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ARTICLE

In vitro callus induction and identification of DNA variation in *Etlingera elatior* using Inter-simple sequence repeat (ISSR) markers

Indução *in vitro* de calos e identificação de variação de DNA em *Etlingera elatior* usando marcadores de sequências simples repetidas (ISSR)

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Abstract: *Etlingera elatior* is a promising ornamental horticultural species with various purposes such as medicinal, antibacterial agent, culinary, ornamental, and floral arrangement. The increasing demand for more variation has led to the improvements of *E. elatior* via tissue culture technology. Somaclonal variation helps to overcome the lack of variation of this species due to asexual propagation. The aims of this study are to induce callus and shoot and to detect genetic variations using ISSR markers. The results showed that the Murashige & Skoog (MS) basal medium supplemented with glucose and 1.5 mg L^{-1} 6-benzylaminopurine (BAP) and 3 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) produced significantly higher callus percentage, 50% after 20 weeks of culture. The friable calluses were then transferred to shoot induction media containing different concentrations of different BAP, 1-naphthaleneacetic acid (NAA) and thidiazuron (TDZ). After 12 weeks in shoot induction media, a root-like structure was observed in calluses masses T11 (0.1 mg L^{-1} NAA and 0.3 mg L^{-1} TDZ). Seven ISSR primers were used to evaluate the genetic variation of calluses. Seventy-two bands were generated, of which 51 bands were polymorphic with an average percentage of polymorphic bands of 72%. Jaccard's coefficient of similarity values recorded between 0.3529 and 0.4762 exhibited the level of genetic variation among calluses. In short, the explants were affected by different concentrations of auxin and cytokinin for callus induction. ISSR markers revealed the occurrence of genetic variation during callus and shoot induction processes, suggesting a potential to generate new variants using tissue culture.
Keywords: genetic variations, horticulture, plant growth regulators, polymorphism, somaclonal variation.

Resumo: *Etlingera elatior* é uma espécie ornamental promissora para a horticultura, com vários propósitos, tais como medicamentos, agentes antibacterianos, culinários e ornamentais. O aumento da demanda por mais variedades levou a melhorias da *E. elatior* por meio da tecnologia de cultura de tecidos. A variação somaclonal ajuda a superar a falta de variedades desta espécie, devido à propagação assexual. Os objetivos deste estudo foram induzir calos e brotos e detectar variações genéticas usando marcadores ISSR. Os resultados mostraram que o meio basal Murashige & Skoog (MS) suplementado com glicose e 1,5 mg L^{-1} de 6-benzilaminopurina (BAP) e 3 mg L^{-1} de ácido 2,4-diclorofenoxiacético (2,4-D) produziu uma percentagem de calos significativamente maior, 50% após 20 semanas de cultura. Em seguida, os calos friáveis foram transferidos para meios de indução de brotos com diferentes concentrações de BAP, ácido 1-naphthaleneáctico (NAA) e thidiazuron (TDZ). Após 12 semanas em meios de indução de brotos, uma estrutura semelhante a raiz foi observada em massas de calos T11 (0,1 mg L^{-1} NAA e 0,3 mg L^{-1} TDZ). As variações genéticas dos calos foram avaliadas com base em sete primers ISSR. Setenta e duas bandas foram geradas, das quais 51 foram polimórficas, com a percentagem média de bandas polimórficas de 72%. O coeficiente de Jaccard de valores de semelhança registrados entre 0,3529 e 0,4762 mostrou o nível de variação genética entre os calos. Em resumo, os explantes foram afetados por várias concentrações de auxina e citocinina para indução de calos. Os marcadors ISSR revelaram a ocorrência de variações genéticas durante os processos de indução de calos e brotos, sugerindo um potencial para gerar novas variantes utilizando cultura de tecidos.
Palavras-chave: horticultura, polimorfismo, reguladores de crescimento de plantas, variação somaclonal, variações genéticas.

Introduction

Etlingera elatior (Jack) R.M. Sm. (Zingiberaceae) or torch ginger is an ornamental horticultural species with high economic potential. All parts of *E. elatior*, including inflorescences, rhizomes, and leaves have their own economic potential (Cunha Neto et al., 2023). In Southeast Asia, the inflorescence bud is popular for flavouring in many Malay, Nyonya, Indonesia and Thai dishes. Besides, *E. elatior* is commonly used as a medicine due to the presence of biochemical substances such as phenols, flavonoids, glycosides, saponins, tannins, steroids, and terpenoids (Yunus et al., 2022). This species is gaining popularity as a decorative and landscaping element, such as floral arrangements, in both gardens and urban areas worldwide (Yunus et al., 2021; Atmaja et al., 2024).

In the competitive ornamental and horticultural industries, there is a growing need to obtain attractive, economically valuable cultivars (Machanda et al., 2024; Patel et al., 2024; Tapia et al., 2024). For now, *E. elatior* is still being propagated traditionally by asexual propagation via rhizomes and the less successful using seeds. However, both methods require more time for flowering, which is inadequate for mass production. To overcome the limitations of conventional propagation of *E. elatior*, *in vitro* cultivation is a promising technique to achieve large-scale multiplication, using samples obtained by genetic improvement, somaclonal variation, genetic engineering and mutation induction.

