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Research Article

CURBING ACTINOMYCETES AND THIDIAZURON ENHANCED MICROPROPAGATION IN THE RARE *ALPINIA GALANGA* - A MEDICINAL ZINGIBER

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ABSTRACT

Objective: Elimination of endophytic actinomycetes before micropropagation using antibiotic pre-treatment in rhizome bud explants of *Alpinia galanga*. Formulation of an operative protocol for micropropagation from the disinfected rhizome bud explants in *Alpinia galanga*.

Methods: A treatment of mercury chloride and carbendazim, alone and in combination was used as surface sterilants. A pre-treatment of rifampicin and fusidic acid was used against actinomycete endophyte disinfection of rhizome bud explants. Then, Murashige and Skoog (MS) medium supplemented with various concentrations of cytokinins was used for micropropagation of disinfected explants.

Results: A treatment of 0.1% (w/v) mercury chloride and 0.1% (w/v) carbendazim, one after the other for 5 minutes gave the best sterility of 83.3%. A pre-treatment of Rifampicin 100 mg/l and fusidic acid 100 mg/l for 2 hrs gave the best disinfection of 70% against actinomycete endophytes. A combination of thidiazuron (TDZ) 0.45 µM and 6-benzyladenine 13.32 µM in MS medium resulted in 9.4 shoots per explant. MS medium fortified with 10.74 µMof 1-naphthaleneacetic acid gave the best rooting of 20 roots/shoot. Inter simple sequence repeat marker genetic similarity of regenerants with the mother plant was confirmed.

Conclusion: This study shows the potency of Rifampicin and Fusidic acid to disinfect explants from actinomycete endophytes and is significant as the first report on curbing actinomycetes endophytes in plant tissue culture of *A. galanga.* This is also the first report conferring the dissimilar regeneration capabilities of TDZ in comparison to other cytokinins in Zingiberaceae.

Keywords: *Alpinia galanga*, Rifampicin, Fusidic acid, Actinomycetes, Thidiazuron, Inter simple sequence repeat.

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INTRODUCTION

Effective *in vitro* plant regeneration is greatly essential for application of most modern biotechnological practices in crop enhancement [1]. *Alpinia galanga* has been propagated *via* tissue culture using micropropagation, direct and indirect organogenesis with rhizome buds as explants [2-5], but the success of these efforts varies significantly, possibly due to the presence of endophytic actinomycetes in the rhizomes of *A. galanga* [6-8]. This interference in regeneration capability could be caused by the plant growth promoting the activity of indole-3-pyruvic acid and indole-3-acetic acid (IAA) produced by the endophytic Actinomycetes [9,10]. The presence of endophytic actinomycetes greatly affects commercial tissue culture both qualitatively and quantitatively; moreover, tissue cultured plants harboring these endophytes are not suitable for export due to plant quarantine.

A. galanga (L.) Willd. (Zingiberaceae) occurring in the Western Ghats of south India, northeastern parts of India and Thailand is the type species of the genus *Alpinia* Roxb. It is an important cultivated medicinal crop in India and is recognized in different traditional systems of medicine to treat microbial infections, inflammations, rheumatic pains, chest pain, dyspepsia, fever, diabetes, burning of the liver, kidney disease, tumors, and even HIV [11]. Galangin, a flavone present in *A. galanga*, has exhibited potent cardioprotective activity against doxorubicin-induced cardiomyopathy in Wistar rats [12]. The plant comprises many such pharmacologically important phytochemicals, namely, 1'S'-1'-acetoychavicol acetate, 1'S'-1'-acetoyeugenol acetate, 1'S'-1'-hydroxychavicol acetate, trans-p-coumaryl alcohol, trans-p-hydroxycinnamyl acetate, trans-p- coumaryl diacetate and nortrachelogenin, caryophyllene, cubenol and eucalyptol [13-18].

