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MICROPROPAGATION OF SELECTED MEDICINAL PLANTS

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ABSTRACT

Micropropagation of selected important medicinal plants viz. ***Alpinia calcarata, Kaempferia galanga, K. rotunda*** *and Malaxis rheedei was carried out successfully. Plantlet regeneration was achieved in all the species through enhanced axillary bud development and multiple shoot formation.*

In ***Kaempferia galanga, K. rotunda*** *and* ***Alpinia calcarata*** *the mode of regeneration was similar. On media containing a wide range of the cytokinins, Benzylaminopurine (BAP) and Kinetin (Kin). multiple shoots were induced Rooting also occurred on all the shoots in the multiplication medium. Liquid medium was found to be better than solid medium for multiplication and plantlet formation. Inexpensive polypropylene (PP) bags were used as culture containers for micropropagation. Natural light was found to be sufficient for illumination of cultures.*

Plantlets of all three species were transferred to soil and more than 80% survival recorded The micropropagated plants in all the three species showed morphological variations. The leaves of micropropagated plants had a higher length to breadth ratio than control plants in the first year. Although higher number of rhizome buds were produced in micropropagated plants than in controls the total biomass of rhizomes was reduced Near-normal morphology was restored and the yield of rhizomes increased in the second generation plants, in both pot cultures as well as in the field.

In ***M. rheedei*** *explants containing arxillary buds taken from pseudobulbs were cultured on media containing cytokinins. Sprouting of the buds occurred on a wide range of cytokinins. Multiple bud formation at high frequency was observed on media containing higher levels of cytokinins when the explants were longitudinally split. Highest multiplication was obtained on 2 µM BAP. Elongation of buds occurred on* lower *levels of cytokinins. Rooting of the shoots was obtained on 3 µM Naphthalene acetic acid (NAA).*

1. INTRODUCTION

Medicinal plants have for long been utilized in different parts of the world through collection from the wild populations. The Ayurveda system is principally based on herbal drugs. Herbs were either collected by the physicians themselves or by people and the requirement was readily met this way until recently. During the past decade mass production of herbal medicines has increased greatly especially in Kerala which has a rich tradition of ayurveda.The increased demand has resulted in large scale manufacture of the traditional formulations and to meet the requirement for raw drugs. the wild plants were overexploited without any regard for sustainability. In the absence of any regulation or standards in the industry, the use of spurious raw material has become prevalent because of the scarcity of several plant species in the wild.

It has been recognized recently that there is a need for cultivating many of our medicinal plants to ensure their availability in the future. In recent years several farmers have switched over to planting medicinal plants on agricultural land. The immediate problem that became evident was the shortage of the planting material and the need for developing cultural practices suitable for the crop. There is a need today for large scale propagation of several of our medicinal plant species. Clonal propagation is a method which not only facilitates large scale propagation but also ensures uniformity in the propagules. Maintenance of superior clones would also be an advantage. Cloning by conventional means on a large scale is best done by rooted stem cuttings. With the use of mist propagation units, plant hormones and artificial rooting media it should be possible to efficiently propagate several medicinal plants.

Several medicinal plants are propagated in nature through vegetative means viz. rhizomes. In many of these plants especially those belonging to Zingiberaceae the rhizome is the medicinally used plant part. Hence. a part of the harvest has to be retained by the farmer for the next season's planting. Conventional vegetative propagation using small cuttings is not possible in this category of plants because of their morphology.

In vitro techniques have been successfully applied to solve some of the problems of conventional clonal propagation in a large number of medicinal plants. In vitro methods have the advantage of requiring very little plant tissue to initiate propagation and very high multiplication rates are typically obtained. Propagation can be carried out at any time of the year and small. uniform and disease-free plantlets suitable for planting produced on a large scale.

One of the limitations to wider application of micropropagation to economically important plants is the high cost of production. Since conventional micropropagation requires relatively sophisticated and energy intensive infrastructure and technically trained labour, it is not cost effective for several crops. Reduction of the costs at every step of the technique needs to be brought about. This can be done by use of cheaper alternatives to the equipment as well as recurring costs such as chemicals and other consumables.

Scope of this study

In this study micropropagation of four species of medicinal plants was attempted. Three of the species viz. *Kaempferia galanga* L., *K. rotunda* L. and *Alpinia calcarata* Roscoe. belong to the Family Zingiberaceae and have a similar growth habit and morphology. Micropropagation of *Malaxis rheedei* Heyne ex Wall. - a terrestrial orchid with medicinal properties was also attempted. The conventional propagule in all the four species is also the commercially important part. Cost of planting material in these species is thus high. Attempts have been made here to develop cost effective simple micropropagation methods and to field test the plants so as to evaluate the applicability of the method to large scale commercial plant production.

Micropropagation of *Kaempferia galanga* from rhizomes and through callus has been described by Vincent *et al.* (1991. 1992). Micropropagation of *K. rotunda*, *A. calcarata* and *M. rheedei* has not been reported so far.