To achieve the demand for *E. elatior*, *in vitro* cultivation technology has been chosen as the primary way in the genetic improvement program of this ornamental horticultural species. This technique can be used to produce disease-free and rapidly multiplying plants. This could help in genomes manipulation aimed at producing commercially valuable compounds derived from improved varieties (Pakum et al., 2020). Callus induction, produced through indirect regeneration is one of the plant tissue culture techniques that could be used (Wu et al., 2024). Generally, calluses are significant sources for somaclonal variation, plant regeneration, secondary metabolites, and starting material for cell suspension culture.

Somaclonal variation is referred to as a variation in genetic content that commonly occurs in plant tissue culture (Orłowska et al., 2024; Ferreira et al., 2023). The results of these variations can be used to obtain desirable ornamental and medicinal properties, pest and disease resistance, and biotic and abiotic tolerance (Duta-Cornescu et al., 2023). Somaclonal variation may be a useful tool for plant breeders, especially when plants are propagated asexually, i.e., when morphological variation is minimal. Furthermore, somaclonal variation is one of the most financially efficient

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strategies for developing a new variety of plants without any sophisticated tools (Eeckhaut et al., 2020;). In addition, somaclonal variation can be detected by molecular markers such as ISSR (Ferreira et al., 2023).

The use of *in vitro* cultivation to obtain an *E. elatior* callus collection is a promising strategy for future work involving genetic transformation based on mutation breeding and the production of biochemical compounds by suspension culture. Therefore, the development of an effective protocol for plant regeneration by callus formation is required. The objectives of this study were to investigate the optimal concentration and combination of multiple plant growth regulators (PGRs), with two carbon sources for callus induction, shoot induction and to determine the genetic variation of the callus using ISSR markers. In brief, this is the first report of the successful callus formation of *E. elatior* from the young, closed bud explants and the first detection of somaclonal variation in *E. elatior* callus using ISSR markers.

Materials and Methods

Explant Selection

Healthy young, closed buds of *E. elatior* without any disease symptoms were obtained from the Glasshouse and Nursery Complex, Kulliyyah of Science (KOS), International Islamic University Malaysia (IIUM), Kuantan, Malaysia. A single flower from the closed bud was used as explant. The closed bud was excised approximately 15 cm long from the plant peduncle and taken to the Plant Tissue Culture Laboratory, KOS, IIUM.

Surface Sterilization

The closed bud was cleaned under running tap water and immersed in 5 mL Teepol and 5 drops of Tween 20 for 10 min to remove adherent dirt and contaminants. It was then placed in a sterile beaker in a sterilized laminar airflow. The bud was immersed in 20% Chlorox® solution with four drops of Tween 20 for 10 min. It was then washed once with sterile distilled water. The bud was then immersed in 98% ethanol for 10 min, rinsed three times with sterile distilled water. The bud was cut in half using a sterile blade on sterile paper. Finally, the individual flower was excised and transferred to callus induction medium.

Callus Induction and Proliferation

MS medium (Murashige and Skoog, 1962) supplemented with either 30 g L^{-1} sucrose or glucose, 0.1 g L^{-1} myoinositol, and 0.1 g L^{-1} ascorbic acid was used for this step. Different concentrations and combinations of BAP (0.0, 1.0, and 1.5 mg L^{-1}) and 2, 4-D (0.0, 3.0, 6.0, and 9.0 mg L^{-1}) were added to the media according to the treatments. A total of 24 treatments were conducted for both sucrose and glucose with different combinations of 2,4-D and BAP, where 0.0, 3.0, 6.0, and 9.0 mg L^{-1} 2-4-D were used either alone or in combination with 0, 1.0 and, 1.5 mg L^{-1} BAP. MS medium without PGR (MSO) was used for the control treatment. The pH was then adjusted to a range of 5.7 to 5.8. Before solidification, the media were mixed with 2 g L^{-1} Phytagel and autoclaved at 121 ºC for 15 min at 103 kPa. Incubation was performed at 25 ± 1 °C in the dark.

Three biological replicates were performed with each replicate consisting of four explants. For callus scoring by visual estimation: - = no callus; + = poor or the callus covered less than half of the explant; ++ = average or the callus covered half of the explant; +++ = good or the callus covered more than half of the explant but not the whole explant; ++++ = excellent or the callus covered the whole explant. To proliferate more callus, the induced calluses were subcultured on the similar MS treatment consisting of the same PGRs. The callus cultures were maintained by subculturing at 4-week intervals for 12 weeks.

Shoot Induction

The calluses were transferred to MSO for two weeks to remove the effects of the previous treatment. The shoot induction medium was supplemented with 30 g L^{-1} glucose, 0.1 g L^{-1} myo-inositol, and 0.1 g L^{-1} ascorbic acid. Then, BAP (0.0, 1.0, 2.0, 3.0, and 5.0 mg L^{-1}), 1-naphthaleneacetic acid (NAA) (0.0, 0.1, 0.5, and 1 mg L^{-1}) and thidiazuron (TDZ) (0.0, 0.3, and 1.0 mg L^{-1}) were then added to the media. MS medium (T1) without addition of PGR was used for the control. Then, calluses (50 mg) were transferred to the shoot induction medium. The shoot induction treatments were adapted from the *Curcuma attenuata* shoot induction study by Kou et al. (2012). Three replicates were performed, with each replicate consisting of three callus clumps.

Experimental Design and Statistical Analysis

A completely randomized design (CRD) was used for the analysis of callus and shoot induction. Analysis of variance (ANOVA) was used for the data analysis by using SAS 9.2 software. When significant differences between treatments were detected, means were compared using Duncan's Multiple Range Test (DMRT) at the 5% significance level.

