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Direct and Indirect Organogenesis of *Alpinia galanga* and the Phytochemical Analysis

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Abstract *Alpinia galanga* is a rhizomatous herb rich in essential oils and various other significant phytoconstituents. Rapid direct regeneration was obtained from the rhizome explants (15.66±0.57 shoots) on MS media supplemented with zeatin at a concentration of 2 mg/l. The callus cultures of *A. galanga* were initiated from the rhizome explants on MS media supplemented with 2 mg/l each of BAP, 2,4-D, and NAA. The callus was analyzed for the presence of a vital phytoconstituent—acetoxychavicol acetate (ACA) associated with various biological properties. ACA was detected in the young friable callus as well as the stationary phase callus. Moreover, the induction of morphogenetic response in callus resulted in higher accumulation of ACA. The phytohormone withdrawal from the propagation media and the subsequent transfer of callus to BAP (2 mg/l) containing MS media has resulted in multiple shoot induction. The regenerated (indirect) plants have shown 1.6-fold higher ACA content (1.253%) when compared to the control plant (0.783%). Micropropagation of such conventionally propagated plants is very essential to meet the commercial demand as well as to ensure easy storage and transportation of disease free stocks.

Keywords *Alpinia galanga* · Rhizome · Callus · Micropropagation · Acetoxychavicol acetate · HPLC

Abbreviations

MS	Murashige and Skoog
BAP	Benzyl amino purine
2,4-D	2,4-Dichlorophenoxy acetic acid
TDZ	Thidiazuron
NAA	α-Naphthalene acetic acid

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IAA Indole-3-acetic acid
IBA Indole-3-butyric acid
ACA Acetoxychavicol acetate

Introduction

Plants have become an essential part of the daily routine in the form of phyto-pharmaceuticals, cosmetics, aromas and essences, food preservatives, dietary supplements, colouring agents, etc. The biological properties are attributed to plant secondary metabolites—a diverse group of molecules that are involved in the adaptation of plants to their environment. To date, the best available source of plant secondary metabolite extraction is the wild plant harvest leading to the loss of genetic diversity and habitat destruction [1]. Around 4,000 species of medicinal plants are globally threatened [2].

Alpinia galanga (L) Willd syn. *Languas galanga* commonly called greater galangal (*Zingiberaceae*), is distributed in various parts of India and Southeast Asia. It has been in use as food additive in Thailand and other parts of Asia. The rhizome is used against rheumatism, bronchial catarrh, bad breath, ulcers, whooping cough, throat infections, incontinence, fever and microbial infections. The compounds present in the plant viz. camphene, myrcene, 1, 8-cineole, α-fenchol, camphor, α-fenchyl acetate, carotol, guaiol etc. are known to be responsible for the characteristic odour as well as the medicinal properties [3]. It also contains phenylpropanoids like acetoxychavicol acetate (ACA), acetoxyeugenol acetate and *p*-coumaryl diacetate [4], responsible for the anti-tumour, anti-HIV and anti-parasitic activities [5–9]. Galangal root oil and root oleoresin are given the regulatory status: generally regarded as safe (GRAS) in the US. India is a major supplier of galangal along with Thailand and Indonesia.

The immense medicinal value of *A. galanga* and its use in food industry, makes it vulnerable to wild harvestation that jeopardizes its survival in the natural habitat. Monocotyledonous plants do not allow much explant (rhizome and shoot tips) diversity for propagation and the conventional propagation method of splitting the rhizome is not sufficiently rapid to meet the commercial demand. The rhizome explants are often infected with pathogens such as rhizome rot [10] and the leaf rot, in which case, conservation and germplasm storage becomes difficult in *Zingiberaceae* [11]. The opportunity to tailor-make the chemical profile of a metabolite by manipulating the chemical or physical environment for the production of a valuable compound can be achieved by plant tissue culture techniques [12, 13]. The simplest way to induce somaclonal variations is by shoot regeneration via the callus phase thus paving the way for improvement of the species [14]. Though multiple shoot induction has been attempted by Borthakur et al. [15] from the rhizome explants of *A. galanga*, this study is the first attempt to achieve mass multiplication on a large scale via the callus phase.

Materials and Methods

Materials

A. galanga plant material was obtained from A.G Biotek, Hyderabad. The chemicals were purchased from various sources—acetoxychavicol acetate (LKT laboratories, USA); MS media (Hi media); plant-growth regulators (Duchefa, Netherlands); HPLC-grade solvents (Merck); analytical-grade chemicals (Himedia).