2. MATERIALS AND METHODS

2.1. Plant Material

Plants of *Kaempferia galanga, K. rotunda, Alpinia calcarata* and *Malaxis rheedei* growing in the Medicinal Plant Garden in KFRI, Peechi were used as the source of plant material for this study. Rhizomes of the first three species were collected at the beginning of the growing season (May to August). In *M. rheedei* pseudobulbils were collected from plants with several fully opened leaves.

2.2. Surface sterilization

Explants were first prepared for culture by removal of dead tissue and cleaning with tap water containing a few drops of detergent (Extran. Merck) followed by 1-2 washes with distilled water to remove the detergent.

Mercuric chloride ($HgCl_2$) solution (0.1% (w/v)). prepared in double distilled water was used for surface sterilization of all the plants used in this study. The duration of the $HgC1_2$ treatment varied from 5-10 min. according to the nature of the plant material.

2.3. Glassware/Culture containers

Borosilicate glassware was used in this study for preparation of culture media and for use as culture vessels. Test tubes (150 mm x 25mm.) were plugged with non-adsorbent cotton plugs. All glassware and water for surface sterilization of explants were autoclaved ($1.5 kg^{-1} cm^{-2}$, 121°C) for 20 min.

As an alternative to the borosilicate glassware the following were also tested as containers for shoot multiplication of *Kaempferia* spp. and *Alpinia* 1. Glass bottles of 400 ml and 600 ml were used either with polypropylene (PP) or stainless steel caps. 2. Polypropylene bags (22 cm x 25 cm) were used as another alternative. These bags were of the type commonly in use for packing groceries and confectionery. By using a heat sealer it was possible to modify the shape and size of the bag whenever required. PP bags are autoclavable and hence can be used several times if needed. However since the thickness of the bag material used in this study was less. the bags were discarded after two uses.

2.4. Culture media

The composition of basal medium used in this study is given in Table 1. The mineral salts and vitamins used in the basal medium were according to Murashige and Skoog (1962). Sucrose was added at 2 % (w/v) as the carbon source in all the media. All chemicals used in this study were of analytical grade (Merck or Qualigens). The medium was prepared using stock solutions of the mineral salts and vitamins (stored frozen) in distilled water. After addition of sucrose, necessary additions of growth regulators and other additives were made from stock solutions stored below 5° C and the pH adjusted to 5.7 with 1N NaOH or HCl. For preparation of solid medium, agar (Hi-Media) was added at 0.5 %(w/v) and melted in a microwave oven before dispensing into the culture vessels. Aliquots of 20 ml were dispensed into each test tube. Culture media were autoclaved at 1.5 kg^{-1} cm^{-2} at 121° C for 15 min. The composition of different media used in this study is given in Table 2.

Table 1. Composition of Basal Medium* (MS)

Sl. No.	Chemical	Concentration in mg/l
1.	KNO_3	1900.00
2.	NH_4NO_3	1650.00
3.	$CaCl_2$. 2 H_2O	440.00
4.	$MgSO_4$. 7 H_2O	370.00
5.	KH_2PO_4	170.00
6.	$MnSO_4.4H_2O$	22.30
7.	$ZnSO_4$.7 H_2O	8.60
8.	H_3BO_3	6.20
9.	KI	0.83
10.	$CuSO_4.5H_2O$	0.02
11.	$Na_2MoO_4.2H_2O$	0.25
12.	$CoCl_2$.6 H_2O	0.02
13.	$FeSO_4.7H_2O$	27.80
14.	Na_2EDTA.2 H_2O	37.30
15.	Myo inositol	100.00
16.	ThiamineHCl	0.10
17.	Nicotinic acid	0.50
18.	Pyndoxine	0.50

* Mineral salts and vitamins of Murashige and Skoog's Medium (1962)

Table 2. Composition of media used in this study

Basal Medium : MS + Sucrose 2 % (w/v)

Response	Composition *	
	Growth regulators	Concentration
Sprouting of Buds	BAP	0.02 M
Shoot Multiplication	BAP	1, 2, 4, 8 and 32 µM
	Kin	1, 2, 4, 8 and 32 µM
	BAP + Kin	1, 2, 4, 8 and 32 µM each

*Agar (0.5% w/v) added to obtain solid media wherever needed.

2.5. Culture conditions

The effect of different light sources on shoot multiplication was tested under the following conditions.

i. Fluorescent illumination: Cultures were incubated in glass bottles with stainless steel screw caps and illuminated by fluorescent tubes (120cm) of 40 W and 8 h light/16 h dark cycle. The net Photosynthetic Proton Flux (PPF) measured at culture level was 18µ E m^{-2} s^{-1} (in the Photosynthetically Active Radiation (PAR) range).

ii. Daylight illumination: Cultures were kept on a window ledge facing north next to the glass pane. Sunlight was diffuse except for a few minutes in the afternoon when direct sunlight was available to the cultures. The average PPF at the window was 14.4 µ E $m^{-2}s^{-1}$.