Protocols were based on Yunus et al., (2013). A DNA integrity assessment was conducted to verify the DNA quality.

Genomic DNA Extraction and quantification

Gel electrophoresis analysis, the resulting gels were documented using a Molecular Imager Gel Doc XR+ system (Bio-Rad, USA). Each sample was evaluated for purity and concentration using a Nanodrop spectrophotometer. DNA samples were diluted to 50 ng uL^{-1} for ISSR analysis.

Primer Selection and Polymerase Chain Reaction (PCR)

Eight ISSR primers were used for primer selection; UBC 808, UBC 809, UBC 811, UBC 830, UBC 855, UBC 880, UBC 888, and UBC 891. These primers were based on the methodology described by Ismail et al. (2019). The PCR reaction was performed twice for each primer to confirm their reproducibility. PTC Tempo Thermal Cycler Bio-Rad was used for PCR analysis. The mixture consisted of 12.5 µL of 2 × Taq Master Mix (Vivantis Technologies Sdn Bhd, Malaysia), 0.5 mM $MgCl_2$ (Vivantis), 0.4 µM of primer (Integrated DNA Technologies, Singapore), and 50 ng of DNA for a final volume of 25 µL. No template DNA was used as a positive control, while mother plant leaves were used as a negative control.

The PCR cycle was follows initial denaturation at 95 ºC for 3 min, followed by 34 cycles of denaturation at 95 ºC for 30 s, annealing at 51.4 ºC to 53.7 ºC for 30 s, and extension at 72 ºC for 1 min. A final extension step was performed at 72 ºC for 10 min, followed by cooling to 4 ºC. Electrophoresis was performed to separate amplicons on 1.2% agarose gels in 1 × TAE buffer at 70V for 75 min. The gels were stained with ViSafe Green Gel Stain (Vivantis SD0101) and visualized. All PCR cycles were performed twice for each primer.

Scoring and ISSR Data Analysis

The assessment of DNA fragments displayed was scored using a binary system, denoted by 0 for absence and 1 for presence. Jaccard's coefficient of similarity was used to determine the genetic relatedness among the nine regenerants. Cluster analysis was then performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). For genetic similarity calculations, Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc), version 2.11a software was used to calculate genetic similarity and construct the dendrogram.

Results and Discussion

Effect of Different Carbon Sources and Concentrations of 2,4-D and BAP on Callus Induction

In this study, two different carbon sources, sucrose and glucose have been examined for their effect on callus induction of *E. elatior*. Table 1 exhibits that the medium containing 3% glucose showed higher callus induction compared to sucrose. Furthermore, three glucose treatments; C14 (3 % glucose with 3.0 mg L^{-1} 2,4-D), C18 (3 % glucose with 3.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BAP), and C22 (3% glucose with 3.0 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BAP) were successful in inducing friable callus compared to the 3% sucrose treatment. In contrast, only treatment C2 (3% sucrose with 3.0 mg L^{-1} 2,4-D) was successful in inducing friable callus. This result agrees with a study by Hossain et al. (2013), which specified that the highest rate of callus formation from *Musa acuminata* was obtained in MS medium containing 3% glucose compared to sucrose and sorbitol. Glucose is more efficient due to its nature as a monosaccharide, consisting of a simple structure compared to the disaccharide structure of sucrose (Marir, 2024). This simpler structure of glucose makes it easier for plant cells to metabolize, resulting in more efficient use of the carbon source. In addition, glucose produced better results for callus formation, callus proliferation, and organogenesis due to its osmotic potential as reported by Oliviero et al. (2022).

Callus induction of *E. elatior* was successfully initiated after 17 weeks of culture on media. This result is analogous with Gomes-Dias et al. (2014), as they found that the application of 2,4-D and picloram proved

to be successful in inducing friable embryogenic callus after 16 weeks of *in vitro* inoculation of *E. elatior*. In our study, the explants from all treatments showed survival during the first four weeks of culture, with swollen, enlarged explants and a change in color from whitish to white creamy (Fig. 1). White friable calluses were developed from the wounded area and gradually extended over the entire surface of the explants (Fig. 1). Using young, closed buds or immature inflorescence as an explant was a common practice, especially in monocotyledonous plants. Immature inflorescence of five different *Curcuma* hybrid cultivars (Yoosumran et al., 2022) and four different *Begonia* L. species (Nabieva and Fershalova, 2023) have been successful in producing friable callus. Both groups of researchers reported that the immature inflorescence explants have a low contamination rate where the explants swollen, enlarge and change colour before the formation of callus.

Of the 24 media tested, only four media; C2 (3% sucrose with 3.0 mg L^{-1} 2,4-D), C14 (3% glucose with 3.0 mg L^{-1} 2,4-D), C18 (3% glucose with 3.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BAP), and C22 (3% glucose with 3.0 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BAP) showed successful callus induction (Fig. 2). The 3 mg L^{-1} 2,4-D was the most effective in inducing callus as observed in these four treatments. Treatment C22 was the best treatment with significantly higher callus induction based on the statistical analysis. 50% of the explants from this treatment produced friable callus with average (++) callus intensity compared to other treatments. Meanwhile, C14 showed 25% callus induction with excellent (++++) callus intensity. Otherwise, both C2 and C18 treatments exhibited similar callus induction success rate (16.67%) and callus intensity (+++). After 20 weeks of callus induction, all friable calluses were detached from the explants and transferred to the new media. Our results showed that supplementing friable calluses on the same MS medium with PGRs from previous successful callus induction treatments (C2, C14, C18 and C22) was optimum optimal for callus propagation.