The validating genetic integrity of *in vitro* regenerated plants with regard to the mother plant is indispensable for the maintenance of certain horticultural and agronomic traits [19]. There are odds of getting somaclonal variations among the regenerants in plant tissue culture due to the impact of factors such as donor genotype, explant type, culture medium composition, physical culture conditions, the duration between successive subcultures and particular reagents used for various techniques which are possibly heritable [20]. Thus, there ascends the need to appraise whether the regenerants are genetically identical to the mother plant.

Due to the increased therapeutic and pharmaceutical demand, the status of *A. galanga* in the wild has been significantly affected, and it has gained a threatened to endangered status in the Western Ghats of India [21]. Therefore, to fulfill the demand of pharmaceutical industries and as a preliminary prerequisite for reintroduction or cultivation of the medicinal crop, the establishment of an efficient *in vitro* regeneration system void of glitches is required. The past reports of *in vitro* plant regeneration in this important medicinal crop are at best ineffective and unreproducible due to the non-elimination of endophytic actinomycetes pedant in the rhizome of the plant as mentioned above. The short-term use of antibiotics such as rifampicin has long been used in plant tissue culture for disinfecting plants without affecting their growth [22]. As there are no methods for successful disinfection of actinomycete in plant tissue culture yet and since the maintenance of genetic characters of the high-class mother plant in *in vitro* raised plants is imperative, the present study was devised to use antibiotic pre-treatments as an effective technique to avail actinomycete free explants of *A. galanga* for micropropagation and to assert the inter-simple sequence repeat (ISSR) genetic fidelity of *in vitro* produced plantlets.

METHODS

Plant material, surface sterilization, and antibiotic pre-treatment

Mature rhizomes of *A. galanga* were collected from Kerala Forest Research Institute, Peechi, Kerala, India, and plants were retained in the department of Plant science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India, and used as explant source. The plant was identified through standard herbarium specimen submitted to the Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India, and was identified as *A. galanga* var. *Galanga* (accession number-177088).

Rhizome bud explants (0.5-1 cm) were rinsed with a few drops of teepol and then subjected to running tap water for 30 minutes to remove surface debris. Further, the explants were surface sterilized with mercury chloride 0.1% (w/v), carbendazim 0.1% (w/v), and both, one after the other in sequence for 2, 5, and 7 minutes. There were 10 explants per treatment, and the experiment was conducted in triplicate. The explants were washed twice with sterile distilled water after each sterilization treatment for 2 minutes.

The surface sterilized explants were pre-treated with antibiotics such as Rifampicin ([Rif] 25, 50, 100, and 200 mg/l), fusidic acid ([Fus] 50 and 100 mg/l), and their combinations. The treatments were carried out for time periods ½ hr, 1 hr, and 2 hrs. Finally, the explants were washed twice with sterile distilled water for 15 minutes after the treatment.

Culture conditions and contamination screening

The explants were inoculated aseptically using a laminar air flow chamber on separate glass test tubes (25 cm × 1.5 cm) containing Murashige and Skoog (MS) medium [23] gelled with 0.7% (w/v) agar and 3% (w/v) sucrose as carbon source (Basal MS). The pH of the medium was adjusted to 5.8 before it was autoclaved (121°C and 100 kPa for 15 minutes). The medium was supplemented with plant growth regulators of different concentrations before adjusting its pH, according to the experiments. The cultures were maintained in a 16 hrs photoperiod (25 μMol/m s^{-1}, 400-700 nm, cool white fluorescent light) (Phillips, India) with a culture room temperature of 25±2°C.

The surface sterilized explants were screened on basal MS medium for 1 week for inferring any contamination. The data on sterility and necrosis of the explants were documented, and percentage sterility and necrosis were calculated for the various treatments and time periods. Then, the explants were subjected to antibiotic pre-treatment and were screened on MS medium with 6% (w/v) sucrose and 0.1% (w/v) casein hydrolysate. The data on sterility of explants were noted after 2 weeks, and the percentage sterility was calculated.