Subcultures were normally carried out every 4-5 weeks to fresh medium. In cultures of *K. galanga, K. rotunda* and *A. calcarata* the multiple shoots were separated into single shoots, the roots removed and the leaves were cut 2 cm from the base. This facilitated easy subculture and did not affect the further growth of the cultures.

2.6. Transfer to soil

K. galanga, K. rotunda and *A. calcarata* plantlets were transferred to soil directly from the multiplication medium since roots were present at this stage. Plantlets were removed from the culture vessel and washed free of the medium in running tap water. Plantlets were prepared by separation of the suckers,

each having at least one elongated shoot. Often two or more short shoots were present at the base of a longer shoot which were retained when transferred to soil. Plants were transferred to polybags of size 20 cm x 12 cm filled with garden soil and kept covered with an inverted polybag of the same size to maintain high humidity. Watering was done dally with tap water.

Plantlets of *M. rheedei* were transferred to a mixture of charcoal and brick pieces and soil in earthen pots, kept covered with an inverted polythene bag. Water was sprayed frequently to maintain humidity inside the bags.

2.7. Pot and field trials

Micropropagated plantlets hardened and growing in polybags were out-planted in the field or transplanted to earthen pots at the onset of monsoon. Rhizome pieces, which are the conventional planting material were planted under similar conditions to act as controls. Raised beds of about 30 cm high and measuring 1 m x 3 m were prepared in the field which had a partial shade of trees. Plantlets as well as rhizomes were planted at 30 cm spacing. Beds with rhizomes were covered with dry leaves to prevent drying out of the young sprouts. Plantlets were also planted in earthenware pots of 30 cm (dia) x 25 cm filled with garden soil. The planting was'repeated for the second year in the same way using rhizomes collected from the first year plants.

Measurements of leaf length and breadth, and petiole length were recorded in *Kaempferia* spp. and *Alpinia* plants in soil after two months of transfer. The mean of measurements of five leaves per plant and five plants per treatment was recorded. Rhizomes were harvested after one season of growth, that is, in February which is also the harvesting time for *Kaempferia*. Soil was loosened and plants were uprooted taking care to avoid damage to the rhizomes. The roots and aerial parts were excised after washing the plants in running tap water. The total below soil biomass, the number and weight of the rhizomes were recorded and the mean of five plants per treatment was used for the comparison.

3. RESULTS AND DISCUSSION

3.1. Sprouting of buds

3.1.1. *Kaempferia* spp. and *Alpinia calcarata*

Fungal contamination of upto 60% was observed in rhizome explants of *Kaempferia* spp. and *Alpinia* placed on various media containing cytokinins. In the remaining explants. sprouting of the buds was observed on all the media in 2-3 weeks. Buds of *K. galanga* sprouted first followed by *K. rotunda* and *A. calcarata*

3.1.2. *Malaxis rheedei*

In 80% of the nodal explants. the axillary buds sprouted to form unelongated shoots in 4-8 weeks. Shoot tip explants did not produce any response whereas the base of the pseudobulbil often produced upto three shoots. The sprouting response appeared to be independent of the hormone type and concentration.

3.2. Shoot multiplication

3.2.1. *K. galanga, K. rotunda* and *A. calcarata*

Sprouted rhizome buds were excised after 4 weeks of growth and placed on different media in the cytokinin series to induce shoot multiplication. Induction of multiple shoots started after 2-3 weeks of transfer. At 8 weeks maximum shoot development was observed. At each subsequent subculture every 8 weeks, the individual shoots were separated and the leaves were cut away 2 cm from the base to facilitate subculture. The different stages of development of shoot cultures from rhizome buds of the three species were similar in many respects (Figs.1-3). The shoot multiplication rates on different media were however different.

The effect of two cytokinins viz. BAP and Kin on multiple shoot formation is given in Table 3. The data were recorded after 3 subcultures on the same composition. Higher concentrations of cytokinins (both BAP and Kin) gave higher rates of multiple shoot formation whereas at lower levels the elongation of the shoots and root production was enhanced. The strategy that could be derived from the experiments is that a media with 0.01 µ M each of BAP and Kin is used to induce sprouting from rhizome buds, followed by transfer of