The result showed that explants in the absence of PGRs; treatment C1 (3% sucrose) and treatment C13 (3% glucose) had no morphogenetic response, turned brown and dead after 5 weeks of incubation while explants with PGRs continuously swell and grow. For this study, 2,4-D alone or in combination with BAP was effective in inducing friable embryogenic callus in different treatments. Our findings were also consistent with the previous findings from Gomes-Dias et al., (2014) who specified that the culture medium augmented with 2,4-D induced the embryogenic callus formation of *E. elatior.* Furthermore, Abd et al. (2016) reported the same observation where the supplementation of 1 mg L^{-1} 2,4-D on *Zingiber officinale* shoot tip explants significantly induced the optimal fresh callus weight.

From the results obtained, 2,4-D played a key role in callus induction of *E. elatior*. The result of the current study agrees with other findings in Zingiberaceae which indicated the application of 2,4-D at lower concentrations for callus induction. For example, Tan et al. (2005) indicated that MS supplemented with 3 mg L^{-1} 2,4-D was the only medium that produced embryogenic callus of *Boesenbergia rotunda*. It was reported that 3 mg L^{-1} 2,4-D produced better results (50%) of callus induction in *Z. officinale* (Taha, 2013). Furthermore, Abd et al. (2016) reported that callus growth of *Z. officinale* was optimal when 2,4-D was used at 0.5 – 3.0 mg L^{-1} in the culture medium.

Table 1. Effect of different carbon sources and concentrations of 2,4-D and BAP on callus induction of *E. elatior* after 20 weeks of culture.

Treatments	Carbon sources (3%)	2,4-D (mg L^{-1})	BAP (mg L^{-1})	Percentage of callus induction ± SE	Intensity of callus induction
C1	Sucrose	0	0	0c	-
C2	Sucrose	3.0	0	16.67 ± 8.33bc	+++
C3	Sucrose	6.0	0	0c	-
C4	Sucrose	9.0	0	0c	-
C5	Sucrose	0	1.0	0c	-
C6	Sucrose	3.0	1.0	0c	-
C7	Sucrose	6.0	1.0	0c	-
C8	Sucrose	9.0	1.0	0c	-
C9	Sucrose	0	1.5	0c	-
C10	Sucrose	3.0	1.5	0c	-
C11	Sucrose	6.0	1.5	0c	-
C12	Sucrose	9.0	1.5	0c	-
C13	Glucose	0	0	0c	-
C14	Glucose	3.0	0	25 ± 14.43b	++++
C15	Glucose	6.0	0	0c	-
C16	Glucose	9.0	0	0c	-
C17	Glucose	0	1.0	0c	-
C18	Glucose	3.0	1.0	16.67 ± 16.67bc	+++
C19	Glucose	6.0	1.0	0c	-
C20	Glucose	9.0	1.0	0c	-
C21	Glucose	0	1.5	0c	-
C22	Glucose	3.0	1.5	50 ± 0a	++
C23	Glucose	6.0	1.5	0c	-
C24	Glucose	9.0	1.5	0c	-

Values are the mean of three replicates where every replicate consists of four explants. Means followed by the same alphabet are not significantly different based on DMRT (p = 0.05). Visual estimation: - = non; + = poor; ++ = average; +++ = good; ++++ = excellent

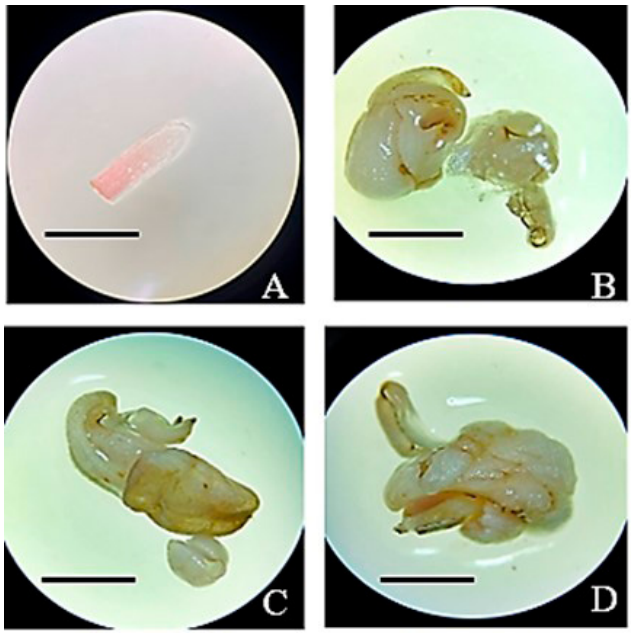

Fig. 1. Early development of young, closed bud explants. A) Fresh explant used for callus induction experiment. Swollen and white creamy explants after 4 weeks of culture on treatment; B) C14 (3% glucose with 3.0 mg L^{-1} 2,4-D) C) C18 (3% glucose with 3.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BAP) and D) C22 (3% glucose with 3.0 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BAP) respectively. Scale bar: 1 cm.

