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Effect of glutamine for high frequency *In-vitro* regeneration of *Aquilaria malaccensis* Lam. through nodal culture

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Abstract
Aquilaria malaccensis is a most commercially valuable non timber indigenous tree species of north eastern region. The aim of the present investigation was to develop a high frequency clonal propagation through *in vitro* culture from non embryogenic tissue. Stem segment having one node inoculated in the MS media supplemented with different hormonal concentration and optimum shoot regeneration was obtained from BAP 0.5and NAA0.5 mg/l with glutamine fortified media. Glutamine with lesser concentration 20mg/l produced highest (24.81±0.74) multiplied shoots and enhancing up to 32 number of shoots in course of fifth sub culture without producing any callus. Glutamine plays an important role in shoot regeneration and early leaf drop. Among the several rooting experiment shoots with highest (64.74±0.87) percentage of well developed root system was observed within 8 weeks of culture in vermiculite of IBA 1.5 mg/l. Healthy rooted plants were established in the field condition after proper hardening.

Keywords: nodal culture, vermiculite, glutamine, mass multiplication

1. Introduction
Aquilaria malaccensis belongs to the family Thymelaeaceae is a medium to large sized commercially valuable non timber indigenous tree species of north eastern India and locally known as *'Sasi* '. The genus is distributed in southern Asia from India to China and throughout most of Southeast Asia, Whitmore 1972 [38]. In India it is distributed in the hills of Assam, Meghalaya, Nagaland, Manipur and Tripura, Annon 1948) [2]. The tree grows in natural forests at an altitude of 1000 meters above in Nagaland, Mizoram, Manipur, Arunachal Pradesh, Tripura and West Bengal and especially in Assam and Meghalaya. Sporadically it occurs in the district of Sibsagar, Sadiya, Nowgong, Darrang, Goalpara, Garo Hills and Cachar by Atal and Kapoor, 1982 [3]. The wood of the tree forms 'agar oil' which is one of the most valuable essential oil infected some fungi and the tree is famous for this black oleo resinous deposit of the valuable essential oil which is the most expensive stabilizing agent used in perfume industry Soehartono and Newton 2000 [32]. Trees were harvested from the tropical forest which is confined to a few forest areas and some home stead gardens of upper Assam in the North east. High economic value of agar wood is the main reason for collecting it by several traders for manufacture of agar oil. Most of all agar being traded and are collected from the natural forest. Due to the increasing interest in agar and the over exploitation it is an urgent measure required to conserve and replenish the plants growing in the wild. Hence, plantation is steadily increasing among the local people in Assam. It can be propagate through seed but seeds are recalcitrant Kunduand Kachari, 2000 [18] and start losing viability under ordinary storage in July to August and completely after 30 days Ahmed and Gogoi 2000 [1]. Good seed years are infrequent hence, not expanded to commercial scale due to seed scarcity and poor seed viability Bhaskar 1984 and Beniwal 1989 [7, 4]. Beside this, the attack of leaf defoliator from April to July at the time of flowering is a massive problem in Upper Assam area and sometimes it happens for three to four times and there is no flowering at all and there by slowing down the natural regeneration. Due to huge business interest of agar traders, there is a problem of over exploitation of agar trees. So, the natural resource made stock exhausted and also the agar bearing trees in the forest became scarce. Hence, considering the importance of this valuable species the present study was conducted for clonal multiplication through *in vitro* culture and to establish under field condition and tissue culture may be employed as a

successful tool for rapid and mass propagation of selected genotypes for future plantation programme.