Multiple shoots induction

Two experiments were done one based on the other, wherein the first experiment the outcome of individual cytokinins 6-benzyladenine (BA), kinetin (KN), thidiazuron (TDZ), zeatin and N6-[2-isopentenyl]adenine (2ip) were studied on induction of multiple shoots. The explants were inoculated on MS medium fortified with the above cytokinins, except for TDZ all the other cytokinins were screened at higher concentrations in the range between 4.44 and 14.76 μM and TDZ was screened at concentrations between 0.22 and 2.25 μM. In the second experiment, MS medium sublimated with TDZ 0.45 μM in combination with BA 04.44, 08.87, and 13.32 μM was studied. Data were analyzed after 8 weeks of culture, and the number of shoots, roots, and leaves per shoot for each explant was documented.

Rooting and hardening

Rooting was studied separately on MS medium fortified with auxins IAA, indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) at concentrations ranging from 4.90 to 17.13 μM as auxins produced much better rooting than cytokinins, NAA in particular. The data were analyzed after 8 weeks of culture. Hardening was done on autoclaved sand, soil, and coco peat (1:1:1[w/w/w]) after separating individual shoots with copious roots. The hardening process was done inside a closed plastic container to simulate high humidity for 2 months and then grown plants were further hardened on the same mixture covered with transparent polythene bags (12 × 8 cm, Tarson, New Delhi, India) for four further months; then, they were potted on normal garden soil. The chemicals used in the above experiments were purchased from Hi-Media Pvt. Ltd. (Mumbai, India).

Genetic fidelity testing

Fidelity of *in vitro* raised clones was tested using ISSR markers. Total genomic DNA was extracted from the leaves of the mother plant and randomly selected micropropagated plantlets using the modified CTAB method [24]. Quality and concentration of DNA were assessed visually by band intensity in contrast with Lambda DNA of fixed concentration using a 0.8% agarose gel. The DNA samples were maintained in tris-ethylenediaminetetraacetic acid buffer and stored at 4°C for further analysis. ISSR analysis was initiated with 10 primers, namely, University of British Columbia (UBC-807), UBC-808, UBC-809, UBC-835, UBC-842, UBC-855, UBC-856, UBC-857, UBC-866, and $(ACTG)_4$. The polymerase chain reaction polymerase chain reaction (PCR) was carried out in 25 μl reactions (12.5 μl 2X TaKaRa Taq™ Premix, 2.5 μl primer (0.5 μM), 2 μl of template DNA and 8 μl of double distilled water). The PCR were run in a DNA thermal cycler (MJ Mini™ 48-well personal thermal cycler, Bio-Rad, USA) with one primer in each reaction. The PCR amplification profile for all the primers was 98°C for 120 seconds, 35 cycles of 98°C for 30 seconds, 50°C for 90 seconds, 72°C for 60 seconds, final extension 72°C for 300 seconds, and 4°C for infinity. The PCR products were run with 4 μl of 6X DNA loading buffer on 1, 1.2, and 1.5% agarose gels for ISSR which was prepared in 1X TBE buffer containing 0.5 μg/ml of Ethidium bromide. A GeneRuler™, Thermo Scientific, Massachusetts, USA, 1 kb Plus DNA Ladder was used. The amplified products were electrophoresed and after separation gels were documented using a ChemiDoc™ XRS+ imaging system, Bio-Rad, USA.

Only clear and reproducible bands were scored, but a prominent band conforming to a feeble band in other repeats was also considered in the analysis. Each and every band was considered as a marker. Bands with identical mobility and molecular weight were reflected as a single locus.

Statistical analysis

All experiments were setup in a completely random design and conducted in quintuplicate with the same culture conditions unless otherwise specified. The mean values of results obtained from various plant growth regulators were subjected to a one-way ANOVA, and Duncan's Multiple Range test was used for comparing means (SPSS 16.0 software, SPSS Inc., Chicago, IL, USA) in the multiple shoots induction and rooting experiments. The statistical significance was set to p=0.05.

RESULTS

Surface sterilization

In the present study, it was found that surface sterilization with 0.1% (w/v) mercury chloride alone and in combination with 0.1% (w/v) carbendazim, one after the other for 7 minutes gave 96.7% and 100% sterility, but it was observed that the necrosis of explants was too high, which was 76.7% and 96.7%, respectively. Therefore, based on the surface sterilization experiment, it was concluded that a treatment of 0.1% (w/v) mercury chloride and 0.1% (w/v) carbendazim, one after the other for 5 minutes, which gave a sterility of 83.3% and had a necrosis of 16.7% was considered as the best sterilization treatment for the rhizome bud explants (Fig. 1).

