American Journal of Pharmacology and Pharmacotherapeutics

Original Article

In vitro Conservation Strategies for the Propagation of *Alpinia calcarata* Roscoe (Zingiberaceae) – A Valuable Medicinal Plant

Silvy Mathew*, S. John Britto and Sinjumol Thomas

The Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College (Autonomous), Tiruchirappalli -620002, Tamilnadu (St), India

*Corresponding author e-mail: silvymathew110@gmail.com

ABSTRACT

Objective: To develop an efficient protocol for the *in vitro* conservation of *Alpinia calcarata*
Methods: Actively growing axillary shoot buds derived from the rhizomes (1-1.5 cm length) was used for the *in vitro* studies. The surface disinfestations of explants were accomplished by dipping rhizomes in 2% mercuric chloride for 3min., 70% ethanol for 2 minutes and 30% NaOCl for 15min and distilled water. After proper sterilization, the culturing were done under laminar air flow chamber and Cultures were maintained at 25 ± 2°C under 16/8 h photoperiod.
Results: The results indicated that MS medium supplemented with BA and KN was the best medium for shoot regeneration. The multiple shoots were cultured from rhizome bud explants of *Alpinia calcarata* on MS solid medium supplemented with BAP 2.5 mg / L-1 and 2.5mg/L-1 of kinetin. Maximum rooting was obtained in ½ MS medium supplemented with IAA (2mg/l-1) and NAA (2mg/l-1).
Conclusion: The *in vitro* derived plants were morphologically identical to the mother plant. So, this protocol proves its utility for rapid propagation of *Alpinia calcarata* which can be exploited for pharmaceutical and commercial purposes.

Keywords: *Alpinia calcarata*, Rhizome bud, Shoot multiplication, Regeneration.

INTRODUCTION

Medicinal plants have been the subjects of man's curiosity since time immemorial. Conservation of medicinal plants and capability to utilize them in a sustained manner are essential for the well being and continued survival of man[1]. Realizing the threat of extinction, there is a need to develop conservation strategies and quick propagation protocol[2-4]. Medicinal plants have been used for centuries as remedies for disease because they contain a component of therapeutic values[5]. During

the present exploration, only medicinal plant species having a broad spectrum of use in healing rheumatism were considered[6]. Natural products from plant, animal and mineral sources have the capacity of treatment of different human diseases[7].

In vitro regeneration holds tremendous potential for the production of high-quality plant-based medicine[8-9]. Tissue culturing of medicinal plants is widely used to produce active compounds for herbal and pharmaceutical industries[10].

Among the different approaches, the *in vitro* culture provides new means of conserving and rapid propagation of valuable, rare and endangered medicinal plants[11-12]. Tissue culture is a very influential component of plant biotechnology, which has opened an existing frontier in the field of agriculture and offers opportunity for the increase in productivity, profitability, stability and sustainability.

Qualitative and quantitative analysis of secondary metabolites is advantageous for the micropropagation of medicinal plants and it has been reported in many medicinal plant species[13-15]. The auxin and cytokinin mediated mechanism play an important role in tissue culture[16-17].

Alpinia calcarata is an important medicinal plant in Ayurveda. Hence, the need to develop tissue culture protocols for medicinally important species such as *Alpinia calcarata* using different explants for the conservation and mass production of this useful plant in India. This investigation, therefore, deals with the standardization of protocol for the *in vitro* propagation of the species from rhizome buds.

The plant is propagated conventionally by rhizome cuttings which are insufficient for a commercial scale production to meet the present day demand. Furthermore, improvement through conventional breeding will be difficult due to its rare flowering and lack of seed set. The isolated cultivation of the plant will not meet the present day demand within the country.

MATERIALS AND METHODS

Actively growing axillary shoot buds derived from the rhizomes of 12 month-old stock plants raised in the herb garden of the St. Joseph's Training College, Mannanam, Kerala were used as explants. Outer 3-4 scale leaves and cut basal surfaces of the explants were removed after a thorough wash with running tap water and emerging rhizome buds were trimmed to 1-1.5 cm length. The surface disinfestations of explants were accomplished by dipping rhizomes in 2% mercuric chloride for 3min., 70% ethanol for 2 minutes and 30% NaOCl for 15min.Afterwards washed with 2% Bavistin for 10 minutes and then washed thoroughly with double distilled water. Then the explants were thoroughly wiped with 70% alcohol to remove remnant contaminants if any.