2. Materials and Methods

For present investigation, preliminary study was completed through survey and selection of genetically diversified plants of *Aquilaria malaccensis* from natural populations of different locations of upper Assam as per considering their infection frequency and established seedling seed orchard (SSO) at RFRI, Jorhat, Assam. First step for initiation of cultures, sterilization of explants were done with two different sterilizing agents namely sodium hypochlorite solutions and mercuric chloride with different concentration (0.1- 0.5) and dipping time (5-10 min.) and then washed thoroughly with running tap water for 10-30 min to remove the impurities and dust particles. Then washed with 0.1% Tween 20 solution and at last rinsed with distilled water. Explants were prepared into appropriate sizes like shoot apices 5-6 mm, node 8-9 mm respectively. Following surface sterilization both exposed ends were trimmed and the remaining segments were inoculated singly and vertically into the prepared medium. The preparation of MMS medium was exactly same proportion of MS basal media only difference with the supplementation of vitamin i.e. the thiamine HCl was 10 mg/l in place of the three standard vitamins used.½ strength of MS medium was prepared by adding equal volume of DDH_20. The pH was adjusted at 5.6- 5.8 by using 1N KOH or 1N HCL prior to addition of agar and autoclaved at 15 p.s. 121^0C for 15 minutes. Observations were taken after 7 days interval for an incubation period of 30 days. Replicates of 5-10 cultures were set up for every experiment. Each experiment was repeated at least twice. Incubation condition was maintained at 25 ± 2^0C and 60-70 per cent relative humidity. The light source was florescent tubes, Bajaj, 40 Watts emitting at an intensity of 3000 lux approximately at culture levels for 16 hours light and 8 hours dark in a 24 hours cycle. Controlling of phenol with PVP 1.0-2.5g/l, activated charcoal in different concentration of 0.5-2.0g/l was applied. After successful induction of shoots, they were excised from initial explants and aseptically inoculated to sterile nutrient media. Regenerated shoots were maintained and sub cultured in the same media after 6 weeks of culture.

First experiment was set up for the selection of optimal basal media viz. MS, MMS, WPM, and 1/2MS supplemented with BAP 0.25 mg/l and the results were evaluated on the basis of nature and morphology of shoots, shoot induction period, percentage of culture response and number of shoots per explants cultured. After selection of optimal basal media with best source of explants, combined and singular effect of auxins and cytokinin were tried for optimum shoot regeneration and growth and results recorded for effect of different hormonal concentration of shoot induction after 4 weeks on incubation. For multiple shoot initiation experiment was conducted with different hormonal concentration in MS basal medium such as various levels of BAP alone 0.5, 1.0, 1.5 and 2.0, BAP; NAA 0.5, 1.0, 1.5 and 2.0; BAP and NAA 0.5, 1.0, 1.5 and 2.0 with 50 mg/l glutamine and without PGR. The observation was made in relation with culture response on multiple shoots, intensity of shoot development, total number of multiple shoots per culture and their growth. Regenerated multiple shoot clumps were separated and each clump containing 3-4 shoots were transferred to fresh medium of same composition in and the process was repeated up to one year for obtaining large number of shoots. For shoot growth and elongation MS+ $GA_3$1.0+adenine0.2 fortified medium was used. Selection of best nodal position and best season for collection of explants for mass multiplication in optimal media concentration MS+BAP0.5+NAA0.5+ glutamine 20 mg/l was laid out to study the potentiality of different nodes on shoot regeneration. For best season nodes were collected from new shoots on pruned branches during the period of Feb-April, May-July, August – Oct, Nov-Jan. Data recorded at the end of the 4^{th} week. For rooting four sets of experiments 4.0-5.0 cm with 3-4 leaves in MS semi solid medium in agar 7% was supplemented with IBA, IAA and NAA alone 1.0-3.0 mg/l, second set with higher concentrations with 5.0, 10.0, 15.0, 20.0, 25.0 mg/l, in third set shoots were pre treated for 24 and 48 hrs with the following conc. IBA 0.5, 1.0, 1.5; IAA 0.5, 1.0, 1.5 and NAA 0.5, 1.0, 1.5 and in the last experiment IBA 0.5, 1.0, 1.5; IAA 0.5, 1.01.5; NAA 0.5, 1.0, and 1.5 were inoculated in sterilized vermiculite. All the experimental culture tubes were incubated with 16 hrs photo period light. The percentage of root induction, number of roots and root length were recorded at the end of the ten weeks and individual shoots grown successfully transplanted into the soil.

2.1 Statistical analysis

Data collection and analysis during the experiments of shoot initiation as well as shoot multiplication, observation on percentage of nodal segments sprouting, number of shoots per node explants and shoot length were recorded. During rooting, data collected were percentage of rooting, number of roots per shoot and root length after root initiation. Ten explants were cultured per treatments and all experiments were quantified and data were subjected to l analysis using 'standard error of the mean'.

Considering the explants sources like shoot apices, node and petiole secretion of phenol possess much more.

