1	7-10	good (callus occur at base)
4	0.1	6-9	stunted (callus occur at base)

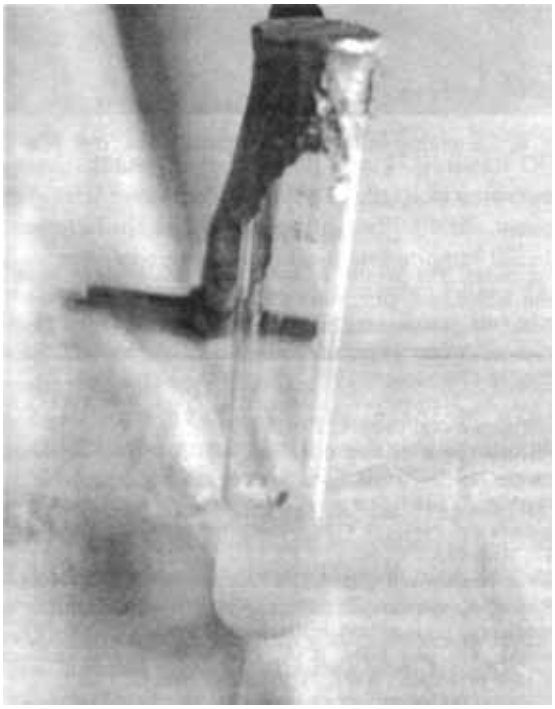

Fig. 1 : Explant in medium

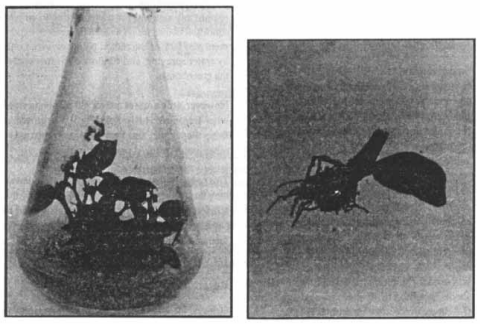

Fig. 2 : Multiple shoots formation in MS medium wiih BAP 1.0mg/lit + NAA 0.1mg/lit

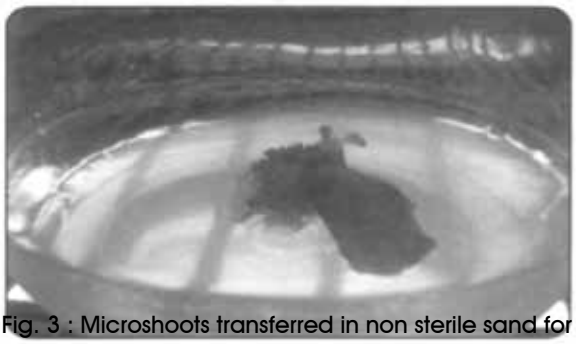

Fig. 3 : Microshoots transferred in non sterile sand for rooting

Fig. 5 : Micropropagated plants in pot

Fig. 4 : Rooted Plants

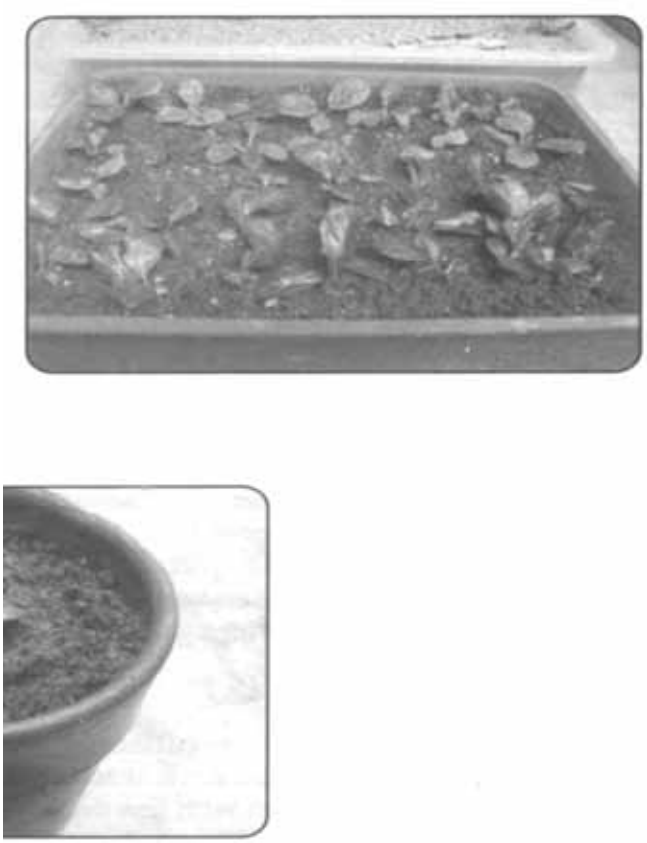

Fig. 6 : Flowering in mocropropagated plant (one year old)

Conclusion

This present result are consistent with that of Mathur *et al.,* but opposed to V.M. Patil and M. Jayanthi. They used media for induction of root but in this experiment the root were induced in non sterile sand.

Thus it is apparent that by this technique, conservation and clonal multiplication of endangered *Rauvolfia serpentina* plant is possible by *in vitro* method.

Acknowledgments

The authors are very much grateful to Mr. M.S. Bista, Director General, Department of Plant Resources for providing facilities for the study.

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Micropropagation of *Valeriana jatamansi* Jones

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Abstract

Plants within the Valerianaceae family have a long tradition in herbal medicines. *Valeriana wallichi, V officinalis* and *Nordostachys jatamansii* have been widely used in India, China and Nepal. *Valeriana jatamansii* (Sugandhawal), a perennial herb is ban for export outside the country as raw material under the Forest Act 1993 in Nepal. In order to propagate it in mass scale, tissue culture has been successfully carried out of this medicinal plant. The multiple shoots were regenerated from the young shoot tips as an explant cultured in Murashige and Skoog medium supplemented with Benzyl Amino Purine (BAP) 1.0mg/l and Naphthalene Acetic Acid (NAA) 0.1mg/l. 90% microshoots rooted in non sterile sand and these plants were successfully established in the field.

Introduction

Plants are the most important source of medicines and many other pharmaceutical products. Men have used herbal medicines from time immemorial. Ancient Egyptians, Indians, Chinese, Nepalese and many others use variety of plants and products for curing different kinds of diseases. At present, due to the identification of plant compounds effective against some dangerous diseases, the interest in herbal medicines have increased.

Plants within the Valerianaceae family have a long tradition in herbal medicines. *Valeriana wallichi DC, V. officinalis* and *Nardostachys jatamansd DC* have been widely used in India, China and Nepal. In china the use of *Nardostachys chinensis* was reported as early as Tang dynesty (Becker and Chavadej, 1988). *Valeriana jatamansi* Jones (Sugundhawal) Syn. V *Wallichii DC is* widely used medicinal plant due to chemical constituents as essential oil and valepotriate and its trade name is known as Valerian. In Nepal the distribution of the plant is in Himalaya region at an altitude between 1200-3000m. This is, a perennial herb growing in moist and shady places. The therapeutic use of its roots and rhizomes is stimulant, carminative, antipasmodic, useful in hysteria, cpilepsy and nuerosis. The plant is use in hypochondriasis, nervous unrest and similar emotional state. In India, the dried rhizomes are used in perfumes and hair preparations and as incense (The wealth of India). A large number of plants are collected with roots and rhizomes from the natural resources. In Nepal approximate quantity of plant collected annually is one hundred and eight metric tons and approximate quantity of raw material annually exported from Nepal is sixty metric tons. Due to over exploitation, the natural resources are declining. To protect the wild sources His Majesty's Government has notified restriction and *V. jatamansi* is ban for export outside the country as raw material under the Forest Act, 1993 (Shrestha and Joshi, 1996). In order to conserve the natural resources and to meet the increasing demand for plant based drugs certain alternative method has become more important. The biotechnological approach such as tissue culture is available method for the development of medicinal plants. *In vitro* propagation of plants offers the advantages of fast multiplication rate of genetically identical, disease free plants and germplasm conservation.

Mathur *et al.,* 1988 have reported *in vitro* propagation of *V. Walllichii.* Valepotriates in tissue culture were first described for *V. wallichii* DC by Becker *et al.* in 1977 (Becker and Chavadej, 1988). The present study concerns the technique of *in vitro* propagation and mass production procedure of this plant.

Materials and Methods

Plant materials were collected from the field of herbal section at Godawari. The roots and older leaves were removed and shoot tips excised were thoroughly washed under running tap water for an

Fig. 1 : Shoot tip explant

Fig. 3 : Established plants in pot (one year old)

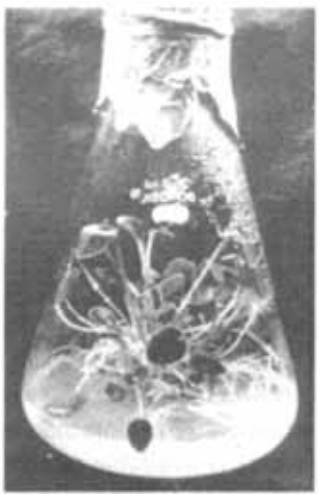

Fig. 2 : Multiple shoot formation

(Tissue culture plant)

(Non tissue culture plant)

Fig. 4 : Field trial cultivation

hour and in detergent solution for few minutes. The shoot tips were surface sterilized with 0.1 percent mercuric chloride solution for five minutes followed by rinsing with sterile distilled water for four times to remove chlorine completely. Then in each explant, outer leaves were removed and inner most shoot tips measuring 0.5 cm in length having one or two leaf primordia were cultured in MS medium (Murashige and Skoog, 1962) in combination with auxin and cytokinin. The combination of auxin and cytokinin is Napthalene Acetic Acid (NAA) 0.1 mg/l and Benzyl Aminopurine (BAP) 1.0 mg/l. The pH of the medium was adjusted to 5.8 before autoclaving. The culture were incubated at 25±2°C under 16 hour photo period at a light intensity of 3000 lux. In 4-6 weeks of explant culture, the shoots were proliferated and *were* about 2-4 cm long. The shootlets of about 2 cm in length were sub cultured in the same medium for multiple shoot production.

For rooting, the non sterile sand rooting procedure (Rajbhandari and Bajaj, 1991) was applied for the microshoots.

Results andDiscussion

After 1-2 weeks of culture, the explants show response and become green in colour (Fig. 1) and shoot proliferation was observed after 4 weeks of culture. The mode of differentiation is organogenesis that is the direct formation of plants from the excised segments shoot tips. By this method identical plant with desired characters are obtained. The number of axillary shoot proliferation was 2-4 from the initial explant. On sub culture, the number of shoot proliferation was increased, and optimum number was 20-30 shoots after 4-6 sub culture (Fig. 2). The microshoot transferred in non sterile sand produced

roots within 3-4 weeks at day night temperature 35°C and 15°C. Ninety percent microshoots rooted in non sterile sand. These sand rooted plants were successfully established in soil (Fig. 3). The field trial cultivation of in vitro propagated plants (Fig, 4) is being continued to observe the yield of rhizome as well as essential oil content in comparison with non tissue culture plants.

The result of percent work demonstrate a technique lot the *in vitro* propagation of perennial medicinal herb *V. jatamansi* from shoot tips and the advantages of this method have direct implication in mass propagation of the species. The high rate of rooting percentage of microshoots and establishment in soil ensures the production of millions of plants from a few mother plants. In addition the present result indicate that the *in vitro* propagation will play an important role in developinp cultivation and preserving the germplasm resources of different medicinal herbs.

Acknowledgments

We are very much thankful to Mr. M.S. Bista, Director General, Department of Plant Resources for facilitating the work.

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Tissue Culture of *Elaeocarpus sphaericus* (Gaertn.) K. Schum.

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Abstract

Elaeocarpus sphaericus (Gaertn.) K. Schum. (Utrasum Bead tree) is an important religious tree for Hindus. It also has medicinal value. Tissue culture of this tree has been possible for clonal propagation. The nodal cuttings from 5 years old plant were used as the explants for culture in liquid MS medium. The established green explants were then sub cultured in solid MS medium supplemented by different concentration of BAP and NAA as well as in MS medium with different concentration of BAP only. MS medium supplemented with BAP 0.25 mg/l was found to be very good for proliferation and elongation of microshoots. The proliferated microshoots when subcultured in MS medium with less ammonium nitrate (300 mg/l) supplemented with BAP 0.25 mg/l at the interval of 2 months, showed good result of proliferation with no browning of microshoots. The microshoots developed roots successfully in non sterile sand within 15 to 20 days in glass house.

Key words: *Elaeocarpus sphaericus,* explant, microshoots, subculture, tissue culture

Introduction

Elaeocarpus sphaericus (Gaertn) K. Schum., (Utrasum bead tree) belongs to Elaeocarpaceae. The plant is a medium sized tree found in south and south east Asia, Australia and Pacific islands. The status of tree is vulnerable in Nepal (Shrestha and Joshi 1996). It is cultivated in eastern Nepal between 650 to 1700m altitude for harvesting religious seeds. The leaves of the plant are elliptic, 15cm long, acute, sub entire, nearly globose, flowers white 1.25cm across in drooping raceme, stamens 35 to 40 with bearded anthers; fruits globose, 1 seeded stone tubercled, grooved, mostly 5 celled. Flowering and fruiting time is May to October. The important part of the plant is the fruit. The fruit is used in the cure of diseases of head and epileptic fits (DMP 1970). The seeds have religious value for Hindus. Commonly seeds are five grooved. The seed without a groove is considered the most valuable one. The Hindus wear the garland of the seeds for its religious purpose (Fig. 1).

Materials and Methods

Nodal cuttings of young branches from 5 years old plant were used as the explant for tissue culture. The five cm long explants were washed for half an hour on tap water. Then they were washed with liquid detergent and rinsed with distilled water. Surface sterilization of the explants was done with $HgCl_2$ (0.1%) solution for 3 minutes.

MS medium (Murashige and Skoog 1962) in liquid form was used to establish the 5 cm long explant. After solving the browning problem, the explants were cultured on solid MS medium supplemented with different concentration of BAP only as well as BAP in combination with NAA. The medium was solidified with 0.8% agar supplemented with sucrose 3%, 1.0 g/l casein hydrolysate. The pH of the nutrient medium was adjusted to 5.8. It was also tried on MS medium with little ammonium nitrate (330mg/l). The cultures were incubated at 25±2°C and 16 hours of photo period for 4 weeks. Then they were again subcultured to find out the appropriate proliferation medium in every 4 weeks period.

The flasks with microshoots were brought to room temperature for 2 weeks for acclimatization before *ex vitro* rooting (Kozai 1991). The sand was washed with water and sun dried for 2 days, sieved through 2x2mm pore size wire net. The sand was filled in box and wetted evenly with 10% water. The microshoots were cut into 10cm long pieces and planted in sand box. The temperature of the sand

box was maintained at 30°C. The sunlight light intensity varied from 8-25 kilo lux. Watering at the microshoots was done regularly to maintain 80% humidity.

Results andDiscussion

The explant cultured on filter paper bridge in MS medium established well. In this method the phenolic compound exudates in filter paper bridge. It takes 2 to 3 weeks to remove phenolic compounds from the explant. Using filter paper bridge for explant establishment in liquid medium was found to be a good method for removing phenolic compounds from it. The established explants subcultured on solid MS medium supplemented with different

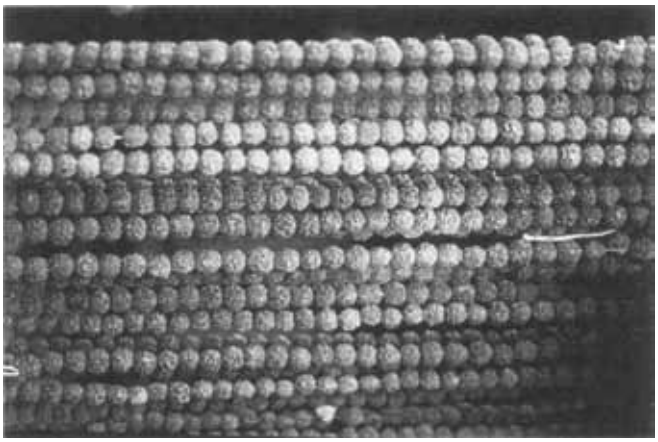

Fig. 1 : Garland made of Elaeocarpus sphaericus seeds.

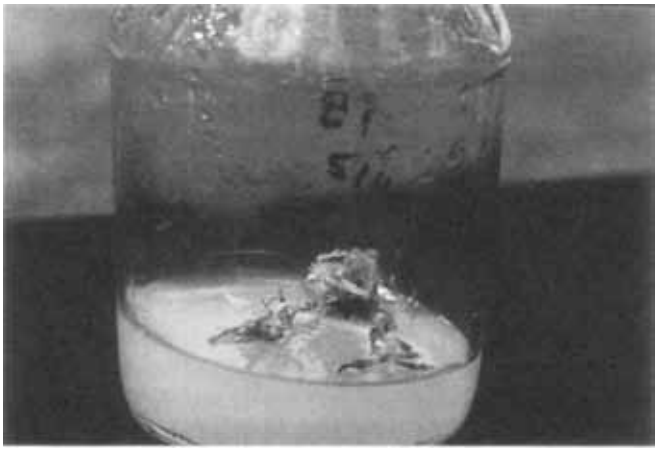

Fig. 2 : Microshoots proliferation in MS medium supplemented with BAP (0.5 mg/l) and NAA (0.01 mg/l).

Fig. 3 : Microshoots proliferation in MS medium supplemented with BAP (0.25 mg/l).

Table 1 : Proliferation of microshoots of *Elaeocarpus sphaericus* in MS medium supplemented with different concentration of BAP and NAA and BAP only after 4 weeks of culture.

Composition of Media	No. of microshoots in the culture flask	Remarks
1. MS+ BAP 1.0 mg/l + NAA 0.01 mg/l		No shoot proliferation; explant became brown
2. MS+BAP 0.5mg/l+NAA 0.01 mg/l	2 to 3	Proliferation and elongation of microshoots; but later changing to brown
3. MS control	2 to 4	Proliferation and elongation of microshoots
4. MS + BAP 0.5 mg/l	4 to 8	Proliferation and elongation of microshoots; no browning
5. MS+ BAP 0.25 mg/l	4 to 10	Good proliferation and elongation of microshoots; no browning

Table 2 : Microshoots formation and elongation in MS medium with less ammonium nitrate after 2 months.

Composition of Media	No. of microshoots in the culture flask	Remarks
1. MS + BAP 0.5 mg/l	8 to 10	Good elongation of microshoots
2. MS + BAP 0.25 mg/l	12 to 15	Very good elongation of microshoots; appropriate size for ex vitro rooting

concentration of BAP (1.0; 0.5; 0.25mg/l) and NAA (0.01 mg/l) or without NAA. After 4 weeks the result was checked. In the MS medium supplemented with BAP (1.0 mg/l) and NAA (0.01 mg/l) the explants did not proliferate and turned brown; in MS medium supplemented with BAP (0.5 mg/l) and NAA (0.01 mg/l) only 2 to 3 microshoots proliferated which later turned brown (Fig. 2); in MS control medium only 2 to 4 microshoots proliferated; in MS medium supplemented only with BAP (0.5mg/l) 8 to 10 microshoots proliferated and in MS medium supplemented with BAP (0.25mg/l) 10-14 microshoots proliferated (Fig. 3). In the medium without NAA, the shoot browning was not a problem. It is possible that NAA might have been responsible for browning of microshoots (Table 1). Further subculture of microshoots in MS medium with low ammonium nitrate (300mg/l) supplemented with BAP (0.25mg/l) resulted in 12 to 15 microshoots whereas in MS medium with BAP (0.5mg/l) only 8 to 10 microshoots developed (Table 2). This shows that low quantity of BAP is enough for more shoots proliferation rather than high. In the medium devoid of NAA, the shoot browning was not the problem. It indicated that NAA might have been responsible for browning of microshoots. In MS medium supplemented with BAP 0.5 mg/l, 4 to 8 microshoots were formed and there was no browning problem. In MS medium with 0.25 mg/l BAP the microshoots were with very good elongation, devoid of browning (Table 1). The

Fig. 4. Long microshoots proliferation in MS medium supplemented only with reduced ammonium nitrate (300 mg/l) and BAP (0.25 mg/l).

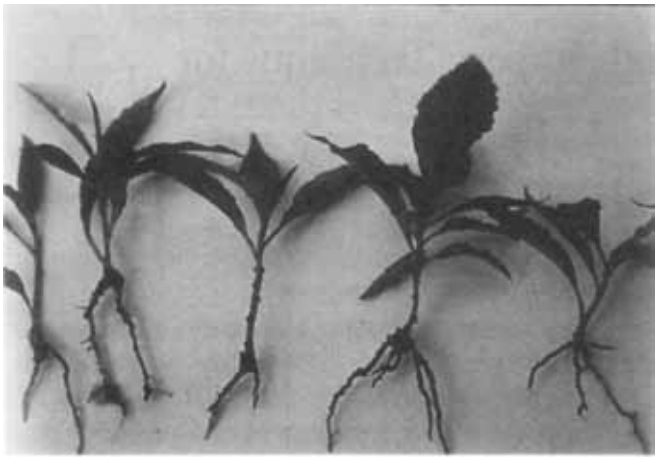

Fig. 5 : Roots developed *ex vitro* in sand.

Fig. 6 : *Elaeocarpus sphaericus* plants produced through tissue cul-ture.

microshoots sub cultured in MS medium with reduced BAP (0.25 mg/l) and low ammonium nitrate (300 mg/l) produced 15 microshoots after 2 months which were long, healthy for sand rooting (Fig. 4 and Table 2). These microshoots when transferred in sand for *ex vitro* rooting developed roots in 15 20 days (Fig. 5). This method *ex vitro* rooting in sand was also successful in other plant species (Saiju, 1997). The rooted plants established well in clay pots (Fig. 6).

Conclusion

The best steps for the micro propagation of *Aleocarpus sphaericus* are:

1. Explant establishment on filter paper bridge in liquid Murashige and Skoog (MS) control medium.

2. Explant transfer to solid *MS* medium supplemented with BAP (0.5 mg/l).

3. Continue subculture in *MS* with less ammonium, nitrate (300 mg/l) and BAP (0.25 mg/l).

4. Microshoots rooting *ex vitro* in sand.

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Pilot scale production of Nepal cardamom (*Amomum subulatum* Roxb. cultivar dambersay) through tissue culture

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Abstract

Nepal cardamom (*Amomum subulatum* Roxb. cultivar Dambersay) is a popular cash crop in Nepal. The multiple shoots were regenerated from this cultivar when young shoot tips were used as an explant for tissue culture in Murashige and Skoog's medium supplemented with 6-Benzylearninopurine (BAP) 1.0 mg/l. and Naphthalene acetic acid (NAA) 0.1mg/l. In the moss 90% micro shoots developed healthy roots. These plants were successfully established in the field.

Introduction

Cardamom (*Amomum subulatum* Roxb.) belongs to the family Zingiberaceae. It is distributed in Nepal, Bhutan and Sikkim. It attains 2 to 2.5 meter height. It is cultivated between 500 to 2000 meters on hill slopes under shades of Nepalese alder plants along the streams. Dambersay cultivar is a natural cross between cultivars, Ramsay and Golsay. It has bigger capsule than Ramsay and Golsay.

Cardamom seeds are one of the major cash crops of eastern hilly regions of Nepal. Nowadays this plant has been introduced in other hills of the country. The contribution of spices to over all socio-economic development of our society is obvious and very significant. Cardamom is suitable for agro-forestry. It also helps, soil conservation in fragile hills. Unproductive land for other crops can be used, in farming of cardamom. Cultivation of this plant has also helped to some extent to check human migration from hills to Terai and urban areas.

Cardamom seeds have a pleasant aroma and a characteristic, warm, slightly pungent taste. Nepal Cardamom seeds are used as spices and medicine. It is used for flavoring curry, cakes, bread and liquors. It is prescribed for Indigestion, vomiting and abdominal pain diseases.

Cardamom seeds or cuttings of rhizome are used for its propagation. Splitting of one year old young shoots with rhizome is used for vegetative propagation. However it is a slow process of multiplication and allows pathogens. Seed germination is also a slow process.

Tissue culture method of propagation of cardamom however is found to be useful for rapid clonal propagation to over come the conventional and slow method of propagation by splitting of stems and seed germination.

The present research work has successfully developed tissue culture technique for pilot scale production of Nepal cardamom plants.

Materials and Methods

Dambersay cultivar of cardamom was brought from Cardamom Development Center, Phikkal, and Iiam. The shoot tip explant 1-2cm. of cardamom was taken out by removing the scale and kept in running tap water for 2 hours with 4 drops of Teepol, (liquid detergent) for surface cleaning. The shoot tips were then sterilized with 0.1%. Mercuric chloride for 20 minutes and rinsed 4-5 times with sterilized distilled water. Trimmed innermost shoot tips, 1-2mm. in size was cultured in sterilized 250ml flasks containing 70ml of solidified MS medium. The culture medium was composed of Murashige and Skoog's (1962) supplemented with 3% sucrose. The medium was solidified with 0.7%, agar powder and pH was

Table1 : Growth response of Cardamom CV. Dambersay on MS liquid medium with different Concentration of BAP and NAA.

MS medium		No. of shoots	Growth Response
BAP mg/l	NAA mg/l		
5.0	0.1	2-4	Less shoots (not so good)
2.5	0.1	4-6	Less shoots (not so good)
1.0	0.1	15-20	Well developed shoots with root (good)
0.5	0.1	8-10	Less shoots (not so good)
0.1	0.1	1-2	Few shoots (Not so good)
Control	-	-	No response

adjusted to 5.8 prior to autoclaving at 15-lb./sq. in for 20 minutes. Cultures were incubated at 25°C±2°C under fluorescent light of 3000 lux for 16 hrs. The observations for all experiments were made 4-6 weeks after culture. After 4 weeks of culture the green shoots were again transferred in the fresh medium. Micro shoots were proliferated after 6 weeks. In the medium different concentration of BAP (5.0, 2.5, 1.0, 0.5, 0.1mg/l) and NAA (0.1mg/l) were added. The explants were cultured to see the response on the growth and development of micro shoots. The sub culture was done every six weeks in the fresh solid medium with BAP 1.0mg/l and NAA 0.1 mg/l. After the 6th subculture the micro shoots were subcultured in liquid media and incubated for 8 weeks.