Fig. 1. Multiple shoots of *Kaempferia rotunda* growing in liquid culture

Fig. 2. Multiple shoots of *Alpinia calcarata* in liquid culture

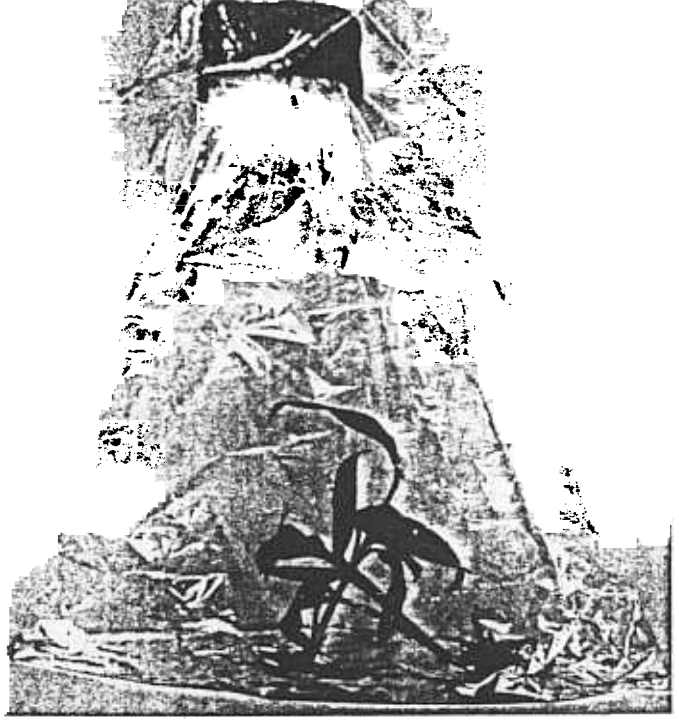

Fig. 3. Shoot cultures of *K. galanga* in PP bags and liquid media

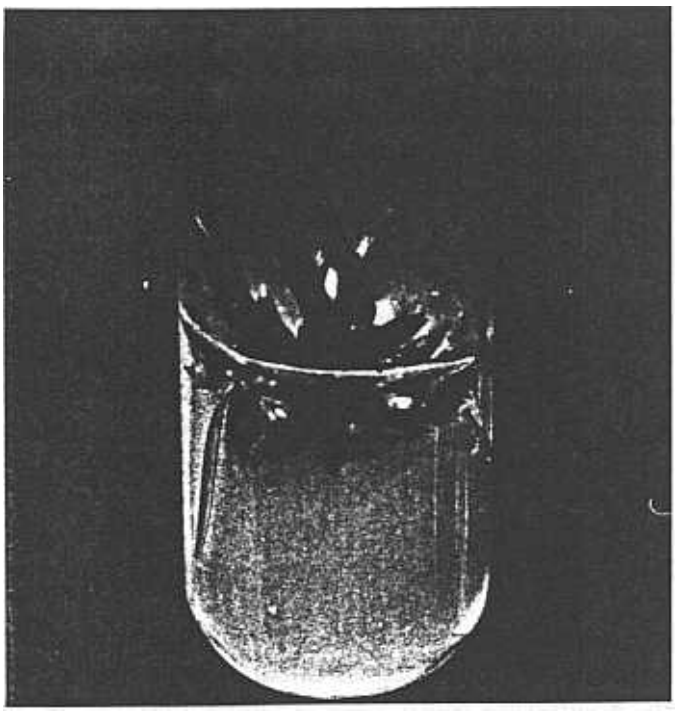

Fig. 4. Multiple shoots of *M. rheedei*

explants to a medium containing 8 µ M each of BAP and Kin for a sufficient number of passages to obtain multiplication. followed by transfer of the shoots to a low cytokinin media to obtain further development of the shoots before transfer to soil.

Table 3. Effect of different cytokinins on shoot multiplication

Hormone	Concentration in mM	Shoot multiplication		
		K. galanga	*K. rotunda*	*A. calcarata*
BAP	2	+	+	+
	4	+	+	+
	8	++	+++	++
	16	++	+++	+++
	32	++	++	++
Kin	2	+	+	+
	4	+	+	+
	8	++	++	++
	16	+++	+++	++
	32	++	++	++
				++
BAP + Kin	2	+	+	+
	4	+	+	+
	8	++++	+++	+++
	16	++++	+++	+++
	32	+++	+++	+++

+. 2 or < 2shoots: ++. 3-4 shoots: +++, 4-6 shoots: ++++, >7 shoots.

3.2.2. *M. rheedei*

Newly sprouted shoots were slow to multiply on the cytokinin series. Multiple shoot formation was enhanced when a longitudinal cut was made on the sprouts. On media containing 2 µ M BAP the multiple shoot formation was maximum (Fig. 4). Elongation of the shoots occurred on media containing lower levels of cytokinins although growth was very slow.

3.3. Rooting

The notable feature of the mode of regeneration in *Kaempferia* spp. and *Alpinia* was that rooting of shoots occurred in the multiplication medium itself. A separate rooting step involving change of medium and transfer to a new container was thus avoided. In *Kaempferia* spp. each individual shoot had 3-4 roots while in *Alpinia* most shoots had formed only a single root.

In *M. rheedei* a few elongated shoots placed on media supplemented with 3 µ M NAA formed roots in two weeks. Roots were initiated at the lower nodes and only one root formed per shoot.

