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IN VITRO PROPAGATION OF *Alpinia galanga* (L.) Willd. (CHITHARATHAI)

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Abstract

This study was conducted to develop an efficient protocol for *in vitro* propagation of *Alpinia galanga*. Explants from rhizomes were cultured on Murashige and Skoog medium supplemented with NAA 0.75mg of and Kinetin 0.1mg of and produced highest mean number of shoots per ex-plant as compared to other concentrations. This study examined the role of different cytokinin and auxin for the shoot and root induction from the rhizome bud explants. The multiple shoots were obtained on the medium containing NAA 0.75mg and Kinetin 0.1mg. The combined effects of BAP and Kinetin improved shoot growth and further proliferation. However, MS medium supplemented with a combination of 0.5 mg BAP and 2.0 mg of Kinetin gave the highest number shoots. The proliferated shoots were green and healthy in appearance. Finally, healthy and mature shoots with well-developed roots were hardened and planted in the field with a survival rate of 80 %. This protocol can be used as a basis for the mass propagation of *A.galanga.*

INTRODUCTION

Plants have fed and caused the world since life began. Medicinal plants play an important role in the lives of rural people, particularly in remote parts of developing countries with a few health facilities. Approximately 80% of the world's population depend on herbal medicine as a primary health care (WHO, 1999). This causes considerable pressure on many species, since the majority of the material is collected from wild plants. Globally, approximately two third of the total of 50,000– 70,000 plant species used for medicinal purposes are collected from the wild condition (Schippmann et al., 2006). Unfortunately, the limited quantity of active metabolites in the plant, slow growth rates and destruction of natural supplies are the few problems encountered when exploiting plants for medical needs.

Plant derived drug research has become more promising in the recent years and also a better alternative for synthetic medicine and therapeutics, in spite of many challenges. It has been found that treatment of many diseases with chemosynthetic drugs shows frequent side effects, toxicity, severe mental and physical abnormalities which are not acceptable by the patients. The plants, which are used raw material for herbal drug formulation are now requires in large quantities (Natesh and Mohan Ram, 1999). Herbal medicine represents probably the first, and certainly the oldest system of human health care. Almost all civilization and cultures have employed plants in the treatment of human sickness. The Indian system of Ayurvedha is probably 5000 Years old which is based on the knowledge contained in Rigvedha and Atharvanavedha, the treatise charaka samhita (1900 BC) and Susruta Samhita (1500 BC). Hence conservative mode of medical treatments and synthetic drugs available "off the shell" appears to be a serious concern (Vanwyk and Wink., 2009). Thus, alternative sustainable and renewable production systems are urgently needed to protect and preserve plant diversity.

Tissue culture technique bears immense potential to overcome the natural hurdles and provides all the year availability of plants. Rapid multiplication of valuable genotypes and release of improved varieties is made possible. The major objective of plant cell, tissue and organ culture is the potential of any living cell to regenerate into whole plant (Bajaj, 1982). The plant tissue culture techniques are being exploited globally for mass propagation of many plants for improving afforestation, woody biomass and conservation of rare and endangered plants (Bonga and Durzam, 1982).

Plant tissue culture techniques offer a viable solution for the production of standardized quality phytopharmaceuticals through mass production of consistent plant material for physiological characterization and analysis of active ingredients. Micropropagation protocols for cloning a variety of medicinal and aromatic plants have been developed over the years (Rout et al., 2000; Nalawade & Tsay, 2004; Rathore et al., 2010). Integrated approaches of micropropagation are needed to provide a basis for the development of novel, safe, effective and high-quality commercial products. Micropropagation can be utilized for rapid multiplication of elite clones. The advantages offered by micropropagation of medicinal plants are many. *In vitro* propagation (micropropagation) offers a number of clear advances, as recently summarized by Denabth (2006). Basically, these include (i) the production of large number of plantlets in a comparably short time, due to usually high multiplication rates; (ii) micropropagation is feasible in all the seasons; and (iii) plants produced *in vitro* are usually free from diseases.

Hence the purpose of this investigation is to develop a cost effective reproducible protocol for *in vitro* regeneration of *Alpinia galanga* for its medicinal potential (Jain Monica., *et al*., 2017).