Antibiotic pre-treatment and contamination screening

In the present study, instead of adding antibiotics to the medium, a pre-treatment of antibiotics for a shorter stipulated time was followed. Rifampicin 200 mg/l for 2 hrs gave a disinfection of 70% against actinomycetes but is a very high concentration of the antibiotic, thus rifampicin 100 mg/l and fusidic acid 100 mg/l for 2 hrs which also gave 70% disinfection against actinomycetes was considered (Fig. 2), since a combination can screen a wider range. The treated explants were inoculated on MS medium fortified with 6% sucrose and 0.1% casein hydrolysate since they are common constituents of actinomycete

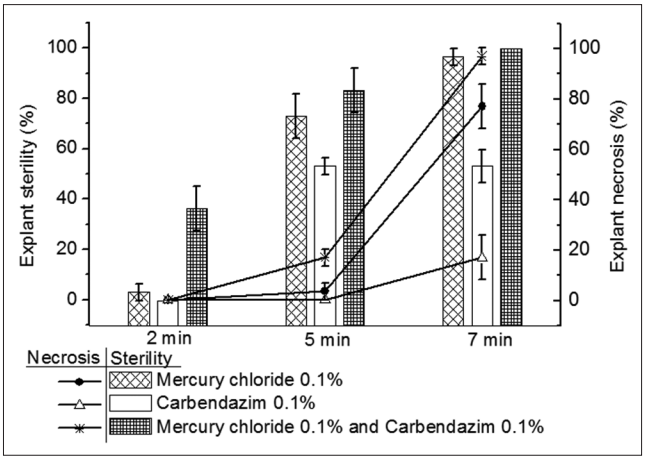

Fig. 1 Percentage sterility versus necrosis of *Alpinia galanga* explants after surface sterilization

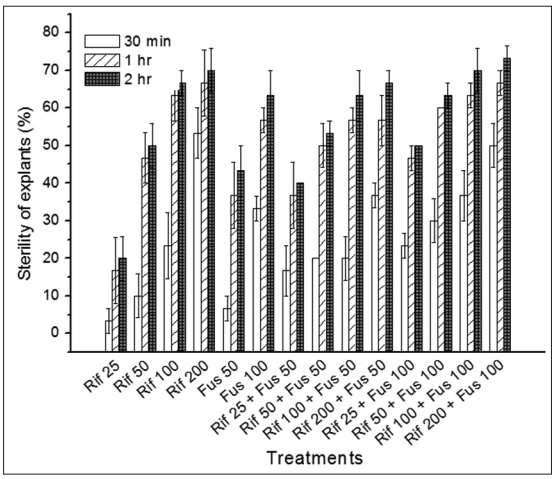

Fig. 2 Percentage sterility of *Alpinia galanga* explants after surface sterilization and antibiotic treatment

medium preparations, which promoted exiting of endogenous actinomycetes on to the medium (Fig. 3a).

Cytokinins on multiple shoots induction

The maximum number of shoots after 8 weeks' time was observed on MS medium fortified with BA 13.32 µM (4.6), followed by TDZ 0.45 µM (4.2) (Fig. 3d and e). Multiple shoot induction in the presence of BA and TDZ was significantly higher compared to control (basal MS) after 8 weeks of culture (Table 1). The minimum multiple shoots in a cytokinin were observed in 2ip which was as low as the control (data not shown). All the cytokinins simultaneously produced roots, with a maximum of 6.4 in KN 13.94 µM and a minimum of 1.6 in TDZ 0.22 µM. Maximum number of leaves (4/shoot) was observed in 2ip 14.76 µM (data not shown) and the minimum was in TDZ 0.22 µM (Table 1).