3.4. Effect of solid and liquid media on shoot multiplication

When solid and liquid media supplemented with BAP and Kin at 8 µ M were compared for shoot multiplication, higher rates were obtained in the latter in all the three species (Table 4). No difference in development of the plantlets were observed except that roots on solidified media were shorter than those formed in liquid cultures. Shoots were trimmed for subculture as in the solid media and even when submerged when inoculated in the liquid media, shoot growth was normal.

Table 4. Comparison of shoot multiplication of *Kaempferia* spp. and Alpinia in solid and liquid media

Medium: MS + BAP (8 µ M) + Kin (8 µ M) + 2 %sucrose

Species	Multiple shoots/culture*	
	Solid medium	Liquid medium
K. galanga	6.8 ± 0.32	7.2 ± 0.25
K. rotunda	4.6 ± 0.30	4.8 ± 0.32
A. calcarata	3.8 ± 0.29	4.4 ± 0.16

* Mean of 10 replicates ± SE

The use of liquid media accompanied by shaking for aeration has been widespread in micropropagation of several plants often with remarkable improvement in multiplication. The better results are attributed to the improved plant-medium contact in liquid cultures leading to better availability of nutrients and hormones. Shaking is considered essential to aerate the cultures. However in the present studies no differences were observed in cultures kept stationary and those kept on a shaker (at 50 rpm), hence all the experiments were carried out without agitation of the cultures.

The advantages of using a liquid medium for shoot cultures are many. Besides the savings in the cost of agar, the time and effort required in dispensing a liquid medium is less compared to the solid medium which requires heating to melt the agar. Shoot cultures in liquid media are much more easily amenable to transfer during subculture than those growing on a solid media.

Likewise, rooted in vitro plantlets are more prone to mechanical root damage and microbial infections when taken out of a solid medium since traces of the medium remain and are difficult to clean whereas a simple wash is enough to remove a liquid medium. Yet another advantage is that cleaning of containers after culture requires less effort in the case of the liquid medium. It is also much easier to detect contamination of cultures in a liquid medium.

3.5. Culture in PP bags

Shoots of *Kaempferia* spp. and *A. calcarata* were cultured in PP bags containing 30 ml of a liquid medium with 8 µM each of BAP and Kin. Three shoots were placed per bag which was kept hanging on a wire against the light source using plastic clips. Since the bags were transparent it was possible to clip together upto three bags without cutting off light significantly.

Shoot cultures of all three species showed no differences when grown in PP bags as compared with the controls in bottles. As in the liquid media in bottles, shoots grew erect (Fig. 4) even when the explants were submerged when inoculated.

The advantages of using PP bags are that they are an inexpensive and easily available material and being transparent permits effective illumination even through stacks of 3-4 bags kept near the light source. The number of plantlets that could be raised this way is thus much higher than when conventional containers are used.

The disadvantages of using PP bags as culture containers are the following:

i. The type of bags used in this study was such that leaks could easily develop unless extra care was taken during the various procedures. Checking each bag for leaks when it is reused is also difficult and hence discarding the bag is advocated. The other alternative is to use bags of a higher gauge. This is not commonly available in the market.

ii. The other drawback in the use of PP bags as culture containers is the difficulty encountered at various culture stages. As mentioned already. on autoclaving, the sides of PP bags tend to slick together and is difficult to open for culture. However, this problem can be eliminated, by placing a paper sheet inside the bags before autoclaving and removing it at the time of culture.

iii. The culture procedures like inoculation and subculture are rendered relatively difficult because of the collapsible nature of the bags. Keeping the mouth of the bag open to permit introduction or removal of tissue under sterile conditions requires some

iv. Although heat sealing of the PP bags will maintain sterility this is undesirable since air exchange is blocked. Folding of the mouth of the bags and retaining the fold by means of paper clips has been observed to be sufficient. Evaporation of the liquid media is, however, relatively rapid.

3.6. Effect of source of illumination

Cultures in liquid medium maintained in ambient conditions and culture room showed no differences in multiplication rates or in growth rates. Condensation of moisture took place on the sides of the culture containers maintained in ambient conditions. Although this probably will reduce the light available to the shoots no effect on the growth of the cultures was observed.

Raju *et al.* (1994) have routinely carried out micropropagation of orchids and other ornamental plants utilizing only the solar radiation available under shade of trees. Muralidharan (1995) also carried out micropropagation of different species under ambient conditions where solar radiation available through the window was sufficient to maintain the shoot cultures. Hayashi *et al.* (1988) succeeded in growing and acclimatizing carnations *in vitro* under solar radiation but probably with temperature control.

Ambient culture conditions if successful could result in large savings in cost of production of micropropagated plants. In fact the capital costs of the culture room and the running costs of maintaining the cultures on the shelf will contribute to a major share of the cost of micropropagation.