Alpinia galanga belongs to Zingiberaceae family. It bears perennial rhizomes and occurs in Pakistan, Indonesia and Europe. Chitharathai is traditionally used as a home remedy for cough, sore throat, congestion and for removing phlegm. This plant is commonly known as greater galangal in English. It is 6 to 7 ft. high and bears perennial rhizomes which are deep orange – brown in color, aromatic, pungent and bitter. The rhizome is from 3.5 – 7.5 cm in length. Flowers are greenish white in colour. Fruits are like small cherry and orange red in colour. During the past, *A. galanga* is gaining lot of interest according to researcher's point of view. Recently many pharmacological studies have been **carried** on *A. galanga*.

MATERIALS AND METHODS:

Plant material

The healthy plants of *Alpinia galanga* were collected form Pachaiyappa's College campus, Chennai – 30. Rhizomes of *A. galanga* found in the campus were utilized to raise plantlets through *in vitro* propagation.

Preparation of Ex-plants

The ex-plants were excised from the healthy mother plants which are maintained in the green house (Fig: 1). The ex-plants namely rhizomes were washed thoroughly in running tap water overnight and then add few drops of soap solution like teepol, twin 20 etc., and washed thoroughly in running tap water and wash with 70% ethanol.

The washed ex-plants were transferred in to laminar Air flow chamber where the surface sterilization was carried out with the help of surface sterilizing agent mercuric chloride ($HgCl_2$).

STERLIZATION OF EX-PLANTS

The rhizome ex-plants were selected to study the morphogenetic response. They were washed thoroughly in running tap water for 10 min. And then it was washed by using Tween – 20 for 10 min. These were washed thoroughly with running tap water. After the washing, the ex-plants were subjected for different concentrations of mercuric chloride. Three different concentrations viz; 0.05, 0.1, 0.15 for 8 minutes were used to standardize the surface sterilization of various ex-plants. The inoculations of ex-plants were carried out under aseptic condition in Laminar flow chamber. The UV light in the chamber was switched on for an hour prior to use.

Inoculation

Inoculation was done under the Laminar Air flow chamber. The work area of LAF was thoroughly wiped with 80% alcohol and UV lamp keep switched on for 30 minutes to kill or denature the microbes before sterilizing function. The inoculation processes was carried out with the help of sterilized forceps and scalpel. The ex-plants were sized to 0.5 cm – 1.0 cm length and placed on culture medium. Before going to each and every operation forceps and

knife were flame heat sterilized with the help of spirit lamp. After inoculation the culture tube were closed tightly by sterilized cotton plugs and allowed for incubation.

Incubation

Inoculated culture were incubated in the culture room, where essential incubation parameters are maintained. The incubation parameters are as follows;

Temperature - 25 ± 2°C

Light intensity - 2000 – 4000 Lux

Photo period - 16/8 hr [Light/ Dark]

Subculture & Maintenance

All the cultures were incubated for 4 – 5 weeks. After that the cultures were taken for next stage in the fresh media.

To study the morphogenetic potential of the culture at 15 replicates were maintained for each individual treatment.

Plant Growth Hormones:

Following are the combinations of growth regulators used:

NAA:0.75mg–Used mainly for root differentiation.

Kinetin: 0.1mg – A specifically high active cell division inducing factor.

The above mentioned growth hormones are supplemented as Auxin and Cytokinin and were used as stock solution in MS medium for shoot induction.

ABBREVIATIONS USED:

BAP: Benzyl Amino Purine
2,4 D: 2,4 Dichlorophenoxyaceticacid
KN: Kinetin
MS: Murashige and Skoog

Culture medium and condition

The rhizome explant were cultured on Murashige and Skoog[10] medium supplemented with BAP (1 - 5 mg/l) alone or in combination with auxin (IAA and NAA) for the production of multiple shoots. Healthy elongated shoots (≥ 2.5 cm) were separated individually and cultured on MS medium supplemented different auxins (IAA, NAA, IBA) at 0.5 - 2.0 mg/l for root induction. The cultures were incubated in a culture room under the temperature of 25 ± 2°C and the photoperiod of 16 hours light and 8 hours dark. Artificial illumination (45

μ mol m^{-2} S^{-1} irradiance level) was provided with help of white fluorescent tubes and 55 - 60 % of Relative Humidity was maintained to facilitate the favorable growth of the cultures.

Subculture and maintenance

The cultures were incubated for 3-4weeks after the establishment and there after routinely sub cultured on fresh media depending on the nature of the experiments. During the subcultures, care was taken to maintain the size of the clumps to get the uniform multiplication ratio. To study the morphogenic potential of the culture 6 replicates were maintained for each individual treatment. After the standardization of the media combination, the particular hormone combination was used in subsequent experiments. The same method was followed at every subsequent passage to assess the morphogenic potential in long term cultures.