Interaction between BA and TDZ on multiple shoots induction

The MS medium supplemented with 0.45 µM TDZ in synergy with 13.32 µM BA showed the best multiple shoot producing (9.4) (Fig. 3f). Number of leaves produced were not much affected but rooting significantly increased when TDZ was combined with BA forming a maximum of 6.4 roots per shoot in contrast to 2.2 roots which was maximum in TDZ 0.22 µM.

Rooting and hardening

In the present study, IAA and IBA also produced roots consistent with other studies on Zingiberaceae members [25,26] but when shoots were cultivated on MS medium with NAA they produced significantly greater number of roots among which the maximum was on 10.74 µM NAA (20) (Table 2) (Fig. 3g and i). Initial hardening was done in plastic cups filled with soil, sand, and coco peat (1:1:1), the potted plants were kept in plastic containers for maintaining high moisture content (Fig. 3j and k). This hardening process was extended for 4 months after which the rhizomes were thick and prominent enough for transfer to the field (Fig. 3l and m). The survival rate of the hardened plants was 80%.

Genetic fidelity testing

Among the 10 ISSR primers employed in the initial screening, 7 produced clear and reproducible bands. The annealing temperature for all the ISSR markers used was 55°C. The 7 ISSR primers formed 51 distinct and scorable bands ranging from 200 bp to 2000 bp in size. The scorable bands for the various primers varied from 3 (UBC-856) to 14 (UBC-842) (Fig. 4) with a mean of 7 bands per primer. The banding profiles from the various micropropagated plants exhibited complete monomorphicity and exhibited 100% similarity to those of the mother plant.

DISCUSSION

Surface sterilization

Since the rhizome bud explants are of underground origin rinsing them thoroughly with running tap water was a must to remove surface debris and to reduce the inoculum of surface microbes [25]. Mercury chloride was subjected as a general purpose sterilant as it works against a broad range of microbes especially bacteria, it attacks the protein sulfhydryl groups and disrupts normal enzyme functioning [27]. Carbendazim also called as Bavistin is a broad range systemic fungicide which works against fungi by causing microtubule disrupting [28]. Mercury chloride and carbendazim were used in concentrations 0.1% (w/v) and more for above 5 minutes to avail good sterile explants in the genus *Alpinia* and species *A. galanga* for the rhizome bud explants [2-5], but in contrary, it was found in the current study that mercury chloride and carbendazim caused greater percentage of necrosis in explants when the treatment exceeded 5 minutes (Fig. 2).

Antibiotic pre-treatment and contamination screening

Antibiotics have long been used in plant tissue culture as media supplements but since some of them are known inhibitors of nucleic acid metabolism, protein synthesis or even plant growth they are not mainstream. Rifampicin is a competitive inhibitor of RNA polymerase and is well known for its wide range activity against actinomycetes [29]. Fusidic acid hinders protein synthesis by inhibition of an elongation factor at the level of the ribosome and is found to be very effective against actinomycetes in synergy with rifampicin [30]. This is the first report of using rifampicin and fusidic acid pre-treatments for actinomycete elimination in plant tissue culture.

Cytokinins on multiple shoots induction

The motive of a micropropagation protocol is basically to produce a large number of genotypically uniform plantlets analogous to the original plant; this is evident using meristematic regions. In the present study, the rhizome bud explant which has a prominent meristematic region was used, and regeneration from such meristematic regions is considered as micropropagation (Fig. 3b and c). Shoot initiation began in 2 weeks' period in all treatments except for TDZ in which the shoots started forming in around 3 weeks' time.

Micropropagation studies in *Alpinia zerumbet* and *Alpinia purpurata* have already shown that BA is very effective in producing shoots from rhizome bud explants [26,31], which concurs with the current study. *Aframomum corrorima* also a Zingiberaceae member has shown good micropropagation of rhizome buds by supplementing BA and TDZ in MS medium as in the present study, but they have not discussed their synergistic effect [32].

Interaction between BA and TDZ on multiple shoots induction

Comparing Fig. 3d and e and f an interaction is clear between BA and TDZ on multiple shoot initiation in *A. galanga* after 8 weeks of culture.