Inoculation of explants

Surface decontamination was performed by immersing the explants in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 8 - 10 min. Each step of sterilization was followed by 5-6 rinses in sterile distilled water. Then the explants were finally dissected. The material was again treated with 0.1 % mercuric chloride for 2 minutes. The explants were cleansed with sterile distilled water to remove the traces of mercuric chloride under a laminar air flow cabinet. After cutting explants into suitable size (2-3cm), explants initially were implanted vertically on the culture medium in test tubes (150 x 25nm) and plugged tightly with non-absorbent cotton.

After proper labelling clearly mentioning media code, date of inoculation etc. the Cultured tubes were transferred to growth room. Cultures were maintained at

25 ± 2°C under 16/8 h photoperiod provided by cool white fluorescent tubes (60 µ mol m-2 s-1) with 55–60% relative humidity.

Shoot proliferation

For shoot proliferation, BA (0.5-3mg/l), Kn (0.5-3mg/l), combination of BA (0.5-3mg/l) with 0.5 NAA, combination of BA (0.5-3mg/l) with 2.5 Kn and mixing of Kn (0.5-3mg/l) with 0.5 NAA used. The successfully regenerated micro-shoots were cultured for 30 days and then used as explants source for the next set of experiments. Combinations of cytokinins were used to examine their effect on shoot multiplication.

After 28 days of culture period of the explants with newly formed shoots were taken out under strict aseptic conditions and were excised from the parent plant with help of a sterile scalpel blade and sterile forceps and inoculated into new bottles containing solid MS basal medium with a different set of growth hormones as mentioned earlier. Data were recorded after 28 days of culture and only shoots greater than 2 cm were considered for taking data. Every possible care has been taken to prevent any further contamination.

Statistical analysis

The results were expressed as the mean ± SD for all experiments. The data were subjected to statistical analysis using one way analysis of variance (ANOVA) and significance of means was assessed by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ using the statistical software SPSS.

RESULTS AND DISCUSSION

Half strength MS medium supplemented with auxin was most effective for rooting of shoots in *Terminalia bellirica*[18]. Earlier investigations concur the observations made in the present study[19].

Shoot induction response for rhizome bud explants was satisfactory on MS basal media. The results indicated that MS medium supplemented with BA 2.5mg/l and KN 2.5mg/l was the best medium for shoot regeneration. *In vitro* root induction was also observed in this study using MS basal media supplemented with different concentrations.

MS media were found to support a greater response for shoot regeneration and more effective than other medium for medicinal plants[20-21]. The pH of culture medium plays an important role in nutrient uptake and shoot proliferation.

The effect of IAA or combination of IAA and NAA was observed in *Alpinia calcarata* (Table. 1&2). MS basal medium without growth regulator did not show any promising results and produced only a few shoots per explants. Multiple shoot proliferation in rhizome buds could not be observed in the basal MS medium. MS medium augmented with BAP (2.5mg/l) induced shoots within 6 weeks of culture. Incorporation of BAP showed better responses at 2.5 mg/L. The explants cultured on MS medium with 2.5 mg/l BAP initiated single shoot bud after 30 days from each axil.

The induced shoots were sub cultured on the same concentrations of cytokinins on which the multiple shoots were induced. The second highest response was observed in MS with 2.5mg / l of Kn. In this supplement 70% of plants regenerated and in average, about 4.33±0.58 shoot buds were regenerated from each explant.

Successful establishment of regenerated plantlets with 93% survival rate was reported in *A. officinarum*[22]. A similar micropropagation protocol for *Alpinia calcarata* from rhizome has been also reported[23]. The multiple shoots were cultured from rhizome bud explants of *Alpinia calcarata* on MS solid medium

supplemented with BAP (2.5 mg / L-1) in combination with Kn (2.5mg/L-1).