3. Results

Selection of optimal basal media and the best source for shoot regeneration**,** the first set of experiments with tender nodal segment and their pre existing axillary meristems were induced to proliferate 2- 3 shoot buds after 2 weeks of incubation, when cultured on different basal medium supplemented with BAP 0.1mg/l (Table 1). This initial regeneration rate of shoots was varied as per explants and node had shown better regeneration in comparison to others. Repeated culture of axillary and adventitious shoots, cutting with nodes organs from first set of experiments leads to multiplication of propagules in large numbers and produced at this stage can be further used for multiplication by their repeated culture. So far nutrient basal is concerned, efficient organogenesis was recorded from both the media tried i.e. MS and WPM. WPM performed better in shoot induction in case of node explant culture. However, leaves were pale green and good growth of shoots as compared to MS. In half strength of MS media, callusing was observed from the lower part of the shoots touches the media.

3.1 Shoots regeneration

Initial experiment to determine the influence of explants collection in different season, bud breaking shows a variation in terms of regeneration which was observed very promisingly. The best period Feb- April was selected for the collection of explants from the donor plant with highest % of bud breaking 90.32±0.41. In the month of May- July shoots also possess very good result with 81.45±0.66. Bud breaking of drastic reduction of four fold was observed in the month of

Aug-Oct 25.63±0.44 and very poor regeneration in the month of Nov-Jan i.e. 10.23±0.39. The results plotted in the (Fig.2) have clearly indicated that the collection period of explants have played a major role in bud breaking of the species.

Shoot having one node inoculated in MS media supplemented with different concentration of plant growth regulators results presented in the (Table2). New shoots emerged after 15-20 days of incubation from different replicates. In consideration with the application of BAP alone possesses lesser number of shoots and increased conc. from 2.0- 4.0 mg/l showed abnormal shoots growth. In combine effect MS+BAP0.5+NAA0.5+glutamine 20 mg/l was recorded maximum number of shoots 2.41 ± 0.74. Shoot regeneration in BAP0.5+NAA0.5 without glutamine shows 1.53 ± 0.62 number of shoots but after 3 weeks of continuous incubation of culture shows early leaf drop and shoots did not possesses further regeneration. In combination BAP0.5+$GA_3$1.0 when supplemented in place of NAA shoots regenerated as 0.84 ± 0.81 possesses lower number of shoots confirming that application of GA_3 did not played a major role in shoots regeneration. But addition of NAA with GA_3 i.e. BAP0.5+$GA_3$1.0+NAA0.5 possesses 1.23 ± 0.90 number of shoots. Among the different media concentration, without BAP i.e. $GA_3$1.0+Kn0.2 shows 1.31 ± 0.71 and shows callusing at the lower part of the shoots touches the media. In case of NAA with BAP possesses similar type regeneration but later shoots was inflamed and glassy type. In case of shoot elongation and growth GA_3 in combination with adenine $GA_3$1.0+ adenine 0.2 without BAP recorded as highest 1.52 ± 0.23.

3.2 Multiplication

Shoot multiplication from nodal shoot segments isolated from *in- vitro* grown shoots is inoculated with different concentration (Table 3). But in case of singular effect BAP was not responded for satisfactory result. Shoots regeneration was recorded lower in the combinations of BAP alone and in auxin and cytokinin with increased conc. of BAP 1.0-2.0 mg/l. Singular effect of cytokinin with BAP shows lesser regeneration than in combination with the auxin NAA. Incorporation of NAA along with BAP has been possessing better result than in application of BAP alone. But incubation of culture in the above media shows immature leaf dropping of shoots after 4 weeks and gradually died. The addition of glutamine in the media possesses nice, green and healthy growth of shoots and multiplied to highest 4.51±0.42 number of shoots in the media containing MS+ BAP0.5+NAA0.5+Glutamine20mg/l and a total number of shoots 24.81±0.74 with a shoot length 1.12±0.56 were observed. The proliferation rate did not decrease in the successive subculture of individual shoots or clump of shoots (3-5 shoots) with multiple shoots 18-20 on the same medium even after more than a year. The best node for mass multiplication was observed highest number of shoots induction 4.31±0.43, nodes/shoot 5.12±0.33, internodal length 0.23±0.33 (Table 4 and Fig.1). The decreasing position of nodes has shown better performance in shoot proliferation, which was recorded up to the 4^{th} node only. A drastic reduction in regeneration potentiality was recorded from 5^{th} node explants.