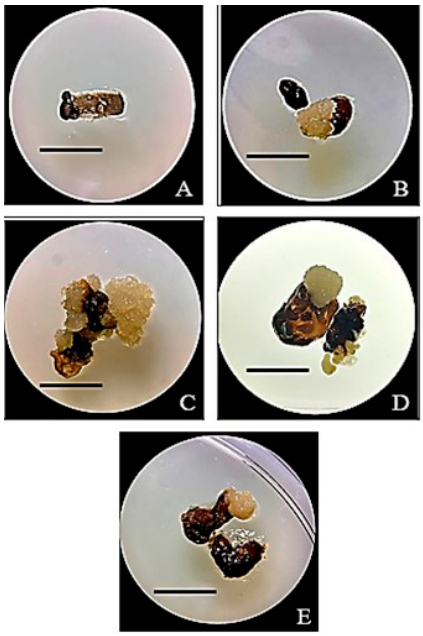

Fig. 2. Explants of different treatments on induction of friable callus after 20 weeks of culture. A) C1, MS media without PGR as a control treatment. Explants turned black and failed to survive. B) Friable callus induced from C2 (3% sucrose with 3 mg L^{-1} 2,4-D). C) Friable callus induced from C14 (3% glucose with 3 mg L^{-1} 2,4-D). D) Friable callus induced from C18 (3% glucose with 3 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP). E) Friable callus induced from C22 (3% glucose with 3 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BAP). Scale bar: 1 cm.

Effect of Different Concentrations of Auxin and Cytokinin on Shoot Induction.

Proliferating calluses were transferred onto MS media without any PGR for two weeks before being transferred to shoot induction treatments. Calluses of *E. elatior* were inoculated on MS media containing 3% glucose, BAP, NAA and TDZ, either alone or in combination. From previous experiment, it was shown that callus induction was maximal on glucose treatment. Thus, all further experiments on shoot induction were performed using glucose.

In this study, proliferated white friable calluses were subcultured on fresh medium every 4 weeks for a period of 12 weeks. The result showed that some part of the white friable calluses changed color to yellowish green and spherical shape as early as 8 weeks of culture in the shoot induction media (Table 2) (Fig. 3). The observed variations in callus color may be due to several factors, including the different PGRs and the specific type of explant used. Instead of applying an auxin alone, applying a combination of auxin and cytokinin resulted in green calluses, induced by cytokinin, which tends to stimulate chlorophyll production (Hudeček et al., 2023). During subculture, all the potential green spots and globular shape of callus were isolated from the clump of white calluses to further observe their morphogenetic changes.

Table 2. Effects of different concentrations of BAP, NAA and TDZ on shoot induction of *E. elatior* after culturing for 8 weeks under light condition.

Treatments	BAP (mg L^{-1})	NAA (mg L^{-1})	TDZ (mg L^{-1})	Observed results
T1	0	0	0	Calluses proliferated
T2	1.0	0	0	Some calluses turned green, globular shape calluses
T3	0	1.0	0	Calluses proliferated
T4	0	0	1.0	Some calluses turned green, globular shape calluses
T5	2.0	0	0.3	Some calluses turned green, globular shape calluses
T6	3.0	0.5	0	Calluses proliferated
T7	5.0	0.1	0	Calluses proliferated
T8	3.0	0.5	0.3	Calluses proliferated
T9	5.0	0.1	0.3	Calluses proliferated
T10	2.0	0.5	0.3	Some calluses turned green, globular shape calluses
T11	0	0.1	0.3	Some calluses turned green, globular shape calluses

The treatment T11 (MS medium with 0.1 mg L^{-1} NAA and 0.3 mg L^{-1}) showed a potential for organ induction of *E. elatior* (Fig. 3F). All replicates from T11 managed to induce green spot or spherical callus after 12 weeks of culture. In addition, only one replicate from each treatment of T2 (1.0 mg L^{-1} BAP), T4 (1.0 mg L^{-1} TDZ), T5 (2.0 mg L^{-1} BAP and 0.3 mg L^{-1} TDZ) and T10 (0.5 mg L^{-1} NAA, 2.0 mg L^{-1} BAP and 0.3 mg L^{-1} TDZ) showed positive signs of green spot and spherical calluses. A root-like structure was observed in callus masses from T11 after culturing for 12 weeks (Fig. 3F). This result is in accordance with that of Gomes-Dias et al. (2014) who obtained root-like structure from callus masses of *E. elatior* inoculated on medium supplemented with 4.0 mg L^{-1} 2.4-D. Our result was in contrast with Kou et al. (2012) who indicated that 33.1% of all callus cultures from *C. attenuata* could differentiate into adventitious shoots on MS medium supplemented with 5.0 mg L^{-1} BAP, 0.1 mg L^{-1} NAA and 0.3 mg L^{-1} TDZ after 60 days of culture.