Establishment of In Vitro Cultures

Explants were washed thoroughly under running tap water for 15 min. Initially, they were treated with alcohol (5 min) and later with 0.1% mercuric chloride for 8 min, followed by eight to ten rinses with sterile distilled water. Different parts of *A. galanga* like leaf, leaf sheath, rhizome and root explants (3 cm) were excised and cultured on semisolid MS media supplemented with different concentrations (0.5–5 mg/l) of cytokinins viz. kinetin, zeatin, thidiazuran, benzyl aminopurine, for multiple shoot induction and auxins viz. α-naphthalene acetic acid, indole-3-acetic acid, indole-3-butyric acid for root induction. The cultures were incubated under the standard culture conditions [25±2 °C; 16/8 h (light/dark) regime with 40–50 mol m^{-2} S^{-1} light].

Callus Induction

Callus initiation was tried with various explants like leaf, root (1 cm) and rhizome (circular discs of 3 mm thick) on MS media fortified with different combinations of auxins (2,4-D and NAA) and cytokinins (BAP). The explants were incubated under standard culture conditions. The callus obtained from the explants was transferred onto MS basal media and sub-cultured every fortnight.

Indirect Organogenesis

The compact mass of cells thus formed was transferred to MS media supplemented with different cytokinin (BAP, Kinetin and TDZ) concentrations for shoot induction and later to auxin (IBA) containing media for root initiation respectively.

Histological Studies

Histological studies were carried out by the process recommended by Johansen [16]. The samples were fixed in a solution consisting of formaldehyde, glacial acetic acid and ethyl alcohol in the ratio of 5:5:90 *v/v*. Following the fixation, tissues were dehydrated in an ethanolic graded series and then embedded in paraffin. Serial sections of 6–10 μm in thickness were cut using a Leitz Rotary Microtome (1512). The sections were obtained from freshly prepared wax blocks with tissue specimen by adjusting the microtome and stained with Saffranine.

Phytochemical Analysis

The phytochemical analysis was carried out by HPLC [17]. The in vitro plantlets and callus cultures (various stages) were oven dried (45 °C) to obtain a constant weight. One gram of dried in vitro plantlets as well as callus were macerated with methanol (HPLC grade) to obtain a fine homogenate and filtered using a 0.22 μm membrane filter before subjecting it to HPLC.

The analysis was carried out using the Shimadzu—LC-10AT VP series HPLC system equipped with a Supelco column (250×4.6 mm, C18, ODS with particle size of 5 μm). The sample was detected using a UV–Vis detector (Shimadzu UV–Visible SPD-LC 10A VP series) at 210 nm. The mobile phase consisted of methanol and water (60:40, isocratic) at a flow rate of 1 ml/min, with an injection volume of 20 μl.

Acclimatization

The regenerated plantlets were transferred to the plastic pots for hardening in a mixture containing autoclaved vermiculite and soil (1:1) under controlled conditions. The potted plants were irrigated with MS basal salt solution (1/8 strength) every 4 days continuously for 3 weeks [18]. Plants were sprayed with 0.1% bavistin once a week. The poly pots were then covered with a rigid plastic cover and water was sprayed to maintain 70–80% relative humidity. The plants were maintained under green house conditions.

Statistical Analysis

Results calculated from triplicate data were expressed as means ± standard deviations. The data were compared by least significant difference ($P \leq 0.05$) test using Statistical Analysis System (SAS, ver. 9.1).

Results and Discussion

In Vitro Culture Establishment by Direct Regeneration

In vitro propagation is one of the key approaches for proliferation of medicinal plants in a large scale. Traditionally, *A. galanga* is propagated through rhizome that is susceptible to fungal diseases, making it difficult for maintenance and storage of quality rhizomes. Pathogen free planting material is vital to accomplish high quality, homogeneity and productivity. Different explants like leaf, sheath, rhizome, and root were tried for in vitro propagation of *A. galanga,* of which only the rhizome explants responded unveiling the importance of resident meristem and its magnitude in in vitro multiplication. The shoots appeared to arise directly from the base of the rhizome. It takes about 3–5 weeks for multiple shoot initiation from the rhizome explants. The rate of shoot bud regeneration varied with each of the cytokinin that could be due to the endogenous hormone levels of *A. galanga*.