3.3 Rooting of shoots

Rooting was observed in the conc. of IBA 1.5 mg/l 64.74±0.87 with 6.41±0.61number of roots and a length of roots 2.42±0.56. The other lower conc. of IBA i.e. 0.5 mg/l not responded in rooting and 1.0 mg/l also possess poor result. In case of IAA failed to root. NAA 0.5 mg/l possesses satisfactory result where rooting % was 60.23±0.78 with 4.22±0.75 number of roots with 2.54±0.74 in length (Table 5). Rooted shoots were transferred into the field after proper hardening. No plantlet mortality was observed after transplanting

4. Discussion

Selection of optimal basal media and best explants source for auxillary bud breaking based on earlier report on *A. malaccensis* from seedling sources by Meng Ling *et al.* 2005 [21]. He studied the plant regeneration via organogenesis from shoots developed from *in-vitro* seedlings sources and plantlets were survived, acclimatized and grew well. Tissue culture of *Aquilaria* species was reported by Hassan *et al.* 2011 [13] studied on *A. hirta* for the effect of 6-benzylaminopurine in different basal media on shoot multiplication of *in vitro* shoots. Tiengtum, P 1995 [36] in his findings reported on **i**n vitro culture of agar wood trees**.** Shoot tips and lateral buds from 2 species of agar wood trees of *A. crassna* and *A. malaccensis* were cultured on WPM and modified MS medium with half-strength of nitrate supplemented with BA, 2iP and kinetin. Both media supplemented with cytokinin promoted growth and shoot proliferation. BA was the most effective for stimulating shoot multiplication, followed by kinetin. The well rooted *A. crassna* plantlets was 90 percent survival rate of when transplanted in nursery. But shoots of *A. malaccensis* failed to root. The age of the explants is more important for the induction of shoots. Physiological age of source of explants played an important role for obtaining the successful regeneration results. Murthy and Saxena 1994 [23] recorded the age of the explants in *Arachis hypogea*. In our investigation season of explants collection influenced shoot development from individual explants, this fact may due to long flowering and seed setting habit of trees. In our study explants collected from 1 to 2 years old established mother plants produced almost similar results whereas the explants collected beyond this age failed to induce bud breaking even if cultured in the selected optimal media for the species. Similar to this, Sharma *et al.* 2003 [23] observed in accordance with in *Casurina andansoni* where initiation is highly influenced by season. Composition and strength of the basal medium plays an important role in shoot multiplication and elongation as well as root induction reported by Borthakur *et al*., 2000 [9]. This is also applied to *A. hirta* cultures. The difference observed between MS and WPM medium could be attributed to the high content of nitrogen in the MS medium Fracaro and Echeverrigaray, 2001 [10]. MS was higher level of nitrogen, phosphorus as compared to WPM medium. Nitrogen may function as a single molecule of plant growth through increased gene expression for enzyme responsible for the uptake and utilization of nitrate Mashayekhi 2000 [20]. Hence, result in case of half strength of MS basal salts do not possesses any shoot regeneration it may be due to low concentration of basal salts. In the present investigations, it has been optimizes the shoot multiplication conditions and novel rooting techniques for much clonal propagation without interference of callus phase. This method is quite common for the propagation of several species like *Fragaria indica* by Indre and Dhar 2000 [14], *Accacia mearnsi* by Marguerite *et al.* 2001 [19] etc. Addition of low conc. of NAA promoted shoot multiplication and growth by counteracting the inhibitory effect of BA on shoot elongation. The combine use of BA and NAA also emphasized in micropropagation of