The drawback of this method is that ambient conditions ***vary*** between geographic locations and also change throughout the year. The actual design of a laboratory using such a method on a bigger scale would have to be very diffirent from that used in the present studies. The design should permit sufficient light and avoid heating and build up of high humidity. Supplementary lighting during cloudy days would also be required.

3.7. Morphology of micropropagated plantlets

The most striking feature of micropropagated plants of *Kaempferia* spp. and *Alpinia* was the abnormal leaf morphology. Plantlets of *K. galanga* had leaves that were less broad, erect and longer than in control plants where leaves were aligned horizontal to the plant axis. The petiole was also longer, whereas in the normal plants it is hardly visible above the soil. In *K. rotunda* the leaves were narrower than in controls and did not have the variegation found in normal leaves. In *Alpinia* the plants did not form a pseudostem as in controls and the plantlets were similar to the other two species in habit.

An explanation for the difference in leaf morphology of micropropagated plants is that plants are not growing under autotrophic conditions and consequently can dispense with functional leaves with normal morphology. Plants grown under in *vitro* conditions differ in several aspects of morphology as well as anatomy (George and Sherrington, 1984). Such differences are the reasons for the poor survival rates of plants when transferred to soil.

The use of photoautotrophic conditions for micropropagation as a means for production of high quality plants, has been the subject of research by Kozai *et al.* (1991).

3.8. Transfer to soil

Very high survival rates of above 85% was obtained in plantlets of *K. galanga* *K. rotunda* and *A. calcarata* transferred to soil in polybags. By keeping the plants covered by an inverted polybag sufficient humidity could be maintained. New and thicker roots had formed in the plantlets after two months of transfer. At this stage more than one sucker had developed into shoots in most of the plants.

High rates of survival of plantlets of the three species without any particular hardening steps is an unexpected result since the susceptibility of in ***vitro*** grown plantlets to water loss is a widespread observation in a large number of plants. The factors that favour good survival *ex vitro* of plants here are the early formation of roots (in the multiplication stage itself) and the presence of a short compressed stem covered by the several leaf bases. The presence of thick fleshy roots at the time of field planting could contribute to favourable water relations of the plants. It is necessary also to examine if functional and normal stomata are formed in culture in these species and whether the wax deposition on the leaf cuticlar surface is normal at the plantlet stage. High rates of mortality of micropropagated plants in several species has been attributed to the abnormal stomata and cuticular wax deposits (Sutter. 1985: Sutter and Laughans. 1982).

Plantlets of *M. rheedei* transferred to soil containing pieces of charcoal block and bricks survived only for about three weeks and showed no further growth.

3.9. Pot and field trials

In all the plantlets of *K. galanga* and *K. rotunda* transferred to pots and to the field at the onset of rains, leaves withered and dried up within the first week. The leaves in A. calcarata were. however, more hardy though eventually they were also lost after about two weeks of planting. New leaves which were

more normal were formed in all the three species within one week of shedding of the older leaves. In *K. galanga* the petiole length and the width of the leaves had increased. The alignment of the leaves were, however, still erect whereas the control plants had horizontally aligned ones. In *K. rotunda* leaves were still narrower than normal but the variegation was apparent in the middle of the leaves as in the control plants. In *A. calcarata* normal plants have a elongated pseudostem formed by the leaf' bases. In the newly emerging leaves of micropropagated plants in the pot and field trials, the pseudostem was formed, although it was highly condensed.

Table 5. Comparison of leaf morphology of micropropagated plants of *K. galanga, K. rotunda* and *A. calcarata* with control

Species	Leaf length (l) in cm	Leaf breadth (b) in cm	l/b ratio	Petiole length (cm)	Total height (cm)
K. galanga					
1. TC I	14.84	5.36	2.76	4.95	-
2. TC II	12.46	5.52	2.25	4.10	-
3. TCF II	12.44	5.48	2.27	4.55	-
4. Control	12.72	6.38	1.99	4.34	-
K. rotunda					
1. TC I	15.05	2.68	5.61	-	-
2. TC II	30.27	5.81	5.20	-	-
3. TCF II	32.00	5.84	5.49	-	-
4. Control	57.80	7.50	7.70	-	-
A. calcarata					
1. TC I	12.09	1.92	6.29	-	7.87
2. TC II	20.10	2.50	8.04	-	42.30
3. TCF II	23.12	2.67	8.65	-	45.75
4. Control	35.58	3.82	9.31	-	61.81

TC I - Tissue cultured 1st year, TCF II - Tissue cultured (Field)
TC II - Tissue cultured 2nd year
* Mean of measurements taken from five plants and five leaves per plant

In the second year's growth all the three species exhibited a near to normal morphology and growth habit. In *K. galanga* the leaf length to breadth ratio had reduced (Table 5.) and leaves were horizontally aligned as in the controls

(Fig.5). Similarly in *K. rotunda* the length to breadth ratio reduced although the length of the leaves had increased (Fig.6). In *A. calcarata* the pseudostem was prominent by the second year (Fig.7)but the interpetiolar distance was much shorter than those of the controls or of tissue culture plants in the field.