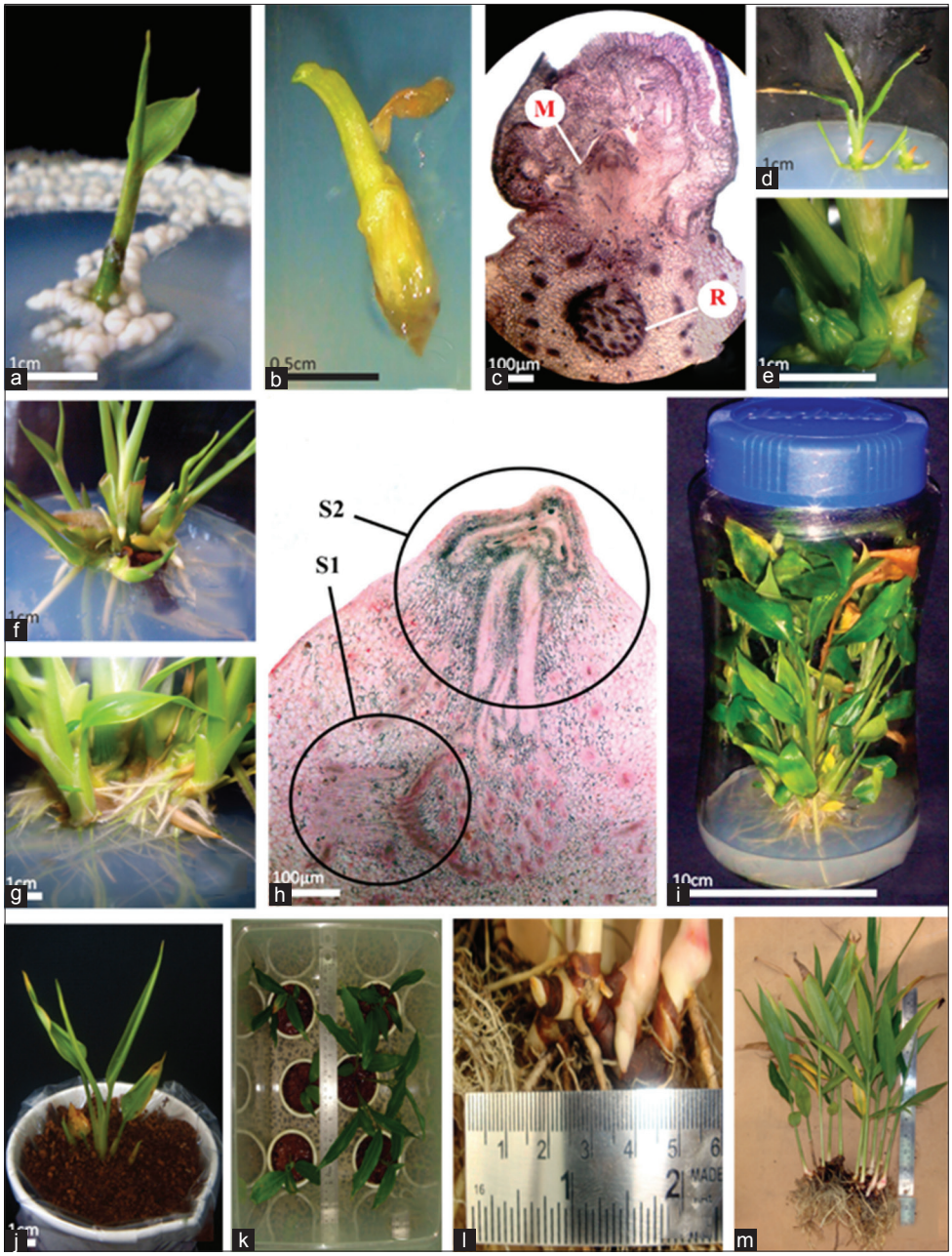

Fig. 3 Micropropagation of *Alpinia galanga*. (a) Infected rhizome bud explant, (b) sterilized rhizome bud explant on Murashige and Skoog medium, (c) longitudinal section of rhizome bud (stained in Safranin) showing Rhizome (R) and meristem (M), (d) multiple shoots on Murashige and Skoog (MS) medium with BA 13.32 µM, (e) multiple shoots on MS with thidiazuron (TDZ) 00.45 µM, (f) multiple shoots on MS medium with TDZ 00.45 + BA 13.32 µM, (g) rooting on MS medium with 1-naphthaleneacetic acid 10.74 µM, (h) handmade cross section of regenerating rhizome (stained in Safranin) showing vascular system of stem 1 (S1) and vascular system of newly forming apical meristem (S2), (i) multiple shoots of *A. galanga* after rooting, (j) initial hardening of separated stems on sand, soil and coco peat (1:1:1[w:w:w]), (k) hardening of plants inside plastic containers to maintain high moisture content, (l) rhizomes of 4 month hardened plant, and (m) 4 month hardened plants

It was inferred that with rising concentration of BA in synergy with 0.45 µM of TDZ, the shoot number significantly increased (from 4 to 9). A combination of TDZ and BA showed not only increase in shoot number but also a significant increase in shoot development which is usually hampered by TDZ. All concentrations of TDZ showed lower multiple shoot production than TDZ 0.45 µM in combination with BA (Table 1).