Ficus carica, Kumar *et al.* 1998 [17] and *Syzygium travancorium*, Ajith *et al.*, 1999 [5]. Use of glutamine in the medium proved to be superior by producing maximum number of shoots per proliferated explants it is significantly higher than that other growth regulator concentration. Reduced nitrogen forms especially the glutamine present in the medium and is absent in the other medium is growing evidence for the usefulness of supplemented amino compounds in the culture medium, which can enhance cell division, differentiation, growth and development of multiple shoots *in vitro* Ramage 1999 and Sotiropoulos *et al*. 2005 [25, 33]. It has been reported that the number of shoots produced by explants was correlated with the amount of phosphorus absorbed by the explants of many tree species Sharma and Thorpe, 1999 [28]. The effects of amino acids on regeneration of cultures were raised on MS medium containing different concentration and combinations of growth regulators BA and NAA. Vasanth *et al*. 2006 [37] when comparing the individual concentrations of BA and NAA the addition of glutamine enhanced maximum number of multiple shoots. The enhancement of the growth rate by glutamine could be explained on the basis that glutamine provided a readily available source of nitrogen, the implication being that the formation of the necessary carbon skeleton or the reduction of nitrate to ammonia is a limiting factor in the cells Gamborg, 1970 [12]. Addition of glutamine which is relatively non-toxic by Gamborg and Miller, 1968 [11] would enable the cells to maintain a high growth rate for a longer period. Glutamine was metabolized extensively to glutamate in the supplied leaf and recovery of L-glutamate as the main component of the young leaves transport in this form. In the present investigation, amino acids may play a vital role in the induction and development of maximum number of multiple shoots. Culture media plays considerable influence in successful plant regeneration and multiplication. Ahmed *et al*. 2002 [4] reported the effect of growth regulators can be strongly modified by the medium on which the cultures were grown. The effect of plant growth regulator BAP plays a major role. Hassan *et al.* 2011 [13] reported the effect of BAP in different basal media on shoot multiplication of *A. hirta*. In his study, he reported the effect of BAP in different basal media like MS and WPM on shoot multiplications of *A. hirta* using nodal segments from young seedlings. He also reported that MS medium supplemented with 0.1 mg/l BAP was the most effective for multiple shoot formation. In our study the combine effect of BAP along with NAA shows better result with new shoots produced within 30 days of culture in MS. It was observed that the number of shoots produced decreased as the BAP concentration in the medium was increased. A reduction in shoot number with increasing cytokinin concentration has also been reported by Joshi and Dhar 2003 [15] proliferated shoots of *A. hirta* in MS supplemented with 0.1 mg/l BAP. According to them shoot elongation is retarded in cultures with increased number of shoots. In this study, it was observed that MS medium favoured the shoots than other medium for shoot multiplication. The need of MS salts for shoot sprouting and multiplication shows the high salt requirement for the growth and similar with *A. hirta* shoots. Combined effect of cytokinin towards increase of shoot buds was reported earlier by Anderson 1984 and Sriskandarajah *et al*. 1990. Tiengtum P 1995 [6, 34, 36] in his findings reported on in vitro culture of agar wood trees of *Aquilaria* species from shoot tips and lateral buds from 2 species of agar wood trees *Aquilaria crassna* and *A. malaccensis* were cultured on woody plant medium and modified MS medium with half-strength of nitrate supplemented with BA, 2iP and kinetin at different conc. He reported that BA at high levels 1-4 mg/l produced more number of shoots that did not elongate. In our findings also possess the high concentration of BAP 4-6 mg/l produced no shoots. Several earlier workers reported about the specificity on the effect of various growth regulators which varies from plant to plant. This is in conformity with Monotoro *et al*. 1993 [22] who working with *Hevea brasiliensis* reported that the basic mineral media used could also modify shoots in which media components and plant growth regulators are quite often responsible. However, where shoot growth is concern BAP was not suitable for shoot elongation and their growth. A similar finding was also reported by Kumar and Bhavananda 1988 [17] in case of shoot differentiation from stem tissue. Frequent sub culturing for control of phenol of explants is widely accepted. Explants collected in the month of Feb-March shows less phenolic accumulation in the medium of the material. Results indicated in our study that browning of tissues was observed when explants picked off in different growing seasons and control of this was only done through frequent sub culturing the explants collected in the month of Feb-April. Tao F. *et al* 2007 [35] also reported in his study that twigs of 2 to 3 year old *Platanus occidentalis* L. were used as experimental material to find the causes for contamination and browning in the initial stages of tissue cultures to compare the degree of browning of from trees on each of the first ten days in January, March, May and July. The results indicated that the contamination and browning rates of the material cut off in January and March were somewhat lower than those in July. Tiengtum 1995 [36] was reported on *A. malaccensis* as failed to root. In the present investigation, the rejuvenation of shoots was achieved under *in vitro* conditions by repeated subculture, and rejuvenated shoots better for rooting condition tested. In *Prosopis cineria*, Sheakawat *et al.* 1993 [30] demonstrated the effect of rejuvenation on *in vitro* rooting by using sub cultured shoots. The application of IBA by a pulse treatment was used in adventitious rooting of *Maytenus emarginata* by Rathore *et al.* 1992 [26] and *Tectona grandis* by Siril and Tiwari 1999 [31] and in *Chionanthus retusus* were obtained best results in vermiculite. The *in-vitro* regenerated shoots were rooted in IBA in the medium vermiculite. A similar report of i*n -vitro* regenerated plantlets by Sanjaya *et al.* 2006 reported in their study of endangered Indian sandalwood *Santalum album* L. with higher percentage of rooting in soilrite from micro shoots than that in agar medium with pulsed pretreated IBA for 30 min. Rooting achieved in vermiculite medium it is likely that better aeration, high concentration if IBA and short exposure might have triggered the genes responsible for adventitious root formation.