Subculture of proliferated micro shoots was done at intervals of 6 weeks. After 2 weeks of acclimatization, the micro plants were separated individually. These micro plants were wrapped with clean moss and kept in a propagator (Fig 3).

Results and Discussions

The colour of explant changed from reddish white to green within 4 weeks of culture. In the beginning, only single green shoot was produced. After 4 weeks the green shoot was again transferred in the fresh medium. Micro shoots were proliferated after 6 weeks (Fig 1). In our observation it was found that liquid media is better for shoot and root proliferation than solid media. In this experiment 10% coconut milk was used. But no significant difference was observed in multiplication of shoots while comparing with or without coconut milk. In our experiment the response of 6 Benzyl Amino Purine and NAA was observed in all concentration. The multiplication rate was very low in BAP 0.1 mg/l and NAA 0.1 mg/l, where only 1-2 micro shoots were produced But in higher concentration of BAP 5.0 mg/l and 2.5 mg/l BAP the number of micro shoots was only 4-6. In the culture media supplemented with BAP 1.0 mg/l and NAA 0.1 mg/l the numbers of micro shoots were increased unto-20. Karki and Saiju (2000) also reported that liquid MS medium supplemented 6-benzylaminopurine (BAP) 1.0 mg/l and Naphthalene acetic acid (NAA) 0.5mg/l showed better proliferation in Ramsay and Golsay cultivars of cardamom. However in our present research liquid MS medium supplemented with 6-benzylaminopurine (BAP) 1.0 mg/l and Naphthalene acetic acid (NAA) 0.1mg/l was best for the proliferation of multiple shoots and roots in Dambersay Cultivar of Cardamom (Fig 2). In the moss the plants developed healthy roots within 4 weeks (Fig. 3 and 4). They survived well in soil sand mixture in poly bags (Fig. 5). Nadgauda et al. (1980) has cultured three cultivars *Elettaria cardamom* (Vazhukkai, Malbar and Mysore) and compared yield with seed raised plants. They observed that tissue cultured plants had significantly higher yield than seed raised plants. Later Nadgauda et al. 1983 has reported that clonal multiplication of *Elettaria cardamom* by using shoot tip culture for plant regeneration. Priyadarshan *et al.* (1992) reported that Schenk and Hilderbrand (SH medium) showed better results in comparison to Murashige and Skoog's medium. Rao et al. (1982) reported the regeneration of plantlets from callus of *Elettaria cardamom*. In this experiment there is no callus formation from the explant. It produces plantlets from shoot tip culture.

Two thousand plantlets were produced through tissue culture. Some of the plantlets were distributed to the farmers and Tistung Botanical Garden. The present result indicates that tissue culture technique can play important role in pilot scale production of Nepal cardamom.

Acknowledgement

We are thankful to Dr. U.R. Sharma, Director General and Mr.Yogesh Nandan Vaidya, Deputy Director General, Department of Plant Resources for facilitating the research work and Devendra Saraf (Cardamom Development Center, Khumaltar) for providing the mother plants of Nepal Cardamom.

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Fig. 1 : Cardamom micro shoots proliferation in solid MS medium

Fig. 2 : Cardamom plants in liquid MS medium

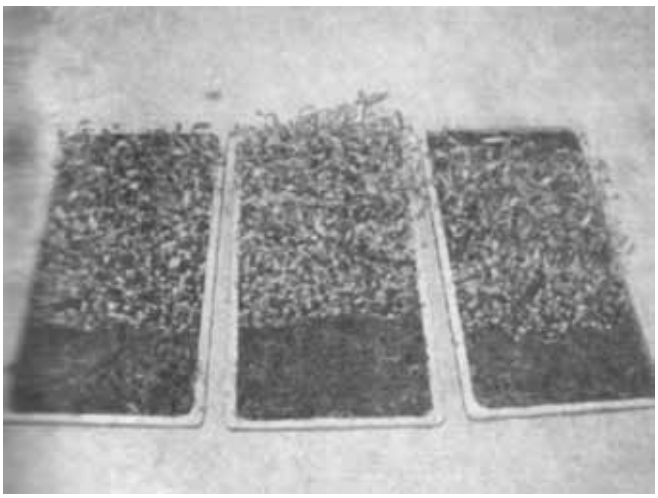

Fig. 3 Cardamom plants rooting in moss

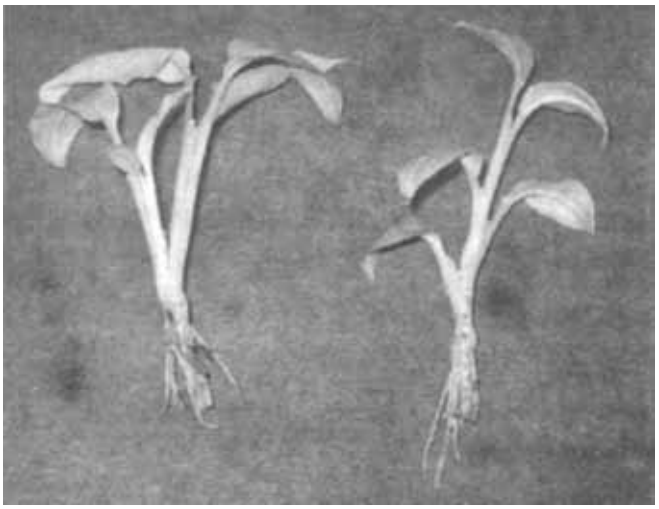

Fig. 4 Rooted Cardamom plants

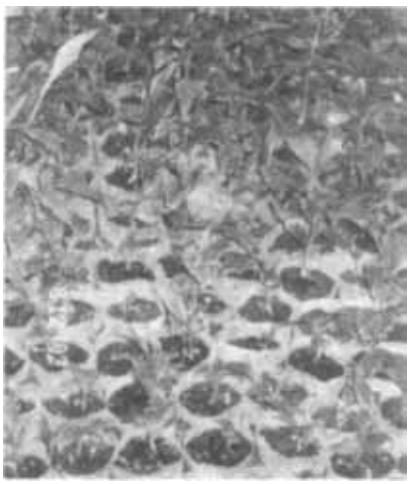

Fig. 5 Cardamom Plants in polythen Bags

Micro propagation of *Dendrobium fimbriatum* Hook.

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Abstract

Dendrobium fimbriatum belongs to family Orchidaceae. It occurs as an epiphytic herb from tropical to temperate region. Protocorms of *Dendrobium fimbriatum* were initiated from shoot tip explant in the MS (Murashige and Skoog's medium, 1962) medium fortified with 5mg/l BAP (Benzyl amino purine), 1mg/l NAA (Napththalene acetic acid) and 10% coconut milk. Protocorms were sub cultured in MS medium with 1mg/l BAP, 1mg/l NAA and 10% coconut milk for multiple shoots and protocorms production.

micro shoots transferred on MS medium with 0.5mg/l NAA produce roots.

Introduction

Orchids belong to family Orchidaceae. There are 361 species of Orchids belonging to 96 genera, in Nepal. Orchids are endangered plants, banned for export and collection from natural habitat. In floriculture, it is the main floral crop because it has long lasting, multiform and multicolour flowers.

Seeds and vegetative parts are generally used to propagate orchids. Seed grown plant progeny is not identical to parent plants because they are heterozygous. In vegetative propagation of plants, the progeny is identical to parent stock. Orchid production in vivo is a very slow process, requiring ten years to obtain a clone of suitable size. We can obtain large number of plants within a short period of time from shoot tip and meristern culture

Tissue culture of orchids was attempted first by Morel (1960, 1962) to obtain virus free cymbidiums by meristem culture. He produced more than 4,000,000 plants of cymbidium in one year from single shoot apex. Sagawa and Shoji (1967) first described the procedure for shoot tip culture of *Dendrobium.* Later on, meristem culture used for *Dendrobium, Catteleya* and other plants.

This paper describes the shoot tip culture of *Dendrobium fimbriatum* Hook. This plant is a temperate, epiphytic with pendulous inflorescence with golden yellow colours flowers with purple blotch in lip blooming 20 to 25 days. The plant flowered in April rarely in June. This species is found in India, Nepal, Bhutan, Myanmar, South China and South East Asia.

Material and Method

Tender shoots about 3.5 to 5cm from mature plant were taken from Green house. Decontamination of these juvenile shoot was performed as thoroughly as possible. First, all the visible dirts were removed by washing the tender shoot, and washed for one hour in running tap water. This process was followed by surface disinfections with detergent, teepol for 5 minutes (few drop of teepol with 300ml of water) and rinsed with distilled water for several times. Finally, in a sterile chamber, these shoots were rinsed with 0.1% mercuric chloride solution for 15 to 20 minutes, then rinsed several times with sterile distilled water to remove all traces of mercuric chloride and transferred to sterile petri dish. The outer leaves were removed one by one. The excisions of shoot tip were done with the help of small forceps. The excised shoot tips (2 to 5mm) were inoculated on Murashige and Skoog's medium fortified with 1g/l casein hydrolysate, 3% sucrose, 10% coconut milk and different concentration of BAP and NAA. The medium was solidified with 0.7% agar and pH adjusted to 5.5 before autoclave at a pressure of 151b/ sq inch for 15 minutes.

The cultures were incubated at 25 ± 2°C under 16 hr photoperiod provided by white fluorescent tubes at 3000 lux.

For shoot tip culture the following combination of cytokinin and auxin were used

MS medium +	Growth responses
No hormone	No responses
BAP 0.5 mg/l + NAA 1 mg/l	No response
SAP 1mg/l +NAA 1 mg/l	Green, no multiplication
BAP 2.5mg/l+NAA 1 mg/l	Very few protocorms at the base of explants
BAP 5mg/l+ NAA 1 mg/l	Protocorms and shoot at the base of explants

Result and Discussion

The shoot tip cultured on MS medium with BAP 5mg/l and NAA 1mg/l showed green protocorms with few microshoots after 6-8 weeks. Initiation of protocorms and micro shoot were seen around the base of shoot tip then towards the tip. Sagawa and Shoji used simple low salt medium, modified VW medium, for protocorms proliferation and plantlets growth. P.G. Latha and Seeni (1992) cultured *Dendrobium hybrid* meristem into the liquid medium on a gyratory shaker. They also found initiation of protocorms from base to tip of the explants (1992).

The protocorms and micro shoots proliferated in MS medium added with 5 mg/l BAP and 1 mg/l NAA were again subcultured in above different concentrations. Among them, MS medium with BAP 1mg/l +NAA1, mg/l was seen best medium for proliferation of protocorms and micro shoot (Fig-2). Latha and Seeni (1992) transferred differentiated protocorms from explant to solid medium for plantlets regeneration. The subculture process was repeated 8-12 weeks. On repeated sub culturing, the number of micro shoots increased from 5-8 to 12-18.

The micro shoot rooted within 6-8 weeks, when these shoot transferred to the MS medium with 0.5 mg/l NAA. The complete seedlings were developed within 6-7 months after initial excision of the shoot tip.

The flasks with rooted plantlets were acclimatized for 8-15 days in green house condition. Then plantslets were removed from flasks, washed in

Fig. 1 : Dendrobium timbriatum plant.

Fig. 2 : Micro shoots multiplication in MS medum

Fig. 3 : Micro propagated plants

Fig. 4 : Six months old micro propagated plant

water to remove agar from plantlets and finally transferred to comunity pots containing tree fern roots, charcoal, broken bricks and compost. Then these grown plants were reported individually on same medium (Fig 3, 4).

Conclusion

From this study, it is apparent that conservation and clonal multiplication of *Dendrobium fimbriatum* is possible by micropagation method.

Acknowledgement

The author is very much grateful to Dr. Uday Raj Sharma, Director General, and Dr. Mahesh Kumar Adhikari, (Chief of NHPL) for providing facilities for this work.

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Micropropagation of *Azadirachta indica*

K. M. Rajkarnikar and G. D. Bhatt

Abstract

Azadiracta indica A. Juss, the most valuable medicinal and economic plant, was selected for its micropopagation. Among the node and leaf explants of the tree, the node explant responded in MS medium supplemented with 0.25mg/l BAP, 0.01mg/l *NAA,* 0.5mg/l charcoals and 50mg/l Adenine sulphate as most suitable for the regeneration of multiple shoots. *In vitro* raised microshoots developed roots in MS medium fortified with 0.5mg/l NAA and 0.5gm/l charcoal.

Key words: *Azadiracta indica,* Micropropagation, Medium and Hormones, Charcoal.

Introduction

Azadiracta indica A. Juss, locally known as Neem, belongs to the family Meliaceae. It is an evergreen fast growing timber tree with broad canopy. The trunk is used as timber and in various purposes. In Nepal it occurs in central and eastern phytogeographic regions in between 300 and 1700m altitude (Bista, Adhikari, Rajbhandari, 2001). It is cultivated widely in Tropical and Subtropical region of Indian subcontinent. It is distributed in south east Asia, tropical Australia and Africa. Each and every part of the tree is used in medicinal purposes for its effective bitter principle "Azadirachtin". The highest concentration of the chemical is found in seed kernels. Except *Azadirachtin,* the other phytochemicals are Salanin, Mellantriol, Nimbin and Nimbbidin.

The bark is tonic, astringent and antiperiodic. The leaves are used as poultice to apply in boils. The decoction of leaves is antiseptic and also used in ulcers and eczema (Anonymous, 1997). The gums are demulcent and tonic in catarrhal affections. The dry flowers are used as tonic and in stomachic. The oil extracted from seeds is stimulant. It is used as antiseptic, alterative in rheumatism and skin disease. The bark, gum, leaf and seed are used in snakebite and scorpion sting. Recently neem is used in agriculture as a bio control pesticide agent, in the pharmaceutical and soap industries (Opender *et al.,* 1990).

Generally, it is propagated from the seeds, but the viability is very low (Gurumurti & Jagadees, 1992). The propagation through cutting is quite low (Narayan & Jaiswal, 1985). So, tissue culture is the only alternative and effective method to multiply the plants in short time. The present work is an investigation to establish *in vitro* clonal propagation of *Azadirachta indica.*

Material and Method

The nodes and leaves were taken from 5 years old (seed grown) plants from the conservation section of National Herbarium and plant Laboratories, Godawari. These materials were washed for one hour in flowing tap water, teepol for 5 minutes and then with distilled water. The explants were treated in 0.1% mercuric chloride solution for 3 4 minutes for surface sterilization. Then they were rinsed 4 times in sterilized distilled water in aseptic condition.

The explant of appropriate size (1 to 2mm) was cultured in MS medium (Murashige & Skoog, 1962). The medium was fortified with 0.8% agar, 0.1% casein hydrolysate, 3% sucrose and different concentration of hormone to determine their effect on shoot proliferation and growth. The medium was adjusted to 5.8 pH before autoclaving. The cultures were incubated at 25±2°C under 16 hours photoperiod of white fluorescent tubes. The nodal segments from regenerated microshoots were subculture into fresh medium in every 8 to 10 weeks, after optimum growth.

The regenerated microshoots were subcultured in MS medium with charcoal and in different concentration of auxin for rooting.

Result and Discussion

The explants produced the callus after three to four weeks. Then in 6 to 8 weeks of interval the explant initiated microshoots. The size of callus, which appeared at the base of the explant differed in each concentration of hormone. No callus was produced in the medium without hormone (Table 1).

The microshoots initiated in 0.25mg/l BAP were cut down into node. These were subcultred in MS medium in following concentrations (Fig. 1).

Fig. 1: Nodal explant in medium.

- 0.25mg/l BAP, 0.0lmg/l NAA, 0.5gm/l charcoal, 50mg/l Adenine sulphate
- 0.25mg/l BAP, 0.0lmg/l NAA, 0.5gm/l charcoal, 100mg/l Adenine sulphate.

After 8 weeks, 8 12 microshoots measuring 0.8 to 1 cm were seen developed from nodal portion in both medium. In the medium containing 100mg/l Adenine sulphate, microshoots as well as callus appeared at the base of nodal segment and the medium containing 50mg/l Adenine sulphate did not regenerated callus. The proliferated nodal portion of microshoots were again subjected to MS medium supplemented with 0.25mg/l BAP, 0.01mg/l NAA, 0.5gm/l charcoal and 50mg/l adenine sulphate in every 8 to 10 weeks for further multiplication (Fig. 2).

Fig. 2 : Microshoots multiplications in MS medium supplemented with 0.25 mg/l BAP, 0.01 mg/l NAA, 50mg/l Adenine sulphate and 0.5gm/l charcoal.

For rooting, the regenerated microshoots were again subcultured in MS medium, supplemented with NAA in different concentration (i.e. 0.01, 0.1, 0.5mg/l) and with charcoal (0.5gm/l) or without charcoal. The microshoot developed callus first at the cut end and then roots regenerated on the medium without charcoal. But in the charcoal-containing medium, the microshoot regenerated roots without callus. The MS medium with 0.5mg/l NAA with 0.5gm/l charcoal is observed as the best medium for rooting *in vitro* (Fig. 3).

Fig. 3 : Root developed on microshoots in MS medium supplemented with 0.5mg/l NAA and 0.5gm/l charcoal.

Roy, Assaduzzaman & Hussain (1996) reported that the best initiation of microshoots was in MS medium, 0.1mg/l NAA and 1.0mg/lit BAP with the addition of CM 10% and 150mg/lit CH. In this experiment, regeneration of microshoots was improved with the addition of Adenine sulphate. They also found that the number of shoots increased in number with the increase of subcultures. Similar result was obtained with present investigations. Gill, Gill & Gosal (1996) also found maximum shoot production on MS medium supplemented with 1mg/lit BAP and 0.5mg/

Table 1 : Response of node explants in MS medium with different concentration of BAP and NAA (mg/l).

Condition of hormone concentration in mg/l		Number of shoots	Responses by shoot tip (explant)	
			Callus initiation	Length
Without Hormone		Only elongate	No callus	4 cm. long
BAP	NAA	-	-	-
0.25		4-6	Little callus	2-3 cm. long
0.5	-	2-5	Little callus	2-3 cm. long
0.5	0.01	3-4	More callus	0.5-1 cm. long
1	0.1	2	More callus	0.5-1 cm. long

l IBA. Medha *et al.* (1993) also obtained callus before plant regeneration. In the present experiment callus was initiated first and then plantlets but formation of callus was inhibited by using 0.5g/l charcoal. Ramesh & Padhya (1990) reported the clonal propagation of Neem from leaf disc. The leaf explants were discarded in the present experiment, because of more callus regeneration from leaf explants.

For rooting, Roy *et al.* (1996) used 1/2 strength MS salts supplemented with 1.0mg/lit each of IBA and IAA. An initial 7 days dark period accelerates the root induction within 14 days, which was different from the present experiment. In the present experiment the full strength MS medium supplemented with 0.5mg/l NAA and 0.5g/lit charcoal were used for rooting. Gill, Gill & Gosal (1996) obtained maximum rooting percentage in 1/2 strength MS medium supplemented with 1mg/l IBA and 0.2mg/l NAA without dark treatment.

Conclusion

In the present investigation it was found that the MS medium supplemented with 0.25mg/l BAP, 0.01mg/l NAA, 50m.g/l Adenine sulphate and 0.5g/l of charcoal was best for multiplication of microshoots. While the MS medium with 0.5g/l charcoal and 0.5mg/l NAA was best for rooting.

Acknowledgement

The authors are grateful to Dr. U. R. Sharma, Director General and Dr. P. R. Sthapit, DDG, Department of Plant Resources, Thapathali for their encouragement and providing facilities to work in this field. We offer sincere thanks to Dr. M. K. Adhikari, Chief, National Herbarium and Plant Laboratories, Godawari for providing necessary facilities and valuable comments during the preparation of this manuscript. Our thank goes to Mr. H. K. Saiju, Scientific Officer, Biotechnology Section, DPR, Thapathali, for suggestion.

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Micropropagation *of Cephaelis ipecacuanha*

K. M. Rajkarnikar, M. Kayastha and G. D. Bhatt

Abstract

The shoot tips of *Cephaelis ipecacuanha* were used as initial explants. In *in vitro* multiple microshoots were obtained from *in vitro* regenerated node segments in MS medium supplemented with 2mg/l BAP and 0.1mg/l NAA. These microshoots produced roots in non-sterile sand within 2 3 weeks. The regenerated plants were successfully grown in soil in the green house.

Key words: Ipecac, Micropropagation, Medium and Hormones, Sand rooting.

Introduction

Cephaelis ipecacuanha A. Richard is the native plant of Brazil and belongs to the family Rubiaceae. It is commonly known as Ipecac. It is a perennial plant and grows in humid forests. It generally survives for five to six years and starts flowering from second year. It prefers shade, humus rich and acidic soil. The plant contains many alkaloids among them emetin and cephaeline are the main active phyotchernicals, which are used for medicinal purposes. The maximum percentage of these chemicals is found in roots. The roots are harvested after three years of cultivation, when there is maximum storage of the alkaloids (Chatterjee *et al.* 1982). It is used as expectorant, an emetic and amoebacide.

Conventionally, this plant is propagated by stem or root cutting and or from seeds. The viability of the seed is 40-45% and the growth is very slow (Chatterjee *et al.* 1982). Though, the propagation of plant done from stem and root cutting is slow, an especial agroclimatic conditions are necessary for slightly faster and proper growth of the plants (Yonzone & Chatterjee, 1986), So, the micropropagation is an essential alternative method for propagation of such plant. In this paper, the micropropation technique of Ipecac attempted through shoot tip and node culture is highlighted.

Material and Method

Two years old plant grown in field of Brindavan Botanical Farm at Hetauda, Makawanpur, was used as the source of mother stock. The shoot tips measuring 0.5cm to 1 cm long were taken out and carried to laboratory They were washed thoroughly in running water for half an hour. Then dipped in teepol for five minutes and rinsed with distilled water. Aseptically, these shoots were sterilized with 0.1% $Hgcl_2$ solution for 3-4 minutes. The sterilized shoots were thoroughly washed in sterilized distilled water. The shoot tips about 2-3mm were excised and inoculated in Murashige & Skoog's (1962) medium. The medium was fortified with 3% sucrose, 0.8% agar and different combination of Naphthalene Acetic Acid (NAA) and Benzyl Amino Purine (BAP). The pH of the medium was adjusted to 5.6 to 5.8 before autoclaving. The medium was autoclaved at a pressure of 151b/ in 2 for 15 minutes. The cultures were incubated at 25±2°C under 16 hours of photoperiod. The experiment was repeated twice involving ten to twelve replicates for each combination.

The microshoots proliferated, in medium; from shoot tip explants were again subcultured as node (Fig. 1) and shoot tip explant in various concentration and combinations of hormone to investigate the mass proliferation of microshoots. The proliferated microshoots were subculture in fresh medium in every 8 to 10 weeks.

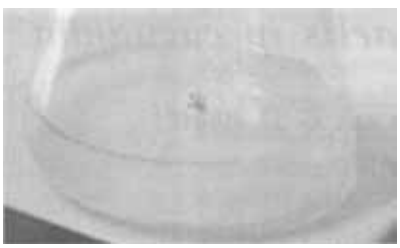

Fig. 1 : Shoot tip explant in medium

For the initiation of roots, 3 to 4 cm long microshoots were transferred in non sterile sand

(Rajbhandary & Bajaj, 1991). The rooted juvenile shoots were transferred to clay pots.

Result and Discussion

For shoot tip culture, the following combination of BAP and NAA were used (Table 1).

Among the combinations of hormone, the MS medium supplemented with 0.1mg/l NAA and 0.5mg/l BAP did not regenerate multiple shoots from explant but the medium with 0.1mg/l NAA and high concentration of BAP (1, 2 and 4mg/l) produced only 2-3 microshoots after 6 weeks. Thus the *in vitro* proliferated microshoots were again subcultured in different combination of BAP and NAA (Table 2).

The MS medium supplemented with lower concentration of BAP showed less number of multiplication of shoots from shoot tip and node. In the medium with higher concentration of BAP, explants regenerated microshoots after 10 to 12 weeks (Table 2). The best proliferation of shoots were seen in MS medium supplemented with 2mg/l BAP and 0.1mg/l NAA that is 6 to 8 microshoots per node and 3-4 microshoots per shoot tip explants. These regenerated microshoots also developed roots after long time incubation of 18 to 20 weeks in same medium (Fig. 2).

Yoshimatsu & Shinomura (1991) reported the adventitious shoots developed from internodal segment in hormone free MS, Gamborg B5, Woody plant, Thomas and Davey medium in dark condition after 7 weeks. They reported that highest number of shoot formation per segment occured in MS medium but they turned brown and did not grow well. They also reported that 0.5mg/l BAP or 1mg/l Kinetin inhibited shoot formation and segment cultured with 3mg/l BAP and Kinetin turned brown. The media containing 0.1mg/l BAP or 0.1mg/l Kinetin cultured under 16 hr. light increased the number of shoots per segment.

Fig. 2 : Proliferation of microshoots in MS medium supplemented with 2mg/l BAP and 0.1mg/l NAA.

In this experiment, explants did not regenerate in hormone free and in lower concentration of MS medium. Jha & Jha (1989) found that 8-12 microshoots were obtained from seedling nodal explants on MS medium supplemented with 8mg/l Kinetin, 0.05mg/l NAA and 200mg/l Adenine after 10-12 weeks incubation. Ideda *et al.* (1988) in their preliminary experiments reported that only one shoot regenerated from apical shoot and two from the node. They got multiple shoots (4 shoots per node segment) on B5 medium supplemented with 0.01mg/l NAA and 3 or 5mg/l BAP after two months from in vitro regenerated node culture. The opinion expressed by Jha & Jha (1989) and Ideda *et al.* (1988) are similar to this experiment where the regeneration of microshoots were initiated for node explants. Ideda *et al.* (1988) used B5 medium supplemented with 3

Table 1. Growth responses of explants in MS medium with different combination of BAP and NAA.