Multiple shoots were induced on MS media supplemented with BAP, kinetin, TDZ and zeatin, among which, zeatin at 2 mg/l concentration gave high frequency regeneration (15.66±0.57 shoots) (Fig. 1). Other cytokinins i.e. BAP and kinetin were successful in

Fig. 1 Multiple shoot induction in *A. galanga* with zeatin 2 mg/l

Table 1 Effect of different cytokinins on multiple shoot induction in *Alpinia galanga*

Cytokinin concentration (mg/l)	No. shoots			
	Kinetin	Zeatin	TDZ	BAP
0.5	–	9.66±1.00	4.66±0.57	–
1.0	4.66±0.57	11.33±1.00	6.00±1.00	5.66±0.52
2.0	8.00±1.00	15.66±0.57	9.66±1.15	11.00±1.00
3.0	10.33±0.63	12.00±0.02	4.33±0.57	11.66±1.15
4.0	6.33±0.57	8.00±1.15	–	5.00±1.00
5.0	4.66±1.15	7.00±1.00	–	2.33±0.57

Observations were taken after 4 weeks of culture

TDZ thidiazuron, *BAP* benzyl amino purine

inducing 11.66±1.15 and 10.33±0.63 shoots at concentrations of 3 mg/l respectively (Table 1). Since there was no significant difference ($P>0.05$) in the number of shoots induced with BAP at 2 mg/l and 3 mg/l and also due to the economic feasibility, BAP at 2 mg/l was preferred to zeatin for multiple shoot induction. The efficacy of BAP in shoot multiplication of *Zingiberaceae* species such as turmeric [19] and ginger [20] was reported earlier. On the other hand, the maximum number of shoots obtained with TDZ was 9.66± 1.15 at a concentration of 2 mg/l. TDZ at higher concentrations is known to cause inhibition of shoot proliferation [21]. The higher concentration of cytokinins do not always yield higher shoot numbers, instead there was a decrease in number. This could be due to the hyperhydricity observed at higher concentrations of cytokinins [22, 23].

Borthakur et al. [15] have reported multiple shoot initiation from the rhizome explants of *A. galanga*, wherein, a maximum of eight shoots were obtained in kinetin (3 mg/l), in contrast to the present finding of 15.66±0.57 shoots on zeatin (2 mg/l) containing media. While most growth regulators exert a direct effect on the cellular mechanisms, many synthetic regulators modify the level of endogenous growth substances [24]. The present study indicates the positive role of zeatin in the regulation of endogenous plant hormones in multiple shoot generation. Similar results were achieved in *Andrographis paniculata* on zeatin supplemented media by Soma Roy et al. [25].

These in vitro shoots were tried for root induction on MS media supplemented with auxins (NAA, IAA and IBA). IBA augmented MS media at a concentration of 2 mg/l gave the maximum

Fig. 2 Callus on MS media supplemented with 2,4-D, BAP and NAA

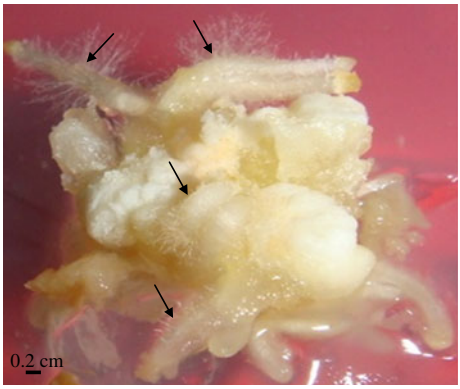

Fig. 3 Sturdy root like structures with root hairs emerging from the compact mass of cells on MS basal media

(7.66±0.57) number of roots. As the concentration of auxins increased, there was a marked decrease in root initiation response. The stimulatory effect of IBA on root formation is similar to the reports in other *Zingiberaceae* species like *Alpinia officinarum* Hance [26], *Kaempferia galanga* L. [27] and other medicinal plants like *Ruta graveolens* [28], *Andrographis paniculata* [25].

Callus Initiation

Initiation of callus from the in vitro regenerated plants of *A. galanga* was reported for the first time in the present study. Among the different plant parts used for the evaluation of callus induction, the rhizome explants proved to be a better choice. Initially, when 2,4-D alone was employed for callus induction, no response was observed. The tissue seemed to be recalcitrant, even when a combination of auxin (2,4-D) and cytokinin (BAP) was used. Only after the inclusion of another auxin (NAA), the explant gave a positive response for callus initiation. However, the lower concentrations (2,4-D+BAP+NAA) could yield a partial response (swelling and curling) that could not develop into a full blown callus. The callus was obtained only on MS media supplemented with BAP, 2,4-D, and NAA at a concentration of 2 mg/l each after 40 days of inoculation. Chirangini et al. [29] have confirmed the efficacy of this combination for callus induction, in *K. galanga* and *Kaempferia rotunda*. Though the callus was slow growing initially, the growth accelerated after repeated subcultures (21 day intervals). The callus obtained from the rhizome explants of *A. galanga* was highly friable with a creamy white crystalline appearance (Fig. 2). The

