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USAID Research Grant 5:399 in vitro Propagation of Clove and Nutmeg

Progress Report

Half-yearly report

(a) Report for half-year 10 Aug 1985 - 10 Feb 1986

(b) Grantee: Mr Y.M.H.B.Yapa Bandara

(c) Grant No: 5: 399

(d) Title of Project: in vitro propagation of clove and nutmeg

(e) Date of award of grant by NARESA: 10 Aug 1985

(f) Description of work done during half-year:

Laboratory studies:

Nutmeg shoot tip culture.

Explants were taken from 2-year old, greenhouse-maintained, regularly pruned nutmeg plants, sterilised, and placed in an appropriate medium. All cultures were maintained in an incubator with apppopriate illumination.

Culture establishment, sprouting of axillary buds, growth behaviour, callus formation, rooting and physical changes were observed.

Shoot-tip culture of Allspice.

Shoot-tips were obtaindd from 2-month-old plants, sterilised, and cultured as above.

Culture establishment, sprouting of axillary and terminal buds, callus formation, and other physiological changes were observed.

Nutmeg leaf cultures.

Explants were taken from young leaves, sterilised, and cultured as above.

Callus formation, type of callus, type of root, number of roots per explant and root length were observed.

In all cases, the effects of different hormones, separately and in combination, on bud break and bud growth and rooting ability were observed.

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Attempts to culture shoot-tip explants from mature field-grown trees of allspice and from other members of the Myrtaceae (Eugenia mallacensis) and leaves of Cinnamomum cassia were unsuccessful.

Data collected: A large volume of data was collected from the above experiments.

(g) Is the work on schedule: yes

(h) Plan of work for the next half-year: continuation of present work, trials with seedling material and young plants of nutmeg, trials with clove.

Any other remarks:

During this period Mr Yapa Bandara attended a Tissue abd Cell Culture course in Tennessee, U.S.A. There was instruction in both theory and practical work, and he found it very useful. He also participated in 2 conferences: (1) on Tissue Culture Propagation of Horticultural Plants at Beltsville, Maryland, U.S.A. and (2) the first International Conference on Molecular Biology at Savannah, Georgia, U.S.A. Here he had the opportunity to meet some eminent workers in this field and discuss his work with them.

(j) Signature of Grantee: [illegible] 2/4/86

(k) Signature of Head of the Institution: [illegible]

date [illegible]

MINOR EXPORT CROPS RESEARCH STATION MATALE

5 JAN 1987

5. 397

USAID/ARESA RESEARCH GRANTS

HALF YEARLY REPORT - SECOND HALF, 1986

1. Grantee I: Y.M.H.B. Yapabandara

Address: Minor Export Crops Research Station, Matale.

II Prof. M.D. Dassanayake
Department of Botany,
Faculty of Science,
University of Peradeniya,
Peradeniya.

2. Grant No.: RG/AID/7

3. Date of award: 10th Aug. 1985

4. Title of Project: In vitro propagation of clove and nutmeg

5. Brief statement of the methodology that was used:

NUTMEG

A) Experiments with in vitro multiplied shoots

Experiment No. 1

Objective: To find out the hormonal requirement to achieve optimum proliferation rate.

Method & Materials: In vitro grown, randomly selected 1 cm long shoot pieces was used for this study. Prepared medium contained the range of concentration of Benzyl-adenine as 0, 0.5, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.50, 2.75, 3.0, 3.5, 4.0, 5.0 mg/l.

Results: Table - 1

Average no. of axillary bud production per 1 cm shoot piece in medium contain various concentrations of BA

BA mg/l	No. of axillary buds
0	0
0.5	2.2
1.0	2.0
1.25	2.2
1.5	3.0
1.75	2.2
2.0	1.5
2.25	1.2
2.50	1.0

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BA mg/l	No. of axillary buds
2.75	.80
3.0	.80
3.5	0.8
4.0	0.6
5.0	0.4

The medium containing 1.5 mg/l BA gave maximum number of axillary buds per stem piece.

Experiment No. 11

Objective: Selection of the appropriate portion of the shoot which regenerates to give the maximum number of axillary buds.