5. Conclusion

The use of preexisting auxiliary buds for propagation reduces the possibility of variation among the progeny and therefore in the present study it was safely applied for rapid propagation of field grown selected trees of agar trees. Hence, the species has the potential of aromatic plant of this region and rural based cottage industries may developed and could place the achievement in the international market to contribute a great deal towards the economy of Assam, particularly to the wage earning sections of the local people. Mass production through supplementing glutamine in the media may be a new finding for the species. Again, i*n vitro* rooting in vermiculite is a cost effective, innovative method for the rapid clonal propagation of *A. malaccensis*.

Table 1: Selection of optimal basal media and source of explants for shoot regeneration BAP 1.0 mg/l

BM	Source of explants	%CR	No. shoots/explants	Observation
	Node	20.4±3.1	1.1±0.5	Pale shoot development from node explants
WPM	Shoot tip	40.4±2.6	-	Shoot tip explants died gradually
	petiole	-	-	-
MS	Node	79.2±1.9	1.3±2.1	Shoots initiated from nodes possesses good growth
	Shoot tip	75.3±1.5	-	shoot tips dried
	Petiole	-	-	-
MMS	Node	25.4±0.7	-	Nodes remain healthy for one month no shoot production
	Shoot tip	18.7±0.6	-	Yellow, shoot tip has died after first sub culture
	Petiole	-	-	-
½ MS	Shoot tip	-	-	On shoot tip no shoots, only callusing at the basal part.
	Node	-	-	Nodes not responded
	Petiole	-	-	-

Data represent means ± SE

Table 2: Combined effect of different hormonal conc. on shoot induction from node explants in MS media (mg/l) after 4 weeks of inoculation

Treatments	No. of Shoots/node	Shoot length (in cm)
BAP0.5	0.55 ±0.68	0.69±0.20
BAP0.5+$GA_3$1.0	0.84±0.81	1.02±0.15
BAP0.5+NAA0.5	1.53 ± 0.62	0.36±0.61
BAP0.5+$GA_3$1.0+NAA0.5	1.23 ± 0.90	1.31 ±0.52
- +$GA_3$1.0+Kn 0.2	1.31 ± 0.71	1.20 ±0.34
- +$GA_3$1.0+adenine 0.2	0.65 ±0.45	1.52±0.23
BAP0.5+NAA0.5+glutamine 50	2.41 ± 0.74	0.88 ±0.33
BAP0.5+NAA0.5+glutamine100	1.12 ± 1.16	0.92 ±0.66
Data represent means ± SE		

Table 3: Effect of different conc. of optimal media for optimal shoot multiplication of node explants in combination of BAP, NAA and glutamine (mg/l) after 5th subculture

Treatments			% of culture on multiple shoots	No. of Shoots/ node	Total no. of multiple shoots	Shoot length (in cm)
BAP	NAA	glutamine				
0.0	0. 0	0.0	10.00 ±0.58	0.30±1.1	0.00	0.00
0.5	0. 0	0.0	51.34±2.17	0.74±0.04	0.84±0.33	0.93±0.05
1.0	0. 0	0.0	92.10±2.23	0.86±0.07	0.75±0.47	0.63±0.30
1.5	0. 0	0.0	78.67±1.44	0.82±0.24	0.68±0.92	0.89±0.04
2.0	0. 0	0.0	62.33±3.51	0.42±0.87	0.54±0.56	0.95±0.22
0.5	0. 5	0.0	74.83±2.24	0.60 ±0.59	3.11±0.12	1.62±0.32
1.0	1. 0	0.0	64.22±3.27	2.21±0.88	3.45±0.84	1.31±0.46
1.5	1.5	0.0	62.33±2.13	2.45±0..36	2.35±0.42	1.75±0.25
2.0	2. 0	0.0	51.30±2.14	0.31±0.18	0.57±0.46	1.51±0.16
0.5	0.5	20	88.10±2.16	4.51±0.42	24.81±0.74	1.12±0.56
1.0	1.0	20	74.67±1.42	2.12±0.57	6.21±0.43	0.77±0.45
1.5	1.5	20	62.33±3.31	1.44± 0.66	4.12±0.63	0.83±0.25
2.0	2.0	20	37.83±2.22	1.65±0.25	4.25±0.88	0.66±0.60

Data represent means ± SE

Table 4: and Fig 1 Positional effects of nodes on shoot induction and multiplication in optimal media conc. MS+BAP0.5+NAA0.5+glutamine 20(mg/l)