Maximum rooting was obtained in ½ MS medium supplemented with IAA (2mg/l-1) and NAA (2mg/l-1) after five weeks with an average of 3 roots / shoot. The shoots inoculated on medium supplemented with IAA and NAA showed rooting response within 30 days. Among two auxins tested, NAA and IAA exhibited best rooting response.

After root initiation, the caps of bottles were gradually opened over a period of 2 weeks and finally removed. After subculture, for hardening of plants, About 90% of the rooted plantlets were acclimatized after hardening and successfully transferred to the field with a 90 % survival rate. Almost all regenerants acclimatized well in the green house and then under outdoor conditions (Plate. 2). Morphologically, there was no variation between the *in vivo* plants and *in vitro* grown plants.

CONCLUSION

Plant tissue culture is necessary to get maximum number of plants for propagation and exploitation. This method also is useful to regenerate *in vitro* plants for conservation of endangered/rare plants. Our protocol has established a reproducible, quick and large scale micropropagation method for *Alpinia calcarata*. This study aimed at establishing an efficient, fast and reliable protocol for the direct organogenesis of the *Alpinia calcarata* plant from the rhizome buds. This protocol could be used for conservation of this important medicinal plant and also for mass propagation in the near future.

REFERENCES

1. Ghimire S, McKey D, Aumeeruddy TY (2004). Heterogeneity in ethnoecological knowledge and management of medicinal plants in the Himalayas of Nepal: implications for conservation. *Ecology and Society*. 96.
2. Purohit S D, Dave A, Kukda G (1994). Micropropagation of safed musli (*Chlorophytum borivilianum*), a rare Indian medicinal plant. *Plant cell Tissue and Organ Culture*.39:93-96.
3. Sudha C, George M, Rameshkumar K, Nair G M (2012). Improved Clonal Propagation of *Alpinia calcarata* Rosc., a Commercially Important Medicinal Plant and Evaluation of Chemical Fidelity through Comparison of Volatile Compounds. *American Journal of Plant Sciences*. 3(7): 930-940.
4. Ahuja Ashok, Sambyal Manju, Koul Sushma (2002). *In vitro* propagation and conservation of *Atropa acuminata* Royle ex Lindi - An Indigenous threatened medicinal plant. *J Plant Biotechnol*. 2:121-124.
5. Kumar T, Chandrashekar K S (2011). *Bauhinia purpurea* Linn. A review of its ethnobotany, Phytochemical and pharmacological profile. *Res J Med Plants*.5:420-431.
6. Panda S P, Sahoo H K, Subudhi H N, Sahu A K (2014). Potential medicinal plants of Odisha used in rheumatism and conservation. *American Journal of Ethnomedicine*. 1(4):260-265.
7. Prasanta Dev, Haruna D S, Tejendra Bhakta (2014).Medicinal plants used as anti-cancer agents by tribal and non-tribal people of Tripura, India. *American Journal of Phytomedicine and Clinical Therapeutics*. 2(5):556-557.
8. Leena T, Jaindra NT (2003). Role of biotechnology in medicinal plants. *Tropical Journal of Pharmaceutical Research*. 2(2):243-253.
9. Murch S J, Krishna R S, Saxena P K (2000).Tryptophan as a precursor for melatonin and serotonin biosynthesis in *in vitro* regenerated St. John's ort (*Hypericum perforatum*. cv. Anthos) plants. *Plant Cell Rep*.19:698-704.
10. Sidhu Y (2010). *In vitro* micropropagation of medicinal plants by tissue culture. *The Plymouth Student Scientist*. 4(1):432-449.
11. Karuppusamy S, Pullaiah T (2007). *In vitro* shoot multiplication of *Bupleurum distichophyllum* Wight. A native medicinal