Method & Materials: Four numbers of one cm long sections of shoot pieces were taken from single in vitro grown shoots leaving out 1.5 cm from the apex and individually labelled (1st, 2nd, 3rd, and 4th) and placed in medium containing 1.5 mg/l BA. Treatments were replicated five times.

Results:

Table - 11

Production of axillary buds from various portions of the shoot tips in medium containing 1.5 mg/l BA

Length from the apex	Average no. of axillary buds produced
1.5 - 2.5 cm	3.4
2.5 - 3.5 cm	4.6
3.5 - 4.5 cm	1.6
4.5 - 5.5 cm	0.8

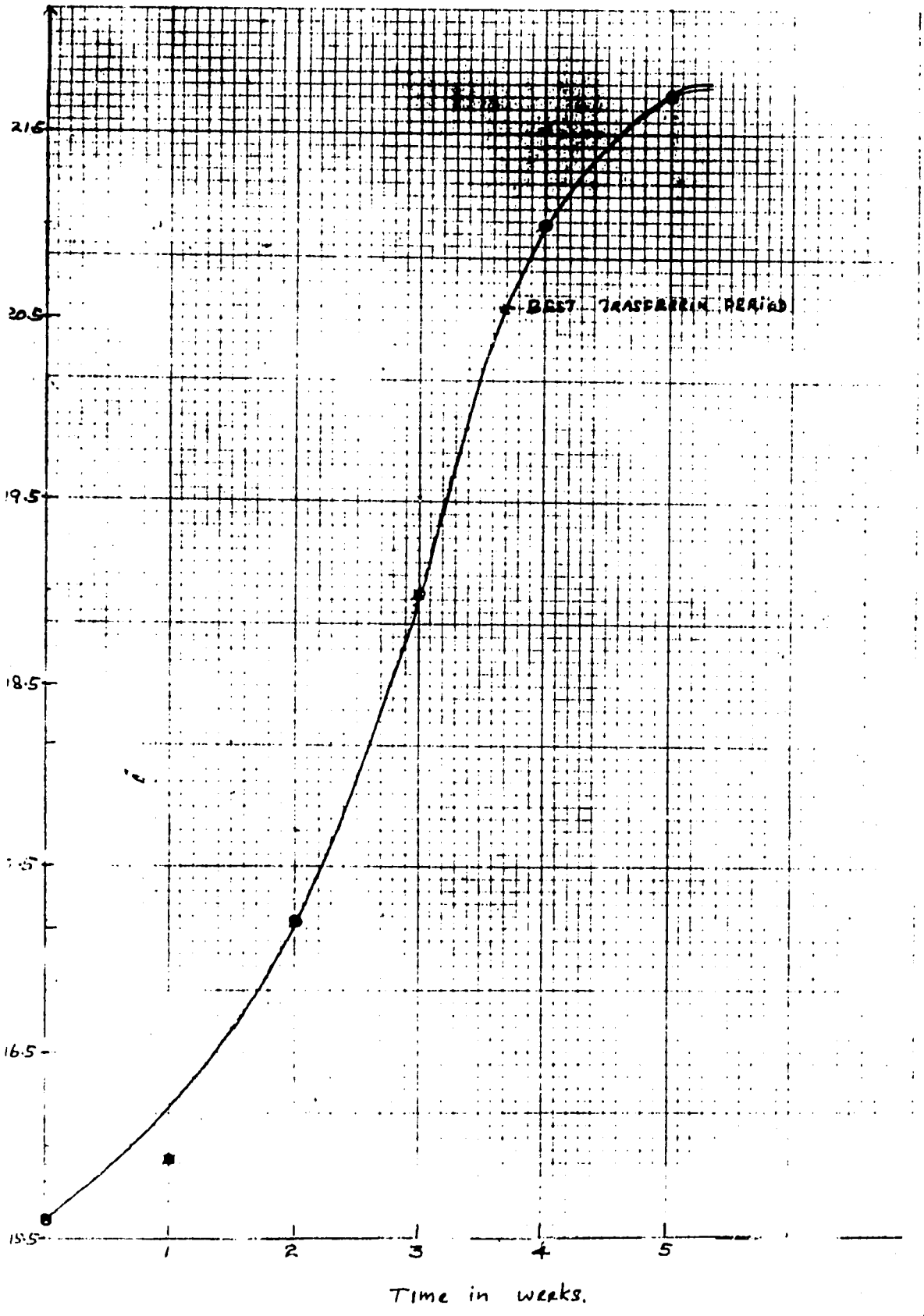
Second portion from the apex showed the highest number of axillary buds, and the first portion showed second highest.