Condition of hormone concentration in mg/1		Growth responses	No. of shoots
No Hormone		No response	----
BAP	NAA		
0.5	0.1	Green, elongation	----
1	0.1	Less shoot	1-2
2	0.1	hoots	3-4
4	0.1	Stunted shoots, brown after few weeks	3-4

Table 2 : Regeneration of microshoots from *in vitro* grown explants in different combination of BAP and NAA.

Explant	4mg/1 BAP+ 0.1mg/1 NAA	2mg/1 BAP+ 0.1mg/1 NAA	1mg/l BAP+ 0.1mg/1 NAA	0.5mg/1 BAP+ 0.1mg/1 NAA	Medium without hormone
Shoot-tip	34	34	Up to 2	Elongation	No response
Node	Stunted 5-6	6-8	2-3	Elongation	No response

or 5mg/l BAP and 0.01mg/l NAA for microshoots multiplication. Jha and Jha (1989) used MS medium supplemented with 8mg/l kinetin, 0.05mg/l NAA and 200mg/l Adenine for microshoots multiplication but in the present experiment, MS medium supplemented with 2mg/l BAP and 0.1mg/l NAA was found best for multiplication of microshoots.

For rooting, the microshoots were transferred in non sterile sands in the propagator. These shoots developed roots within 2 to 3 weeks. Yoshimatsu & Shimomura (1991) and Jha & Jha (1989) reported that microshoots developed roots in hormone free medium but Idecla *et al.* (1988) developed roots in B5 or 1/2 MS medium supplemented with 3mg/l IAA. These rooted microshoots transferred to clay pots for further growth (Fig. 3).

Fig. 3. Rooted plants transfer into clay pots.

Conclusion

From this experiment, it can be concluded that the mass propagation can be done from node culture in MS medium supplemented with 2mg/l BAP and 0.1mg/l NAA. The sand-rooting technique would be useful for reduction of cost in clonal propagation and conservation of Ipecac.

Acknowledgement

The authors are grateful to Dr. U. R. Sharma, Director General and Dr. P. R. Sthapit, DDG, Department of Plant Resources, Thapathali for their encouragement and providing facilities to work in this field. Sincere thanks are offered to Dr. M. K. Adhikari, Chief, National Herbarium and Plant Laboratories, Godawari for providing necessary facilities and valuable comments during the preparation of this manuscript. Thanks are due to Mr. H. K. Saiju, Scientific Officer, Biotechnology Section, DPR, Thapathali, for suggestion.

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Micropropagation of *Swertia ciliata*

K. M, Rajkarnikay, G. D, Bhatt and M. K. Adhikari

Abstract

The explants (1 2mm shoot tips and leaves) of *Swertia ciliata* were cultured in MS medium supplemented with 1mg/l BAP and 0.01mg/l NAA for multiplication of microshoots. The developed microshoots were transferred in non sterile sands for rooting. The roots were initiated within 2 to 3 weeks of transplantation. The rooted plants were established successfully in field.

Key words: *Swertia cilita,* Micropropagation, Medium and Hormones, Sand rooting.

Introduction

The genus *Swertia* (family Gentianaceae) is used in traditional Ayurvedic medicine since a long time. About 100 species of *Swertia* are recorded (Bhattarai, 1997) from the world. *Swertia spp.* (locally known as Chiraito), the wild medicinal plant, occupies highly prioritized position in trade. Thirty four districts of Nepal from east to west have been found to contribute the supply of Chiraito (Bhattrai & Acharya, 1998). In Nepal 29 species *viz Swertia acaulis* H.Sm. (C. & E. Nep, 3700-5500m), *S. alata* (Royle ex D.Don) C.B. Clarke (W. & C. Nep, 2000 2600m), S. *alternifolia* Royle (C. Nep, 3000-4000m), S. *angustifolia* Buch.-Ham. ex D.Don (W., C. & E. Nep, 600-2600m), *S. bimaculata* (Sieb. & Zucc.) C.B. Clarke (E. Nep, 900-2700m), S. *candelabruin* H.Sm. (C. Nep, 4800m), S. *chirayita* (Roxb. ex Fleming) Karstrn (C. & E. Nep, 1500-2500m), S. *ciliata* (D.Don ex G.Don) B.L. Burtt (W., C. & E. Nep, 2800-4000m), S. *cordata* (G.Don) C.B. Clarke (C. & E. Nep, 2000-3000m), S. *cuneata* D.Don (E. Nep, 3900-5000m), S. *dilatata* C.B. Clarke (W., C. & E. Nep, 1800-4000m), S. *gracilescens* H.Sm. (C. Nep, 2000-3700m), S. *hispidicalyx* Burkill (C. Nep, 4100m), S. *hookeri* C.B. Clarke (E. Nep, 3800-4300m), S. *kingii* Hook. f. (C. & E. Nep, 3100-4500m), S. *lurida* (D.Don ex G.Don) C.B. Clarke (W. & C. Nep, 2500m), S. *macrosperina* C.B. Clarke (C. & E. Nep, 2000-3200m), S. *inulticaulis* D.Don (C. & E. Nep, 4000-4900m), S. *nepalensis J.* Shah (C. Nep, 3850m), S. *nervosa* (G.Don) C.B. Clarke (W., C. & E. Nep, 700-3800m), S. *paniculata* Wall (W. & C. Nep, 1500-3000m), S. *pedicellata* Banerji (E. Nep), S. *petiolata* D.Don (W. Nep, 5600m), S. *raceniosa* (Griseb.) C.B. Clarke (W., C. & E. Nep, 3000-5000m), S. *raniosa W.W.* Sm. (E. Nep, 4100m), S. *speciosa* D.Don (W., C. & E. Nep, 1400-3000m), S. *staintonii* H.Sm. (E. Nep, 3800-4400m), S. *tercs J.* Shal (W., C. & E. Nep, 3000-5000m) and S. *tetragona* Edgew. (W. Nep, 2400-3000m) are recorded (Bista, Adhikari & Rajbhandari, 2001). Among these, the species like *Szvertia alata, S. angustifolia, S. chirayita, S. ciliata, S. dilatata, S. inulticaulis, S. nervosa, S. raceinosa* and S. *tetragona* are traded from Nepal (Barakoti 2001). Among these species *Swertia chirayita is* considered as the most important species for its medicinal value, while other species of *Swertia* are used as substitutes and adulterants of *Swertia chirayita* (in *The Wealth of India, Vol. X)*

Swertia ciliata (D.Don ex G.Don) B.L. Burtt is a perennial herb growing on the slopes of central Himalaya, near forest edges, in moist shady slopes up to 4000m. This species is distributed throughout temperate and alpine region. The leaves of this plant are used in medicinal purposes for fever (Bhattacharjee, 1998). Conventionally, this plant is propagated by seeds and cutting of roots in well drained soil (Bhattacharjee 1998). The bitter principle has been a source of medicinal value.

The present paper attempts to highlight on the technique of *in vitro* culture of *Swertia ciliata* for its mass propagation of pathogen free plantlets at reduced cost.

Material and Method

Few plants were brought to the green house of National Herbarium and Plant Laboratory, Godawari from the Pilot section (Medicinal Plant Screening) of Department of Plant Resources. The shoot tip from five month old plants were taken out and washed in running water for one hour followed by teepol for few minutes. Then these shoots were rinsed 3 to 4 times with in distilled water. They were surface sterilized with 0.1% $Hgcl_2$ for 5 minutes and washed with sterilized distilled water. Aseptically the sterilized explants were trimmed 1 to 2mm size shoot tip and leaf explants. These explants; were cultured in MS medium (Murashige & Skoog, 1962) supplemented with BAP and NAA. The medium was solidified with 0.8% agar. The pH was adjusted to 5.8 before autoclave at 15lbs/in_2. for 15 minutes. The cultures were incubated at 25±2°C under fluorescent light of 3000 lux (ca.) for 16 hrs. Each experiment was carried out in ten replicates. The subcultures of regenerated shoots were done in every 4 to 6 weeks.

For rooting, acclimatized microshoots; of about 3 to 4 cm size were excised and transferred in non sterile sand under glass house condition (Rajbhandary & Bajaj, 1991). The rooted plants were transferred to polybags with soil for field establishment.

Result and Discussion

Initially all explants responded in all concentration of BAP and NAA. Later on differentiation of roots and shoots were observed varying in different combination of BAP and NAA (Table 1).

The shoot tips of Sivertia ciliata were cultured in MS medium with different concentration. The MS medium supplemented with 1mg/l BAP and NAA concentration (1mg/l and 0.1mg/l) showed development of callus, shoots and roots. The MS medium supplemented with BAP (0.5 and 1mg/l) and NAA (0.01mg/l) developed only shoots within three weeks. But, in the MS medium supplemented with BAP (1mg/l) and NAA (1mg/l), the explants regenerated callus at first after two weeks, and then shoots and roots simultaneously after four weeks.

Table 1 : Responses of shoot tip explants in different combination of BAP and NAA.

MS medium + BAP+NAA (in mg/l)		No. of shoots	Growth responses
1	1	6-8	Stunted shoots + callus
1	0.1	6-8	Shoots + roots
1	0.01	10-12	Shoots
0.5	0.1	7-10	Shoots + roots
0.5	0.01	8-10	Shoots

Among these tested mediums, the MS medium supplemented with 1mg/l BAP and 0.01mg/l NAA was found best for initiation of good quality of microshoots (Fig. 2). This result showed that NAA plays great role in differentiations of callus, shoots and roots (Table 1).

Fig. 1 : Regeneration of callus and embryoids from leaf explant.

The explants from leaves regenerated callus in MS medium supplemented with high concentration of NAA (1mg/l) and 1mg/l BAP but in medium with 1mg/l BAP and low concentrations of NAA (0.1mg/l and 0.01mg/l), the explants regenerated embroids after 5 to 6 weeks of culture (Table 2, Fig. 1). The result shows the gradual differentiation of tissues from caulogenesis, embryogenesis to organogenesis from the explant of leaf.

The plants developed from direct organogenesis were identical to the mother plants. So, the callus derived microshoots were discarded. The microplants developed on the MS medium supplemented with 1mg1l BAP and O Olmg/l NAA were subcultured for further multiplication at an

Table 2 : Responses of leaf explant in different combination of BAP and NAA.

MS medium + BAP + NAA (in mg/l)		Growth responses
	1	Callus
	0.1	Embroids
	0.01	Embroids

interval of 4-6 weeks. The proliferation of microshoots increased in numbers (18-20) as they were subcultured (4-5 times) in the same medium. The micropropagation technique used in the present species showed similar result as in Swertia chirayita (Kayastha, 2000), where the callus and roots were developed in MS medium supplemented with 1mg/l BAP and 0.1mg/l NAA.

Fig. 2 : Microshoots proliferation in the MS medium supplemented with 1mg/l BAP and 0.01mg/l NAA.

For rooting, 3-4 cm long microshoots were transferred in non sterile sand, in propagator under green house conditions. These shoots developed roots after 2 to 3 weeks (Fig. 3). The rooted plants were well established in pots.

Fig. 3. Sand rooted plant.

Conclusion

The experiment thus carried out concludes that mass propagation of Swertia ciliata through tissue culture can be successfully done on the MS media supplemented with 1mg/l BAP and 0.01mg/l NAA.

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In vitro Propagation of Hybrid of Asiatic Lily

G. D. Bhatt, K. M. Rajkarnikar and M. K. Adhikari

Abstract

The MS medium supplemented with 0.5mg/l Benzylaminopurine (BAP) and 0.1mg/l NapLhaleneacetic acid (NAA) developed plantlets with in 5 to 6 weeks from the nodal explants of hybrid of Asiatic Lily. The established shoot tips were subcultured in the same medium for four weeks. 8 to 10 microshoots developed in the 3rd subculture. These microshoots rooted in non sterile sand within 3 to 4 weeks.

Key words: Asiatic Lily, Micropropagation, Explant, MS medium and Hormones, Sand **rooting**

Introduction

Six species of Lilium [*(Lilium bakerianion* Collet & Hemsely (E. Nep,), *L. nanim* Klotzsch (W., C. & E. Nep, 3700-4600m), *L. neplaense* D.Don (W., C. & E. Nep, 2300-3400m), *L. oxypetaluin* (D.Don) Baker (W. Nep, 3400-5100m), *L. sherriffiae* Stearn (E. Nep, 3800m), and *L. wallichianum* J.A. & J.H. Schultes (W. & C. Nep, 1100-2400m)] are recorded from Nepal (Bista, Adhikari and Rajbhandari, 2001). Among these *Lilitun nepalense* (Saiju *et al.,* 1994) and *Liliuin longiflorum* (Saiju & Rajbhandary, 1992) have already been taken into trial for micropropagation studies.

The hybrids of Asiatic Lily are largely traded in the international market. The genus *Lilium* (family Liliaceae) in floriculture practices ranks in the seventh status among the cut flowers. It is a very popular pot plant (Anonymous, 2000) for indoor and outdoor decorations. The hybrids are derived from nine species of Asian Lily *(L. aniabile, L. cermum, L. dauricum, L. davidii, L. maculatum, L. auratum, L. rebidlum, L. concolor,* and *L. nobillissimum* (Aartrijk *et al.,* 1990).

According to Aartrijk and coworkers (1990) the main constraints in conventional propagation of Lily includes the inadequate availability of healthy, disease free planting material, and slow multiplication of bulbs. Thus there is a great need to develop mass propagation methods for commercially important varieties and to make available the disease free planting stocks.

Material and Method

The bulbs of hybrids of Asiatic Lily possessing different colours of flowers were bought from market and grown in green house. The young nodal segments were selected as explants for micropropagation.

The explants; were washed in running tap water for 15 minutes to half an hour followed by washing with few drops of teepol for 2 to 3 minutes shaking vigorously. They were finally washed with distilled water for 4 to 5 times. The explants were sterilized in 0.1% mercuric chloride solution for 5 minutes and rinsed thoroughly with sterile distilled water 4 to 5 times. The explants were cut down to desirable size with the help of sterilized forceps and knife.

The excised node (0.5 to 0.7 cm) were inoculated on Murashige & Skoog's medium (1962) supplemented with 1gm/l casein acid hydrolysate in several flask containing different concentration of hormone. The pH was adjusted between 5.6 and 5.8 before autoclaving. Then media was autoclaved at a pressure of 15lb/in2 for 15 minutes. The cultures were incubated at 25±2°C temperature for 16 photoperiodic hours in 3000 Lux (ca.) of florescent light.

For rooting, the elongated shoots were transferred to non sterile sand under glasshouse condition. The rooted plantlets were transferred to polybags with soil for establishment.

Result and Discussion

The swelling in the nodal segment was observed after 10 to 16 days of culture, followed by initiation of shoots. Healthy microshoots were developed with in 5 to 6 weeks (Table 1, Fig. 1).

In both the concentration of MS media, (0.5mg/l BAP + 0.1mg/l NAA and 1mg/l BAP+0.1mg/l NAA) the shoots were equally produced in quantity. Though, the Table 1 shows the best medium to be the MS medium supplemented with 0.5mg/l BAP, 0.1mg/l NAA and 1mg/l BAP, 0.1mg/l NAA but for the cheaper production the MS media supplemented with 0.5mg/BAP and 0.1mg/l NAA is preferred. The subculturing process was repeated at an interval of 6 weeks. In third subculture 8 to 10 microshoots were developed (Fig. 2).

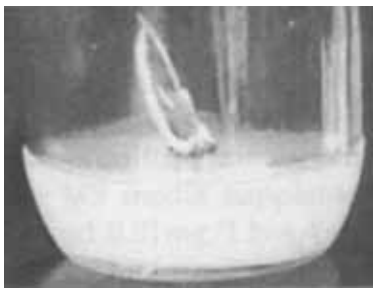

Fig. 1 : Nodal explant growing in medium.

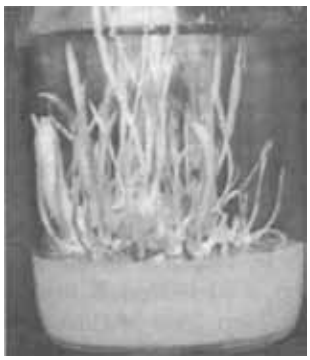

Fig. 2 : Microshoots proliferation in the MS medium supplemented with 0.5mg/l BAP and 0.1mg/l NAA after 3rd subculture.

Fig. 3 : Sand rooted plant transfer into propagator containing soil.

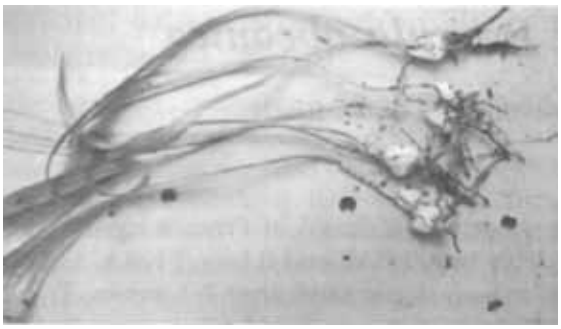

Fig. 4 : Bulblets developed in sand rooted plants after 8-10 weeks.

Varshney *et al.* (2000) reported the bulb scale segment culture of hybrid of Asiatic lily in Murashige & Skoog (MS) medium with 3% sucrose and 0.5µ M naphthalene acetic acid. They found the optimal condition for formation of smaller bulbs on 16/8 hour of photoperiodic condition.

In vitro shoot regeneration has been reported from nodal segments of *Lilhan nepalensis* (Saiju *et al.,* 1994) and shoot tip of *Lilium longiflorum* (Saiju & Rajbhandary, 1992). They reported MS medium with supplement to 0.lmg/l NAA was for the embryoid initiation. They also reported the multiplication of shoots in MS medium supplemented with 0.5mg/l BAP, 0.01mg/l NAA and 10% coconut milk can be done.

Table 1 : Nodal explants, response on MS medium with different concentration of hormone.

BAP mg/l	NAA mg/l	No. of shoots	Growth patterns
0.5	0.01	3-4	Thin shoots
0.5	0.1	5-7	Uniform healthy shoots
-	0.5	2-4	Base embroids, thin shoots
1	0.01	3-5	Thin shoots
1	0.1	5-7	Uniform healthy shoots
1	1	5-7	Base callus and stunted shoots

Flasks with mature microshoots were transferred in the green house and kept for 10 to 14 days to acclimatize. The plantlets were taken out by forceps and washed with clean water. These washed microshoots were trimmed for 3 4 cm long and transplanted in sand box, covered with polythene sheet. The water was sprayed regularly for rooting. Roots were visible after 10 days of transferring of shoots to sand. A healthy root developed in microshoots in 3 to 4 weeks. The percentage of rooting was observed very good all year around except in winter season (60 to 80%). The rooted plants were transferred to the pots or polythene bags for field plantation.

Conclusion

The study indicated that MS medium was better for shoot multiplication of Asiatic hybrids of Lily in MS medium supplemented with 0.5mg/l BAP and 0.1mg/l NAA.

Acknowledgement

The authors are grateful to Dr. U. R. Sharma Director General and Dr. P. R. Sthapit, DDG, Department of Plant Resources, Thapathali for their encouragement and providing facilities to work in this field. Our thank goes to Mr. H. K. Saiju, Scientific Officer, Biotechnology Section, DPR, Thapathali, for suggestion. Special thanks go to Mr. S. K. Dhungana for helping in many ways.

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Micropropagation of *Primula obconica*

K. M. Rajkarnikar, M. Kayastha and G. D. Bhatt

Abstract

The multiple shoots were regenerated from the shoot tips (2-3mm) of *Primula obconica,* an ornamental plant, in MS medium supplemented with 1mg/l BAP and 0.1mg/l NAA. Eighty to ninety percent of the plantlets produced roots in non sterile sand after 2 3 weeks. These rooted plants flourished well. No any morphological variation was noted when compared to mother stock.

Key words: *Primula obconica,* Micropropagation, Medium and Hormones, Sand rooting.

Introduction

The name *Primula* was derived from the Latin word *Primus* referring to the early flowering of some of the species, such as Primrose. It is a large genus and widely distributed in high Alps. *Primula obconica* belongs to the family Primulaceae. It is an exotic species.

Primula obconica has broad, heart shaped leaves with the covering of short glandular hairs and pungent scent. It blooms during spring to summer. The flowers vary from pale pink to blue purple. They are borne in clusters on long stalk.

Splitting the mother plant into plantlets soon after they have finished flowering follows propagation. The quantity of plantlets is quite low for the commercial propagation of plants. Therefore micropropation method is used for mass production of this plant.

The present investigation involves *in vitro* propagation of the plant through shoot tip culture for commercial production.

Material and Method

The mother stock of *Primula obconica* was bought from a nursery in Kathmandu valley and grown in the green house of National Herbarium and Plant Labotories, Godawari. The excised shoot tips were washed in running water for one hour. Then dipped in teepol for few minutes and rinsed in distilled water. The surface sterilization was done by 0.1% Mercuric chloride by dipping for five to seven minutes and washed for 3-4 times in sterilized distilled water. Aseptically, these explants were trimmed to 2-3mm sizes and cultured in Murashige & Skoog (1962) medium supplemented with different concentration (Table 1) of Benzyl Amino Purine (BAP) and Napthalene acetic acid (NAA). The medium was also supplemented with 3% sucrose, 0.1% casein hydrolysate, 0.8% agar. The pH ranged 5.6 to 5.8 before autoclaving. Each composition consisted of 10 to 12 replicates and each treatment was repeated twice. The cultures were incubated at 25±2°C under the fluorescent light of 3000 lux for 16 hrs.

Table 1 : Response of shoot tip explants in different concentration of BAP and NAA.

MS medium + BAP + NAA (in mg/l)		Growth responses	No. of microshoots
1	1	Callus+few shoot	3-4
1	0.1	Best shoots	6-8
1	0.01	Thin shoots	4-6

The microshoots developed were acclimatized first, washed thoroughly with water and then transferred to non sterile sand for rooting.

Result and Discussion

After 1-2 week, the explants; were green in all combination but after 4 weeks of culture, a different pattern of growth in different combination was observed.

The medium containing BAP 1mg/l + NAA 1mg/l regenerated callus well with 3-4 microshoot from

the base of the explant. But the medium containing BAP 1mg/l + NAA 0.1mg/l and BAP 1mg/l +NAA 0.01mg/l regenerated microshoots only from base of explant. The quantity of microshoot proliferation was less in the medium containing BAP 1mg/l + NAA 0.01mg/l with poor in quality. The medium containing BAP 1mg/l + NAA 0.1mg/l showed high number and better quality of microshoots (Fig. 1). There was direct formation of plants from excised explantlets. By this method true to type clone plants were obtained. The proliferated microshoots were again sub cultured in the medium with BAP 1mg/l + NAA 0.1mg/l for further multiplication. The number of microshoots proliferation was found increased (up to 30 to 40), after 6 8 subcultures.

The microshoots were transferred in non-sterile sand (Rajbhandary & Bajaj, 1991) (Fig. 2), which developed roots within 2 to 3 weeks. Eighty to Ninety percent of microshoots were rooted in nonsterile sands. These sand rooted plants were successfully established in soil and flowering took place as mother stocks (Fig. 3). Michie (1992) reported that variation occurred in flower colour of the plants, which were regenerated from leaf explants. But there were no any variation in plants which were derived from shoot tip explants.

Fig. 1 : Multiplication of microshoots in MS medium supplemented with 1mg/l BAP and 0.1mg/l NAA.

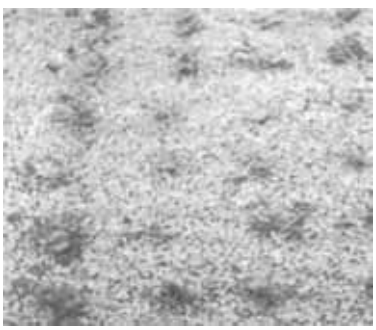

Fig. 2 : Microshoots in sand.

Fig. 3 : Flowering micropropagated plants.

Conclusion

The result indicates that the technique of clonal multiplication of *Primula obconica* plant is possible to fulfill the increasing demand of plant in the market.

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Tissue Culture of Banana and its Field Plantation

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Abstract

Banana is one of the most popular fruits in Nepal. In Nepalese context this fruit plays quite an important role not only for its consumption value but both cultural and religious values. Both the plant and the fruit are used in special occasion, especially for worshipping of the deities.

For tissue culture the suckers were collected from healthy mother plants of banana. The inner most shoot tips were taken out aseptically and cultured on Murashige and Skoog medium (1962) supplemented with 5.0 mg/l 6-Benzylaminopurine and 10% coconut milk. The cultured flasks were kept in incubation room under the flurescent light with 3000 lux for sixteen-hour and room temperature 25°C. The shoot tip was swollen within six weeks of culture. The swollen shoot was cut vertically into two pieces and subcultured in the new fresh medium with same concentration of growth hormone and coconut milk. The microshoots were sub-cultured in every interval of six week in the same concentration of nutrient medium.

The flasks with mature multiple shoot were brought out to ordinary room condition for acclimatization. After two weeks, the microshoots were taken out of the flasks and cleaned with water thoroughly. The microshoots were transplanted in clean sand in propagation for root development. The roots developed within four weeks. The rooted microplants were transplanted in soil in polyethylene bag. One feet tall banana plants with six leaves were distributed. Up to now ten thousand banana plants were distributed for field plantation in different districts of Nepal.

Key words: banana, microshoots, Murashige and Skoog medium, plantation, suckers and tissue culture

Introduction

Banana the most important tropical fruits was originated in South East Asia & spread all over the world as important cash crop. Banana and plantation make a major contribution towards the world's total food production (FAO 1987) and are an important staple for several million of people in inhabiting humid and sub humid tropics. Throughout the tropical and subtropical regions, more than 100 countries cultivated the bananas (Sharrock & Frison 1999, Sharrock & Frison 1998). Among them India is the world largest banana producer (Singhal 1999).

Banana is used as staple food due to its rich carbohydrates. It is rich source of vitamins and several minerals such as calcium, magnesium, potassium, and phosphorus and has several medicinal properties (Singh & Uma 1996).

The usually employed method for vegetative propagation of Musa Cultivars by suckers is limited due to its low multiplication rate. Researchers like Barker (1959) De Langhe (1961) Hamilton (1965) have attempted to overcome this problem by increasing the number of adventitious buds & suckers.