plant of Southern India. *Plant Tiss. Cult. Biotech*. 17(2):115-124.
12. Batra A (2001). *Fundamentals of plant biotechnology*. Capital publishing company. New Delhi.India.p.3-4.
13. Mohanty S, Parida R, Singh S, Joshi R K, Subudhi E, Nayak S (2010). Biochemical and Molecular profiling of micropropagated and conventionally grown *Kaempferia galanga*. *Plant cell and organ culture*. 106(1):39-46.
14. Singh M, Chaturvedi R (2010).Improved clonal propagation of *Splilanthes acmella* Murr. For production of scopoletin. *Plant cell tissue and organ culture*.103 (2):243-253.
15. Rathore M S, Shekhawat N S (2009). Micropropagation of *Pueraria tuberose* (Roxb. Ex Willd.) and Determination of Puerarin content in different tissues. *Plant cell and organ culture*.99 (3):327-334.
16. Miller C O, Skoog F, Okumura F S, Von Saltza M H, Strong F M (1955). Structure and synthesis of kinetin. *J Am Chem Soc*.77:2662.
17. Tiwari S K, Kashyap M K, Ujjaini M M, Agarwal A P (2002). *In vitro* propagation of *Lagerstromia parviflora* Roxb. From adult tree. *Ind J Exp Biol*. 40: 212-215.
18. Phulwaria M, Rai MK, Harish, Gupta AK, Ram K, Shekhawat NS (2012). An improved micropropagation of *Terminalia bellirica* from nodal explants of mature tree. *Acta Physiol. Plant*. 34:299-305.
19. Gopalan Sudha, Mathew George, Bahuleyan Rameshkumar, Govindapillai Mohanadasan Nair (2012).Improved Clonal Propagation of *Alpinia calcarata* Rosc., a Commercially Important Medicinal Plant and Evaluation of Chemical Fidelity through Comparison of Volatile Compounds, *American Journal of Plant Sciences*. 3:930-940.
20. Komalavalli N, Rao MV (2000). *In vitro* micropropagation of *Gymnema sylvestre* - a multipurpose medicinal plant. *Plant cell Tiss. Organ Cult*. 61:97-105.
21. Monsor N, Ismaila D, Yaye KGD (2003). *In vitro* multiplication of the semi-arid forest tree. Balanites *aegyptiaca* (L) Del. *African j. Biotechnol*.2:421-424.
22. Selvakumar C, Balakrishnan A, Lakshmi B S (2007). Rapid *in vitro* Micropropagation of *Alpinia officinarum* Hance., An Important Medicinal Plant, Through Rhizome Bud Explants. *Asian Journal of Plant Sciences*. 6(8):1251-1255.
23. Asha K I, Indira Devi A, Dwivedi N K, Asokan Nair R (2012). *In vitro* propagation of Lesser Galangal (*Alpinia calcarata* Rosc.) -a commercially important medicinal plant through rhizome bud culture, *Research in Plant Biology*. 2(5): 13-17.

Table 1. Number of roots and root length due to the effect of IAA (ANOVA)

		Sum of squares	df	Mean square	F	Sig.
Number of roots	Between Groups	1.667	3	.556	1.111	.400
	Within Groups	4.000	8	.500		
	Total	5.667	11			
Root length	Between Groups	3.703	3	1.234	19.487	.000
	Within Groups	.507	8	.063		
	Total	4.209	11			

Table 2. Number of roots and root length due to the effect of NAA (ANOVA)

		Sum of squares	df	Mean square	F	Sig.
Number of Roots	Between Groups	2.917	3	.972	.055	.055
	Within Groups	2.000	8	.250		
	Total	4.917	11			
Root Length	Between Groups	1.403	3	.468	.075	.075
	Within Groups	1.107	8	.138		
	Total	2.509	11			