Experiment No. 111

Objective: To find out a suitable transferring period.

Materials & Methods: Ten numbers of shoot tips were allowed to grow in medium containing 1.5 mg/l BA and individual length was measured at weekly intervals.

Results: Growth rate showed normal sigmoidal curve.


21.5
20.5
19.5
18.5
16.5
15.5
1
2
3
4
5
BEST TRANSPLANTING PERIOD
Time in weeks.

Experiment No. IV

Objective: To find out a suitable medium for root initiation and elongation

Materials & Method: In vitro grown about 2 cm long shoots were placed in medium containing concentrations of I BA or NAA with or without 2% charcoal (0, 0.2, 0.5, 1.0, 2.0 mg/l).

Number of roots initiated, length and physical characters were recorded.

Results:

Table - 111

Effect of auxins and activated charcoal on rooting of nutmeg

Auxin	Concentration mg/l	Shoot length cm	Without Charcoal		With 2% Charcoal		
			No. of roots	Root length cm	Root length cm	No. of roots	Root length cm
NAA	0	2.9	Nil	-	3.2	3	2.14,2.0,3.2
	0.2	2.8	Nil	-	2.9	2 (Thick)	2.2,1.2
	0.5	1.2	Nil	-	2.2	1	1.1
	1.0	1.3	Nil	-	2.9	2 (Thick)	2.8,1.6
	2.0	1.6	Nil	-	2.5	2 (1 Thick)	1.6,1.8
IBA	0	1.5	Nil	-	1.7	1	0.8
	0.2	1.5	Nil	-	2.8	4(2 Thick)	2.3,1.9,1.5, 0.5
	0.5	2.4	Two	0.78,0.58	1.5	1	0.9
	1.0	2.8	Nil	-	1.9	1(Thick)	0.5
	2.0	2.6	Nil	-	2.6	1(Thick)	4.9

Better root initiation and elongation was seen in medium with charcoal & either NAA or IBA.

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B) Experiments with shoot tips from Matale Research Station, about seven year old plants.

Experiment No. 1

Objective: To find out basic hormonal requirements for establishment, proliferation and rooting.

Materials & Methods: Shoot tips about 2-3 cm long were introduced to medium containing all possible combinations of BA (0, 0.5, 1.0, 2.0, 5.0 mg/l) with NAA (0, 0.2, 0.5 mg/l)

Results: Showed response quite different from earlier (see report, 1986 first half year). Axillary bud break and very slow growth observed in medium containing BA 2.0 with NAA 0.5. But axillary buds didn't grew further.

Experiment No. 11

Objective: To find out the effect of GA_3 for bud elongation.

Materials & Method: Five concentrations of GA_3 (0, 0.1, 0.2, 0.5, 1.0 mg/l) were used for this study. Basal medium selected was Anderson's with BA 2 mg/l and NAA 0.5 mg/l. Results as in above experiment.

Results: No elongation observed in any of the combinations. These experiments will be repeated using lower concentrations of GA_3.

C) Experiments with grafted plants

Experiment No. 1

Objective: To find out basic hormonal requirements for establishment, proliferation and rooting.

Materials & Method: Prepared medium with all possible combinations of BA (0, 0.5, 1.0, 2.0, 5.0 mg/l) with NAA (0, 0.2, 0.5 mg/l) and introduced GA_3 pre-treated shoot tips.

Results: Trial is continuing

Experiment No. 11 - Leaf culture experiment is continuing

D) Shoot tips directly from mother plants

Difficult to establish in medium, main reason being the large amount of contaminants.