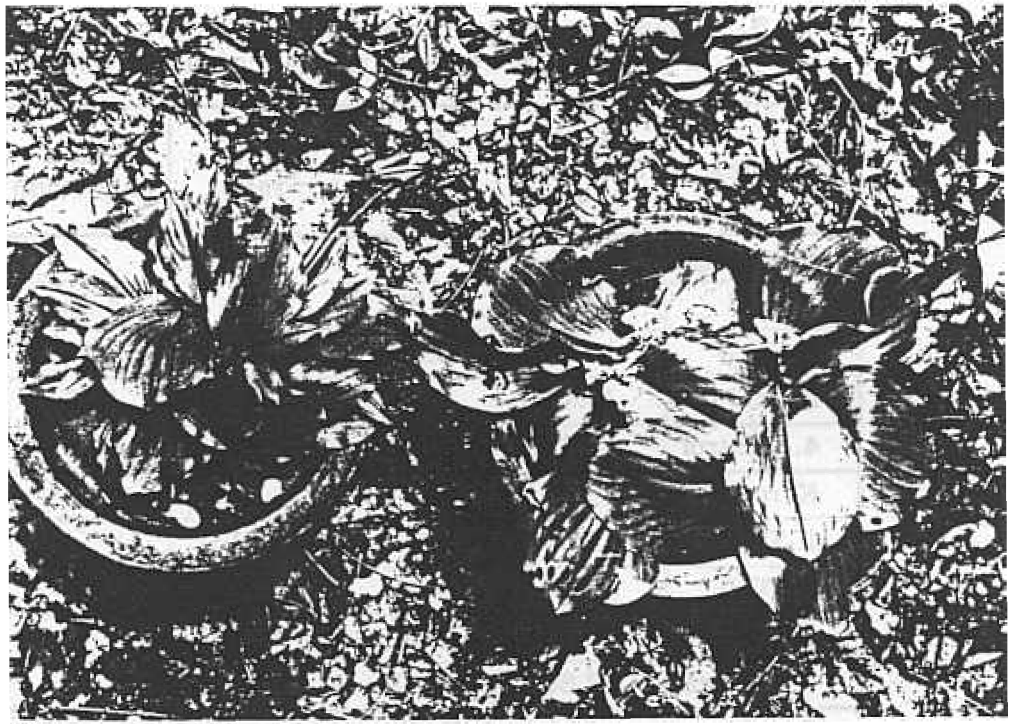

Fig. 5 *K. galanga* plants in pots - 2nd year, Left - micropropagated plant Right - control (note flowering in control plant)

Leaves began to dry up in plants of *Kaempferia* spp. in the pot and field trials in the month of November as in the control plants. In *A. calcarata* the leaves were retained much longer and newer shoots emerged before the older ones dried up. The control plants of *Alpinia* growing in the field had large clumps having upto 50 shoots which remained evergreen.

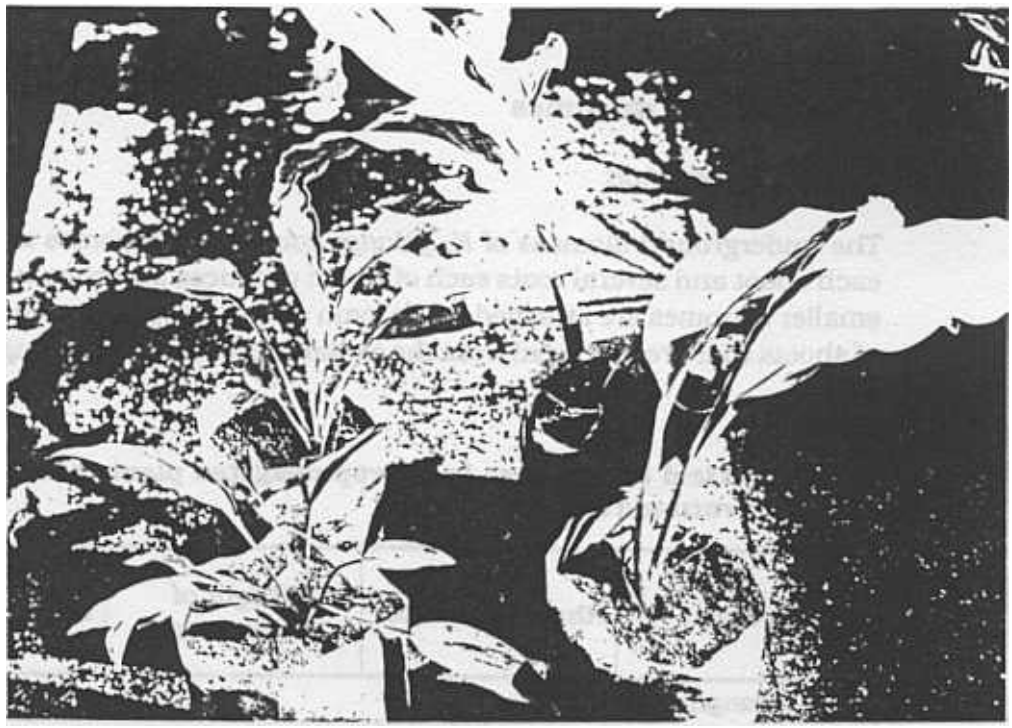

Fig. 6. *K. rotunda* plants in soil. Left - 1st year. Right - 2nd year.