Table 2 Multiple shoot induction in plantlets formed through indirect organogenesis on different cytokinin containing media

Cytokinin concentration (mg/l)	No. shoots		
	BAP	Kinetin	TDZ
0.5	–	–	3.66±0.57
1.0	7.66±0.57	–	4.33±0.57
2.0	18.33±1.52	4.75±0.50	6.33±0.57
3.0	15.75±0.95	6.00±0.33	–
4.0	16.00±0.81	8.00±1.00	–
5.0	12.75±0.95	5.75±0.50	–

BAP Benzyl amino purine, *TDZ* thidiazuron

Fig. 4 Shoot emerging from the compact mass on MS media with BAP (2 mg/l)

efficiency of 2,4-D and BAP/Kn for callus induction from the internode and leaf explants of *Ceropegia candelabrum* L. was reported by Beena and Martin [30].

Multiple Shoot Induction by Indirect Organogenesis

Shoot regeneration via a callus phase is the simplest way to induce somaclonal variations, thus paving the way for improvement of the species [31]. The friable callus obtained after two to three subcultures, was transferred to MS basal media without phytohormone supplement, so as to initiate differentiation. The callus was repeatedly sub-cultured on MS basal media for two to three cycles, after which a compact mass of cells was obtained with sturdy root-like structures (Fig. 3). This compact mass was then transferred onto MS media supplemented with BAP, kinetin and TDZ at different concentrations separately for shoot induction. Among the different cytokinins used, BAP at a concentration of 2 mg/l proved to be the best for shoot initiation and elongation (Table 2 and Fig. 4). In this study, microshoot formation was observed after 8 weeks of culture. A maximum of 18.33±0.57 shoots were observed from each compact mass of cells (Fig. 5). BAP is considered to be one of the most constructive cytokinins for achieving the multiplication and micropropagation of plants [32]. The role of BAP in shoot induction from callus was in agreement with other species of *Zingiberaceae* [33–35].

Fig. 5 Multiple shoots from the callus of *A. galanga* obtained on BAP containing MS media (2 mg/l)

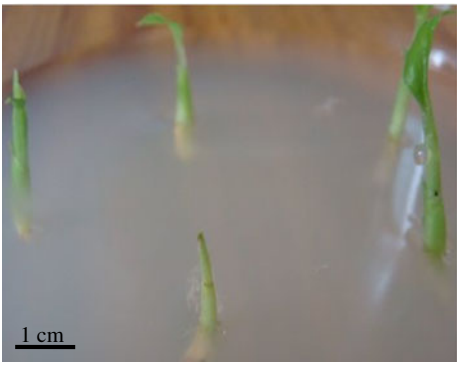

Fig. 6 Excised shoots cultured on BAP and IBA containing MS media at concentrations of 2 mg/l

The well-developed shoots were excised from the shoot clump and transferred onto MS media fortified with IBA (2 mg/l) for root induction along with BAP (2 mg/l; Fig. 6). The microshoots transferred to the MS media later developed into mature plantlets with multiple shoots and a healthy root system (Fig. 7).

Histological Studies

Based on the histological studies, it was shown that the process of differentiation starts after 6 weeks of transfer to the MS basal media. The sections taken during initial stages of morphogenesis have shown the existing meristematic activity (Fig. 8). The longitudinal sections of the explants have shown the typical arrangement of dome-shaped shoot apical meristem and the underlying parenchymatic cells. The plantlets were observed to develop via the indirect organogenesis that has been confirmed by the histological study showing the continuity of tracheal elements throughout the tissue (Fig. 9). Such indirect organogenesis was reported in many medicinal plant species including *Asparagus cooperi* [36], *Plumbago indica* [37], *Holostema ada-kodien* [38], *Gloriosa superba* [39], *Phellodendron amurense* [40] and *Abrus precatorius* [14].