CLOVE

A) Experiments with 1-2 month old seedlings.

Experiment No. 1

Objective: To find out the basic hormonal requirement for establishment.

Materials & Method: Prepared medium with all possible combination of BA (0, 0.5, 1.0, 2.0, 5.0 mg/l) with NAA (0, 0.2, 0.5) and introduced shoot tips about 1-2 months old.

Results: Successful establishment was found in medium containing 2 mg/l BA.

B) Experiments with 2 year old seedlings.

Shoot tips were introduced to
15 possible combination of BA and NAA but failure to establish.
Cultures gradually turn brown and finally dead.

C) Experiments with shoot tips directly from mother trees.

Introduced four different stages of clove shoot tips i.e.

i) Actively growing shoot tips
ii) Induced shoot tips
iii) Shoot tips with two green leaves
iv) Dormant shoot tips

to the medium contained 15 possible combination of BA and NAA.

Results: Observed only dormant buds were alive for a maximum of 7 days.

D) Experiment with Epicotyl/Hypocotyl/Cotyledone of Clove

Epicotyl and hypocotyl segments were introduced to medium containing all possible combination of 2,4 - D (0, 0.25, 0.50, 1.0, 2.0, 5.0 mg/l) with kn (0, 1.0, 2.0, 5.0, 10.0 mg/l). All treatments were kept in dark.

Results: Experiment will continue.

5. Conclusion drawn from the work.

Nutmeg juvenile

1. Anderson's medium with 1.5 mg/l BA showed optimum proliferation for the in vitro grown nutmeg cultures. Fig. 1
2. Suitable transferring period was found to be 3½ - 4 weeks.
3. The shoot portion of 2.5 to 3.5 cm from the apex produced maximum number of axillary buds. Fig. 2

4. IBA or NAA with charcoal produced roots.

Clove - juvenile

1. Juvenile shoot tips of clove could be established in Anderson's medium with 2 mg/l BA. Fig. 3

Clove - Adult trees

1. Comparatively dormant shoot tips showed slightly better establishment than actively growing and induced shoot tips. Fig. 4

Is the work on schedule? Yes

7. Plan of work for the next half year:

Nutmeg

a) Experiments will be carried out to compare the rate of multiplication in solid medium and liquid agitated medium.

b) Rooting ability with Pluroglucind will be evaluated.

c) Tests will be carried out to select best potting medium and acclimatization condition.

d) Investigation of the possibilities of production of somatic embryos, through cultures of epicotyl/hypocotyl sections in appropriate medium.

e) More experiments will be done with mature/grafted materials.

Clove

a) Optimum rate of proliferation, transferring period and rooting experiments will be examined.

b) Production of plantlets through leaf and anther filaments will be examined.

c) More experiments will be done with mature materials.

8 Any other comments: I am experiencing long delays in the supply of chemicals and equipment from foreign sources, and this is affecting my programme adversely.

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Y.M.H.B. YAPABANDARA
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Minor Export Crops Research Station
MATALE.

Signature of grantee/s:

1. Y.M.H.B. Yapabandara .....

2. Pref. M.D. Dassanayake .....

Comments (if any) of Head of Department/Section:

Recommended please

.....
Signature of Head of Department

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Fig. I - Proliferation of nutmeg cultures in Anderson's medium with 1.5 mg/l BA

Fig. 2 - The shoot portion of 2.5 to 3.5 cm from the apex produced maximum number of axillary buds in Anderson's medium with 1.5 mg/l BA

Fig. 3 - Clove shoot tips established in Anderson's medium with 2 mg/l BA

Fig. 4 - Very few number of cultures established with respect to shoot tips from mature clove trees.