Node position	No. of shoots induced	No. of nodes/shoot	Internodal length (in cm)
1st node	0.00	0.00	0.00
2nd node	0.58±0.64	1.41±0.09	0.41±0.25
3rd node	2.10±0.74	2.42±0.42	0.45±0.31
4th node	4.31±0.43	5.12±0.33	0.23±0.33
5th node	0.25±0.22	1.33±0.54	0.40±0.45

Data represents mean ± SE

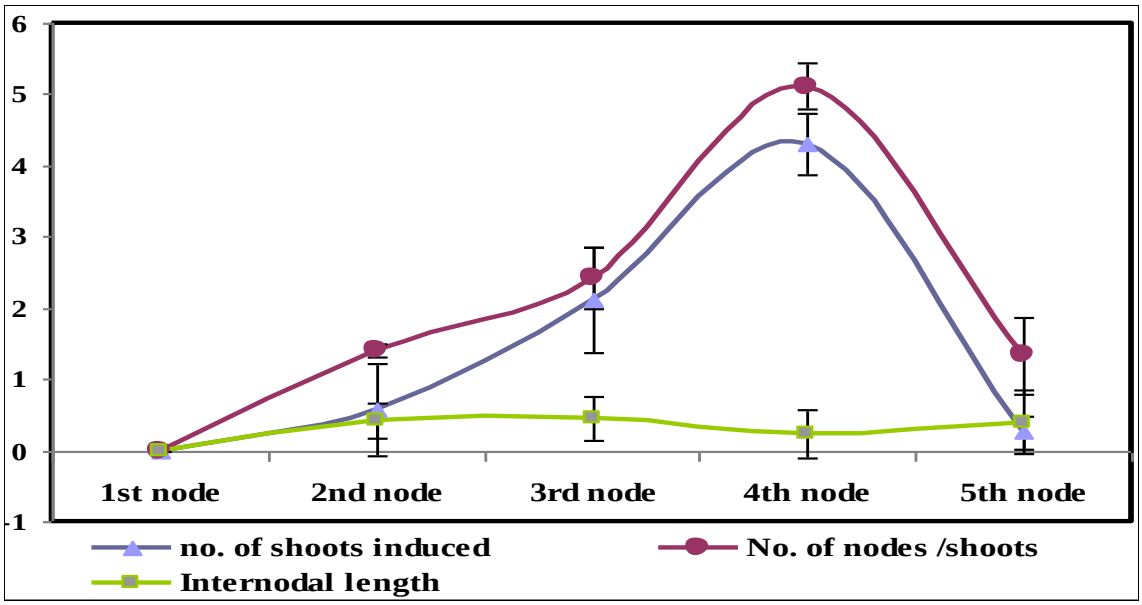

Fig 1: Combine effect different hormonal conc. On shoot induction in MS media after 6 weeks of culture incubation

Table 5: Influence of different auxin in root initiation of regenerated shoots in vermiculite

Treatments			% rooting	No. of roots	Length of roots (cm)
IBA	NAA	IAA			
0.5	-	-	0.00	0.00	0.00
1.0	-	-	10.42±0.74	1.21±0.44	0.23±0.65
1.5	-	-	64.74±0.87	6.41±0.61	2.42±0.56
-	0.5	-	60.23±0.78	4.22±0.75	2.54±0.74
-	1.0	-	15.12±0.66	2.53±0.84	0.35±0.33
-	1.5	-	12.88±0.63	2.67±0.77	0.18±0.71
-	-	0.5	0.00	0.00	0.00
-	-	1.0	0.00	0.00	0.00
-	-	1.5	0.00	0.00	0.00