It was interesting to observe that multiple shoots formed in BA and other cytokinins other than TDZ had a different anatomy when compared to that formed on TDZ. In cytokinins other than TDZ, the multiple shoots formed were observed to be positioned at 180° or 360° (like a bull horn or a two-horned rhino) as in Fig. 3d but the multiple shoots formed on TDZ were positioned at around 90° (Fig. 3h). This signifies that regeneration formed by TDZ is considerably different from other cytokinins. Thus, justifying BA and TDZ cytokinin synergy producing better results than the individual cytokinin in the current study.

The present study is the first to discuss a BA and TDZ cytokinin synergy in the micropropagation of a Zingiberaceae member although BA and TDZ combination has been perceived in micropropagation of *Psoralea corylifolia* a Fabaceae member [33].

Table 1: Effect of cytokinins on multiple shoot formation in *A. galanga*, after 8 weeks

PGR (µM)				Mean shoots/explant (±SD)	Mean roots/shoot (±SD)	Mean leaves/shoot (±SD)
BA	KN	TDZ	ZEA			
0	0	0	0	1.0±0.0^{j}	1.2±0.4^{e}	1.4±0.5^{bcd}
04.44	0	0	0	2.8±0.8^{fgh}	3.4±0.5^{c}	1.5±0.5^{bcd}
08.87	0	0	0	3.4±1.3^{ef}	3.2±0.4^{cd}	1.5±0.4^{bcd}
13.32	0	0	0	4.6±0.9^{cd}	3.6±0.5^{c}	1.4±0.3^{bcd}
0	04.65	0	0	1.4±0.5^{ij}	5.8±0.8^{a}	1.8±0.3^{bc}
0	09.29	0	0	2.2±0.4^{ghi}	6.0±1.2^{a}	1.9±0.6^{bc}
0	13.94	0	0	3.2±0.4^{fg}	6.4±1.3^{a}	1.9±0.5^{bc}
0	0	00.22	0	2.4±0.5^{fghi}	2.2±0.4^{e}	1.2±0.2^{cd}
0	0	00.45	0	4.2±0.8^{de}	2.0±0.0^{e}	0.9±0.2^{d}
0	0	02.25	0	1.0±0.0^{j}	1.6±0.5^{de}	1.4±0.5^{bcd}
0	0	0	04.56	1.0±0.0^{j}	5.8±1.1^{a}	2.6±0.5^{ab}
0	0	0	09.12	1.8±0.4^{hij}	5.4±0.5^{a}	2.0±1.1^{ab}
0	0	0	13.68	2.0±0.0^{hij}	4.2±1.1^{bc}	2.0±0.5^{a}
04.44	0	0.45	0	5.2±1.1^{c}	6.4±1.5^{a}	1.5±0.2^{bcd}
08.87	0	0.45	0	7.4±0.5^{b}	5.2±0.8^{ab}	1.4±0.2^{bcd}
13.32	0	0.45	0	9.4±1.3^{a}	5.6±0.5^{a}	1.4±0.2^{bcd}