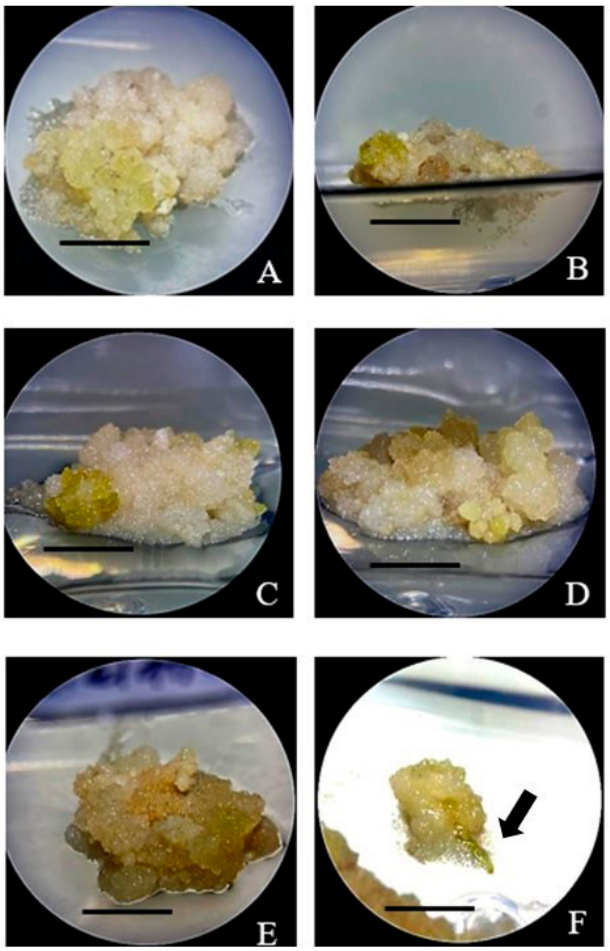

Fig. 3. Differentiation of calluses on MS media supplemented with different PGRs after 12 weeks of culture. A) T2 (1.0 mg L^{-1} BAP). B) T5 (2.0 mg L^{-1} BAP and 0.3 mg L^{-1} TDZ). C-E) T11 (0.1 mg L^{-1} NAA and 0.3 mg L^{-1} TDZ). F) Organ formation from callus masses after 12 weeks of culture at T11. The root-like structure is indicated by the black solid arrow. Scale bar: 1 cm.

Our results show that the ratio of PGRs plays a crucial role in organogenic callus induction in *E. elatior*. The balance between auxin and cytokinin is crucial for shoot formation, as well as for determining the organogenic response, as they are involved in regulation of the cell divisions. It has been postulated that the levels of endogenous plant hormones influence the morphological changes induced by TDZ (Guo et al., 2011).

Although the embryogenic callus formation induced from different parts of *E. elatior* was previously reported by Gomes-Dias et al. (2014), this is the first report of roots regenerated using indirect organogenesis from friable calluses. In this study, organogenic calluses were successfully developed through indirect regeneration. However, after more than 12 weeks of culture on media, the spherical callus did not show any positive response. The friable spherical callus hardened and became creamy white on medium T11 (0.1 mg L^{-1} NAA and 0.3 mg L^{-1} TDZ) after 12 weeks.

Evaluation of Genetic Variation of *E. elatior's* Calluses using ISSR Marker

ISSR analysis of four-week-old white friable calluses from 11 different shoot induction treatments and leaves from mother plants yielded 72 scorable bands, ranging from 100 - 1000 bp. Of these, 51 (72%) were polymorphic (Table 3), with the number of bands ranging from 6 (UBC 830) to 14 (UBC 811) and an average of 10.3 bands per primer. The percentage of polymorphism varied from 44.4% to 100% with an average of 72% per primer. These data indicate a strong ISSR variation among the samples. Figure 4 shows the amplification profiles of different primers of UBC 808, UBC 809, UBC 811, UBC 830, UBC 880, UBC 888, and UBC 891 across samples.

The Jaccard's coefficient, ranging from 0.3529 to 1.000, indicates a high degree of somaclonal variation among 12 samples (Table 4). When comparing the callus treatments with the control, the maximum similarity value (0.4762) resulted from T4 callus (MS medium with 1.0 mg L^{-1} TDZ), while the minimum value (0.3529) resulted from T3 callus (MS medium with 1.0 mg L^{-1} NAA). The highest degree of similarity between two treatments of callus was found between T9 (MS medium with 5.0 mg L^{-1} BAP, 0.1 mg L^{-1} NAA and 0.3 mg L^{-1} TDZ) and T11 (MS medium with 0.1 mg L^{-1} NAA and 0.3 mg L^{-1} TDZ), with a similarity coefficient of 1.000 (no genetic variation). Meanwhile the minimum similarity coefficient or the highest genetic distance (0.7213) was observed between two different treatments T3 (MS medium with 1.0 mg L^{-1} NAA) with T4 (MS medium with 1.0 mg L^{-1} TDZ), and T3 with T6 (MS medium with 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA).

Finding from Ghorbanpour and Khadivi (2015) revealed that the somaclonal variation in 18 different calluses samples of *Plantago major* that were grown *in vitro*. Out of 18 ISSR primers tested, six primers were able to produce bands and collectively generated a total of 60 bands. Among these bands, 59 exhibited polymorphisms across all clones, with an average of 9.83 polymorphic bands per primer. The Jaccard's coefficient calculated among the different samples ranged from 0.16 to 0.71. These values indicate a significant degree of somaclonal variation within the samples, suggesting that the genetic makeup of the callus had undergone considerable changes (Ghorbanpour & Khadivi, 2015). Another study by Hu et al. (2011) found somaclonal diversity in 28 distinct callus derived plants from *Amorphophallus riverieri*. Out of 26 ISSR primers tested, 13 showed distinctive and reproducible band patterns, resulting in 131 bands with an average of 10.1 per primer. Ten primers produced 16 polymorphic bands, with a mean polymorphism of 12.2%. The Jaccard's similarity coefficients ranged from 0.961 to 1.000, thus indicate low somaclonal variation observed in the regenerated plants (Hu et al., 2011).

The dendrogram constructed by the UPGMA method using the data from the 72 bands (Fig. 5) shows two distinct groups: (a) the leaf from the mother plant (control) and (b) all calluses from 11 different shoot induction treatments. The callus group was further separated into two subgroups: T3 and other callus treatments. T3 shows a clear separation with higher genetic distance compared to other callus treatments of calluses. In short, our ISSR analysis allowed to determine somaclonal variation in the calluses as DNA polymorphisms when comparing samples from callus and from leaves of the mother plant.