Phytoconstituent Analysis

Acetoxychavicol acetate has been detected in the callus cultures of *A. galanga* for the first time in the present study. The stepwise HPLC quantification of ACA in the callus cultures as well

Fig. 7 Multiple shoots obtained through indirect organogenesis on MS media supplemented with BAP (2 mg/l)

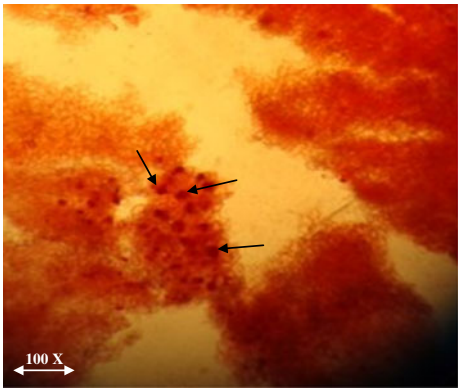

Fig. 8 Darkly stained areas (*arrows*) showing meristematic activity

as the cultures giving morphogenetic response leading to the regeneration of plants was carried out. But the quantity of ACA in callus was found to be very low when compared to the differentiated tissues. The quantity of ACA in young cultures was found to be 0.232% in comparison to the late/stationary callus (0.473%), which was found to be 2.04-fold higher (Fig. 10). This observation could not rule out the possibility of ACA being stress related.

In another study, ACA was analyzed at different stages of differentiation process to observe the relationship between product accumulation and morphological differentiation. An increase in ACA accumulation was seen along the morphogenetic response. The increase in concentration from 0.232% in early callus to 0.761% in compact callus and 1.253% in the regenerated plant (Fig. 11), confirms the role of differentiation in the synthesis of ACA (Table 3). The compact callus (0.761%) and control plant (0.783%) accumulated similar amounts of ACA. The amount of ACA in the regenerated plant was found to be 1.253%, 1.6-fold higher than the control plant. The content of ACA was found to be 5.4-fold higher in the regenerated plant in comparison to the early/nascent callus.

The effect of differentiation on ACA accumulation indicates a complex process being involved in the biosynthesis. This observation of close correlation between the expression of secondary metabolites and morphological cytological differentiation was also reported by Matkowski [41]. Anasori and Asghari [42] have given a similar report on the production of gingerol and zingiberene in *Zingiber officinale* Rosc., along the differentiation process, indicating that, the complex metabolic changes accompanying the differentiation of cells in plants, initiate biosynthetic pathways, which result in the accumulation of new compounds.

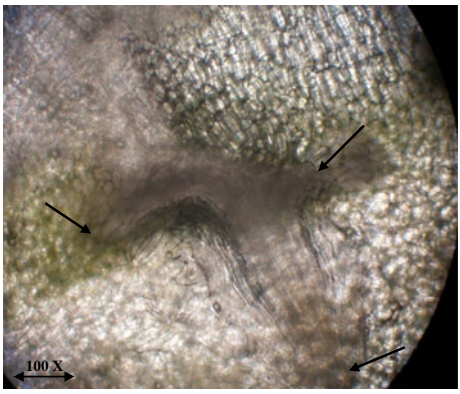

Fig. 9 Shoot buds (*arrows*) arising from the callus and their attachment to the vascular system

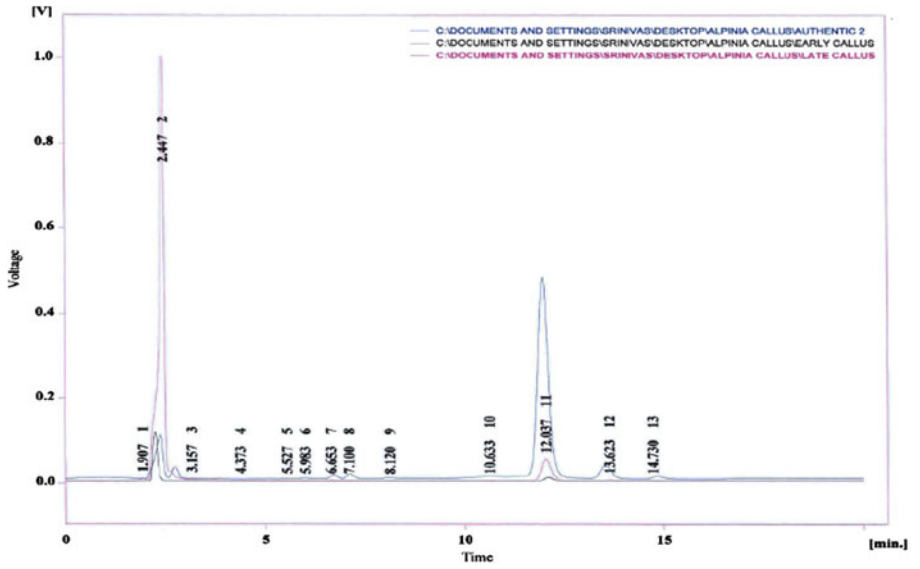

Fig. 10 Chromatogram showing higher ACA accumulation in stationary phase callus in comparison to the early callus