Table 3. Multiple shoot emergence on MS medium supplemented with BAP and Kn

Plant growth regulators (mg/l)			No. of Explants responded	Percentage of explants responded	Number of shoots / plant	Shoot length (cm)	Number of leaves / shoot	Length of Leaves / shoot	Number of plants recovered on acclimatization
BA	KN	NA							
0.5			15	39.47	2.67±0.58^{a}	4.67±0.58^{a}	3.33±0.58^{a}	4.33±0.58^{a}	7.33±1.53^{a}
1			13	46.28	2.33±0.58^{a}	5.33±0.58 ^{a}	3.67±0.58^{a}	4.33±0.58^{a}	6.67±0.58^{a}
2			20	71.42	3±0^{a}	6.33±0.58^{a}	4.67±0.58^{a}	3.67±0.58^{a}	8.33±1.15^{a}
2.5			22	78.57	4.33±0.58^{b}	8.33±0.58^{b}	5±0^{a}	5.67±0.58^{b}	10.67±1.53^{a}
3.0			11	39.28	2.67±0.58^{a}	5.67±0.58^{a}	4.67±0.58^{a}	4.67±0.58^{a}	5±1^{a}
	0.5		10	35.71	3.33±0.58^{c}	5.33±0.58^{c}	3.33±0.58^{b}	4.67±0.58^{c}	5.67±0.58^{b}
	1		9	32.14	2.67±0.58^{c}	5.67±0.58^{c}	3±0^{b}	4±0^{c}	3.67±0.58^{b}
	2		14	50	2.67±0.58^{c}	5±0^{c}	3.67±0.58^{b}	3.67±0.58^{c}	8±1^{c}
	2.5		16	57.14	4.33±0.58^{d}	9±0^{d}	4.33±0.58^{b}	5.33±0.58^{d}	7.67±2.31^{c}
	3.0		11	39.28	2.33±0.58^{c}	5.33±0.58^{c}	2.67±0.58^{b}	3.33±0.58^{c}	5.67±0.58^{b}
0.5		0.5	14	50	2.33±0.58^{e}	5.67±0.58^{e}	3±0^{c}	3.67±0.58^{e}	6.33±0.58^{d}
1		0.5	13	46.28	3±0^{e}	5±1^{e}	3.33±0.58^{c}	3.33±0.58^{e}	6.67±1.15^{d}
2		0.5	12	42.85	2.33±0.58^{e}	5±1^{e}	2.67±0.58^{c}	3.67±0.58^{e}	7±0^{d}
2.5		0.5	15	39.47	4.33±0.58^{f}	7.67±0.58^{f}	4.33±0.58^{d}	5.67±0.58^{f}	7.67±0.58^{e}
3.0		0.5	12	42.85	3.33±0.58^{e}	6.33±0.58^{e}	2.67±0.58^{c}	3.67±0.58^{e}	6.67±0.58^{d}
0.5	2.5		14	50	3±0^{g}	5.33±0.58^{g}	4±0^{e}	3.67±0.58^{g}	7.33±0.58^{fg}
1	2.5		15	39.47	2.33±0.58^{g}	4.33±0.58^{g}	3.33±0.58^{e}	4.33±0.58^{g}	6.67±1.15^{fg}
2	2.5		19	67.85	3.33±0.58^{g}	5.67±0.58^{g}	4±0^{e}	3.67±0.58^{g}	9.33±0.58^{gh}
2.5	2.5		24	85.71	4.33±0.58^{h}	8.67±0.58^{h}	5.33±0.58^{e}	6±0^{h}	13±1^{h}
3.0	2.5		12	42.85	3±1^{g}	5.33±0.58^{g}	3.67±0.58^{e}	3.33±0.58^{g}	7.67±0.58^{f}
	0.5	0.5	13	46.28	2.67±0.58^{i}	4.67±0.58^{i}	3.67±0.58^{f}	3.33±0.58^{i}	6.67±0.58^{i}
	1	0.5	12	42.85	3.33±0.58^{i}	5.67±0.58^{i}	3.67±0.58^{f}	4±0^{i}	7±1^{i}
	2	0.5	14	50	2.67±0.58^{i}	5.33±0.58^{i}	3.33±0.58^{f}	4.33±0.58^{i}	6.67±0.58^{i}
	2.5	0.5	16	57.14	3.67±0.58^{i}	7.33±0.58^{j}	4.33±0.58^{g}	5.33±0.58^{j}	7.33±1.15^{i}
	3.0	0.5	13	46.42	2.67±0.58^{i}	5.33±0.58^{i}	3.33±0.58^{f}	3.67±0.58^{i}	5.33±0.58^{i}

Mean values within a column having the same alphabet are not statistically significant (p=0.05) according to new Duncan's multiple range test.