Bower and Fraser (1982) Cronauer and Krikorian (1984) have also contributed in reporting the *in vitro* propagation of Banana from shoot tip culture.

propagation through vegetative means is through sucker or corms. This is slow process for plant regeneration. Micro propagation through tissue culture has acquired the commercial production owing to uniformity in crops, early ripening, disease free and high yield.

Karki *et al*. 1992 reported the rooting of *in vitro* produced Musa Cultivar William hybrid plantlets on substrate moss. This paper reported the plant regeneration of banana through tissue culture and its field performance.

By tissue culture method 10,000 banana plants were distributed in different districts of Nepal.

Materials and Methods

A healthy sucker of an elite mother plant of banana was chosen for tissue culture. These suckers were brought from different districts of Nepal. The explants (2-3 cm.) shoot tips of banana were taken out by removing the outer leaf sheaths and rhizomatous tissue and washed with tap water continuously for 4-5 hours.

Fig. 1 : Plant Tissue Culture Laboratory, Dept. of Plant Resources, Thapathali, Kathmandu

The explants (2-3 cm.) shoot tips of banana were taken out by removing the outer whorls of overlapping leaf bases and washed with tap water continuously for 4 hours. The explants were again washed with 4 drops of liquid detergent (teepol). the shoot tips were then sterilized with 10% calcium hypochlorite solution for 12 minutes and thoroughly washed (6 times) with sterilized distilled water. Removed the leaf base until dome shaped structure appeared in the centre and isolated it as explants. The shoot tips (1-2 mm.) in size were transferred to the sterile jam bottle (250-ml.) containing 70 ml. solidified medium. The culture medium used was Murashige and Skoog (1962) supplemented with 3% sucrose and 10% coconut milk. The medium was solidified with 0.7% agar and pH was adjusted to 5.8 prior to autoclaving at 15 lb/sq. inch for 15 minutes. The culture flasks were incubated at 250 ± 2 under fluorescent light of 3000 lux (Fig. 1).

Shoot regeneration appeared by the addition of 1-5 mg/l 6-benzylminopurine. Cultures were routinely transferred in every 36 weeks interval of time. Multiple shoots were separated into smaller and larger groups. The smaller groups were subcultured for further shoot multiplication and the rest with 1.5 to 3.00 cm long shoots were excised for rooting.

The flasks with microshoots were transferred to glass-house for two weeks before rooting for acclimatization, hardening and change to autotrophic nutrition. Sand was cleaned with water and dried in sunlight for two days, then sieved through a 2 x 2mm pore sieve, kept in a box and wetted evenly with 10% water. The leafy, single node, 2 cm long microcuttings were transplanted into the sand. The sandbox was covered with polythene sheet. The maximum and minimum temperatures in the box were 30˚C and 20˚C respectively. The sunlight intensity varied from 4-15 kilo-lux. Watering was done with a sprayer to maintain 80% humidity.

After acclimatization, the microshoots were transferred in non-sterile sand in a propagator. The roots were developed within 4 weeks. The rooted micro plants were transplanted in soil in polythene bag. One feet tall banana plants were distributed for field plantation in different districts of Nepal.

Ten thousand banana plants (one foot tall with six leaves) were distributed for field plantation in different districts of Nepal (Table 1). The field performance was observed from Dhanusa and Nuwakot districts (Table 3).

Table 1 : Name of district and number of plants distributed for plantation.

Name of District	No. of Plants
Shyanja	1155
Makwanpur	950
Dhanusha	800
Lalitpur	676
Nawalparasi	670
Kabra	665
Palpa	500
Nuwakot	496
Bara	427
Dhadhing	400
Kaski	290
Kailali	250
Ilam	150

Dolkha	150
Dadeildhura	114
Dang	103
Salyan	100
Jhapa	100
Jumla	100
Baglung	100
Banke	100
Kathmandu	100
Rupandehi	95
Rauthat	88
Chitwan	84
Morang	72
Kapilbastu	67
Saptari	56
Sarlahi	50
Baitadi	50
Siraha	35
Lamjung	10
Total	**10,000**

The present result indicates that the tissue culture technology will play an important role in commercialization of tissue culture banana plant in Nepal.

Result and Discussion

Shoot tips (1-2 mm.) were cultured on Murashige and Skoog medium containing 6-benzlaminpourine with 10 percent coconut milk. The colour of explants changed from creamy white to green within 10-15 days. At time base of excised shoot a blackened portion was seen. The blackened portion at the base was removed at the interval of 6 weeks. The remaining green shoot was cut longitudinally through shoot apex into two and cultured on Murashige and Skoog medium containing BAP (1.0 to 10.0 mg/l) with 10 percent coconut milk. Shoot developed after 6 weeks.

Multiple shoots were developed after subsequent subculture in the same medium. It has been observed that high concentration (10 mg/l) of 6-benziaminopurine prohibited shoot multiplication. Shoot proliferation was observed in all concentration of BAP but number of shoot proliferation was low in BAP (1.5 mg/l). On the other hand BAP (5.0 mg/l) was found best in comparison to other concentration (Table 2) with 6-8 shoots per shoot at each sub-culture (Fig. 2).

The micro shoots were transplanted in clean sand in propagator for root development (Fig. 3). The roots developed within four weeks (Fig. 4). The rooted microplants were transplanted in soil in polybag (Fig.5).

Fig. 2 : Banana microshoot proliferation

Fig. 3 : Sand rooting of banana microshoots

Table 2 : Growth responses by explants on MS medium with different concentration of BAP.

MS medium + BAP mg/1	Expant responses	Number of shoot multiplication
1.5 mg/1	Response in all concentration	1-2
2.5 mg/1		2-2
5.0 mg/1		6-8
10.0 mg/1		4-5
Control		No response

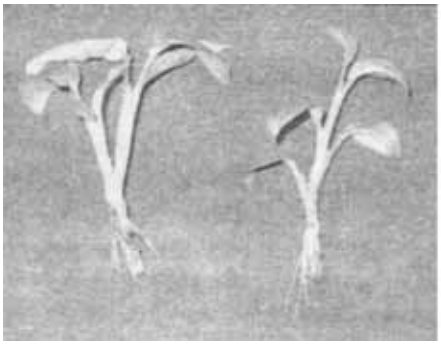

Fig. 4 : Rooted banana plants

Fig. 5 : Banana plants in polybags

Fig. 6 : Measurement of Banana in Trisuli, Nuwakot

Fig. 7 : Banana plantation in Dhanusadham Botanical Garde, Dhanusha

Fig. 8 : Fruitning Banana in Dhanusadham Botanical Garde, Dhanusha

Fig. 9 : Ripe Banana in Dhanusadham Botanical Garde, Dhanusha

Barker (1959) reported that few clones of dessert banana were capable of yielding entire plant by asptic culture. Berg and Bustmante (1974) reported the virus free plants of Cavendish group from meristem and lateral buds of virus infected plants by a combination of heat treatment and aseptic culture. Wong (1986) reported that the Cultivar William hybrid produced a cluster of more than 20 well formed shoots which measured two to twelve mm. in height on the medium with BAP. He reported that proliferation of shoots only occurred when rhizomatous base was included in each shoot.

The field observation of banana plantation was observed in Dhanusa and Nuwakot district (Table 3). The plant height was 330 to 390 cm. and started to flower in 12 to 14 months after plantation (Fig. 6 & 7). The number of hands in each plant were 10 to 12, the number of fruits per hand were 12-15, (Fig. 8 & 9) and weight of each fruit was 80-100 gm. The total weight of fruits in a plant was 9.6-18 kg.

Table 3 : Field Observation of Banana Plantation

S.No.	Characters	Observation
1.	Plant height during plantation	25-30 cm.
2.	Plant diameter during plantation	5 cm.
3.	No. of leaves during plantation	4-6
4.	Months of flower	12-14 months
5.	Height of the plant during flowering	330-390 cm.
6.	No. of hands/plant	10-12
7.	No of fruit/hands	12-15
8.	Length of the fruit	13-14 cm.
9.	Diameter of the fruit	2-4 cm.
10.	Weigh to the fruit	80-100 gm.
11.	Colour of the skin	Yellow
12.	Thickness of the skin	1.5-2.5 mm.
13.	Taste of the fruit	Tasty and good smell
14.	No. of suckers/plant	4-6

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Micro propagation of cactus: *Mammillaria carnea*

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Abstract

In the present paper the method of micro propagation of *Mammillaria carnea is* discussed for clonal multiplication. The micro bulbs were initiated in MS medium supplemented with 1mg/l BAP and 0.1 mg/l NAA from areoles portion of explants. These micro bulbs were transferred to both nonsterile sand and MS medium with 0.5mg/l NAA for rooting. The micro bulbs produced roots after 20 to 25 days in medium and 15 to 20 days in sand.

Introduction

Traditionally cacti are propagated either through seeds, cutting or by offshoots. This conventional propagation method for cacti is inadequate because most of cacti have slow growth, low germination rate and provide very few numbers of offshoots. King (1957) one of the pioneer to attempt the successful cultivation where he used the fragments of cactus for culture. Kolar *et al.* (1976) and Johnson & Emino (1979), on trial in a number of cactus species, regenerated new plantlets through callus cultures by treating hormones.

Mammillarias occurs in Mexico and the adjoining states. This genus is a large one with over 200 species. Till now no micro propagation technique for the investigation on the protocol development of Cacti has been carried so far (Rajkarnikar, Bhatt & Adhikari, 2004) in Nepal. Present paper deals with the micro propagation of *Mammillaria carnea* from aereoles. *M. carnea* is a globose to cylindrical cactus with angular tubercles and central spines in its aeroles. The pink blossoms are rather small but form a colourful ring at the top. It is not indigenous to Nepal and introduced in Nepal long time ago.

Materials and Methods

The bulb of *Mammillaria camea* was collected from the Royal Botanical Garden, Godawari. The spines were trimmed without damaging the areoles. The bulb was washed in running water for half an hour and dipped in diluted teepol solution for five minutes. It was washed thoroughly with distilled water. Aseptically the bulb was sterilized for five minutes in 0.1% $HgCl_2$and again washed with sterile distilled water. The sterile bulb was sliced into smaller pieces, each containing 3 to 4 areoles. These isolated explants were cultured on MS (Murashige and Skoog's, 1962) medium containing various concentration of hormone, (benzyl amino purine, BAP and Naphthalene Acetic Acid, NAA). The medium was further enriched by 0.1% casein acid hydrolysate, 3% sucrose and 0.8% agar for solidification of medium and its pH was adjusted to 5.8 before autoclave. The cultured flasks were incubated in controlled room at 25±20°C under 16 hr. light and 8 hr. dark. For rooting, the micro bulbs were transferred in sand or in MS medium supplemented with 0.5mg/l NAA.

Result

After one to two weeks of inoculation, the explants initiated callus on cut surfaces in all hormone concentration. Then after next two to three weeks, small shoot bud was initiated from aeroles except in control medium. Among in tested BAP and NAA concentration, in 2 mg/l BAP + 1.5 mg/l NAA + 10% coconut milk explants regenerated more callus and more bulbs proliferation. But some of proliferated micro bulbs seemed to be vitrified. In BAP 1mg/l + 0.1mg/l NAA and 1mg/l BAP + 1mg/l NAA regenerated less number of micro bulbs and the quality of micro bulbs were good (see table and fig. 1 below). These proliferated, micro bulbs were sub cultured in medium with 1mg/l BAP and 0.1 mg/l NAA for multiplication. After fourth to fifth subculture, multiplication of micro bulbs increased up to 20-25 in number.

Table: Showing the responses of explants on MS medium with different combination of BAP and NAA

Conc. of hormone in mg/l BAP + NAA		Initiation of micro bulbs in numbers	Condition and Quality of micro bulbs
2	1.5+ 10% coconut milk	10-15	Vitrified
1	1	8-10	Good
1	0.1	8-10	Good
Medium without hormone		No multiplication	x

For rooting, the proliferated microbulbs were separated, washed thoroughly with water and transferred to nonsterile sand in propagator under green house condition. These micro bulbs initiated roots after 15-20 days. The micro bulbs transferred to MS medium with 0.5 mg/l NAA initiated roots within 20-25 days. Finally these rooted plants were transferred into the sand substrate in green house.

Discussion and Conclusion

Vyskot & Jara (1984) reported that the best proliferation of *M. carmenae* was on MS medium supplemental with 1 mg/l NAA + 2mg/l BAP, but the initiation of axillary shoots in *M. prolifera* was achieved best medium in lower concentration of hormone (0.5-1 mg/l NAA and 0.5-1 mg/l BAP). In the case of *Trichocereus uspachianus* and *Astrophytum myriostigma*, they reported that the MS medium enriched with 5 mg/l IAA and 0.5 mg/l Kinetin was best for regeneration of shoots from axillary meristem James & William (1987) reported that the MS medium containing 0.2μ□M adenine + 46.5 μ□M kinetin + 5.4μ□M NAA was best for axillary shoot proliferation from apical part of germinated seedling. For rooting, they used shoot proliferation medium without Kinetin. Vyskot & Jara (1984) used MS medium with the addition of 1 mg/l IAA or MS media without phytohormones. The result shows that the clonal propagation of *M. carnea* through aereoles is easily possible.

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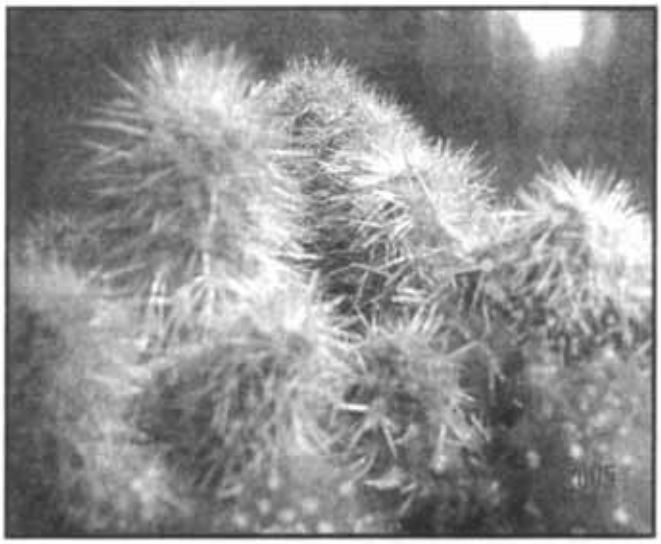
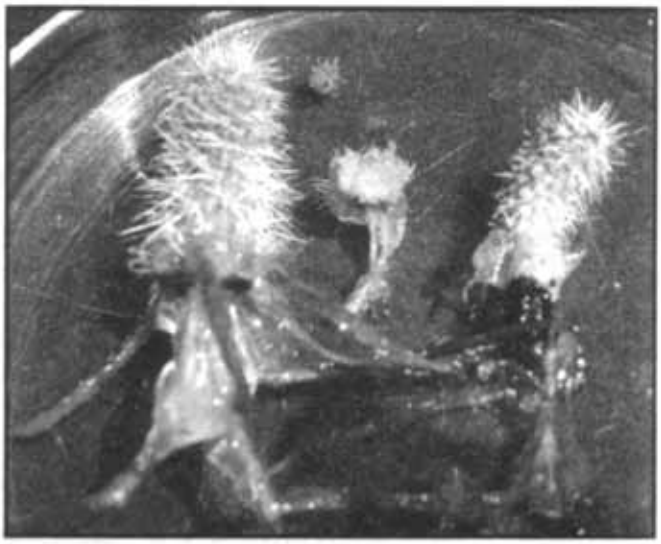
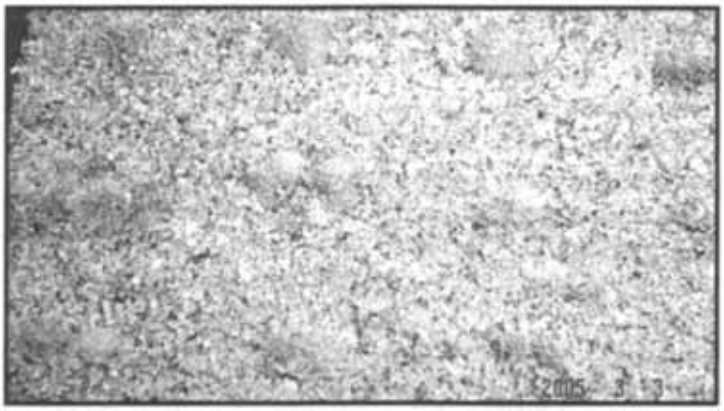


a. Microbulb multiplication on media



b. Rooting after multiplication in media

Fig 1. Micropropagation of Mammillaria



c. Microbulbs on sand

In vitro multiplication of *Vanilla planifolia* Andrews using axillary bud explants

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Abstract

Vanilla, a climbing, branching and terrestrial orchid is being tested for its clonal propagation through tissue culture. The axillary buds (2-3 cm in length) were collected from mother plants. The buds were washed in running tap water with few drops of liquid detergent (Teepol) for half an hour. Then the axillary buds were sterilized with 0.1% mercuric chloride solution for 8 to 10 minutes and rinsed with sterilized distilled water for four times. The tip of the axillary bud about 0.5 -1.0 cm long were cut off aseptically and cultured in MS medium (Murashige & skoog medium 1962) supplemented with 1.0 mg/l Benzyl amino purine, 1.5 mg/l Kinetin along with 10% coconut water. The culture flasks were kept in incubation room under the fluorescent light with 3000 lux and 25±2°C room temperature. Two to three new shoots were developed after 4-6 weeks of culture. The micro shoots were sub cultured in every 6-8 weeks in the same concentration of growth hormone along with coconut water. The mature shoots were transferred in cocopit or moss for rooting.

Introduction

Vanilla is native to the tropical rainforest of southeastern Mexico and Central America. When the Spanish conquistadors were in Mexico in 1520, they observed the emperor Montezuma drinking a beverage of cocoa beans, corn, vanilla pods and honey. They were so impressed that they took vanilla back to Spain and, by the end of the 16th century; factories were established to manufacture chocolate with vanilla flavoring.

Vanilla became popular throughout all of Europe for its flavor and began using it as an ingredient in pastries, cakes and beverages. The ice cream manufacturers and bakeries use vanilla. Vanilla is grown mainly in Madagascar and Indonesia with some production in Mexico, Tonga, French Polynesia, Fiji, Costa Rica, Uganda, India and China.

Vanilla planifolia (Vanilla Orchid) is an evergreen, leafy, and somewhat fleshy vine, growing under a canopy of support trees in the tropics and subtropics. They have thick, adventitious roots with succulent, jointed, green stems that climb or trail and bear stalkless or short-stalked, sometimes absent, ovate to oblong, fleshy green leaves, 6 inches long. In spring, bears auxiliary racemes of many yellow-green flowers, 2 cm across, with yellow-haired lips, followed by pendulous, cylindrical, brown seed pods, 15 to 25 cm long. It climbs 3 to 24 m. It is cultivated commercially for vanilla flavoring, extracted from its seedpods. It can be cultivated in places with Minimum temperature 15°C and Maximum temperature 30°C.

Material and Methods

Vanilla plants were received from University of Tsukuba, Japan by senior volunteer of JICA. The vanilla plants were cut-off in to pieces with minimum 3 to 4 nodes and layering was done in the moss for rooting. After few weeks axillary buds and roots were developed from the nodal portion of the plants. These young axillary buds measuring 1.5-2.0 cm were collected for tissue culture. The axillary buds were washed under running tap water for half an hour with few drops of liquid detergent (Teepol). The cleaned axillary buds were then surface sterilized with 0.1% mercuric chloride solution for 7-10 minutes followed by rinsing with sterilized distilled water for four times. The upper most parts of axillary bud measuring about 0.5-1.0 cm was aseptically excised and cultured in MS medium combination with different concentration of cytokinin and auxin, cytokinins only. The pH of the medium was adjusted to 5.8 before autoclaving.

The culture was incubated at 25±2°C temperature under 16-hour photoperiod at light intensity of 3000 lux. After 4-6 weeks of explant culture the shoot were proliferated. The new micro shoots were then sub cultured in same medium along with coconut water. The explant was also cultured in MS medium supplemented with different concentration of BAP and NAA (Table 2) but the explant remain same and there was only few micro shoots were regenerate. The regenerated mature shoots were acclimatized for a week in green house. Then these shoots were transferred to moss or cocopit for root induction.

Result and Discussion

After 4-6 weeks of culture, the explant shows response and 2-4 numbers of micro shoot were proliferated. The proliferation of shoot was found to be best in the MS medium supplemented with 1.0 mg/l BAP and 1.5 mg/l KN along with 10% coconut water. The number of axillary shoot proliferation was 2-3 from the initial explant. On third sub culture the number of shoot proliferation was increased. After 6-8 weeks of sub culture 8-10 numbers of shoots were developed. (Table 1) the explants also cultured in medium with growth hormone BAP 1, 2,3,4,5 mg/l with NAA 1.0 mg/l. Among them only few shoots were developed in BAP 1.0, 2.0 mg/l, along with 1.0 mg/l NAA. After third, forth sub cultures the numbers of plant remain same in the media. There was no further proliferation in the media supplemented with BAP and NAA.

The roots were also developed from the nodal portion of the shoot. The mature shoots were transferred in green house about 7-10 days for acclimatization. Then the micro shoots were transferred in cocopit or moss for rooting. 80-85% of micro shoot were rooted in cocopit.

There are several research work done for the propagation of Vanilla plant through seed, axillary bud and meristem using different kind of growth hormones along with coconut water. George, P.S. and G.A. Ravishankar (1997) regenerated Vanilla using axillary bud as an initial material and culture in MS medium supplemented with BA, KN (1,2,5 or 10 mg/l) along with NAA, IBA and IAA (1.0 mg/l). Philip, V. J. and J. Paclikkala (1989) reported the role of IAA in the conversion of root meristems to shoot meristems in *Vanilla planifolia*. Plantlet regeneration of *Vanilla planifolia* from callus (Gu.et al. 1987; Daviclonis and Knorr 1991), or root tips (Philip and Nainar 1986). However, regeneration from callus was not desirable in clonal propagation due to the potential for variation. Konowicz and Janick (1984) reported an in vitro propagation method using node explants, but the rate of multiplication was lower. Shrestha M. and Rajbhandari S.B. (1993) reported regenerationof *Cymbidium gradiflorum* from meristem culture.

We found different kinds of explants used for initial material in *in-vitro* propagation of *Vanilla planifolia.* It is observed that axillary bud was easy and best explants for *in-vitro* propagation of *Vanilla planifolia.* In case of growth hormone used for the propagation, some researcher reported cytokinin along with auxin but in our cases we used BAP and Kinetin, both are cytokinin. In case of cytokinin and auxin we do not found satisfactory result.

Among different concentration of BAP and Kinetin the best result was found in BAP 1.0 mg/l and KN 1.5 mg/l. The number of shoot and shoot growth condition was also found to be best in this concentration. The explant also responses in BAP 2.0 mg/l and KN 1.5 mg/l but the number of shoot

Table 1: Growth responses by explants on MS medium with different concentration of BAP and K

S. N.	Growth hormones		Explants responses after 6-8 weeks	Induction of shoots number in average 10-12 weeks	Shoot growth condition
	BAP mg/l	KN mg/l			
1	1.0	1.5	2-3	8-10	Best
2	2.0	1.5	explants response	2-3	not so good
3	3.0	1.5	X	X	X
4	4.0	1.5	X	X	X
5	5.0	1.5	X	X	X -

Table 2 : Growth responses by explants on MS medium with different concentration of BAP and NAA

S. N.	Growth hormones		Explants responses after 6-8 weeks	Induction of shoots number in average 10-12 weeks	Shoot growth condition
	BAP mg/l	NAA mg/l			
1	1.0	1.0	explants response	2-3	not so good
2	2.0	1.0	explants response	1-2	not so good
3	3.0	1.0	explants response	Remain green	not so good
4	4.0	1.0	X	X	X
5	5.0	1.0	X	X	X

was found to be less and shoot growth condition was not also good.

Among different concentration of BAP (1,2,3,4 and 5 mg/l) along with NAA 1.0 mg/l the explants responses 1,2,3 mg/l BAP with 1.0 mg/l NAA but the number of shoot proliferation was very few and shoot growth condition was not so good.

Conclusion

Hence, present research work developed a protocol for in-vitro propagation of Vanilla from axillary bud culture. Among the different concentration of growth hormone used for in vitro propagation of Vanilla, the best result was found in BAP 1.0 mg/l and KN 1.5 mg/l along with 10% of coconut water.

Acknowledgement

The authors are very much grateful to Dr. Uday Raj Sharma, Director General, Department of Plant Resources for providing facilities for the research work. We are also very much thankful to senior volunteer of JICA Mr. H. Shirai for providing the Vanilla plants.