Acclimatization

The transition period of plantlets, from in vitro to in vivo is a very crucial phase. The plantlets with roots were washed under running tap water and transferred to the plastic pots for hardening in a mixture containing autoclaved vermiculite and soil

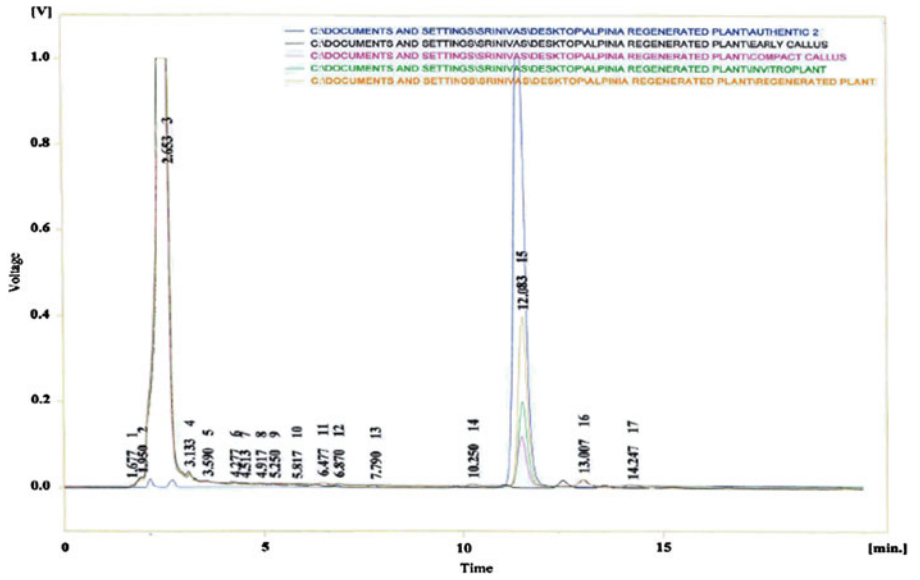

Fig. 11 HPLC chromatogram showing higher ACA production in indirect regenerated plants against the in vitro propagated plant and various stages of callus

Table 3 Comparison of ACA content in different stages of callus and regenerated plantlets

Degree of differentiation	Concentration (% dw)
Early callus (7 days)	0.232±0.164
Late callus (45 days)	0.473±0.034
Compact callus	0.761±0.023
Regenerated plant	1.253±1.346
Control plant	0.783±0.125

(1:1) under controlled conditions (humidity, light and temperature) for a period of 3 weeks (Fig. 12). The potted plants were irrigated with MS basal salt solution (1/8 strength) devoid of sucrose and myo-inositol every 4 days for 3 weeks, to avoid untoward deficiencies [18]. To avoid necrosis caused by fungal growth, plants were sprayed with 0.1% bavistin once a week. The growth of plants regenerated from bigger microrhizomes was found to be better in terms of shoot, leaf and root growth parameters, which was similar to the study conducted by Islam et al. [43]. The plantlets were maintained under green house conditions for getting attuned to natural conditions. 86% of the plantlets were able to survive the in vivo conditions. The remaining plantlets were unable to survive either because of their inadaptability to harsh climatic conditions or microbial overgrowth which caused necrosis of the explants.

Conclusion

In vitro multiplication of the medicinally important herb, *A. galanga* proves to be a difficult task, given the narrow explant choice and the rate of propagation. The presence of acetoxychavicol acetate in the in vitro grown callus cultures and the regenerated plantlets was reported for the first time in the present study. Regenerated plantlets have shown higher levels of ACA accumulation in comparison to the callus cultures and control plants. The enhanced levels of ACA would result in higher yields that can be utilized in the preparation of biopharmaceutical. The micropropagation protocol for regeneration of *A. galanga* from the callus cultures would be advantageous over the conventional methods in large-scale biomass generation in a considerably short span of time. Moreover, the faster regeneration would ensure equilibration of the demand-supply equation, thus preventing the natural habitat destruction. Also, production of

Fig. 12 Plantlets transferred to trays for hardening

rhizomes through indirect organogenesis can be obtained in a considerably short span of time.

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