Fig. 7. Plantlets of *Alpinia calcarata* in the 2nd year

3.10. Yield of rhizomes

3.10.1. *K. galanga*

The underground biomass of *K. galanga* consists of rhizomes at the base of each shoot and several roots each of which produces a swollen tuber. Several smaller rhizomes are attached to the main rhizome depending on the number of shoots that are produced from the axillary rhizome buds during the growth period.

Table 6. Yield of rhizomes in micropropagated plants of *K. galanga, K. rotunda* and *A. calcarata*

Species	Rhizome number	Weight of rhizome (g)	Weight of rhizome + tubers (g)
K. galanga			
1. TC I	**25.4**	**36.22**	**38.00**
2. TC Il	**30.2**	**38.50**	**42.20**
3. TCF Il	**22.0**	**34.23**	**36.02**
4. Control	23.0	37.09	44.00
K. rotunda			
1. TC I	**4.2**	**0.52**	**5.97**
2. TC Il	**2.1**	1.00	**15.43**
3. TCF Il	**3.0**	**3.20**	**18.36**
4. Control	3.6	7.92	32.00
A. calcarata			
1.TCI	**4.0**	**0.910**	-
2. TCII	**6.2**	**18.91**	-
3. TCF II	8.2	17.89	-
4. Control	4.0	34.64	-

TC I - Tissue cultured 1st year TCF II- Tissue cultured(Field).
TC II - Tissue cultured 2nd year.
* data is the mean of five replicates.

In micropropagated plants the rhizome number was higher than the control (Table 6) although the total weight remained more or less the same. Since *in vitro* culture is carried out in the presence of cytokinins the carry over of the

effect of the hormones can be expected in the first generation. The partitioning of available biomass into a larger number of rhizomes and leaves results in the relatively smaller size and weight of the individual organs in micropropagated plants. In the second generation this effect is reduced considerably but not completely. The higher rhizome yields in 2nd year plants grown in pot compared to those in the field was probably because of the increased availability of water (irrigation)and direct sunlight.

3.10.2. *K. rotunda*

In micropropagated plants of K. rotunda the rhizome production is evident even in plants at the time of transfer to soil after hardening and after the first season's growth a thick rhizome was produced. In contrast to *K. galanga* in this species a large part of the bulk of the below ground biomass is contributed by the oblong 'tubers' (Fig. 8).

Fig. 8. Rhizomes and tubers of *K. rotunda* (Left - 1st year, Right -2nd year)

As in *K. galanga* the rhizome number in micropropagated plants was higher (Table 6) than controls. The total biomass produced in the 1st and 2nd year in pots as well as the field was however relatively low. This could be attributed to the lack of vigour of the plants especially the leaves which were prone to falling over.

Fig. 9. Rhizome production in micropropagated plants of *A. calcarata* (Left - 1st year, Right - 2nd year)

3.10.3. *A. calcarata*

The underground biomass of *A. calcarata* consists of a woody horizontal rhizome connecting the different stems and having long fibrous roots. The base of the stems contribute to the bulk of the rhizome biomass. Micropropagated plants develop 2-4 short thin rhizomes after one growth season whereas in the second year upto six thick and long rhizomes had developed (Fig. Control plants in the field were taller and had fewer but bulkier rhizomes than micropropagated plants.

4. CONCLUSIONS

From the results obtained in the above experiments and in the field trials of the plantlets of *Kaempferia* spp. and *Alpinia* it becomes apparent that although high multiplication rates could be achieved through fairly simple procedures of micropropagation it has some limitations in its use for mass production of 'planting material. Results obtained in this study indicate that only the second generation plantlets are suitable as propagules for obtaining a productive crop. Pot culture trials indicate that there is a potential to improve productivity by providing irrigation and control of sunlight. The potential for cost reduction through use of cheaper alternatives has also been demonstrated in this study, especially in the use of polypropylene bags and liquid media and incubation of cultures under ambient conditions of light and temperature.

In *M. rheedei,* although very high shoot multiplication was obtained, the poor rooting and survival rates of plantlets indicate that further experiments are required to improve shoot vigour and root quality. Testing of other potting media used for terrestrial orchids to improve the survival of the plants will also be reauired.

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