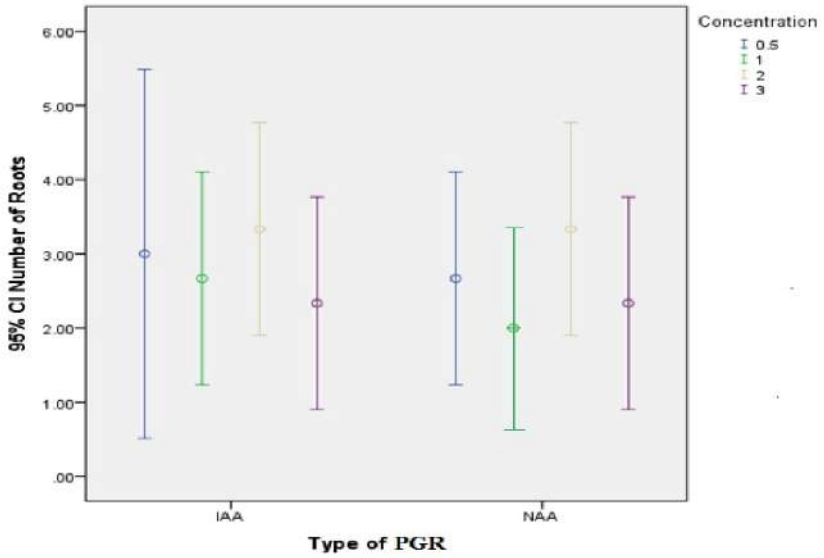

Figure 1. Number of roots produced by the use of IAA and NAA

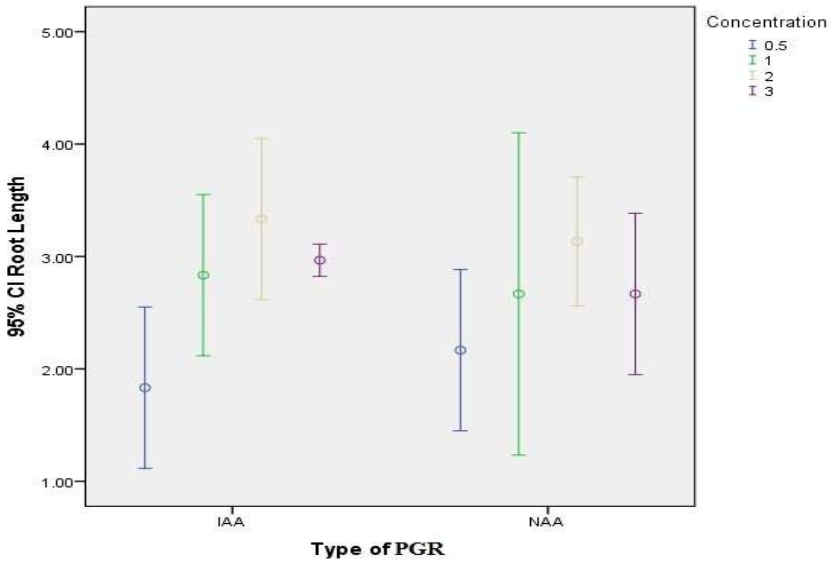

Figure 2. Length of roots produced by the use of IAA and NAA

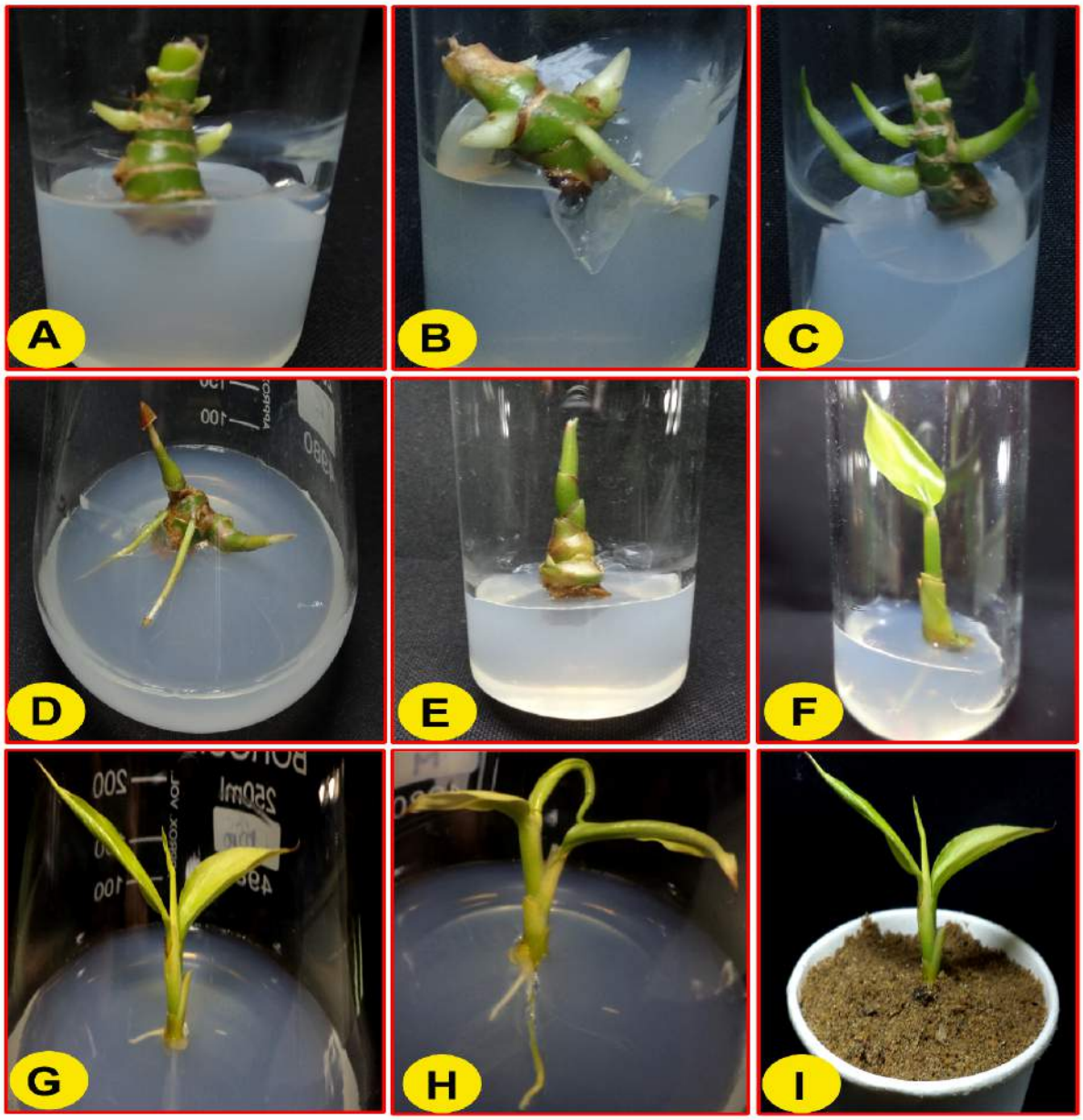

Plate 1. *In vitro* micropropagation of *Alpinia calcarata*: A) Axillary shoot bud initiation of aseptic rhizome explants grown on medium supplemented with BAP 2.5mg/L^{-1}after 6 weeks. B) Vigourous growth of axillary shoot buds on medium containing BAP 2.5 mg/L^{-1} & NAA 0.5 mg/L^{-}1with the formation of roots C) Multiple shoot initiation after 6 weeks of inoculation in MS with BAP2.5mg/L^{-1}&Kn 2.5mg/L^{-1} D) Root initiation on rhizome with the effect of IAA2mg/L-1 &NAA 2mg/L^{-1} E) Shoot initiation from rhizome explants with the Supplementation of MS with BAP 2.5mg/L^{-1} **F)** Shoot growth on rhizome with combinations of MS supplemented with Kn2.5mg/L-1 & NAA 0.5mg/L^{-1} G & H) Shoot emergence on rhizome with the effect of BAP 2.5mg/L-1 & Kn 0.5mg/L-1 I) Hardened plant