Data represent means ± SE

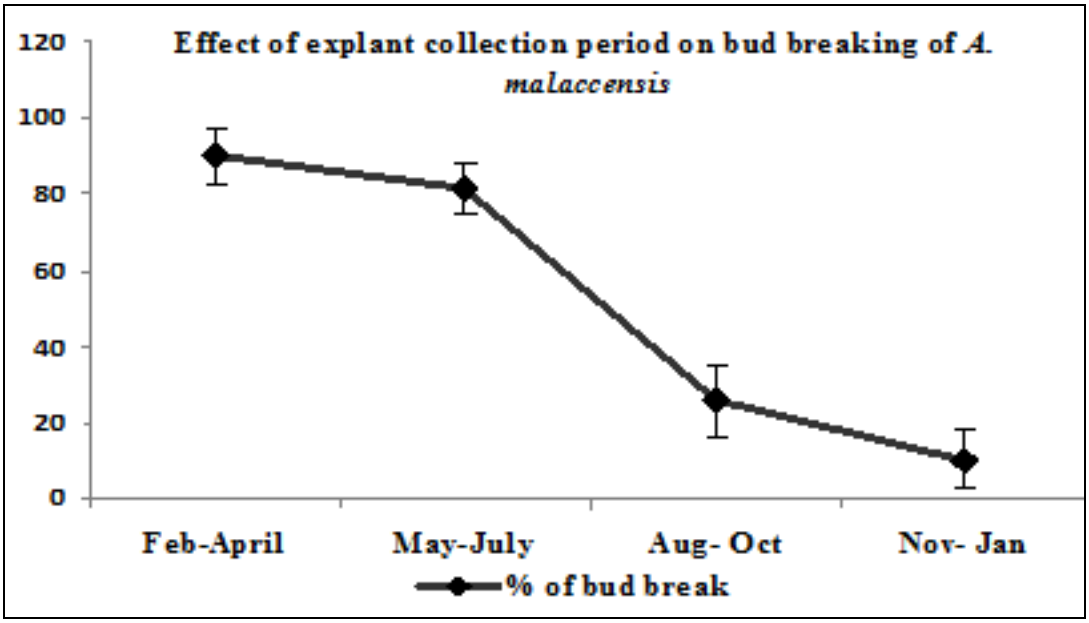

Fig 2: Effect of explants collection period (season) in shoot regeneration on MS media supplemented with BAP0.5+NAA0.5+glutamine 20(mg/l)

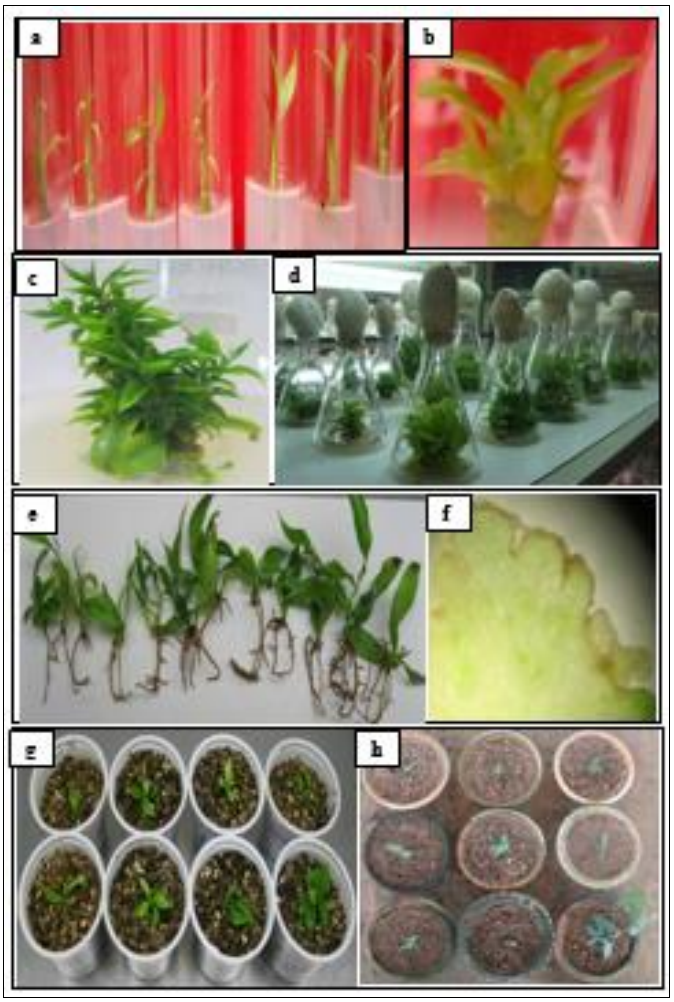

Fig 3: *In-vitro* propagation of *A. malaccensis* from non embryogenic nodal tissues

6. Fig Description: A. Regeneration of shoots from nodal explants, b. well developed multiplied shoots from node explants, c. multiplied shoots after 5th sub culture, d. mass multiplication of shoots e.3months old well developed rooted shoots before establish in mist chamber condition, f. cross section of the multiplied tissue shows distinct shoot bud regeneration, g. Rooted *in-vitro* shoots in the laboratory condition, h. established rooted *in-vitro* shoots in the green house condition.

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