Hardening

Regenerated plants with healthy root system were washed thoroughly (especially the root portion) under running tap water to remove the agar from the roots to avoid the contamination after the transfer. After 45 days, the primary hardened plants were transferred to cocopeat and sand (1:1) mixture for secondary hardening. The plants were transferred to field after 60 days of secondary hardening. The roots were then soaked in a 2% (w/v) Bavistin solution for about 15 minutes and the plantlets were then transferred to protrays containing presoaked sterilized vermiculite medium and kept inside the mist chamber. The timer was set to 1 minute on time and 30 minutes off time with relative humidity at 90 %. After four weeks they were ready to transplantion to the field.

RESULTS AND DISCUSSION

During the first week of culture the explants failed to produce any callus. The rhizome ex-plant did not show any response of bud break when cultured on phytohormone free medium and failed to induce any shoots even after 3 weeks of culture. After the third week, the first visible response was found, which involves the initiation of callus on one of the wounded edge of rhizome. It is because the cut ends of the rhizome provide a way for nutrients and growth regulators to be absorbed efficiently from the medium and started functioning according to the nature of growth regulator *Reynoired* et al, 1993.

Explants cultured on MS medium with various concentration of 2,4-D, NAA and Kinetin for different days showed a discernible response on bud breakage. Multiple shoot induction was observed in the presence of various cytokinins (Figure 1). The callogenesis was found in the presence of Kinetin and NAA, both the PGRs influenced the growth.

The combination of BAP and Kinetin induced shoot induction and proliferation.

In this present study maximum callus induction and growth was recorded in cultures grown with MS media containing 2,4-D (1mg) when compared to NAA (0.75mg) and Kinetin (0.1mg). It was observed that cytokinin was required in optimal quantity for shoot proliferation in some species of Zingiberaceae, but inclusion of low concentration of auxin along with cytokinin triggered the rate of shoot proliferation (Rout and Das, 1997; Sharma and Singh, 1997). Further, Stanly and Keng (2007) reported that MS medium supplemented with 6 mg/L BAP induced the formation of multiple shoots of *Alpinia galanga*. Earlier, it was reported that the highest numbers of shoots were obtained on MS medium supplemented with 5 to 10 mg/ L BAP from *Zingiber officinale* Roscoe (Noguchi and Yamakawa, 1988). Faria and IIIg (1995) noted that the addition of 2.25 mg/l of BAP with 1.0 mg/l of IAA induced a high rate of shoot proliferation of *Zingiber spectabile*. In *Curcuma haritha*, Bejoy *et al.* (2006) found that the best shoot multiplication and root system were achieved on MS medium supplemented with 1.0 mg/l of BAP and 0.5 mg/l of IAA. This results indicate the importance of IAA hormone in the induction of roots. Moreover, Anish *et al.* (2008) reported that MS medium supplemented with IAA at 0.5 mg/l in a combination with BAP seems to be optimum for rooting of *Boesenbergia pulcherrima*. In the same trend, Vincent *et al.* (1992) reported that highest number of shoots per explant was obtained with cultured axillary buds of *Kaempferia galangal* on MS medium supplemented with 0.50 mg/l of BAP and 3.0 mg/l kinetin after 120 days of incubation.

The creeping rhizome of the plant in mud or in muddy water and the ex-plants derived from them were a source of severe contamination and the contaminants included both fungi and bacteria. Several trials and standardization procedures carried out with the rhizome explants revealed that the percentage of infection and survival solely depended on the disinfectant ($HgCl_2$) used and time of its treatment. Nearly 70% of the explants were lost due to microbial contamination.

seventy five tubes were inoculated of which 25 tubes were free from contamination and their weekly observations enlisted below.

8 tubes were contaminated due to difference in duration of the surface sterilizing agent.

12 Tubes got contaminated in the following week due to bacterial contamination.

After proper maintenance of inoculated tubes contamination was minimized to only 5 tubes.

The regenerated shoots were green and healthy in appearance. The role of BAP in shoots proliferation has been reported in other Zingiberaceae species (Ikeda and Tambe, 1989; Balachandran et al., 1990; Smith and Hamil, 1996; Rout et al., 2001; Panda et al., 2007; Mohanty et al., 2011; Abdelmageed et al., 2011).

Table 2: Combination of growth regulators utilized for various responses from rhizome ex-plants of *Alpinia galanga*.