 ISSN 2393-8862

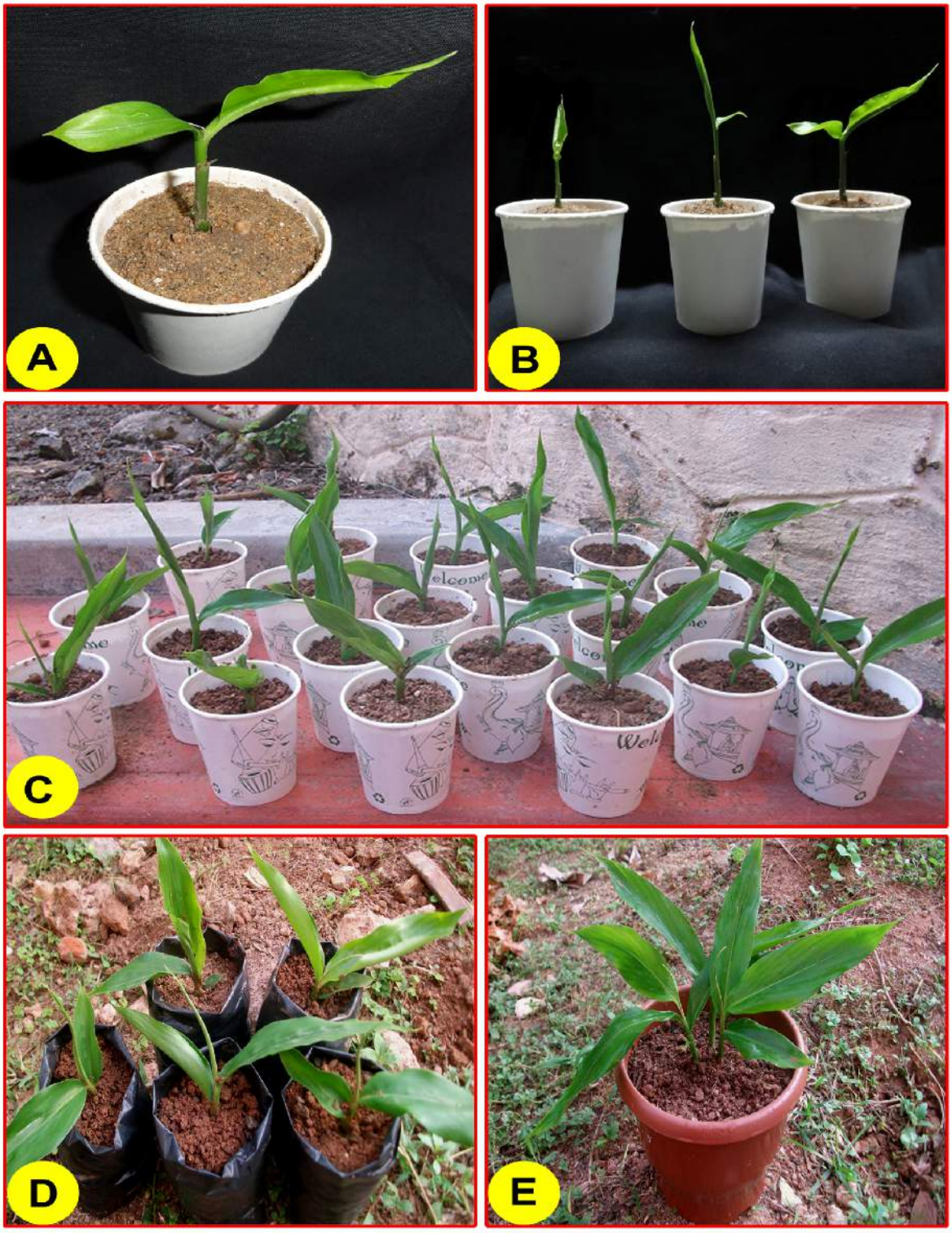

Plate 2. A-C. Hardened plants under the green house. D-F. Acclimatized plants in the field