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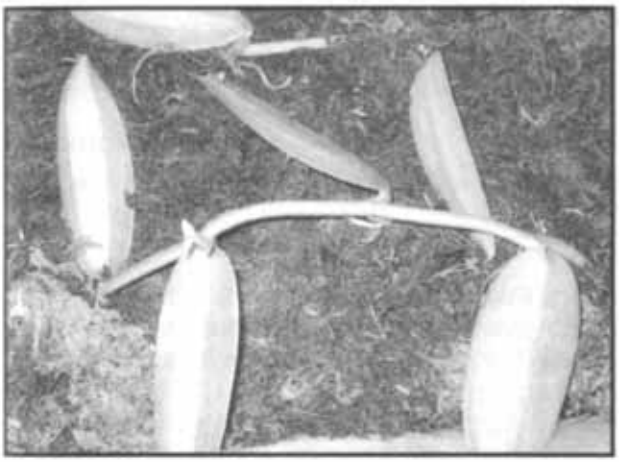
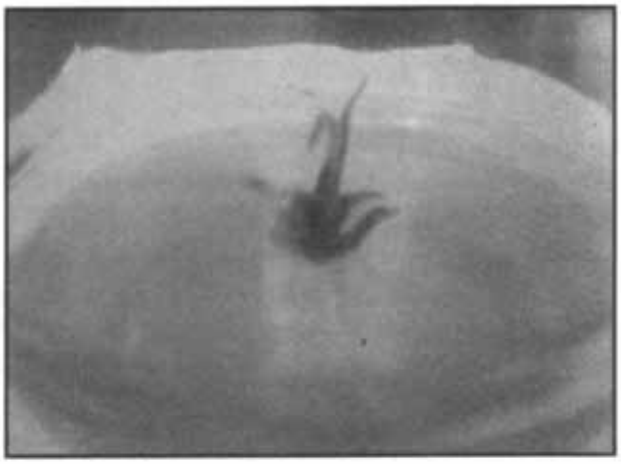
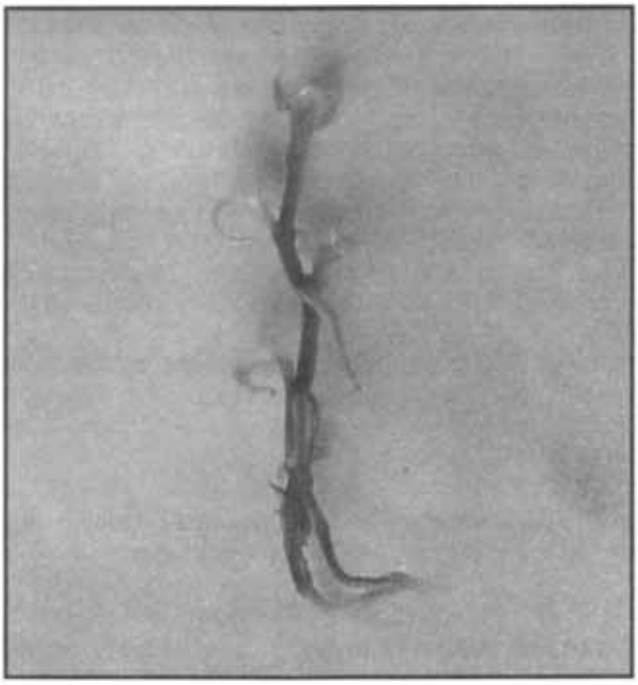


Vanilla plant showing axillary bud

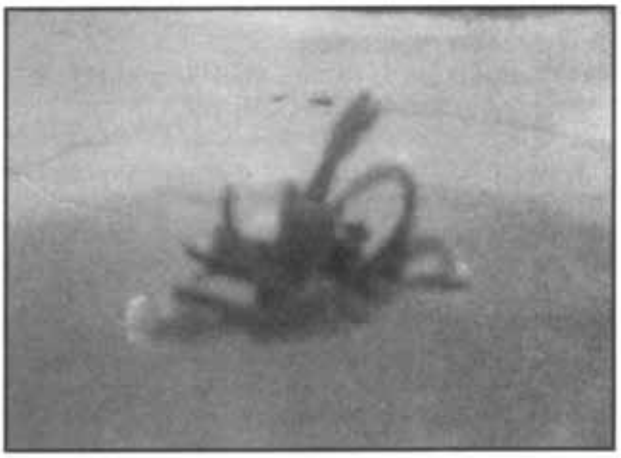


Shoot proliferation after 6-8 weeks of culture



Rooted Vanilla plant

Shoot proliferation after 12 weeks



Shoot proliferation

Tree Tissue Culture and Ex Vitro Sand Rooting for Reforestation

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Introduction

Micropropagation has been routinely used for the clonal multiplication of plants. However in vitro produced shoots often fail to root, or die, due to contamination in the field. The problem to be solved for the mass scale production of plants through micropropagation is how to induce roots that can survive in the field (Nemeth 1986).

Conventionally, microshoots are rooted in vitro in an auxin rich medium. This is followed by the hardening process and finally plantation to the field. The conventional method of in vitro rooting develops weak roots with low survival and growth rates under natural conditions (Kozai 1991). In vitro proliferated potato shoots developed roots in nonsterile sand and dried leaves mixed in an equal proportion by volume (Manandhar and Rajbhandary 1986). In vitro produced plant species have rooted in nonsterile sand (Rajbhandary and Bajaj 1991). Tissue cultured microshoots of forestry species also develop roots ex vitro in sand. These microplants can easily survive in field plantations.

Materials and Methods

Explants (shoot tip, young leaf, node, cotyledon node, seed) from 21 forest tree species and two forest bamboo species were used for tissue culture (Table 1).

Table 1 : Forestry species used for tissue culture

Species	Explant
Tree	
Acacia auriculiformis	Cotyledonary node
Artocarpus heterophyllus	Cotyledonary node
Artocarpus lakoocha	Cotyledonary node
Citrus aurantifolia	Cotyledonary node
Citrus limon	Cotyledonary node
Citrus sinensis	Cotyledonary node
Dalhergia sissoo	Cotyledonary node
Elaeocarpus sphaericus	Nodal segment
Eucalyptus camaldulensis	Shoot tip/Young leaves
Eucalyptus citriodora	Nodal segment
Eucalyptus terecticornis	Nodal segment
Ficus auriculata	Cotyledonary node
Ficus carica	Shoot tip
Ficus elastica	Shoot tip
Ficus lacor	Shoot tip
Ficus nerfifolia	Shoot tip
Ficus semicordata	Shoot tip
Fortunella sp.	Shoot tip/Cotyledon node
Morus alba	Shoot tip/Cotyledon node
Poncirus trifioliata	Shoot tip/Cotyledon node
Populus ciliata	Shoot tip/Cotyledon node
Bamboo	
Dendrocalamus hamiltonii	Seed
Dendroealamus striactus	Seed

Methods

Explants were cultured on Murashige and Skoog (1962) medium supplemented with various concentrations of kinetin, benzylaminopurine (BAP), napthaleneacetic acid (NAA) and/or 2,4 dichlorophenoxyacetic acid (2,4 D). The pH of the medium was adjusted to 5.8 before autoclaving. The culture flasks were incubated in a room maintained at 25°C and 3 kilolux light. The established explants were kept in the same medium for 6 weeks, and then the microshoots were recultured in a cytokinin-enriched medium. Subculturing was done at 8 week intervals. The maximum number of shoots was produced after the sixth subculture, after which the flasks with microshoots were incubated for 3 months for the elongation of microshoots. The flasks with microshoots were transferred to a glasshouse for 2 weeks before rooting, for acclimatization, hardening, and change to an autotrophic mode of nutrition. Sand was cleaned with water, dried in sunlight for 2 days, sieved through a 2 *x* 2 mm pore sieve, put in a box and wetted evenly with water.

The leafy, single node, 2 cm long microcuttings were dipped in I ppm IAA for 10 min and then transplanted in the sand. The sandbox was covered with a polythene sheet. The maximum and minimum temperatures in the box were 30°C and 20°C, respectively. The sunlight intensity varied from 4-15 kilolux. Watering was done using a sprayer to maintain 80% humidity.

Results

The tree and bamboo species cultured in the nutrition medium produced healthy microshoots that developed roots ex vitro in sand within 3 weeks. The microshoots were left in the same box for 3 more weeks to develop healthy roots. The rooted microplants were transferred to a soil sand mixture (1:1) in polythene bags. When the plants were 10-25 cm long, they were ready for field plantation. The sand rooted plants established easily in the field.

Discussion

In vitro produced microshoots of 23 tree and bamboo species transplanted in sand for ex vitro root induction produced healthy roots. These plants were successful as part of a reforestation program.

Glasshouse conditions of 20°-30°C, a sunlight intensity of 4-15 kilolux, and 80% humidity were good for ex vitro root development in Ficus carica (Saiju et al. 1995). These conditions were also suitable for the plant species trialed in the present study (Table 1). Optimal temperature, light, and humidity under natural or greenhouse conditions will have a positive affect on growth (Kozai 1991).

The factor limiting the use of micropropagation of forest tree species is the high cost of production involved in tissue culture (Chu 1989). Ex vitro rooting technique can lower the production cost of tissue cultured plants. During this process, a nutrient medium and incubation room are not needed. This rooting technique may be helpful for taking plants produced in the laboratory to the field. The roles of temperature, humidity and light for ex vitro root development in in vitro produced shoots need further detailed study.

Acknowledgments

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Micropropagation of *Neopicrorhiza scrophulariifolia* (Pennell) Hong

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Abstract

A protocol for the in-vitro multiplication of Neopicrorhiza scrophulariffolia (pennell) Hong was developed by using shoot tip explants. The regeneration of microshoots from explant was found to be best in 0.8% agar -solidified MS (Murashige & Skoog) medium supplemented with 0.5 mg/l BAP, 0.01 mg/l NAA and 10% coconut water. The microshoots regenerated roots on MS medium with 0.5mg/l or with 0.1 mg/l NAA. The experiment is still in investigation for successful establishment of plantlets in the field.

Key words: *Neopicrorhiza scrophulariifolia*, Shoot-tip, Micropropogation, Murashige and Skoog medium, Hormones

Introduction

Tissue culture can be used in the wide range for conservation and sustainable use of medicinal plants. It can be successfully employed for the production of pharmaceutical, food additives (carotenoids, anthocyanins, vanilla), perfumes (monoterpenoids, sandal wood oil, agar wood oil and biopesticides (Hable, 1996). Phytochemicals such as Podophyllotoxin from *Podophyllum peltatum,* Taxol (anticancer drug) from *Taxus* sp., Camptothecin (anticancer drug) from *Camptoteca accuminata* and Castanospermine (AntiAIDS drug) from *Castanospermum australe* are under development for tissue culture production (Ishii, 1998).

In Nepal, the tissue Culture Laboratory was at first established in 1976 at Natonal Herbarium and Plant Laboratories, Godawari under Department of Plant Resources. Since then several protocols have been developed, involving more than 30 species of medicinal plants in Nepal (Bista et al. 1996, Saiju, 1998; Rajkarnakar et al 2004; Rajkarnakar & Bhatt, 2004; Rajkarnikar et al., 2004c; Manandhar & Pant, 2004). Several important medicinal plant species are still awaiting thier commercial production.

The present paper highlights on the *in-vitro* propagation of one of the most important medicinal plant as commercially known as Kutaki *(Neopicrorhiza scrophuariifolia)*. It belongs to the family Scrophhulariaceae. It is a prostrate herb with perennial woody rhizomes covered with old leaves at the base. Leaves are crowded at the tip, each leaf 2-6 cm, long, oblanceolate and narrowed below to a wing leaf stalk with toothed margin. Flowers are borne in a dense terminal spike arising from a rosette of conspicuously toothed leaves, spikes 5-10 cm long with dark blue-purple colour (Watanabe, et al 2005). It is distributed throughout Nepal at 3500-4800m on open rocky pasturelands (Bista, et al., 2001; Manandhar, 2002). It is endemic to Hindu-Kush Himalayas (Tandon, et al. 2001).

Its rhizome is bitter and is useful in dropsy, antiperiodic fever, anaernia and jaundice. It promotes secretion of bile, improves appetite and stimulates gastric secretion. Decoction of rhizome is taken as all antipyretic. The rhizome of this plant is used to cure bile disease, intestinal pain, blood and lung fever, sore throat, eye disease, gastristis. It is also taken as medicine for cough and sometimes to get rid of intestinal worms. In its chemical constituents, vanillic acid, aucubin and catalpol is found (Lama, et al. 2001; Manandhar, 2002 and Watanabe, et al. 2005).

Conventionally it is propagated by rhizome. Due to its high medicinal value, the rhizome of this plant is collected extensively from natural habitats for various purposes and is enlisted in vulnerable species (Shrestha & Joshi, 1996). Manandhar (2002) kept it

in one of the endangered species of Nepal. However, there are no reports on the micropropagation of *Neopicrorhiza scrophuariifolia* (Rajkamikar, et al. 2004) so far in Nepal. To fulfill the increasing demands and to conserve the endangered Kutki plant, all attempt has been taken for mass production of this plant through tissue culture technique, which would be helpful in the domestication of the species.

Fig. 1

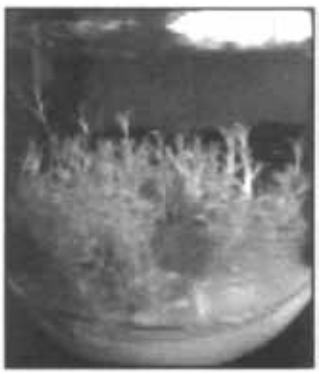

Fig. 2a

Fig. 2b

Fig. 3

Legend

Fig. 1 Explant on MS medium after one week.

Fig. 2 Proliferation of microshoots on MS medium supplemented with
- a) 0.5 mg/l BAP and 0.01mg/l NAA
- b) 0.5 mg/l BAP, 0.01mg/l NAA and 10% coconut water

Fig. 3 Micro-shoots transferred in non-sterile sand for rooting.

Materials and method

The plants of *Neopicrorhiza scrophulariifolia* were collected from Lauribinayak area of Langtang National Park, Rasuwa District. They were grown and conserved in green house of National Herbarium and Plant Laboratory, Godawary. Shoot tips were separated from these plants and used as explants. The explants were washed in running tap water half an hour and then washed thoroughly with teepol for few minutes. After that explants were rinsed three to four times with distilled water. Finally, surface sterilization was done with 0.1% HgCl, for 5 minutes and followed by washing with sterile distilled water. The explants were cultured (Fig. 1) on 0.8% agar gelled MS medium (Murashige and Skoog 1962) supplemented with different concentration of BAP (Benzyl Amino Purine) and NAA (Naphthalene Acetic Acid). The pH of the medium was adjusted to 5.8 prior to autoclaving at 15 1b pressure/inch^2 and 121°C for 20 minutes. The cultures were incubated at 25±2°C under 16 hour photoperiod at light intensity of 3000 lux.

The cultures were regularly Subcultured on fresh medium at 4-6 weeks of interval. All treatments consisted of 10 replicates and experiments were repeated two times. The regenerated plantlets were again subcultured on MS medium supplemented with 0.5 mg/l and 0.1 mg/l NAA for rooting. In the months between December and January, some plantlets *in-vitro* were removed from the culture flasks, washed thoroughly to remove the medium from microshoots and transferred in to non-sterile sands (Rajbhandary & Bajaj, 1991). The rooted plantlets were transferred to polybags with soil.

Results and Discussion

After one to two weeks of culture, the explants showed responses in all treated hormone concentrations. The mode of differentiation of microshoots was organogenesis, that is direct formation of plantlets from excised explants in all combination. Then after 3-4 weeks, the multiplications of microshoots were differing in different combinations (Table 1). Although, the multiple microshoots were seen in all the combination of BAP and NAA, the medium supplemented with 0.5 mg/l BAP and 0.01 mg/l NAA was found to be best for multiplication and growth of microshoots. Then these microshoots were subcultured again in same concentration of hormone

Table 1: Effect of BAP and NAA on shoot-tip explants of Kutki

S.no.	MS medium with Growth hormone conc. (mg/l) BAP + NAA		No. of responses	microshoots
1	1	0.01	Good	8-10
2	0.5	0.01	Best	12-15
3	0.5	0.1	Good	10-12
4	1	0.1	Good	10-12
5	2	0.1	Brown after few week	4-6
6	Without hormone		Not so good	1

with or without 10% coconut water. The coconut water further enhanced multiplication and growth of microshoots *in vitro*. On subculture, the number of microshots multiplication increased up to 40-50 after 6 to 8 subculture (Fig. 2). The microshoots subcultured on medium enriched with NAA for rooting, which regenerated root after 2-3 weeks.

Several members of Scrophulariaceae are known to show successful plant regeneration from various explants as well as cell / callus cultures (Garg & Rangaswamy, 1984). Under a programme for conservation of endangered species through tissue culture, Lal, et al. (1988) developed a procedure for the rapid clonal propagation of *Picrorhiza kurroa* using shoot tip culture.

Conclusion

In-vitro propagation of Kutki from shoot tip was successfully established. The rooting experiments of regenerated shoots from *in vitro* culture are continuously carried on for their successful establishment in field.

Acknowledgement

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Micropropagation of *Bergenia ciliata* (Haw.) Stemb. Through Leaf Culture

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Abstract

The leaf explants of *Bergenia citiata* (Haw.) Sternb. were cultured in Murashige and Skoog medium on filter paper bridge supplemented with BAP (1.0mg/l) and NAA 0.1 mg/l. After 8-12 weeks of culture the leaf explants were transferred from liquid to MS solid medium supplemented with BAP 0.5 mg/l and NAA 0.1 mg/l addition of Adenine sulphate 100 mg/l, The shoot proliferation was observed after 6-8 weeks. The micro-shoot were regularly subcultured in the same medium at intervals of 6 weeks for maintenance. The micro shoots were rooted in sand. After rooting micro shoots they were transferred in polybags.

Introduction

Bergenia ciliata is a plant belonging to the family Saxifragaceae. It is known as Pakhanved in Nepali and Sanskrit. It is perennial rhizomatous herb, with stout and woody rootstock. It is found in the temperate regions of Himalaya between the altitudes of 2100m to 3000m. Though it is a shade loving plant its foliage is more attractive in a sunny position. Flowers are white, pink and purple in color. Flowering season starts from April-May. The flowers are hermaphrodite (having both male and female organs).

Bergenia ciliata has been known to have medicinal value from vedic period. The drug is reported to possess astringent tonic antiacorbic and laxative properties. It is reported to be helpful in dissolving kidney stones. The rhizome extracts is reported to be cardio-toxic in higher doses. The bruised roots are applied in eye disease, cuts & burns. Juice or powder of the whole plant is used to treat urinary troubles. The juice of the leaves is used as drops to relieve earaches (Websites). The root is used as a tonic in the treatment of fever, diarrhea and pulmonary infections. The root juice is used to treat cough and colds, asthma and urinary problems. The root contains 14-16% tannin. Beside medicinal use this plant is useful in floriculture practice, which is used in cut flower due to long inflorescence, blooming slowly and lasting for many days (Shrestha *et al.* 2004).

Plants are commonly collected from natural sites for medicinal purposes which makes the plant endangered in near future. It is therefore, an attempt has been taken for mass propagation through tissue culture technique.

Conventionally this plant is propagated by seeds and stem cutting. Due to slow propagation by stem cutting and seed germination, the micro-propagation is alternative method for plant propagation of *B. ciliata* which is valued for medicinal and ornamental purposes as cut flower in floriculture.

Reports on micropropagation of *B. ciliata* have not been found so far. The present paper highlights on the micro propagation of *Bergenia ciliata* through leaf culture.

Materials and Method

Mother plants of *B. ciliata* were collected from Godawari and planted in pot for establishment. Young leaves of *Bergenia ciliata* were selected as the initial material for culture. The young leaves were kept in running tap water for I hour with 3-5 drops of teepol liquid detergent. Then the leaves were rinsed with distilled water for 4-5 times. These clean leaves were surface sterilized with 0.1% mercuric chloride for five minutes followed by rinsing with sterilized distilled water for 5 times.

These leaves 0.5-1.0 cm were cultured in liquid Murashige and Skoog (1962) medium on filter paper

bridge supplemented with 1.0 mg/l BAP and 0.1 NAA. The culture was incubated at 25°±2°C temperature under 16hr photoperiod. The subcultures were carried out at each and every four weeks interval of times into the solid MS media with 0.5mg/l BAP + 0.1 mg/l NAA along with 100mg adenine sulphate.

For rooting acclimatized micro shoots of about 2-3cm size were excised and transferred in non sterile sand. The rooted plants were transferred in polybags with soil for field establishment.

Result and Discussion

After 8-12 weeks of culture, the leaf explants swell up and shoot proliferation was observed from upper portion of the leaf explants. These proliferated shoots transferred in MS solid media with different combination of BAP and NAA. (Table 1).

These proliferated shoots when transferred in solid media with 0.5 mg/l BAP and 0.1 mg/l NAA with 100 mg/l Adenine sulphate showed more multiplication of micro shoots (Fig.2). The MS medium supplement with lower concentration of BAP showed less number of multiplication of shoots. The best proliferation of shoots were seen in BAP 0.5 mg/l and NAA 0. 1 mg/l with 100 mg/l Adenine sulphate. The micro-shoots were regularly sub-cultured in the best medium ie. MS medium with BAP 0.5 mg/l and NAA 0.1 mg/l and Adenine Sulphate 100 mg/l at interval of 4 weeks.

In this experiment, it was observed that MS medium supplemented with lower concentration of BAP showed less number of shoots and higher concentration of BAP did not response in shoot proliferation. But MS medium with BAP 0.5 mg/l and NAA 0.1 mg/l showed more proliferation with addition of Adenine sulphate 100 mg/l. The microshoots were regularly subcultured in same medium at intervals of 6 weeks.

After 4-6 subculture, the multiplication rate of micro shoots was increased and optimum number was 25-30 shoots (Fig 3). Multiple shoots were separated into smaller and larger size. The smaller sized shoots were again used for sub-culture for further shoot multiplication and 1.5 to 2.0 cm sized shoots were excised and kept for rooting.

Flasks with mature micro shoots were transferred in ordinary room temperature for 10-15 days for acclimatization. The micro shoots were taken out by forceps and transplanted in sand box, covered with polythene cover. The water was sprayed regularly. Roots were visible after 10-15 days of transferring of shoots in sand (Fig.4). These rooted microshoots were transferred to clay pots for further growth. (Fig.5).

Start and Cumming (1976) propagated African violet through leaf culture. They also reported the most suitable explant is leaf lamina because peripheral portion have been shown to be less productive. Grout (1990) also cultured African violet using leaf explant.

Takayama (l990) cultured leaf explant in MS medium supplement with BAP and NAA for micro propagation of *Begonia.* After 3-4 weeks numerous buds formed on the upper surface of the leaf explant. Karki and Rajbhandari, (1983) reported the multiplication of *Solanum laciniatum* plantlets through leaf culture. Rajkarnikar, and Bhatta, (2001) reported micro propagation of *Begonia tuberhybrid*

Table 1 : Responses of leaf explant in MS medium with different concentration of BAP, NAA and Adenine Sulphate (mg/l)

S.N	MS medium + BAP+ NAA and Adenine sulphate mg/l			No. of shoots	Growth responses
1	0.1	0.1	100	No proliferation	No response
2	0.25	0.1	100	2-4 micro shoots	Stunted shoots
3	0.5	0.1	100	30-40 micro shoots	Good shoots number
4	1.0	0.1	100	20-25 micro shoots	Less shoots number
5	2.0	0.1	100	Few number	Cluster form
6	Control			No shoots	No response

through leaf culture. In our research work we also found numerous shoot buds formed on the upper surface of the leaf explant in MS medium along with BAP and NAA.

Conclusion

This experiment thus carried out concludes that micro propagation of *B. ciliata* through tissue culture can be successfully done on the MS media supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA along with 1.00mg/l Adenine sulphate. The most suitable explant was found to be leaf for micro propagation.

Acknowledgments

The authors are very much thankful to Mr. Hari Krishna Saiju former Director General of the Department of Plant Resources for providing facilities for the research work. We would like to express our cordial thanks to former Biotechnology Section Chief Mrs. Radha Niroula for her valuable suggestions during research work.

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Fig. 1-5 : Plants of different stages of growth conditions

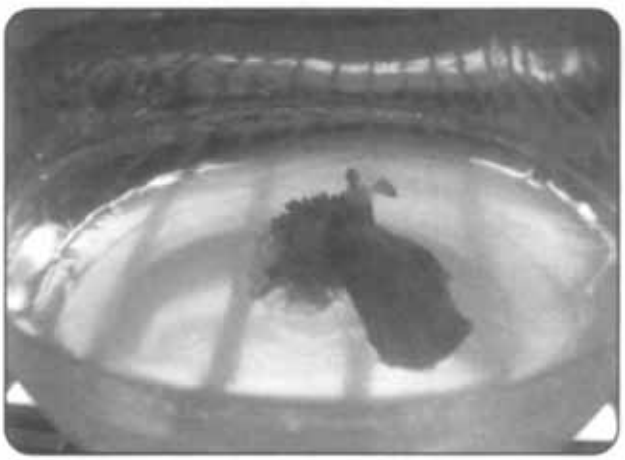

Fig. 1 Shoot proliferation from leaf explant

Fig. 2 Subculture of micro shoots

Fig. 3 Multiplication of micro shoots

Fig. 4 Rooting in Sand

Fig. 5 Rooted plant in pot

Micropropagation of *Rhyncostylis retusa (L.)* Blume From Seeds

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Abstracts

The seeds of *Rhyncostylis retusa (L.)* Blume were cultured on Murashige and Skoog's medium (MS medium) without growth hormone and supplemented with 1mg/l casein hydrolysate, 30gm/l sucrose and 8gm/l agar. The protocorms like bodies (Plbs) were obtained from 6-8 weeks old culture. The Plbs were again subcultured on hormone free MS medium for further growth. Some of Plbs were subcultured on Ms medium supplemented with 1mg/l Benzyl amino purine, 1.5 mg/l Kinetin and 10% coconut milk for further multiplication of plbs and seedlings. The seedlings were transferred in moss for their establishment.

Key words : Protocorms like bodies (Plbs), Growth hormone, Culture and subculture.

Introduction

Rhyncostylis retusa (L.) Blume ranks among the beautiful orchid belonging to family Orchidaceae. It occurs as an epiphytic herb in the tropical and subtropical zones of central and east Nepal at 300 to 1800m and also grown in gardens (Fig. 1). This species is also found in India, Myanmar, Sri Lanka, Malaya, Vietnam, Loas, Cambodia and the Philippines (Rajbhandary and Bhattarai 2001). The inflorescence is pendulous, racemose, densely flowered, cylindric. The flowers are spotted pink or violet. This plant is disappering from its natural habitats due to extensive collection by orchid enthusiasts, deforestation and natural calamities also. Hence tissue culture is an alternative method of growing and conserving it. Orchid produces numerous fragile and non-edospermic seeds of which nearly 80-90% are viable seeds. In nature, orchid seeds depend on suitable mycorrhiza association for germination and therefore only some of them germinate in nature (Prasad and Mitra, 1975). Ever since Knudson (1922) successfully raised *Catteleya* seedlings *in vitro*, this technique of asymbiotic seed germination has made possible the large scale multiplication of orchids for commercial purpose. Conventionally, *Rhyncostylis retusa* is propagated by cutting and produces few side shoots (4-6) per plant per year. The present paper deals with the *in vitro* propagation of this plant by using its seeds and their subsequent development into seedlings.