Evaluation Report by Prof R.E. Litz from University of Florida, USA March/April 1987

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PROJECT : **IN VITRO PROPAGATION OF CLOVE, NUTMEG AND CINNAMON**

PRINCIPAL INVESTIGATOR : **Y.M.H.B. Yapabandara**
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Matale

This Project has made significant progress in the development of in vitro procedures for regenerating clove, nutmeg and allspice. All of these spices are known to be recalcitrant and dificult to regenerate from cultured tissues. Working primarily with juvenile explants that possess lower degree of determination. Mr. Yapabandara has demonstrated that it is feasible to regenerate whole plants from cultured shoot apices and nodes of nutmeg and allspice. He has established the protocols for stimulation of axillary bud proliferation in vitro in similar cultures of juvenile material of nutmeg, allspice and clove. Although he has not yet been able to repeat this controlled pattern of development with clonal selections, he has established the basic parameters for the procedure and has demonstrated the potential for micropropagation of these three spicies.

On the basis of his accomplishments to date, I strongly recommend that Mr. Yapabandara should publish the results of his preliminary studies involving the use of juvenile plants as explant sources. This work is of sufficient quality that it should not be difficult for it to be accepted and published in a major, international refereed journal. As Mr. Yapabandara has accumulated more data in his studies with nutmeg, this research could probably be presented in "Plant Cell, Tissue and Organ Culture". The studies involving allspice could be written as a short communication for "Horticulture Science". After whole plants have been regenerated from cultured model segments of juvenile clove. This research can also be published. Since none of the published articles will appear in the journals for at least a year, and since the techniques do not describe procedures that are appropriate for mature, clonal tree selections,

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Mr. Yapabandara will be able to ensure confidentiality of his current studies involving mature tissues as explants.

At this time this project is attempting to transfer the information that has been gathered using juvenile, i.e., embryonic and young seedling tissues as explants to the more important and more difficult problem of regeneration from explants of mature trees, i.e., trees at the flowering or mature stage of development. In the transition from juvenile to mature phases of development, the vegetative growth of trees becomes fixedly determined along a specific pattern. As the age of the tree increases, this pattern becomes more difficult to reverse to a more juvenile or plastic developmental pattern. This problem, as it is expressed in vitro, is the intransigence of excised shoot tips from mature trees. These explants, unlike comparable explants from juvenile or embryonic sources, do not readily respond to growth regulators which stimulate axillary bud proliferation or subsequent plantlet regeneration. The problem has been defined as the ageing of plant meristems. Mr. Yapabandara must now face this problem with clove, nutmeg and allspice. Is it possible to reverse the natural ageing of meristems in these spices? Is it feasible to induce this phase change in vitro by manipulating the growth regulators or is it necessary to stimulate juvenility in mature trees by other means?

During the next phase of his research Mr. Yapabandara must address this question in order to develop alternative propagation methods for spice trees. In discussions with him, we have concluded that certain experiments should be conducted, and these are briefly discussed and justified.

Nutmeg - Explants from mature trees, including fairly large nodal cuttings, have not survived in vitro. Therefore, it may be necessary to manipulate the stock plant trees in order to induce a degree of phase change.

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1. Using mature scion material grafted successfully onto seedling rootstocks, the scion should be hard pruned to permit vigorous growth as close to the graft union as possible. The newly formed vegetative shoots should then be regrafted onto seedling rootstock. This sequence should be repeated indefinitely. The new growth, after each regrafting cycle, should be tested for the *in vitro* response. Re-establish ment of juvenility in the scion can be measured by the successful establishment of nodal cultures *in vitro* and by the ability to induce rooting of scion cuttings. It is obvious that this study will be labour-intensive and will require considerable plant material, both seedlings and scion cuttings at the outset, and seedlings at appropriate intervals during the course of the investigation.

2. It is possible that the stock plant can be manipulated in other ways. For example, the cut end of detached 1½ foot branch terminals can be inserted into dilute nutrient solution containing benomyl for several days. The foliage should be sprayed at 2-3 day intervals with benzyladinine (approx. 100 p.p.m.). After determining the maximum period of survival under these conditions, remove nodal sections at intervals, and culture *in vitro* on Anderson's medium containing 1.0 - 2.0 mg/liter benzyladinine.