Within each experiment, mean in a column followed by the same lowercase letter are not significantly different (p<0.05), SD: Standard deviation

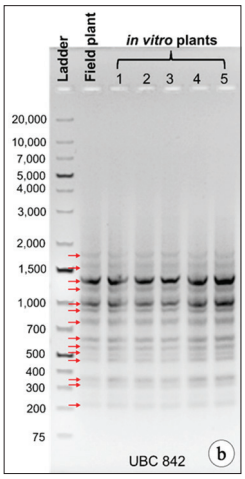

Fig. 4 Agarose gel showing inter simple sequence repeat markers (arrows) of field plant and *in vitro* plants generated from primer University of British Columbia 842

Rooting and hardening

Although all cytokinins simultaneously induced rooting which was consistent with previous studies of Zingiberaceae members [31,34], it was still miniscule when compared to the potential of auxins especially NAA (Table 2). The greater number of roots were ideal since shoots with more number of roots survived well during the hardening process.

Genetic fidelity testing

In vitro regenerated plants have an ambiguity; a considerable degree of somatic mutation can occur in them [35]. ISSR primers have long been effectively used in assessing the genetic integrity of plants and have broadly been used in Zingiberaceae [36]. ISSR markers are preferred against SSR markers in the detection of variations among micropropagated plants since an SSR marker aims at fast evolving hypervariable sequences. ISSR method is similar to the random amplification of polymorphic DNA method, as both are PCR-based methods using appropriate primers, except that ISSR primers are designed from microsatellite regions. They amplify sequences flanking SSR, and the amplifications are of anonymous SSR loci. ISSR loci are predominantly dominant and rarely co-dominant as the size of prevailing space between microsatellites differ. They are most cost-effective, fast, and easy to use markers.

Table 2: Effect of auxins on root formation in *A. galanga*, after 8 weeks

PGR (µM)			Average number of roots/shoot (±SD)
IAA	IBA	NAA	
05.71	0	0	4.4±1.5^{d}
11.42	0	0	4.6±1.3^{d}
17.13	0	0	4.8±1.6^{d}
0	04.90	0	4.0±0.0^{d}
0	09.80	0	5.0±0.0^{d}
0	14.70	0	4.0±0.0^{d}
0	0	05.37	16.2±2.0^{b}
0	0	10.74	20.0±1.2^{a}
0	0	16.11	8.4±1.3^{c}

Within each experiment, mean in a column followed by the same lowercase letter are not significantly different (p<0.05), SD: Standard deviation, IAA: Indole-3-acetic acid, IBA: Indole-3-butyric acid, NAA: 1-naphthaleneacetic acid, *A. galanga: Alpinia galanga*

In the current study, UBC ISSR markers were used for the first time in genetic fidelity testing of *A. galanga.* The UBC ISSR markers are the most widely accepted and consistent in comparison to other ISSR markers. The results from the current study concurred with other ISSR works done in the species although utilization of a much more standard group of marker such as UBC adds weightage in the present scenario [3,37].

CONCLUSION

This is the first report on curbing actinomycetes endophytes in plant tissue culture of *A. galanga* using antibiotic pre-treatments. A pre-treatment of rifampicin 100 mg/l and Fusidic acid 100 mg/l for 2 hrs gave 70% sterile explants against actinomycetes; the combination was successful in defending against a broad array of endophytic actinobacteria from soil source. The initial surface sterilization of 0.1% (w/v) mercury chloride followed by 0.1% (w/v) carbendazim, one after the other for 5 minutes each, gave a sterility of 83.3% with an acceptable necrosis of 16.7%.

This is also the first report conferring the dissimilar regeneration capabilities of TDZ in comparison to other cytokinins in Zingiberaceae. Thus, a synergy of cytokinins TDZ 0.45 µM with BA 13.32 µM resulted in the best multiple shoot production of 9.4 shoots per explant. This protocol is apt for producing microbe-free explants from a rhizome or similar underground stems where actinomycete endophytes are present.

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