Response	BAP	Kinetin	NAA
Callogenesis	-	0.1 mg	0.75 mg
Shoot induction and proliferation	0.5mg	0.2mg	-

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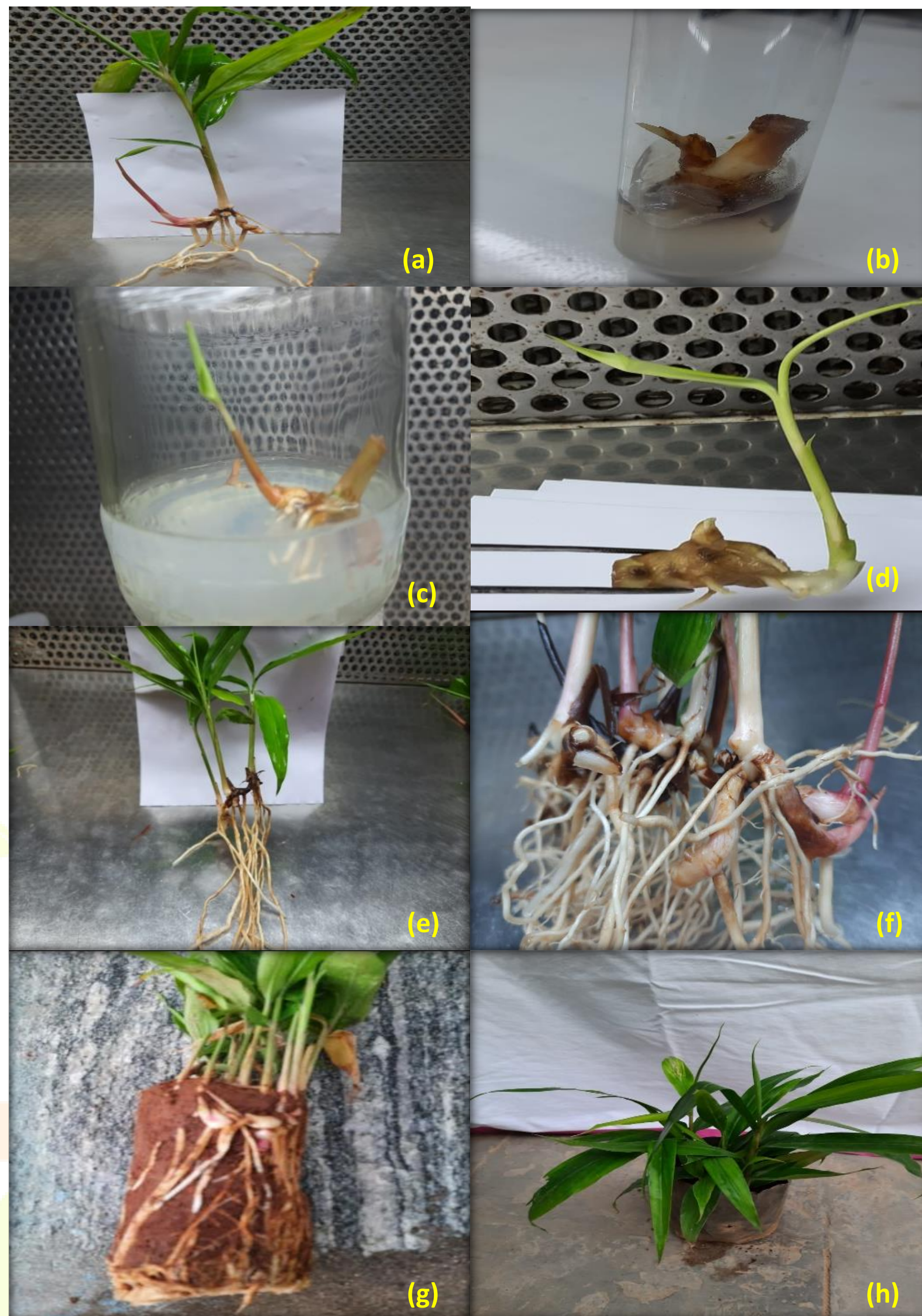

Figure 1. *In vitro* micropropagation of *Alpinia galanga*; (a) Mother Plant, (b) Inoculated ex-plant, (c) Shoot emergence from inoculated rhizome, (d) Single shoot production, (e) Rooted shoots, (f) Multiple shoots and roots, (g) Hardened shoots with rhizomes, (h) Potted plant after hardening.

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