3. A method for rejuvenation of plant material that is being used increasingly involves the technique of micrografting. Nutmeg seeds should be germinated under sterile conditions *in vitro*. Similarly, nodal explants from mature plants should be conditioned *in vitro*. After seeds have germinated the seedling should be decapitated below the cotyledons. Using a simple Cleft-type grafting method, the conditioned shoot tip from the mature tree should be inserted into the prepared hypocotyl. If the micrograft is successful, juvenility in the scion will be expressed as the increased rooting potential (*in vitro*) of cuttings from the scion. Repeated or segmential micrografting of the established scion onto *in vitro* grown seedlings may be necessary.

Because it is possible that rejuvenation of nutmeg may be unsuccessful, it is important that other potential regeneration pathways should be considered. in particular, somatic embryogenesis should be attempted because it may be the only practical alternative and because, unlike shoot tip cultures, somatic embryo derived trees will possess more vigorous root systems that would enhance tree survival under conditions of extreme water stress. Accordingly, we propose that certain research approaches should be followed.

4. Somatic embryogenesis has been described from 2 types of tissues from mature trees, i.e., very young leaves from new vegetative flushes, and from the nucellus. Because of the relative simplicity of studies involving leaves as explants and because of their ready availability, the possibility that somatic embryogenesis can be induced from young leaves should be pursued vigorously. Mr. Yapabandara has already demonstrated that callus and root regeneration is possible from fully expanded nutmeg leaves. Since partial regeneration of this sort is associated with the transition of the explant from juvenility to maturity, it is important that newly emerged leaves, prior to expansion and prior to the stage of development when anthocyanin pigments predominate, should be utilized. Leaflets should be no more than 1.5 - 2.0 cm. in length. Since internal fungal contamination may be problematic, prior treatment of whole, intact leaves in benonyl solution should be considered.

Leaflets should be cultured on a low salt medium such as B5 (Gamborg et.al) containing benomyl. The final culture medium for induction of possibly embryogenic callus should consist of B5 with 60 g/liter sucrose,, 400 mg.liter glutamine, 100 mg/liter ascorbic acid and 2,4-D together with kinetin, each of which should be used at 0, 0.5, 1.0, 2.0 and 3.0 mg/liter in all possible combinations. This procedure has resulted in embryogenic callus production from mature leaves.

of coffee (Sondahl et.al,, 1977), soapnut (Desai et.al, 1986) and longan (Litz, 1987). Embryogenic callus should be recognizable after approximately 3 months.

5.- The nucellus of nutmeg may also provide an emyryogenic explant and may be a practical approach for regenerating nutmeg. It is important for this pattern of embryogenesis that the ovule should be relatively large, and easily extracted from the immature fruits. It is equally important that the stage of ovule development should be followed closely, because the nucellus deteriorates as the zygotic embryo increases in size. Accordingly, the stages of ovule development up to an including that stage at which the zygotic embryo occupies no more than 1/3 - 1/2 of the ovule cavity should be identified and those young ovules used for nucellar culture studies. The condition of the endosperm is also an important determinant and should still be at the liquid phase of development.

Entire fruitlets of the appropriate developmental stage should be surface-sterilised in a strong (20-30% v/v) commercial bleach solution. After washing with sterile water, bisect the fruitlet and remove the ovule. Bisect the ovule, remove and discard the zygotic embryo. It is probable that removal of the mucellus will result in increased polyphenol oxidase activity and death of the tissue. Therefore, the 2 ovule halves should be left intact. It is advisable that initially the ovule halves should be cultured in liquid plant growth medium in test tubes in the dark in order to suppress blackening of the tissue. The medium should be B5 containing 30 g/liter sucrose,10% (v/v) filter-sterilized coconut water, 400 mg/liter glutamine and 100 mg/liter ascorbic acid. After 1-2 weeks, the ovule halves should be transferred onto solid medium of the same composition, but which contains, in addition, 0.5-2.0 mg/liter 2,4-D and 0.7%. The ovule halves should be cultured so that the nucellus is in contact with the solid medium (The nucelluslines the embryo sac). If the medium incontact with the explant blackens, the tissue should be

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resituated on the medium. If nutmeg nucellus is embryogenic, callus and somatic embryogenesis should be evident after approximately 2 months.