Materials and Methods

The mature capsules were collected and preserved until use. The capsule were washed in running tap water for one hour then with few drops of liquid detergent (Teepol) and rinsed in distilled water for 5-6 times. These capsules were then surface sterilized with 0.1% $HgCl_2$ for 10-12 minutes and followed by rinsing with sterilized distilled water repeatedly to ensure complete removal of the disinfectant. The seeds were then scooped out aseptically by splitting the capsule longitudinally with scalpel and inoculated on MS medium (Murashige and skoog, 1962) without growth hormone. The medium was supplemented with 0.1% casein acid hydrolysate, 3% sucrose and 0.8% agar for solidification of medium.

Figure 1 : *Rhyncostylis retusa* plant

The pH of the medium was adjusted to 5.5 before autoclaving. The cultures were incubated at 25±2°C under 16 hrs photoperiod. After formation of protocorms like bodies (Plbs), some of the plbs were

Figure 2 : Seedling ready for transfer

subcultured on MS medium with different concentration of hormone, i.e Benzyl amino purine (BAP) and Kinetin (Kn) for further multiplication. The other Plbs were subcultured on hormone free MS medium for seedlings development having shoots and roots.

The regenerated seedlings were acclimatized for 1-2 weeks in green house. Then these seedlings were transferred to moss for further growth in green house condition.

Results and Discussion

The seeds started swelling after about 10-15 days and within 6-8 weeks of culture period the embryo developed into green globular protocorms like bodies (Plbs) with small absorbing hairs on their bodies. Plbs so obtained were thinned out by subculturing on hormone free MS medium for further growth. These Plbs developed shoot and root primordial within 8-10 week of subculture. The differentiation of shoot and root primordia enhanced by the development of root. The seedlings were thinned out 2-3 times by transferring them aseptically in fresh hormone free medium. Then these seedlings were large enough (3-4 cm long) and ready to transfer in moss substrate (Fig 2). It took 8-10 months to develop full sized seedling from protocorms.

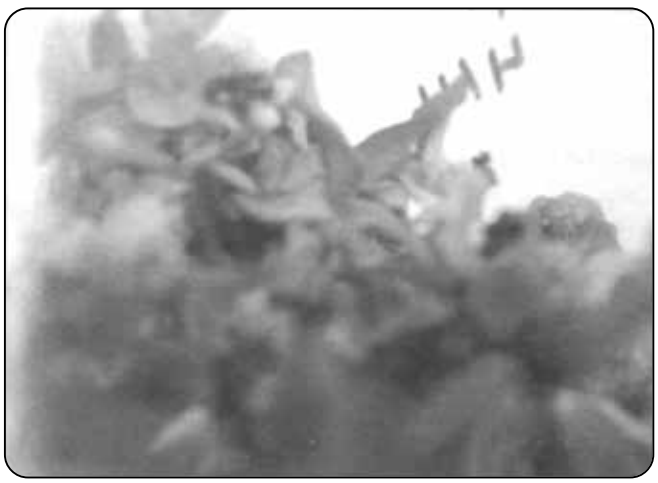

Figure 3 : Proliferation of seedlings and protocorms like bodies

The protocorms were subcultured in MS medium supplemented with different concentration of BAP and Kinetin. Among these concentration, the MS medium supplemented with 1 mg/l BAP and 1.5 mg/l kinetin was found to be best for further differentiation of Plbs and seedlings (Table 1). At high concentration the Plbs did not show good multiplication. The seedlings and plbs multiplication was enhanced by the addition of 10% coconut milk in the medium enriched with 1mg/l BAP and 1.5mg/l NAA (Fig 3). Arditti (1977) recommended the use of banana pulp in medium for transplanting seedlings. The seedling growth and multiplication is promoted when the medium supplement with coconut milk in *Cymbidium aloifolium* (Bopalah and Jorapur, 1986). The Plbs were again differentiated with many protocorms and seedlings within 3-4 months in medium enriched with 1 mg/l BAP, 1.5mg/l Kin and 10% coconut milk (Fig 3). In this case also, the juvenile seedlings were again subcultured in hormone free MS medium for further growth and Plbs were again subcultured in MS medium enriched with 1 mg/l .BAP, 1.5mg/l Kinetin and 10% coconut milk to continue multiplication. Vij *et al.* (1984) reported that the best result with seedlings leaves of *Rhyncosylis retusa* was also obtained in medium containing Kinetin and NAA. Chaturbedi and Sharma (1986) reported that the young leaves and roots differentiated Plbs in modified VW medium supplemented with 1 mg/l BAP, 1 mg/l IAA and 200mg/l casein hydrolysate.

When the seedlings grow about 3-4cm long, the culture bottles were kept 2-3 weeks in green house for acclimatization. The seedlings were taken out by forceps, washed with clean tap water and the roots

Table 1 : Growth responses of seedling on MS medium with different concentration of BAP and Kinetin and 10% coconut water.

S.N.	Conc. of hormone mg/l		Growth responses	Shoot growth
	BAP	Kn	after 16-20 weeks	condition
1	1	1.5	Many protocorms with 15-20 seedling	Best
2	2	1.5	Few protocorms with 10-15 seedling	Not so good
3	4	1.5	Died after few month	No response

were rapped by moss for further growth (Fig 4). After few months, these plantlets were kept in clay pot with tree fern roots, charcoal, dry cow dung and brick pieces as potting medium.

Figure 4 : Seedlings transferred in moss

Conclusion

From the result it is concluded that *in vitro* multiplication of *Rhyncostylis retusa* from seed culture can be applied for mass production and conservation of this plant.

Acknowledgments

The author is very much grateful Mr. H. K. Saiju, former Director General, Department of Plant Resources and Dr. Mahesh Kumar Adhikari, former Chief of National Herbarium and Plant Laboratories for providing facilities for this work. The author is also thankful to Mr. G. D. Bhatt in helping during this work.

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In vitro culture of *Piper longum* Linn.

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Abstract

In vitro clonal propagation of *Piper longum* using shoot tip explants was attempted for rapid multiplication. The explants regenerated micro shoots along with few callus and embryoids on the Murashige and Skoog (1962) medium supplemented with 1mg/l BAP and 0.1mg/l NAA. The NAA concentration of the same medium was lowered to 0.01 mg/l and added 10% coconut milk for subculture. For rooting, these regenerated microshoots were transferred to non-sterile sand after acclimatization. Microshoots initiated roots within 15 to 20 days and were transferred to polybags for further growth and field transfer.

Key words: *Piper longum,* Shoot-tip, Micropropagation, Murashige and Skoog medium, BAP, NAA

Introduction

Piper longum belongs to family Piperaceae. It is a creeping aromatic herb. Flowers are in spike, male spike narrow and female circular. Fruits are berry, crowded in cylindrical spike. Flowering is on September to October and fruiting in November to December. The fruits are collected in January. It is distributed throughout Nepal upto 800m in shady places, also in northern India, Bhutan, Srilanka and Malaysia (Manandhar, 2002).

The dried unripe fruits are useful in cold, cough, chronic bronchitis, diarrhea, obstruction of bile duct, dysentery and leprosy. It also possesses anthelmintic and carminative properties. The fruits are also used as spice, pickle and preservatives. Beside fruits, the roots and thicker parts of the stem cut, dried and used as all important drug Piplamul in the Ayurvedic medicine. The roots also used to ferment rice beer bronchitis, stomachache, disease of spleen and tumors (in The Wealth of India III, 1972). It improves appetite also. The roots and spikes used ill gout lumbago. Water boiled with two or three dried fruits is taken in case of cold and cough (Manandhar, 2002).

The fruits contain piperine and piplartine alkoloids, starch, resin, gum and fat. The dried fruits oil steam distillation yields 0.7% essential oil with spicy odor (Bhattacharjee, 1998).

Since conventional methods of its propagation viz seeds cutting or air layering could neither provide better quality materials nor in mass-scale *in vitro* clonal propagation using shoot tips has been attempted in the present work. This would provide quality planting materials *en-mass* sought after by community forest user groups and individual farmers of Nepal.

Material and method

Piper longum were collected from pilot section of Department of Plant Resources, Godawary. They were grown in green house of National Herbarium and Plant Laboratories, Godawary as stock plants. Shoot tips were cut from these plants and used as explants during. These explants were washed in running tap water half an hour and then washed with teepol for few minutes. After that, these explants were rinsed three to four times with distilled water. Finally, Surface sterilization was done with 0.1% $HgCl_2$ for 5 minutes and followed by washing with sterile distilled water. Then these explants were cultured on 0.8% agar gelled MS medium (Murashige and Skoog, 1962). The medium was supplemented with 1% casein hydrolysate and different concentration of BAP (Benzyl Amino Purine) and NAA (Naphthalene Acetic Acid). Tile pH of the medium was adjusted to 5.8 prior to autoclaving at 151b Pressure/inch^2 and 121°C for 20 minutes. The Cultures were incubated at 24±2°C

under 16 hour photoperiod at light Intensity of 3000 lux. All treatments consisted of 10 replicates and experiments were repeated two times.

After multiplication of microshoots on medium, they were regularly subcultured on fresh medium at 6-8 weeks of interval for further multiplication.

For rooting, the plantlets multiplied *in-vitro* were first acclimatized, then they were removed from the culture flasks, washed thoroughly to remove the medium from microshoots and transferred oil non-sterile sands (Rajbhandary & Bajaj, 1991). The rooted plantlets were transferred to polybags and clay pots with soil for further growth.

Result

The explants initially response in all concentrations of BAP with 0.1mg/l NAA but the explants were grew differently after few weeks. The few explants initiated 2-3 cm. long four to five microshoots along with few callus and embryoids in about 4-5 weeks of inoculation on MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA (fig. 1).

But in other concentrations, most of explants initiated globular embryo and callus along with 2-3 microshoots around the periphery of the explants within about four weeks (Table).

The primarily initiated callus and embryoids when subcultured on same medium and same concentration of hormone ratio, some subcultured piece regenerated embryo, callus and few microshoots. Some piece (mostly callus) turned brown and necrotic within 3-4 weeks of subculture. The microshoots subcultured in the same medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA they again initiated few callus, embryoids and microshoots. For further multiplication, the regenerated microshoots were again and again subcultured on same medium, but the microshoots did not satisfactory multiplied. Then the concentration of NAA level is lowered to 0.01 mg/l for inducing axial growth in regenerated shoots. The microshoots were again subculture on MS medium supplemented with 1 mg/l BAP+0.01 mg/l NAA and 0.5mg/l BAP + 0.01 mg/l NAA with 10% coconut milk. After 4-5 subculture on medium containing 1 mg/l BAP and 0.01 mg/l NAA and 10% coconut milk, the number of microshoots increases upto 15-18 microshoots (fig. 2).

For rootings, the proliferated microshoots after acclimatization were transferred on non sterile sands maintaining the humidity of propagator. After these microshoots initiated roots within 15-20 days (fig3), they were transferred in polybags follow by clay pots for further growth fig. 4,5).

Figure 1 : Explant establishment

Figure 2 : Multiplication of microshoots on medium

Figure 3 : Sand rooted plants

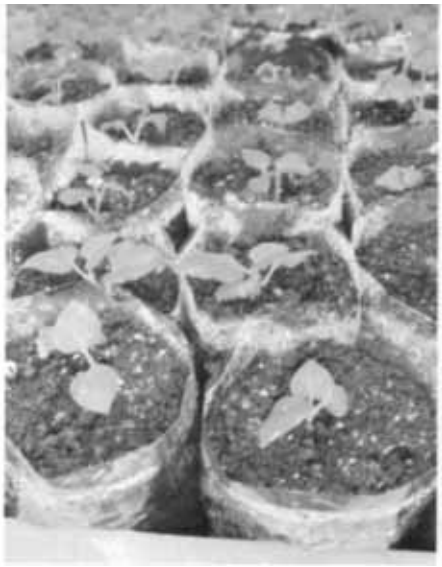

Figure 4 : Rooted plants in soil

Figure 5 : one year plant

Table : Effect of BAP and NAA on differentiation of plantlets, callus and embryoids from explants on MS medium.

Conc. of hormone (mg/l) BAP	+	Growth condition NAA	Growth responses
0.5	0.1	Good	Callus + embryoids
1	0.1	Better	Less Callus + embryoids + 4 to 5 microshoots
2	0.1	Not so good	Callus + embryoids + 2 to microshoots
3	0.1	Worse	Callus + few ernbryoids
No hormone	Worse	No multiplication	

Discussion

Mathew and Rao (1984) obtained profuse callusing on seedling explants of *Piper nigrum* in media containing auxin/ cytokinine concentration. These calli did not regenerate even in media containing higher level of BA. Joseph et al. (1999) reported that prolifieration and embryogenesis induced in absence of exogenous growth regulators in Suspension culture in case of *Piper nigrum.* Soniya and Das (2002) have been used shoot tip culture for *Piper longum* in MS medium supplemented with growth hormones l3cnzyladenine (BA) and Kinetin (K). They found maximum number of shoots on MS medium containing BA & Picloram (P). In our experiment MS medium supplemented with BAP & NAA without Picloram (P) was found to be best.

Conclusion

From this experiment, it is Concluded that MS medium supplemented with 1 mg/l BAP 0.0 1 mg/l NAA with 10% coconut milk is best for multiplication of microshoots. Sand rooting technique is best for regeneration of roots to reduce the costs of micropropagated seedlings. Thus this method is potential for commercial production of *Piper longum.*

Acknowlegdments

The authors are very much grateful to Dr. L. R. Sharma, Director General, Department of Plant Resources and Dr. S. R. Baral, Chief of National Herbarium and Plant Laboratories for providing facilities for this work.

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Clonal propagation of *Stevia rebaudiana* Bertoni through shoot tips culture and antibacterial assay of its leaf extracts

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Abstract

The genus *Stevia* includes 240 species of herbs and shrubs in the sunflower family Asteraceae. Shoot primodia, which were able to propagate vegetatively with a very high rate and to redifferentiate easily to new plants were induced from shoot tips of *Stevia rebaudiana* on Murashige and Skoog agar media supplemented with 1.0 mg/l Benzyl amino purine and 0.1 mg/l Naphthalene acetic add. The multiple shoots originated from a number of adventitious buds formed on the margin of the leaf. Multiple shoots were obtained by repeating the subculture in a same medium. The mature culture bottles were acclimatized for a week and transferred the micro shoots in non sterile sand for rooting. The rooted plants were then transplanted in polybag.

With an objective of understanding the antimicrobial potential of *Sievia rebaudiana,* chemical extracts from its leaves were subjected to bacterial assay using six solvents (petroleum ether, cyclo-hexane, chloroform, water, zoform. Water extract of *Stevia* leaf showed activity against *B. subtilis* and *S. aureus* only. Petroleum ether gave the highest zone of Inhibition against all the tested microorganism. Highest antibacterial Index (A_bI;11mm) was obtained for petroleum ether whereas the lowest antibacterial index (A_b1;2.88mm) was obtained from water extract.

Key words: *Stevia rebaudiana,* Shoot primodia, Clonal propagation, M.S. media, antibacterial activity, leaf extract, z antibacterial index (A_bI).

Introduction

Stevia rebaudiana is a herbaceous perennial herb of the Asteraceae family. It is native to Paraguay where it grows wild in a sandy soil. The plants are slender, grows up to 60-70 cm height. The species commonly known as sweet leaf, sugar leaf, and honey plant and is grown widely for its sweet leaf. As a sugar substitute Stevia's taste has a slower onset and longer duration than that of sugar. The leaves of this plant produce sweetness which is 300 time sweetener than that of sucrose, and hence garnered attention with the rise in demand for low carbohydrate, low sugar food alternatives. This also showed promise in medical research for treating different condition such as obesity and high blood pressure. *Stevia* has a negligible effect on blood glucose; even enhancing glucose tolerance therefore it is attractive as a natural sweetener to diabetics and other on carbohydrate controlled diets. Today the species is cultivated and used in food elsewhere in East Asia including in China since 1984, Korea, Taiwan, Thailand and Malaysia. China is the world's largest exporters of Stevioside. The plant has been used for centuries as a natural sweetener. The Japanese are the largest consumers of *Stevia* leaves and employ the plant to sweeten foods, such as soy sauce, confections, and soft drinks, as a replacement for aspartame and saccharin. The plant has cardio tonic actions, which normalize blood pressure and regulate heartbeat.

Stevia is multipurpose plant and used worldwide in different purposes but it is difficult to propagate by seed. Seeds of *Stevia* show a very low germination percentage and vegetative propagation is limited by lower number of individuals. Tissue culture is the only rapid process for the mass propagation of *Stevia.* The present study was carried out to standardize a suitable protocol for in vitro propagation of *Stevia rebaudiana* Bertoni.

Material and methods

Slevia rebaudiana plants were received from Dabur Herbal nursery, Banepa and planted in clay pots for establishment. Shoot tips measuring 1.0-1.5 cm were collected for tissue culture. The shoot tips were washed under running tap water for half an hour with few drops of liquid detergent (Teepol) and then washed with distilled water. The cleaned shoots were then surface sterilized with 0.1% mercuric chloride solution for 5 minutes followed by rinsing with sterilized distilled water four times. The shoots-tip measuring about 0.5 cm was aseptically excised and cultured in MS medium supplemented with different concentration of cytokinin and auxin. (Fig 1). The pH of the medium was adjusted to 5.8 before autoclaving.

The culture was incubated at 25°C temperature under 16-hour photoperiod at light intensity of 3000 lux. After 4-6 weeks of culture the shoot were proliferated. The new micro shoots were then sub cultured in same medium. The explant was also cultured in MS medium supplemented with different concentration of BAP and NAA (Table 1). The regenerated mature shoots were acclimatized for a week in green house. These shoots then were transferred to non sterile sand for root induction.

Plant Material and microorganisms

Stevia rebaudiana leaves were collected from the young plantlets grown in National Botanical Garden, Godawari were packed in polythene bags.

Six bacterial *(Escherichia coli, Bacillus subtilis, Enterococcus faecalis, Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus aureus)* isolates were obtained from College for Professional Studies, Maitidevi, Kathmandu, Nepal. These bacteria were selected for bacterial assay study as these are common pathogens either to plants or animals or cause food spoilage. The pure cultures were maintained by routine sub-culturing at one week interval in nutrient agar.

Preparation of extracts

Stevia rebaudiana leaves were washed, air dried for 7-8 days and grinded into powder using motor and pestle. 10gm weighed powder was taken for extraction in the flask of Soxhelet apparatus using six different solvents viz; water, ethanol, petroleum ether, cyclo-hexane, acetone and chloroform separately at temperature 20°C for 4-5 hours except in case of chloroform extraction where the leaf sample was submerged in 10% chloroform for 2-3 days at room temperature. The extracts were filtered using Whatman No. 1 filter paper and stored in labeled sterile screw capped bottles at – 20°C until further analysis.

Determination of minimum inhibitory concentration (MIC)

Plate dilution method was followed to determine MIC of petroleum ether extract taking different concentration (100, 250, 500, 750μg/ml) against 0.1ml of 10^{-4} inoculum dilution prepared from 24 hours incubated culture of *E. coli* into a sterile Petri plate followed by pouring of 20ml autoclaved nutrient agar media so as to understand the minimum concentration needed to prevent the growth of bacterial strain and use the obtained MIC from this test for evaluation of inhibition zone diameter for all other extracts against six test bacteria. The seeded plated were incubated at 37°C for 48 hours and the growth was noted down for different volumes of extract separately. The main objective of the present work being comparative evaluation of anti bacterial potential of several extracts along with standard antibiotics by measuring diameter of inhibition zone, MIC was obtained for one extract using *E. coli*.

Antimicrobial assay

All extracts were subjected to antibacterial assay by measuring the diameter of zone of inhibition using disc diffusion technique. Nutrient agar were prepared by pouring 20ml in each sterile Petri dish for bacterial assay and then allowed to solidify. 0.2ml of 10^{-4} dilution of 24 hours old bacterial cultures were used so as to ensure the concentration of these organisms to contain approximately $1X10^6$ CFU/ml. Sterilized

cotton swabs dipped in respective cultures were swabbed on solidified agar surface. Pre sterilized filter paper discs of 5 min diameter which absorbs 10-12µg sample/disc were dipped into individual extract of 250µg/ml concentration separately and placed on the swabbed agar plates before incubation. Similar process is followed for controls using streptomycin discs (10µg drug/disc), obtained from Hi-Media laboratories private limited, Mumbai, India as standard against bacteria. At the end of incubation period diameter of inhibition zones formed in all three replicates were measured in mm using measuring scale and the average of the three was determined. (Barry and Thornsberry, 1985).

Antibacterial Activity Index

Antibacterial index (A_bI) for individual chemical extract for Stevia were calculated as the mean value of zone of inhibition obtained against all individual bacterial test strains.

Result and discussion

After 4-6 weeks of culture, the explant shows response and 4-6 numbers of micro shoot were proliferated. The proliferation of shoot was found to be best in MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA (Fig. 2). On third sub culture the number of shoot proliferation was increased. After 6-8 weeks of sub culture 25-30 numbers of shoots were developed. (Table 1). The explant were also cultured in medium with growth hormone BAP 1.0, 2.0, 3.0, 4.0, 5.0 mg/l with NAA 0.1 mg/l. Among them only few shoots were developed in BAP 2.0-5.0 mg/l, along with 0.1 mg/l NAA. In further subculture, multiplication of shoot was not increase.

The mature shoots were transferred in green house for 7-10 days for acclimatization. The micro shoots were then transferred in sand for rooting maintaining the temperature below 27°C in the green house (Fig. 3). 80-85% of micro shoot were rooted in sand (Fig 4). The rooted micro shoots were than transplanted in soil (Fig. 5).

Although no research has been carried out till now for micro propagation of Stevia and its anti microbial assay in Nepal but lots of research works have already been done in other countries. H. Miyagawa et al. in 1986 regenerated *Stevia rebaudiana* through shoot tips culture on Gamborg B5 medium containing 6-benzylaminopurine (BAP) and alpha-naphthaleneacetic acid (NAA) under light.

Ferreira & Handro (1988), regenerated Stevia by culturing young leaves of adult plants on medium containing 6-benzyladenine (BA) (2.0 mg/l) in the light or BA (2.0 mg/l) plus 1-naphthaleneacetic acid (NAA) (2.0 mg/l) in the dark. Latha Sivaram and Usha Mukundan (2003) regenerated shoots when cultured on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA; 8.87 ì*M*) and indole-3-acetic acid (5.71 ì*M*). A survival rate of 70% was recorded at the hardening phase on the substrate cocopeat. Yukiyoshi Tamura et. al. (1984), established *Stevia rebaudiana* by culturing stem-tips with a few leaf primordia on an agar medium supplemented with a high concentration (10 mg/l) of kinetin. Mousami Debriath, (2008), regenerated *Stevia* plants through in-vitro culture of nodal segments with axillary buds on MS medium supplemented with 0.2 mg/l BAP and 1.3 mg/l IAA.

Different kind of explants such as seed, leaves, shoots tip and meristem were used for initial material

Table 1 : Growth responses by explant on MS, medium with different concentrations of BAP and NAA

S.No.	Growth hormones		Explant responses after 4-6 weeks	Induction of shoots number in average 6-8 weeks	Shoot growth condition
	BAP mg/l	NAA mg/l			
1	1.0	0.1	6-8	25-30	best
2	2.0	0.1	2-5	5-7	not so good
3	3-0	0.1	explants response	only few	not so good
4	4.0	0.1	X	X	X
5	5.0	0.1	X	X	X

Table 2 : Antibacterial activity of Solvent Extracts of Stevia (Diameter zone of inhibition in mm)

Extract	Petroleum ether	Cyclo hexane	Chloroform	Water	Acetone	Ethanol Standard	
E. coli	7	5	6	0	10	5	22
P. aeruginosa	10.5	6.5	4.5	0	6	8	25
P. mirabilis	9.5	5.5	6.5	0	5	10.6	23
E. faecalis	13	6	5	0	8.3	5	18
S. aureus	16	8.5	11	9.3	5	5	23
B-subtilis	10	3	4	8	10.3	5	22.5
Antibacterial index (A_bI)	11	6.08	6.16	2.88	7.4	6.4	22.2

and different kind and concentration of growth hormones were used in in-vitro propagation of *Stevia*. Some researchers used Gamborg media supplemented with BAP and NAA. Most of the researcher used MS media supplemented with BAP and NAA. The present result indicates that shoot tip explant was best for in-vitro propagation of *Stevia*. Concentration of growth hormone, at the rate of, 1.0 mg/l BAP and 0.1 mg/l NAA was found to be best for clonal propagation and number and growth condition of *Stevia*.

The explant also responses in BAP 2.0 and 3.0 mg/l with NAA 0.1 mg/l but the number of shoot was found to be less and shoot growth condition was not also good.

Antibacterial activity

A concentration of 250µ□g/ml of petroleum ether extract was found sufficient enough to inhibit the growth of test microorganism *E. coli* completely in Petri plates. This indicates better effectiveness of the extract at lower concentration level preventing the growth of bacteria.

It is clearly depicted in table 2 that among the bacterial pathogens selected for this study, highest rate of susceptibility was exhibited by *S. aureus* invariably by all four extracts petroleum ether (16mm), cyclohexane (8.5mm), chloroform (11.0mm), water extracts (9.3 mm) except acetone (5.0 mm) and ethanol (5.0 mm) which were not able to inhibit this specific bacteria at all. *P. aeruginosa* and *P. mirabilis* showed highest resistance to different extracts except against petroleum ether (showing zone of inhibition diameter 10.5 mm, 9.5 mm) and ethanol (8 mm, 10.6 mm). Of all the extracts petroleum ether has highest A_bI (11 mm) with highest antimicrobial activity against 3 bacterial species. *S. aureus, E. faecalis, P. aeruginosa* forming diameter of zone of inhibition of 16 mm, 13 mm, 10.5 mm respectively. The A_bI indicates petroleum ether has got the total best antimicrobial property against all six bacterial species. Whereas water extract has lowest A_bI (2.88 mm) and showed antibacterial activity against only two species that we have taken i.e. *S. aureus* (9.3 mm) and *B. subtilis* (8 mm). Similar results were reported by Tadhani & Subhash, (2006) that water extract of Stevia leaf showed activity against *B. subtilis* and *S. aureus* only. But the work done by Sumit Ghosh *et al.,* (2006) showed that water extract of stevia leaf have antibacterial activity against *E. coli, P aeruginosa, P mirabilis* and *E. faecalis.*

Conclusion

Hence, the present research work developed a protocol for clonal propagation of *Stevia rebaudiana* from shoot tip culture. Among the different concentration of growth hormone used for *in vitro* propagation of *Stevia,* the best concentration of growth hormone was found to be BAP 1.0 mg/l and NAA 0.1 mg/l. The present research work developed an efficient method for micro propagation of *Stevia rebaudiana* using shoot tip culture

Likewise present investigations provided the basic information about new non antibiotic drug molecules of plant origin, especially petroleum ether extract of stevia leaves which is found to be potent enough

in exhibiting substantial antimicrobial activity against dreaded animal pathogens like *S. aureus, E faecalis* bacteria and *S. aureus* may be explored for a value addition natural food preservative to sugar substituting property of stevia used now a days for diet restricted package food products. Therefore, these molecules could be proved as future potential candidates.