The somaclonal variation observed in our samples subjected to *in vitro* cultivation may be a response of the *E. elatior* genomes to PGR supplementation, media pH, or culture environment, as reported by Machanda et al. (2024). Somaclonal variation is often observed in *in vitro* culture because of epigenetic effects or genome changes in developing vegetative cells generated by *in vitro* culture conditions (Orłowska et al., 2024). Bansal et al. (2024) revealed that several PGRs play an important role in maintaining somaclonal variation. The difference between the T3 and other callus groups might be due to the application of the auxin NAA and the absence of cytokinin (BAP and TDZ). Thus, in this study, probably the PGRs added to the shoot induction media together with the number of subcultures during the callus and shoot induction period probably caused the high level of somaclonal variation in samples.

Table 3. List of primers, optimised annealing temperature, number of amplified products, polymorphic bands and polymorphism percentage.

No	Primer	Optimised annealing temperature (°C)	Total amplified products	Polymorphic bands	Monomorphic bands	Percentage of polymorphism
1	UBC 808	52	9	9	0	100.00
2	UBC 809	52	11	7	4	63.64
3	UBC 811	52	14	9	5	64.28
4	UBC 830	54	6	5	1	83.33
5	UBC 880	52	11	9	2	81.82
6	UBC 888	54	12	8	4	66.67
7	UBC 891	54	9	4	5	44.44
		Total	72	51	21	504.18
		Mean	10.3	7.3	3	72

Table 4. Jaccard's coefficient of similarity matrix for control and treatments of calluses from *E. elatior* determined from ISSR analysis using seven different primers and analyzed by UPGMA programme.

	Control	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11
Control	1.0000											
T1	0.4627	1.0000										
T2	0.4603	0.8644	1.0000									
T3	0.3529	0.7742	0.7931	1.0000								
T4	0.4762	0.8197	0.8750	0.7213	1.0000							
T5	0.4308	0.8500	0.8421	0.7500	0.8276	1.0000						
T6	0.4308	0.8813	0.8750	0.7213	0.8596	0.9273	1.0000					
T7	0.4355	0.8135	0.9057	0.7414	0.8545	0.8889	0.9245	1.0000				
T8	0.4219	0.8474	0.9074	0.8070	0.8246	0.8909	0.8909	0.8868	1.0000			
T9	0.4545	0.9000	0.8947	0.7419	0.9123	0.8793	0.9123	0.8750	0.9107	1.0000		
T10	0.4461	0.8983	0.9273	0.7667	0.8772	0.8772	0.9454	0.9074	0.9444	0.9643	1.0000	
T11	0.4545	0.9000	0.8947	0.7419	0.9123	0.8793	0.9123	0.8750	0.9107	1.0000	0.9643	1.0000