Cloves - As we have discussed above (with nutmeg), nodal and shoot tip cultures derived from mature clove trees have not survived explanting. Any potential for manipulating stock plants of clove in order to stimulate phase change from maturity to juvenility must be limited by the sheer impossibility of vegetatively propagating clove trees. Marcottage, grafting and rooting of cuttings have met with little success with these spices. Nonetheless, we feel that it is imperative to exhaust all possible approaches for rejuvenation of clove stock plants. Juvenile shoot tips and nodal explants appear to establish themselves readily in vitro, and Mr. Yapabandara has obtained axillary bud proliferation from these explants. At this point, it is of crucial importance to induce adventitious roots from these cultures in order to demonstrate if any clove-juvenile or mature - has this organogenetic potential.

1. Most importantly, because coppicing has been shown to be effective for stimulating dorment vegetative buds from mature trees, shoot tip and nodal cultures should be established from these plants. Because of various factors that seem to interact and inevitably result in nodal explant death, a modified experimental design should be adopted that would permit the investigation of several growth limiting parameters in a single experiment. Accordingly, a three dimensional multi-factorial design approach should be adopted to examine the interaction of dark vs light growing conditions, different anti-oxidants, e.g., ascorbic acid, PVP, activated charcoal, cysteine, nitrogen (nitrate and ammonium) levels, salt concentration, explant size, sterilization procedure,etc.

2. Although the probability of success would appear to be low, micrografting of clove should at least be attempted. If initial trials appear to be fruitless, this approach should not be continued.

Increased emphasis should be placed on attempts to regenerate clove *via* alternate pathways, e.g., somatic embryogenesis and organogenesis from different explant types.

3. Because somatic embryogenesis has already been described from nucellar and/or integumental tissue cultures of two other *Syzygium* species (*S. malaccensis* and *S. jambos*) (Litz, 1984), the morphogenetic potential of clove (*S. aromatium*) nucellus should also be studied. Unlike nutmeg, the seeds of clove are relatively small, and ovules at the appropriate stage of development for bisection and culture of the nucellus are very small. Generally, however, only a single clove ovule is furtilized, whereas there are many ovules within a single ovary. By carefully removing the unfertilized ovules under sterile conditions and culturing them whole,it may be possible to stimulate the formation of embryogenic nucellar or integumental callus.

The procedure should involve the following steps : Initially, it is important to determine the earliest stage of fruit development at which it is possible to recognize and excise easily the fertilized ovule. This ovule will appear swollen. It can be cultured separately as the immature zygotic embryo should be able to form an embryogenic callus. Having recognized the appropriate stage of fruit development, the fruitlets should be surface-sterilized, and the sterile fruitlets subsequently bisected. The mass of unfertilized ovules can be removed with the blunt edge of a spatula, taking special care not to slice or otherwise injure them. The sterile ovules can be cultured on plant growth medium (B5) containing 10% (v/v) filter-sterilized coconut water, 400 mg/liter glutamine, 60 g/liter sucrose, 0.5- 2.0 mg/liter 2,4-D. 60g/liter sucrose and 0.7 g/liter agar. The cultures should be maintained in darkness.

If the nucellus of cultured unfertilized ovules or the zygotic embryo of fertilized ovules forms embryogenic callus, it will burst through the delicate ovule wall. The

time frame should be 2-3 months. Because of the relative ease whereby clove ovules can be excised and cultured, this approach should receive a high priority.

4. The induction of somatic embryogenesis from callus derived from young leaves in newly formed vegetative flushes should also be attempted. The same procedure outlined in the nutmeg section (see 4 above) should be followed.

5. Other explants and the responses of these explants to different growth regulators should be examined, e.g., cotyledons, hypocotyl, etc. It is probable that these explants will form embryogenic or organogenetic callus or medium supplemented with 2,4-D and kinetin.

In summary, this project has made considerable progress with tree species which have been recognised as being difficult to propagate conventionally. The research concerning nutmeg should be published in an international journal as the studies involving juvenile plants have reached certain conclusive results.