Acknowledgement

The authors are very much grateful to Dr. Lokendra Raj Sharma, Director General, Mr. Puspa Raj Shrestha, Deputy Director General of Department of Plant Resources for providing facilities for the research work. We also very much thankful to Scientific Officer Mr. Lalit Prasad Kattel for provided the Stevia plants. At last but not the least, we would like to thank Mr. Niran Maharjan and Mr. Puskar Basnet for their great support during this research work.

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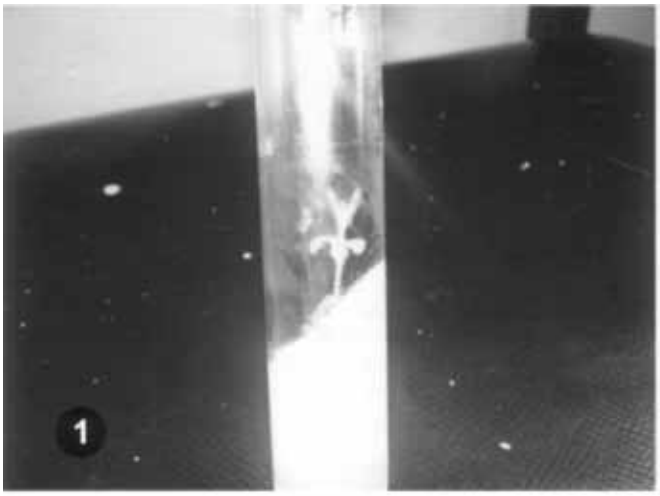

Fig. 1 : Shoot tip Culture

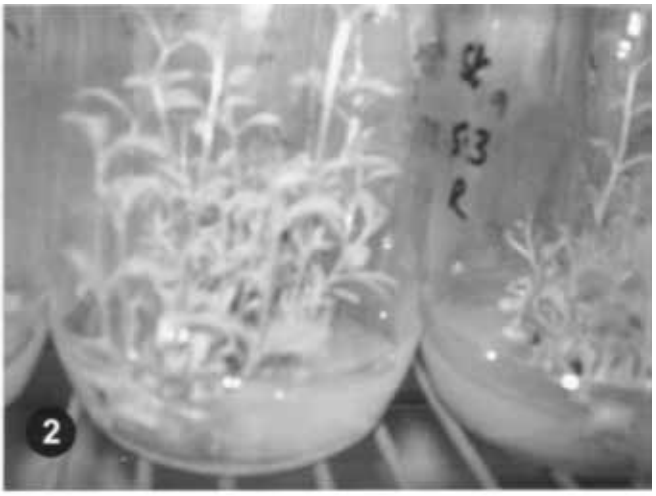

Fig. 2 : Stevia shoot proliferation in culture bottle

Fig. 3 : Sand rooting of Stevia micro shoots

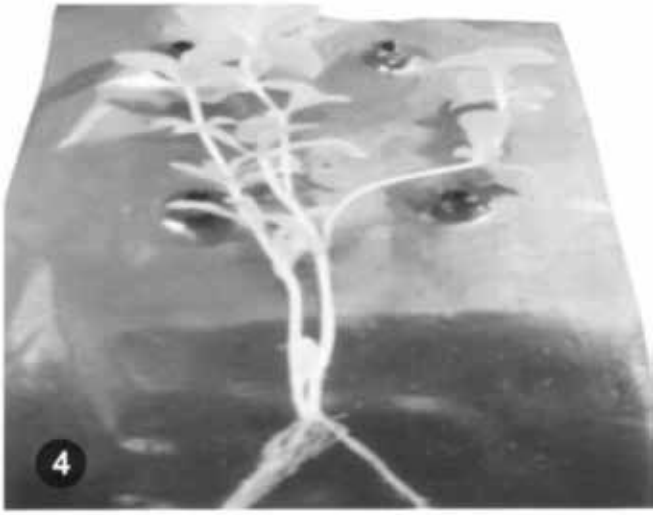

Fig. 4 : Rooted Stevia micro shoot

Fig. 5 : Stevia plants in polybag

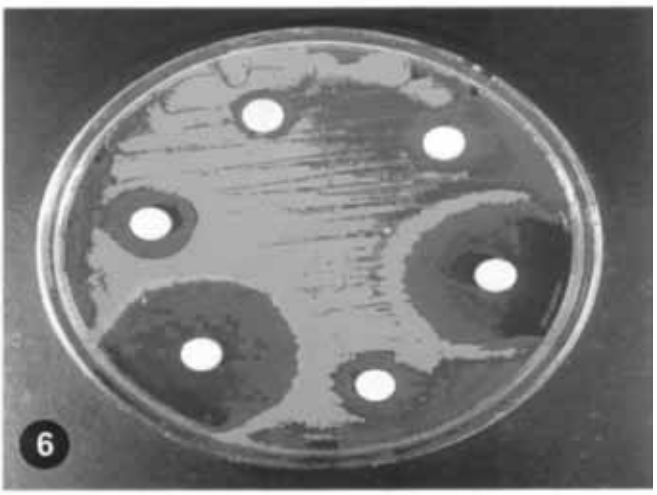

Fig. 6 : Bacterial Assay

Micropropagation of *Spathiphyllum wallisii* Hort.

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Abstract

Spathlphyllum Wallisii Hort. is a beautiful indoor plant because of its glossy pleasant foliage and its white arum like flower, which develops in spring to summer. It belongs to family Araceae. Shoot tips and nodes were used as explants. After sterilization, aseptically these explants were cultured on 0.8% agar gelled Murashige and Skoog's 1962 medium supplemented with different concentration of Benzyl amino purine and Napththalene acetic acid. The pH of the medium was adjusted to 5.8 before autoclave. Among these concentrations, the medium supplemented with 1mg/l BAP and 0.1mg/l NAA was found to be the best hormone combination for multiplication of microshoots. After acclimatization, the multiplied microshoots were transferred in non sterile sands for induction of roots in green house. About 90% of microshoots were rooted in non sterile sand within 15-20 days. These plantlets grew well and flourished normally.

Key words: *Spathiphyllum wallisii, Micropropagation,* Murashige and Skoog's medium, Hormones, Sand rooting.

Introduction

Now-a-days, decorating plants in the room, corridor and drawing room of the hotels and homes have become more popular. Some people even think that drawing rooms are incomplete without potted plants. Many more plants suitable for growing indoors are available in these days. *Spathiphyllum wallisii* is also a beautiful indoor plants because of its glossy pleasant foliage and its white arum like flower. It develops in spring to summer. The spathes change from white to green after a week or so but remain decorative for several weeks. It belongs to family Araceae. The genus *Spathiphyllum* has 36 species, which are rhizomatous, evergreen and perennial. The genus is found in damp tropical forests of Indonesia, Philippines and tropical north, central and South America. It prefers bright location, warmth and moderate humidity but not direct sun.

Conventionally, *Spathiphyllum wallisii* Hort. is propagated by seed sowing or by dividing mature clumps after flowering. The present paper deals with micropropagation of *Spathiphyllum wallisii*. The protocol develops the clone plants which fulfills the demand of this plant.

Material and methods

Spathiphyllum wallisii. plants were bought from commercial nursery and kept in green house of National Herbarium and Plant Laboratories at Godawary. Shoot tips and nodes of these plants were used as explants. These explants were washed in running tap water for half an hour first, then with teepol for few minutes and were rinsed three to four times with distilled water. Finally, surface sterilization was done with 0.1% $HgCl_2$ for 5 minutes followed by washing with sterile distilled water. The explants were cultured on 0.8% agar gelled MS medium (Murashige and Skoog's 1962) supplemented with different concentration of BAP (Benzyl amino purine) and NAA (Napththalene acetic actic). The pH of the medium was adjusted to 5.8 before autoclave. The cultures were incubated at 25±2°C under 16 hour photoperiod at light intensity of 3000 lux.

The multiplied micro-shoots were regularly subcultured on fresh medium at 6-8 weeks of intervals. All treatments consist of 10 replicates and experiment was repeated two times. During the warm summer time, the plantlets *in vitro* were removed from the culture flasks, washed thoroughly to remove the medium from microshoots and transferred into non sterile sands (Rajbhandary and Bajaj, 1991). The rooted plantlets were transferred to clay pots with compost and soil.

Result and discussion

The node and shoot tip explants showed responses in all mediums with hormone. After 2-3 weeks of culture, the node and shoot tip explants became greenish and regenerated many embryoids. These embryoids were developed into microshoots after 6-8 weeks of culture. The node explant developed more microshoots than the shoot tip explants (Table 1). The MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA was found to be the best hormone combination for multiplication of microshoots (Fig-1). The microshoots proliferated upto 20-25 shoots after 6-7 subcultures. They also developed roots in same medium after 4-4.5 months in incubation (Fig-2). Pant *et al.* (2005) also reported that initiation of roots on microshoots in medium supplemented with BAP and NAA in *Valeriana jatamansii.*

For sand rooting, the microshoots were acclimatized first, then removed from bottles, washed thoroughly with tap water to remove the medium from the microshoots and transfer to non sterile sand in propagator under green house (Fig-3). Water was sprayed regularly on these microshoots. About 90% of microshoots were rooted in non sterile sand within 15-20 days after transfer (Fig-4). These plantlets grew well and flourished normally in soil in the green house (Fig-5, 6).

Micropropation through node explants are reported in other plants also. Manandhar and Pant (2004) induced microshoots from node explant of *Heracleum wallichii* in MS medium supplemented with 1 ppm BAP and 0.5 ppm NAA. Bhasker and Subhash (1995) induced multiple shoots from node explant of *Acacia mangium* in MS medium with 3 ppm BAP and 0.1 ppm NAA. Similarly, Sarkar *et. al.* (1996) regenerated microshoots from node explant of *Rauwolfia serpentina* in MS medium supplemented with 1 ppm BAP and and 0.1 ppm NAA.

Conclusion

From this experiment, it is concluded that the node explants are the best for *in vitro* multiplication of *Spathiphyllum wallisii* and this method can be used for mass propagation of this plant which fulfills the demand of *spathiphyllum wallisii* by nursery men.

Acknowledgement

The authors are grateful to Dr. L.R. Sharma, Director General, Dr. M. K. Adhikari, Deputy Director General, Department of Plant Resources and Dr. S. R. Baral, Chief of National Herbarium and Plant Laboratories for providing facilities for this work. We are also thankful to Dr. Keshav Raj Rajbhandari for identifying *Spathiphyllum wallisii.*

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Table 1 : Regeneration of microshoots from the shoot tip and the node explants in MS medium with different combination of BAP and NAA.

Explant	2mg/l BAP+ 0.1mg/l NAA	1 mg/l BA0 + 0.1mg/l NAA	1 mg/l BAP+ 0.01mg/l lNAA	2mg/l BAP+ 0.01mg/l NAA	Medium without hormone
Shoot tip	Shunted microshoots 2-4 in number	Normal microshoot 4-6 in number	Normal microshoot 4-6 in number	Shunted microshoot 3-5 in number	No response Only elongated
Node	Shunted microshoots 5-8 in number	Normal microshoots 10-15 in number	Normal microshoots 4-6 in number	Shunted microshoots 4-6 in number	Normal microshoots 2 in Number

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Fig. 1 : Proliferation of microshoots from node

Fig. 2 : Development of roots in flask

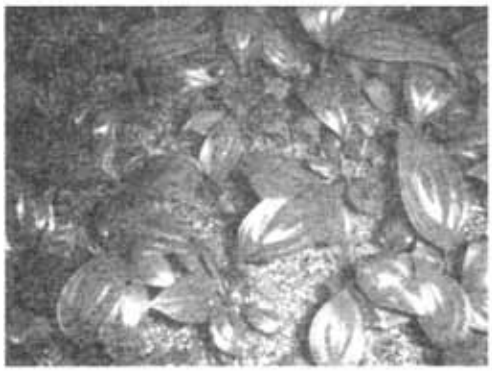

Fig. 3 : Microshoots transferred in sand for rooting

Fig. 4 : Roots developed in microshoots in sand

Fig. 5 : Sand rooted plant transfer in pots

Fig. 6 : Flowering micropropagated plants

Micropropagation of *Withania somnifera* (L.) Dunal from germinating seeds.

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Abstract

An efficient protocol was developed for large scale propagation using seed as explants in *Withania somnifera.* Shoot multiplication was achieved in vitro from shoot tips of aseptically germinating seedlings of *Withania somnifera L.* using Murashige and Skoog's agar media supplemented with 1.0mg/l Benzyl amino purine (BAP) and 0.1mg/l Naphthalene acetic acid (NAA). The multiple shoots were obtained by repeating the subculture in the same medium with addition of 50 mg/l adenine sulphate. The mature culture bottles were acclimatized for a week and then the micro shoots were transferred in non sterile sand for rooting. The rooted plants were then transplanted in polybags.

Keywords: Explants, *in vitro,* Micropropagation, tissue culture.

Introduction

Withania somnifera (L)Dunal, belonging to Solanaceae family commonly known as winter cherry, vegetable rennet, Ashwagandha, Indian ginseng, Ajagandha. It bears yellow flowers and red fruit. Its fruit is berry like in size and shape. It grows prolifically in Nepal, India, Pakistan, Srilanka and Bangladesh. It is widely distributed throughout the dry regions of India up to an attitude of 2000m in Himalayas. *Withania soninifiera* contains flavonoids and many active ingredients of the class withanolides. So far 12 alkaloids, 35 withanolides and several sitoindosides have been isolated from this plant species and thoroughly studied for its medicinal properties (Uma and Akagi 1996, Molian 2004). Much of the pharmacological activities of *Wilhania somnifera* has been attributed to two main withanolides, withaferin A and withanolide withaferin A is the most important of the withanolide isolated from *Withania soninifiera,* to which the curative properties of the leaves are attributed (Uma and Akagi 1996, Mohan 2004). Studies over two decades indicate that withanolide has antiinflammatory, anti-tumor, anti-stress (Archana and Namasivayam 1999), anti-oxidant, mind boosting, rejuvenating and antimicrobial properties (Jaffer and Jawad 1988). Withanolides is thus account for multiple medicinal applications. In addition to the alkaloids the roots are reported to contain starach, reducing sugars, glycosides, dulcitol and withaniol.

The plant prefers acid, neutral as well as basic (alkaline) soil. It cannot grow in shade. It requires moist soil. The whole plant, but especially the leaves and the root bark are abortifacient, adaptogen, antibiotic, aphrodiasiac, deobstruent, diuretic, narcotic, strongly sedative and tonic. Internally, it is used to tone the uterus after a miscarriage and also in treating post-partum difficulties.

It is also used to treat nervous exhaustion, debility, insomnia, wasting diseases, failure to thrive in children, impotence, infertility, multiple sclerosis etc. Externally, it has been applied as a poultice to boils, swellings and other painful parts. The root is harvested in the autumn and dried for later use.

Withania somnifera can be propagated both sexual and asexual method. Seed propagation, however is not always satisfactory, since the' heterogenetically the strain produces a great deal of variation. Again multiplication through cuttings give rise to less ramified plants and is consequently less productive than plants obtained from seeds. The requirement of *Withania somnifera* has sharply raised due to its popularity owing to a large scale unrestricted exploitation. Due to these reasons present study was

carried out under *in vitro* condition with the possibility of developing a protocol for *in vitro* multiplication of *Withania somnifiera.*

Materials and Methods

Seeds of the plants were collected from Salyan, Nepal, and were washed thoroughly with running tap water for half an hour with few drops of liquid detergent (Teepol), again washed with distilled water and then surface sterilized with 0.1% mercuric chloride solution for 5 min followed by 3 times washing in sterilized distilled water to remove traces of mercuric chloride. Then washed seeds were placed in MS medium without hormones with the help of forceps (Fig. 1). The pH of the medium was adjusted to 5.8 before the addition of 0.8% agar. All cultures were incubated at 25°C temperature under continuous 16 hour dark/light period for four weeks. (Fig. 3).

For further elongation and multiplication of regenerated shoots, the primary shoots formed *in vitro* germinating seeds were separated aseptically and Sub cultured in MS medium supplemented with BAP 1.0 mg/l and NAA 0.1mg/l (Table 1). All cultures were incubated at 16 hour photoperiod. The regenerated mature shoots were acclimatized for a week in green house. Then these shoots were transferred to non sterile sand for root induction.

Result and Discussion

After 4-6 weeks of culture, the explant shows response and 4-6 numbers of micro shoot were proliferated. The proliferation of shoot was found to be best in the MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA with addition of 50 mg/l adenine sulphate (Fig. 2). After third sub culture the number of shoot proliferation was increased. After 6-8 weeks of sub culture 30-35 numbers of shoots were developed (Table 1). The explant also cultured in Medium with growth hormone BAP 1.0, 2.0, 3.0, 4.0, 5.0 mg/l with NAA 0.1 mg/l. Among them only few shoots were developed in BAP 2.0-5.0 mg/l, along with 0.1 mg/l NAA. In further subculture multiplication of shoot was not increased.

The mature shoots culture bottle (Figure 4.) were transferred in green house for about 7-10 days for acclimatization. Then the micro shoots were transferred in sand for rooting maintaining the temperature below 27°C in the green house (Fig 5). 80-85% of micro shoot were rooted in sand. The rooted micro shoots (Fig 6.) were then transplanted in poly bags.

There were lots of research works done for the micro propagation of *Withania somnifiera* in many countries. Like Jayanti Sen and A.K. Sharma in 1990 did *in vitro* shoot tips culture from aseptically germinated seedlings *Withania somnifiera* L. using low concentration of 6-benzyladenine (BA), viz. 2.2, 4.4 and 8μ□M. Maximum number of shoots were obtained when 2.3μ□M 2,4dichlorophenoxyacetic acid (2,4-D) or 2.5μ□M indolebutyric acid (IBA) was added to medium containing 4.4μ□M BA during initiation of shoot multiplication, but not when added later. Direct multiple shoot initiation was also obtained from germinating seeds in the presence of BA alone. Ujjwala supe, Fanisha Dhote and M.G. Roymon 2006, in vitro Plant regeneration of *Withania somnifera* through MS media supplemented with BAP 0.6mg/l with 0.4mg/l IAA. About 90% rooting was achieved with 0.4 mg/l of IBA and 0.4 mg/l IAA. N.A. Siddique et al., 2004 plant regeneration of *Withania somnifera* (L.) Dunal frorn nodal segments derived callus through MS medium supplemented with 1mg/l BAP and 2.5mg/l Kinetin. But kinetin was less effective than BA in inducing shoot multiplication. Similar results have been obtained with other specles (Lundergan & Janick 1980; Rahaman & Blake 1988).

Shoot differentiated best from axillary shoot base callus on MS medium supplemented with BA 2 mg/l. Regenerated shoots rooted best on MS medium containing IBA 2mg/l (Gita Rani & I.S. Grover, 1999) and callus induction was noticed in 91% of cultures which showed shoot regeneration on MS medium supplemented with 2mg/l 2,4-D and 0.2mg/l Kinetin. Maximum shoot multiplication was observed after 60 days of the second culture on MS medium containing 2 mg/l BA. These shoots were rooted best (87%) on MS medium containing 2 mg/

l IBA (Gita Rani et al, 2002). The maximum number of shoots (7±0.53 shoots per explant) and elongation (7±0.75cm) was achieved when explants subculture on MS medium containing 2 mg/l BA with combination 0.5mg/l IBA. For rooting, shoots derived were excised and cultured on ½ MS medium with different concentrations of IBA (1,2 or 4mg/l) and Kinetin (1 or 2mg/l) alone.

Different kind of explants such as seed and shoots tip, were used for initial material and different kind and concentration of growth hormones were used as in vitro propagation of *Withania somnifera*. Most of the researcher used MS media supplemented with BAP and NAA. The present result indicates that shoot tip explant was best for in vitro propagation of *Withania somnifiera.* In case of growth hormone used for the propagation, 1.0 mg/l BAP and 0. 1mg/l NAA was found to be best for micro propagation. The number of shoot and shoot growth condition was also found to be best in this concentration. The explant also responses in BAP 2.0 and 3.0 mg/l with NAA 0.1 mg/l but the number of shoot was found to be less and shoot growth condition was not also good.

Conclusion

Hence, the present research work developed a protocol for micro propagation of *Withania somnifera* frorn shoot tip of germinating seeds. Among the different concentration of growth hormone used for *in vitro* propagation of *Withania soninifera,* the best concentration of growth hormone was found to be BAP 1.0 mg/l , NAA 0.1 mg/l and 50 mg/l adenine sulphate. The present research work developed an efficient method for micropropagation of *Withania soninifera* using shoot tip from germinating seeds.

Acknowledgement

The authors are very much grateful Mr. Puspa Raj Shrestha, Deputy Director General of Department of Plant Resources for providing facilities for the research work and Ms. Asha Karki and Mr. Puskar Basnet for their valuable suggestion.

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Table 1 : Growth responses by explants in MS media with different concentration of BAP and NAA.

S.N.	Growth hormones		Explant responses after 4-6 weeks	Induction of shoots number in average 6-8 weeks	Shoot growth condition
	mg/l	NAA mg/l			
1	1.0	0.1	6-8	30-35	best
2	2.0	0.1	2-4	5-7	not so good
3	3.0	0.1	explant response	only few	not so good
4	4.0	0.1	X	X	X
5	5.0	0.1	X	X	V

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Fig. 1 : Withania somnifera seed on MS media without control

Fig. 2 : Withania somnifera shoots proliferation in cluture b

Fig. 3 : Culture bottles in incubation room

Fig. 4 : Mature shoots in culture bottle

Fig. 5 : Sand rooting of Withania somnifera micro shoots

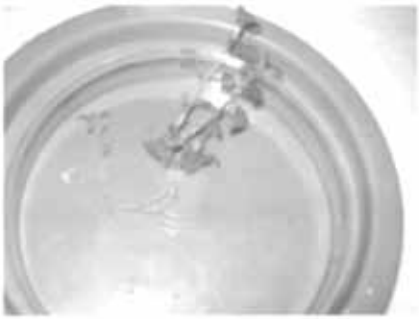

Fig. 6 : Rooted Withania somnifera shoot

Fig. 7 : Rooted plants transfer in Poly bag

In vitro propagation of *Dendrobium amoenum Wall.* ex Lindl. from seed culture

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Abstract

The seeds of *Dendrobium amoenum* were cultured on agar gelled MS (Murashige and skoog) medium supplemented with or without 10% coconut milk, 1% casein hydrolysate and 3% sucrose. The protocorm like bodies (PLBs) obtained from three weeks old culture were subcultured on fresh MS medium for seedlings development. Very few PLBs were subcultured on MS medium with different hormone concentration for further multiplication of microshoots. The medium supplemented with 1.0mg/l BAP, 1.5mg/l Kinetin and 10% coconut milk and 1.0mg/l BAP, 1.0mg/l NAA and 10% coconut milk were most suitable for multiplication of PLBs and for healthy seedling growth. The miroshoots thus obtained were transferred on hormone free medium to regenerate roots and for further growth. Thus, obtained seedlings were washed thoroughly by water and transferred in mosses for further growth of seedlings.

Key words: *Dendrobium amoenum,* Protocorms like bodies (PLBs), Growth hormone, Culture and subculture.

Introduction

Plant tissue culture is commercially successful aspect of biotechnology in plant propagation and breeding. Orchid is the first horticultural plant cloned by tissue culture method on commercial scale (Griesbach, 1986). In nature, the orchid seeds do not grow in absence of appropriate host, fungus, so it remains as a rare species. Symbiotic germination of orchid seeds was first developed by Noel Bernard. He Successfully isolated a number fungi and used them in the germination of orchid seeds between 1900 to 1911 (Breddy, 1991) Later, Hans Burgeff (1909) carried on the work and demonstrated the association of fungal mycelia with orchid root structure and their role in orchid seed germination (Arditti, 1979). He also stressed the need for symbiotic germination. Knudson (1922) showed that orchid seed germination was possible on simple nutrient medium containing minerals and sugars without the help of any fungus or mycorrhiza. His discovery was used in germination of many species of orchid including many hybrids. This became a standard procedure for germinating orchids. Orchid seeds are very small and contain very little food reserves. Because of their small sizes and their need for fungus to germinate makes it difficut to germinate seeds *in vivo*. Germination and development is easier and quicker *in vitro* as there is a conditioned environment and no competition with fungi. Most of orchids are disappearing from its natural habitats due to extensive collection by orchid enthusiasts, deforestation and natural calamities. Most people of Nepal exported these plants by collecting from natural habitats not from Cultivation. Thus tissue culture is an alternative method to propagate these plants. The present research work deals with the *in vitro* propagation *Dendrobium amoenum* by using its seeds and their subsequent development into seedling.

Dendrobium amoenum is an epiphytic orchid and grows as clustered pendulous five to six slender stems. It flowers in May-July. The flowers flourish on older stems in cluster of two to three per node. The flower is delightfully perfumed. It is common in temperate forest and distributed to western Himalaya to Sikkim, Darjeeling, Bhutan, Meghalaya and Burma (Sharma and White, 2000).

Material and method

Mother plants with or without capsule of *Dendrobium amoenum* were collected from Dhampus area of Kaski district and planted in the

green house of National Herbarium Plant Laboratories. The capsules were detached from mother plant and sterilized by alcohol flaming. Then, aseptically these capsules were cut down longitudinally into halves by sharp sterilized blade. The seeds were cultured in MS medium with or without 10% coconut milk. The medium was also fortified with 0.1% casein hydrolysate, 3% sucrose and 8% agar for solidification of medium. The pH of medium was adjusted to 5.5 before autoclaving. The cultures were incubated at 25±2°C tinder 16 hour photoperiod.