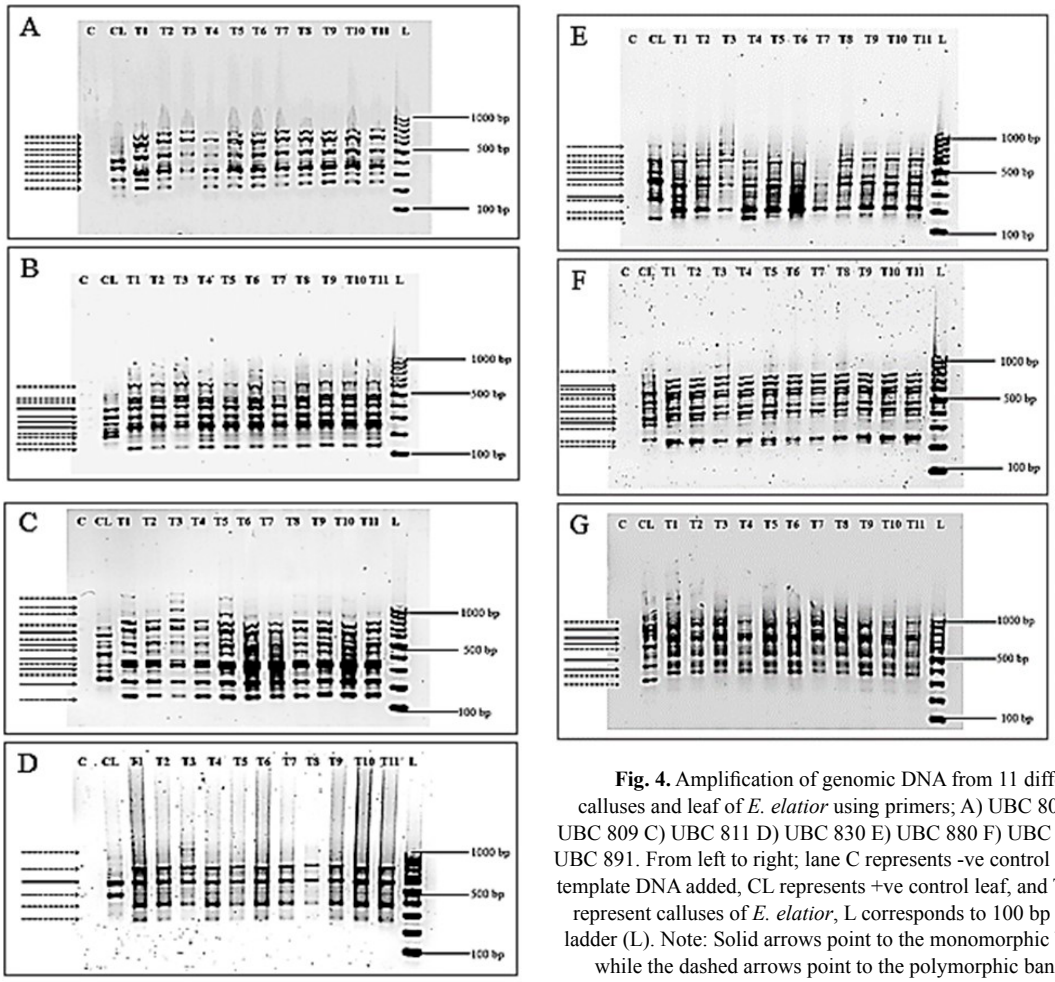

Fig. 4. Amplification of genomic DNA from 11 different calluses and leaf of *E. elatior* using primers; A) UBC 808 B) UBC 809 C) UBC 811 D) UBC 830 E) UBC 880 F) UBC 888 G) UBC 891. From left to right; lane C represents -ve control with no template DNA added, CL represents +ve control leaf, and T1-T11 represent calluses of *E. elatior*, L corresponds to 100 bp DNA ladder (L). Note: Solid arrows point to the monomorphic bands, while the dashed arrows point to the polymorphic bands.

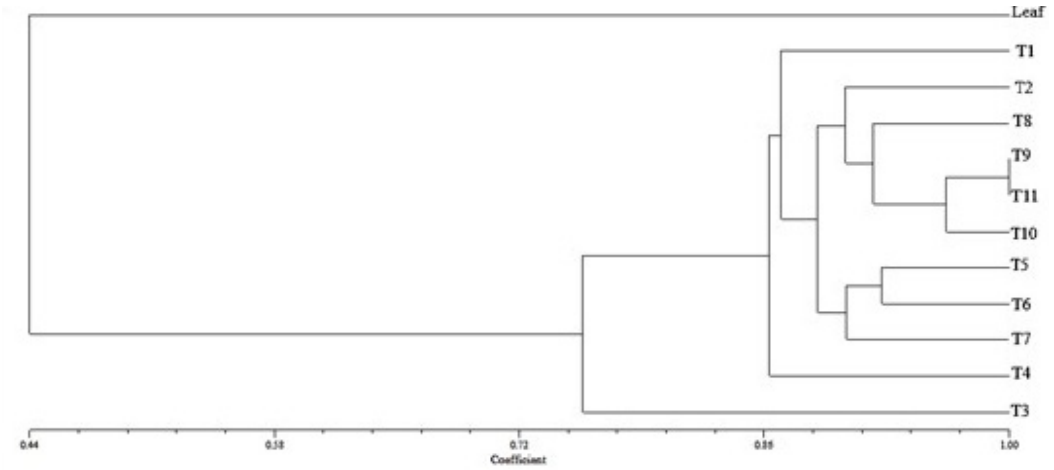

Fig. 5. Dendogram constructed from Jaccard's similarity coefficients from ISSR data, showing the clustering of the 11 different calluses and leaf as a control. (L: Leaf, T1-T11: calluses from 11 different treatments).

Somaclonal variation can be useful for crop improvement, particularly in plants with limited genetic diversity. These variations provide a unique and valuable reservoir of genetic variability. It is important to note that callus undergoes dedifferentiation and redifferentiation during *in vitro* culture while being under stress, which increases genome plasticity. According to Abd El-Hameid et al. (2020), ISSR markers confirmed the presence of genetic variants during the callus induction process in *Z. officinale.* The genetic differences resulting from the *in vitro* condition of callus initiation were investigated using four ISSR primers. The ISSR analysis yielded 36 amplicons with a polymorphism rate of 42.86%. Although Parida and Nayak (2021) who showed that the micropropagated plants of *Curcuma aromatica* did not show any genetic variation after evaluation by ISSR analysis, our results showed that using this molecular marker has success to detect somaclonal variations in *E. elatior*.

Conclusions

This study demonstrates a viable indirect micropropagation protocol for *E. elatior* using young, closed bud explant, supported by evaluation of somaclonal variation using ISSR markers. This is the first report using the young, closed bud of *E. elatior* as an explant. We were able to produce root-like structures (calluses masses T11 using 0.1 mg L^{-1} NAA and 0.3 mg L^{-1} TDZ) after 12 weeks, which probably originated via indirect organogenesis. The follow-up of the somaclonal variation using ISSR and a dendrogram allowed the separation of the samples into two clear groups (callus and mother plant leaves). Our contribution confirms that the application of these low-cost techniques can be a strategy to obtain genetic diversity in *in vitro* culture and to generate new varieties of *E. elatior*.

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Author Contribution

NMR: Conceptualization, Data Cration, Formal Analysis, Investigation, Methodology, Writing – Original Draft. **MFY:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Writing – Review & Editing. **TSMC:** Conceptualization, Supervision, Methodology, Writing – Review & Editing. **ZZ:** Conceptualization, Supervision, Methodology, Writing – Review & Editing. **MRM:** Resources **MHAP:** Resources.

Conflict of Interest

We do not have any conflict of interest with any parties.

Data Availability Statement

The research data is contained in the manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that the use of AI and AI-assisted technologies was not applied in the writing process.

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