After formation of Protocorms like bodies (PLBs), PLBs were subcultured on MS medium without Hormone and coconut milk for the development of seedling having root and shoot. A few of PLbs were subcultured on MS medium supplemented with different concentration of 3 hormones (BAP, Kinetin and NAA). The medium is also fortified with 10% coconut milk for multiplication of microshoots.

The regenerated microshoots on different concentration of hormone were separated into smaller and larger sizes. The smaller size microshoots with few PLBs were subcultured on MS medium containing BAP Kinetin and 10% coconut milk for further multiplication and larger sized microshoots (2-2.5cm. long) were subcultured in MS medium with or without NAA for rooting.

The developed seedlings from the PLBs on MS medium without hormone were thinned out by subculturing seedlings at an interval of two to three months on same medium. This subculturing process was done for two to three times.

Result and Discussion

The immature seeds (white paste like when the capsule cut down) did not germinate either in coconut milk containing MS medium or in MS medium without coconut milk. But the mature seeds, which were white powdery, were germinated well on MS medium and MS medium with 10% coconut milk. Tomato juice was found to be very effective both for seed germination and seedlings development of *Vanda sp*. Jaaquim (Rao and Avadhani, 1964, ONG 1969). Synergistic effects of banana extract, coconut milk, tomato juice and NAA or IBA on seed germination and protocorms development were determined in *Cattleyai, Dendrobium sp., Phalanopsis sp.* and *Vanda sp.* (Pages 1971, Valmayor 1974). Firstly, the seeds imbibed water and swelled within few days, greenish after two weeks and formed a small globular protocorms like bodies (PLBs) within three weeks of seeds culture. The PLBs so obtained were developed subsequently large and healthy seedlings (2.5-3cm.) after subculturing PLBs/seedlings for two/three times on same medium without coconut milk i.e. thinning the PLBs and seedling. It takes 6 to 8 months (fig 1). The differentiation of roots and shoots primordias from PLBs was enhances by development of shoots. After that these seedling were ready to transfer in moss substrate in natural environment.

The protocorms were subcultured in MS medium supplemented with different concentration of BAP, NAA and Kinetin. Among these concentration, the MS medium supplemented with 1.0 mg/l BAP, 1.5mg/l kinetin and 10% coconut milk and the MS medium supplemented with 1.0mg/l BAP, 1.0mg/l NAA and 10% coconut milk were found to be best for differentiation of PLBs and microshoots (Table 1, Fig 2&3). At high concentration the PLBs regenerate callus like undifferentiated PLBs and differentiated few microshoots. The seedling growth and multiplication is promoted when the medium supplement with coconut milk in *Cymbidium aloifolium* (Bopaiah and Jorapur, 1986). The PLBs were again and again subcultured in medium enriched with 1.0mg/l BAP, 1.5mg/l Kin and 10% coconut milk or in medium with 1.0mg/l BAP, 1.0 mg/l NAA and 10% coconut milk to obtained protocorms and microshoots at an interval of 6 to 8 weeks (Fig. 2&3). In every subculture, the long microshoots were subcultured in hormone free MS medium for further growth and development of roots. The smaller microshoots and PLBs were again subcultured in same above medium as to continue multiplication. Vij *et al* (1984) reported that the best result with seedlings leaves of *Rhyncosylis retusa* was also obtained in medium containing Kinetin and

Table 1: Growth responses of PLBs on MS medium with different concentration of NAA, BAP and Kinctin.

S.No.	Concentration of hormone, mg/l NAA	BAP	Kn	Coconut milk	Growth responses by protocorms
1	1.0	2.0		10%	Callus like undifferentiated PLBs, PLBs and few differentiated microshoots.
2	1.0	1.0		10%	Differentiated PLBs and short many microshoots.
3	0.5				Multiplication of few microshoots with roots and few PLBs
4	0.1				Multiply few microshoots with roots.
5		1.0	1.0	10%	Multiplication PLBs and microshots
6		1.0	1.5	10%	Multiplication PLBs and best microshoots.
7	Ms medium only				PLBs differentiated roots and shoots, no multiplication.

NAA. Chaturbedi and Sharma (1986) reported that the young leaves and roots differentiated PLBs in modified VW medium supplemented with 1.0mg/l BAP, 1 mg/l IAA and 200mg/l casein hydrolysate. The protocorms on MS medium supplemented with 0.5 mg/l of NAA regenerated few shoots and protocorms (fig 4). Depending upon the medium and growth promoters used, the callus phase can be maintained or organogenesis can be induced. The differentiation in callus and subsequent developmental changes that lead to plantlets formation (Rao, 1963).

When the seedlings grow about 2-3cm long (Fig 5), the culture bottles were kept 2-3 weeks in green house for acclimatization. The seedlings were taken out by forceps, washed with clean tap water and the roots were rapped by moss for further growth. Seedlings generally grow better in groups than singly, so seedlings were kept in group. After few months, these plantlets were kept in clay pot with tree fern roots, charcoal, dry cow dung and brick pieces as potting medium.

Conclusion

The present research provides a practical method for the production of millions of commercially important orchid, *Dendrobium amoenum* Without damaging the mother plant.

Acknowledgement

The author is very much grateful to Mr. Puspa Raj Shrestha, Director General, Department of Plant Resources and Dr. S. R. Baral, Chief of National Herbarium and Plant Laboratories for providing facilities for this work. The author is also thankful to Mr. G. D. Bhatt in helping during this work.

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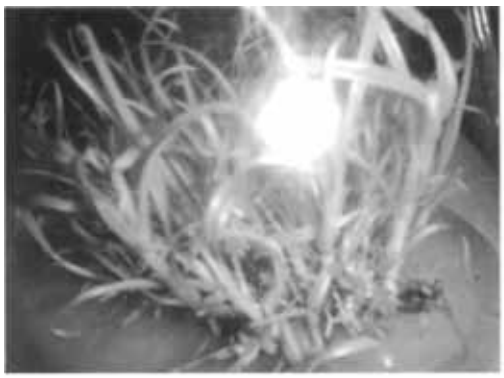

Fig. 1 : Sedlings from protocorms

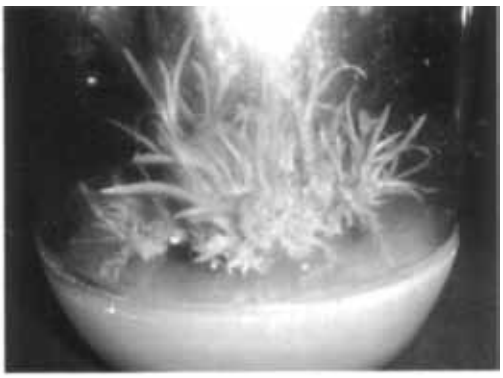

Fig. 2 : Microshoots on medium with 1 mgl BAP+1.5mgl Kinetin = 10% c. milk

Fig. 3 : Protocorms and microshoots on medium 1 mgl BAP+1 mgl NAA + 10% c. milk

Fig. 4 : Microshoots from protocorms in MS medium with 0.5 mgl nNAA

Fig. 5 : Seedlings on MS medium

Flower of Dendrobium amoenum

Microshoots and PLBs on medium 1mgl BAP + 1.5mgl Kinetin + 10% c. milk

Propagation of Cymbldium aloifolium (L.) Sw. In vitro by Seeds

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Abstract

Plant tissue culture is a promising alternative method for conserving and multiplying the plants. The present study deals with the in vitro germination of *Cymbidium aloifolium* seeds and their subsequent development into seedlings for conservation and multiplication. *Cymbidium aloifolium* seeds were cultured on solidified MS medium supplemented with 0.1% casein hydrolysate and 3% sucrose. The protocorm-like bodies (PLBs) were obtained from eight weeks old culture. These PLBs were subcultured again and again (1 to 2 times) on fresh medium to obtain 10 to 12 cm long normal and healthy seedlings. Some PLBs were subcultured on MS medium supplemented with different combinations of Benzyl Amino Purine and Napthalene Acetic Acid for further proliferation of microshoots and protocorms. Among two tested combinations of BAP and NAA, the combination of 2mg/l Benzyl Amino Purine, 1.5 mg/l Naphthalene Acetic Acid and 10% coconut milk was best for further multiplication of PLBs and microshoots. These regenerated seedlings were acclimatized and transferred to moss substrate for further growth. The obtained result showed that In vitro growing of *Cymbidium aloifolium* seeds is a reliable explant for the seedlings growth and multiplication of its seedlings.

Key words: *Cymbidium aloifolium*, seeds, Protocorm-like bodies, seedlings

Introduction

Nepal, having the diverse climatic belts, is highly suitable for growing orchids of various types. It is a orchid rich country representing 363 species under 97 genera (Rajbhandari and Bhattarai, 2001) among an estimated 25000 in the world. In nature, orchid seeds do not grow in absence of appropriate fungus, so it remains as a rare species. Orchid is the first horticultural plant cloned by tissue culture method on commercial scale (Griesbach, 1986). Symbiotic germination of orchid seeds was first developed by Noel Bernard. He successfully isolated a number of fungi and used them in the germination of orchid's seeds between 1900 and 1911 (Breddy, 1991). Later on, Hans Burgeff (1909) carried on that work and demonstrated the association of fungal mycelium with orchid root structure and their role in orchid seed germination (Arditti, 1979). Knudson (1922) showed that orchid seed germination was possible on simple nutrient medium containing minerals and sugars without the help of any fungus. His discovery was used in the germination of many species of orchid including many hybrids. This became a standard procedure for germinating orchid. Mostly a *Cymbidium* orchid capsule contains 2-3 million seeds even though only a few seeds germinate in natural environment because due to its small size, they contain very little food reserves, and they need particular fungal requirement and seeds sown in nursery beds required long period for germination and any disturbances to the soil and physical environment destroyed the whole population. Thus, the germination and development is easier and quicker in vitro as there is a conditioned environment and no competition with fungi. Many orchids are disappearing from their natural habitats due to their reckless collection by orchid enthusiasts, deforestation and natural calamities. Mostly orchid traders of Nepal also exported orchids by collecting them from natural habitats. Therefore, tissue culture is a potential alternative method for conservation and propagation of such plant species.

The present study deals with the germination of *Cynibidium aloifolium* seeds and their subsequent development into seedlings. The conventional method of vegetative propagation of *Cymbidium aloifolium* i.e. division of rhizome, bulbs or by the rooting of offshoots is slow and difficult to obtain

desired number of plants. *Cymbidium aloifolium is* a beautiful, epiphytic orchid found in the subtropical open deciduous forest. It flowers during May-July. The raceme is pendulous, 40+ cm long with a 20 cm scape and well spaced 3cm diameter flowers. These are yellowish with a central broad reddish stripe. It is widely distributed from north to south India, Sri-Lanka, Burma, Malaysia and Thailand (White and Sharma, 2000).

It is also used as a medicinal plant. The rhizome is used as emetic and purgative (Vaidya, Shrestha and Joshee, 2000). The root paste is used on fractures and dislocated bones (Shrestha, 2000).

Materials and Methods

The plant with capsule was collected from near the Fewa lake of Kaski District, The plant was growing as an epiphyte on *Schima wallichii* tree. The collected plant was planted in the Green house of National Herbarium and Plant Laboratories. After one and a half months, the capsule was detached from the mother plant. The capsule was sterilized by alcohol flaming. Then, aseptically the capsule was cut down longitudinally into halves by a sharp blade and was put in a petridish. The seeds were then cultured on MS medium and MS medium supplemented with 10% coconut milk. The medium was also fortified with 0.1% casein hydrolysate, 3% sucrose and 8% agar for solidification of the medium. The pH of the medium was adjusted to 5.5 before autoclaving. These cultures were incubated at 25±2°C under 16 hr photoperiod.

After formation of protocorm, like bodies (PLBs), PLBs were subcultured on MS medium for the development of seedlings having root and shoot. The developed seedlings from PLBs on medium were again thinned out by subculturing. The subculturing process was done for two to three times at an interval of 8 to 10 weeks to develop healthy seedlings which were then ready for transfer into the community pots.

A few protocorms were subcultured on MS medium supplemented with different combinations of BAP and NAA for further multiplication of protocorms and microshoots.

The seedlings thus obtained were acclimatized first and then transferred to community pots or to propagator after wrapping their roots by mosses.

Result and Discussion

The seeds changed into green color after four weeks of culture in both the medium containing coconut milk and without coconut milk. The coconut milk did not show effect on differentiation of seeds into protocorms. However, Krishnamohan and Jorapur (1985) observed that highest percentage of seeds germination and seedling growth in Acampe praemorsa, when the VW medium was supplemented with 1gm/l peptone and 20% coconut milk. Bopalah and Jorapur (1985) found that the normal seedlings of *Cymbidium aloefolium* were developed on modified Knudson medium fortified with NAA, Kinetin, Glycin, thiamine HCI, Niacin at low concentration (1 ppm each). A slight increase in the concentration of this growth adjuvant did not have any positive effect on seedling growth. Alam et.al (2002) also found that MS medium was best for seeds germination of *Dendrobium transparens.*

These seeds developed into green globular protocorms like bobies (PLBs) within 8 weeks of culture (Fig-1). These PLBs when subcultured on MS medium, they grew 5-6 cm long seedlings having shoot and root after two months (Fig-2). Again, these seedlings were thinned out by subculturing on same medium. After that, healthy seedlings were ready to be transferred to the greenhouse (Fig-3).

The protocorms were subcultured on MS medium supplemented with the combination of 1mg/l BAP, 1mg/l NAA and 10% coconut milk and the other combination was 2mg/l BAP, 1.5mg/l NAA and 10% coconut milk. In both mediums, protocorms multiplied into protocorms and microshoots but the latter combination seemed to be best for the multiplication of microshoots and protocorms (Fig-4). These regenerated microshoots were again subcultured on MS medium supplemented with 0.5mg/lNAA for rooting of microshoots. The small microshoots and protocorms were there after subcultured on the tested best medium for further multiplication.

Before transferring the *in vitro* grown seedlings, these were acclimatized first. After acclimatization, the plants were taken out from the bottles and washed thoroughly with water, and then the roots were wrapped with mosses and kept in propagator or in community pots for further growth (Fig-5). After a few months, they developed more new roots and also grew in height.

Conclusion

A single capsule of *Cymbidium aloifolium* contains millions of seeds. Thus, millions of seedlings can be obtained from a single capsule through *in vitro* culture. The microplants can be continuously obtained from PLBs on MS medium supplemented with BAP and NAA. The aseptic method of seeds germination, multiplication and their subsequent development into seedlings helps in the production and conservation of this species.

Acknowledgement

The author is very much grateful to Dr. S. R. Baral former Chief of National Herbarium and Plan Laboratories and now Acting Director General DPR for providing facilities to do this work. The author is also thankful to Mrs. Rose Shrestha for helping in the identification of the plant.

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Fig. 1 : Protocorms

Fig. 2 : 1st subculture

Fig. 3 : 2nd subculture

Fig. 4 : Microshoots on 2mg/l BAP, 1.5mg/l NAA+10% coconut milk

Fig. 5 : Seedling transfer on moss

In vitro shoot tips culture of *Aloe vera* Mill.

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Abstract

Aloe vera Mill. is an important medicinal plant and used worldwide in medicine and cosmetic industry. Micropropagation method for elite selection of *Aloe vera* by axillaries.branching method using shoot tip as explants was standardized. Shoot tips cultures were initiated on MS medium containing the growth regulators BAP and NAA. Maximum shoot proliferation was achieved on medium containing BAP 2.0mg/l with NAA 1.0 mg/l. The mature micro shoots were kept in greenhouse for acclimatization. Micro shoots were transfer in sand for rooting. The roots were initiated after two weeks of transplantation. Sand rooted plants were transferred to soil and they showed 85% survival. The regenerated plants were morphologically similar to mother plants.

Key *words:* *Aloe vera* Mill., shoot tip culture, *in vitro*, plant growth honnone

Introduction

Aloe vera synonyms *barbadensis* Mill. belongs to the family Liliaceae. It is commonly called as 'Burn plant'. There are about more than 40 *Aloe*-based formulations being marketed in the global market. It is a xerophytic medicinal plant and can be grown even in dry lands under rain fed conditions. *Aloe* is a perennial plant with a short stem, found in the semi-wild state in many parts of the country. Phylloclade 30-60 cm long, erect, crowded in a basal rosette, full of juice, glaucous-green, narrow-lanceolate, long-acuminate, smooth except for the spiny teeth on the margins. Flowers are yellow, in dense racemes terminating the scapes. Scapes are longer than leaves, scaly and branched. Propagation is done primarily by means of suckers (or) offshoots, which are separated carefully from mature plants and transplanted. Medium sized suckers are chosen and carefully dugout without damaging the parent plant at the base and can be directly planted in the field (Gui et al., 1990; Meyer and Staden, 1991). Leaves exude a bitter liquid, which is dried and known as "bitter *Aloes.*" Leaves are broken off and the clear gel is applied to the skin as a first aid for burns. Aloe contains cathartic anthra-glycosides and its active principle ranging from 4.5 to 25% of Aloin. These are extensively used as active ingredients in laxative, anti-obesity preparation, as a moisturizer, emollient, wound healer, in various cosmetic and pharmaceutical formulations (Meyer and Staden, 1991). Sexual reproduction by seeds due to male sterility in *Aloe vera is* almost not effective and vegetative propagation through lateral shoots only possible during growing seasons. To overcome this problem *in vitro* cloning and multiplication by means of development of apical meristems as explants cultured in appropriate basal medium in commercial scale can be a solution and further it can provide the propagation materials of superior quality also. Therefore the present investigation was carried out for the mass production of *A. vera*. through tissue culture.

Materials and Methods

Plant material and explants sterilization

The plant material used for present investigation was collected from nursery. The extra leaves were removed and shoot was trimmed to size of 2-3 cm. For the surface sterilization, the trimmed shoot tip explants were kept in running tap water for about one hour with few drops of liquid detergent Tween 20. After washing with detergent the explants were thoroughly rinsed with distilled water for 4-5 times to remove any traces of detergent remain in explants. After these treatments explants were taken inside the laminar air flow for further sterilization. Explants were surface sterilized with freshly prepared 1.0% w/v aqueous solution of Mercuric chloride for 10

minutes. After Mercuric chloride treatment, explants were thoroughly rinsed for 3-4 times with sterilize water to remove any traces of Mercuric chloride.

Culture medium

Shoot tip Explants were inoculated onto MS basal (Murashige and Skoog, 1962) medium supplemented with different concentration of plant growth regulators Benzyl Amino Purine (0.5 mg/l,1.0mg/l and 2.0 mg/l) and Naphthalene Acetic Acid (0.5mg/l, 1.0mg/l) for bud break and shoot proliferation. Sucrose 3% was used as carbon sources and media were adjusted to pH 5.8 using Sodium hydroxide (NaOH) before autoclaving. The media were solidified with 0.8% agar, poured in jam bottle and were autoclaved at 121 C.

Inoculation of explants

Before inoculation, explants were transferred to sterilize petridish with the help of sterile forceps under strict aseptic conditions. The explants were further trimmed and extra outer leaves were removed to make them in appropriate sizes of (1-2cm). Explants are transferred to culture bottles containing MS medium with different concentration and composition of growth hormone, BAP, NAA (Fig. 1). After successful initiation of the multiple shoot, newly formed shoots were excised and further sub cultured on the same media to increase the number of shoots proliferation. The cultures were incubated under 16 h photoperiod with light intensity of 3000 lux and temperature of 25±2°C (Fig. 5). Mature plantlets were shifted to greenhouse for acclimatization for 7-10 days (Fig. 6). The plantlets were taken out carefully with the help of forceps and dipped in water to remove any traces of solidified agar media. The plantlets were carefully planted in sand (Fig.7).

Results

Shoot proliferation

After four weeks of inoculation, explants started to show signs of proliferation. New shoot buds from the axils of leaves of shoot tip explants and buds develop into shoots after 6 weeks of culture. The culture was maintained by regular sub culturing at 4 week intervals to fresh medium with the same composition. All the medium of combination of BAP and NAA produced shoot proliferation (Table 1). Among all combination of growth hormone BAP and NAA, 2.0 mg/l BAP and 1.0 mg/l NAA gave better shoot proliferation than other hormone concentration (Table 2).

This table showed that explants responded in all media containing different concentration of growth hormone BAP and NAA (Fig 2, 3). But after sub culturing of micro shoot in same concentration of BAP and NAA, the best concentration for shoot multiplication was found to be 2.0 mg/l BAP and 1.0 mg/l NAA. In this composition, maximum number of shoot was observed than in other composition.

Sand Rooting

Plants were thoroughly watered and covered with polythene hood having 80% humidity and 30°C temperature for rooting in sand. After 2 weeks of transplant, plantlets initiated to give rise to roots

Table 1 : Effect of different concentration of BAP and NAA on shoot proliferation of *Aloe vera* after 6 and 8 weeks

S.N.	MS + Growth Hormone mg/l		Explant showing shoot formation	Average number of shoot per explants		Condition of shoots	Remarks
	BAP	NAA		after 6 weeks	after 8 weeks		
1	0.5	0.5	responded	few shoots	3-5	Satisfied	
2	1.0	0.5	responded	few shoots	4-6	Satisfied	
3	2.0	0.5	responded	few shoots	7-8	Satisfied	
4	0.5	1.0	responded	4-5	Remain same	Good	
5	1.0	1.0	responded	6-8	Remain same	Good	
6	2.0	1.0	responded	9-12	16-20	Best	

(Fig.8). After 4 weeks of sand rooting, the plants were transferred to soil. 80-90% of plants give rise roots (Fig.9).

Discussion

Aloe vera has been cultured *in vitro* by various researchers (Abrie and Staden, 2001), Chaudhuri and Mukundan (2001) and Aggarwal and Barna (2004). Micropropagation of *Aloe vera Linn* is also reported by use of BAP for shoot proliferation of *Aloe polyphylla* and *A. vera* respectively. At higher levels, cytokinins tend to induce adventitious bud formation. In agreement with these concepts in the present study also, shoot proliferation occurred only in the presence of cytokinin with particular reference to BAP. However, it is in contrast to earlier reports in *Aloe vera* by Meyer and Staden (1991) and Natali *et al.* (1990). They reported that better proliferation occurred on medium containing Kinetin instead of BA. It may be due to the genotypic variation. Shoot initiation was more pronounced in BAP 1.5 mg\I and Adenine sulphate 50 mg\l, respectively followed by another combination of BAP and Adenine sulphate at, 2.0 mg\l and 50 mg\l. Aloes have been cultured *in* vitro with 2, 4-D and cytokinins. (Groenewald *et al.* 1975, Natali *et al.* 1990). Axillary bud development and adventitious bud formation was obtained with decapitated shoot explants of Aloe *barbadensis Mill.* Maximal bud growth and rooting of shoots was obtained on a modified medium of Murashige and Skoog supplemented with IBA. The optimal temperature for bud growth and development was 25°C. Bud growth was slowed at 10°C (Meyer & Staden, 1991). Little work has been done on callus culture of Aloe species because establishment of primary cultures is difficult owing to the secretion of the phenolic substances by explant. There is only one report on callus formation and plant regeneration from seed calli of Aloe *pretoriensis* (Groenewald *et al.,* 1975). Roy and Sarkar (1991) report the rapid propagation by the formation of shoots from calli of Aloe vera. They induced the callus formation in stem segments from young axillary shoots grown on the underground rhizomatous stem. Polyvinylpyrrolidone was used to reduce the secretion of phenolic substances from the explant. Modified MS media with 2,4-D and Kinetin was used for callus induction. (Roy & Sarkar, 1991). Micro propagation of Aloe was carried out by culturing fragments from axillary shoots on MS medium without growth regulators (the presence of which was found to inhibit the first stage of development). Richwine *et al.* (1995) reported the induction of shoot cultures of *Aloe, Gasteria* and *Haworthia species* from immature inflorescence. Shoots were initiated on a modified MS medium containing zeatin and later maintained on medium containing Zeatin and BA. Different researcher used different kind of growth hormone for shoot initiation. Some researchers multiply by callus culture. Rooting response of microshoots is reported to be controlled by growth regulators in the medium (Bhojwani and Razdan, 1992), basal salt composition (Garland and Stoltz. 1981, Zimmerman and Broome, 1981, as well as culture condition (Murashige, 1977). For most of the species auxin is required to induce rooting.

In our research work, Adenine sulphate and Kinetin were not used for shoot initiation. There was direct shoot bud formation from the shoot bud explants. There was no callus formation. Most of the researcher produced *in vitro* rooting plantlets using auxin NAA and IBA. The *in vitro* rooting was economically expensive and takes one more step in tissue culture process. In our research work, micro shoots were transferred in pure sand for initiation of roots (Fig.7). Once plantlets were established, rapid multiplication was observed in sub culture on same concentration of growth hormone BAP and NAA. Single culture bottle contain 16-20 plantlets after third subculture (Fig.3). The best media for shoot initiation was MS media supplemented with 2.0 mg/l BAP and NAA 1.0 mg/l.

Conclusion

Aloe vera syn *barbadensis* Mill. is a xerophytic medicinal plant of considerable importance. It is widely used in cosmetic and drug industry and its demand are increasing day by day. Due to widespread male sterility it propagates only through vegetative mode of reproduction. But its propagation rate is very slow to meet commercial demand of high

quality planting material for its commercial cultivation. The objective of this present research work was to standardize growth hormone concentration for mass propagation and rooting of micro shoots. Benzyl amino purine (BAP) 2.0 mg/l and Naphthalene acetic acid (NAA) 1.0 mg/l promoted higher frequency of shoot proliferation.

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Fig.1 : New explant culture

Fig. 2 : 4-5 Shoots

Fig. 3 : 6-8 Shoots after sub culture

Fig. 4 : 16-20 after third sub culture

Fig. 5 : Acclimatization in green house

Fig. 6 : Sand rooting of *Aloe Vera*

Fig. 7 : Rooted Aloe Vera

Fig. 8 : *Aloe Vera* in polybag

Fig. 9 : *Aloe Vera* in pot after 6 weeks