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Research Article
Micropropagation and Assessment of Genetic Variability of *Cyclanthus bipartitus*

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Abstract

Background and Objective: *Cyclanthus bipartitus* is an indoor ornamental plant. It's fertilization relies on *Cyclocephala* sp. (Scarabaeidae). The absence of these pollinators could lead to lack of production of seed and therefore its vegetative propagation is obligatory. This study was carried out to determine the optimum concentration of benzylaminopurine (BAP), alpha-naphthalene acetic acid (NAA) and sucrose to be used in the Murashige and Skoogs (MS) media for *in vitro* propagation of *Cyclanthus bipartitus* and to evaluate the variation in DNA sequence of regenerated plants. **Materials and Methods:** Explants were cultured on sterilized MS medium containing different combinations of BAP, NAA and sucrose. The preparations were cultured for 10 weeks at temperature 25-28°C under 16 h of photoperiod with light intensity of 30 µmol m^{-2} sec^{-1} and the growth of the plantlets were monitored. Somaclonal variation amongst mother plants, daughter plants and the plantlets generated from the following three subculturing at the DNA level and was analyzed by using random amplified polymorphic DNA (RAPD). Data obtained were subjected to analysis of variance (ANOVA) using PROC GLM of SAS software. **Results:** Multiple adventitious shoots of *Cyclanthus bipartitus* were induced from lamina explants cultured on MS medium supplemented with various combinations of BAP, NAA and sucrose concentrations. The highest number of shoots, tallest shoots, highest number of roots and longest root were recorded in medium with a combination of 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} and 30 g L^{-1} sucrose. Results of DNA analysis showed that 18 out of 26 score able bands were polymorphic and eight were monomorphic, which gave 69.2% polymorphism frequency. **Conclusion:** Optimum concentrations of BAP, NAA and sucrose for shoot and root development *in vitro* propagation of *Cyclanthus bipartitus* was 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 30 g L^{-1} of sucrose. 69.2% polymorphism was detected in regenerated plants indicating genetic variation had occurred amongst somaclones.

Key words: Tissue culture, benzylaminopurine, alpha-naphthalene acetic acid, sucrose, random amplified polymorphic DNA

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cyclanthus bipartitus can be propagated by cutting and division, however, micropropagation seems to be the best method for commercial purposes as mass multiplication can be done at a faster rate compared to the conventional method. Moreover, pollination process for this plant requires a specific pollinator (*Cyclocephala* spp., Scarabaeidae)[1]. The absence of the pollinators could lead to no production of seed.

Preparation of appropriate medium for explants is an important step in tissue culture procedure, as it would affect the ability of explants to fully utilize elements supplemented to initiate the process of organ regeneration. Addition of growth regulators, especially cytokinin and auxin to the medium is crucial in order to enhance and help the growth of the explants. Culture medium supplemented with auxins and cytokinins have been used regularly to propagate plants *in vitro*. Common auxins used for tissue culture procedure are the indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA), which are widely used in combination with cytokinin[2]. When cytokinin and auxin were combined together in optimal concentrations, the synergic influence was evident to both shoots and roots regeneration. The addition of NAA to an auxin manifestly enhanced the percentage of regeneration and number of shoots/explants of *Dianthus cryophyllus*, as seen in a study by Varshney *et al.*[3]. They reported that the highest growth was obtained from leaf explant cultured in medium supplemented 2.5 μM 6-benzyladenine and 0.5 μM α-naphtheleneacetic acid (NAA) which produced 15.30±1.19 shoots produced/explant with shoot length of 6.75±0.63 cm after 8 weeks of culture.

Sugars are required in the media culture to replace the carbon, which plants normally obtain from atmosphere and fixed by *in vivo* photosynthesis for growth and development[4]. Sucrose is also often assumed to be the preferred form of sugar in cell and tissue culture media as it is the most common form of carbohydrate in the phloem sap of many plants apart from being cheap and easily availability[5,6].

All plants regenerated from cell or tissue culture were expected to have genetic materials identical to the parent plant. In spite of this, phenotypic variation was observed to be common amongst regenerated plants[7]. This variation is termed as somaclonal variation and is defined as phenotypic and genetic variation among clonally propagated plants from a mother plant. The presence of somaclonal variation has been associated to growth regulators, variability of cultivar, the age of cultivars in culture, level of ploidy, explants sources and other endogenous culture conditions[8]. The presence of chemicals in medium culture may enhance the rate of this variation. Sahijram *et al.*[9], found that somaclonal variation can also exist in many forms including chromosomal abnormalities that involve changing in chromosome numbers, the activation of transposable elements, DNA methylation changes, point mutations and epigenetic variation. The resulting changes that occur are permanent and heritable. Somaclonal variation could be a useful source of novel variation for plant improvement[8,10]. While some changes that occur could result in plant having unwanted characteristics, other changes might result in plant obtaining good and new characters[11,12]. New plant characteristics such as attractive leaf and flower colours, plant forms and flower numbers and its longevity among somaclones induced following *in vitro* propagation would be beneficial as it would leads to creation of new variety.

The objectives of this study were to evaluate the effects of concentrations of BAP and NAA chosen as plant growth regulators, concentration of sucrose used as a carbon source in the medium and also to evaluate the genetic variability of *Cyclanthus bipartitus* regenerated plants following micro propagation and subculturing.

MATERIALS AND METHODS

Plant materials: One month old stock plants of *Cyclanthus bipartitus* were prepared at Focus Horticulture Sdn. Bhd. nursery (Tanjung Malim, Perak Darul Ridzuan, Malaysia). The materials were prepared in squared flasks containing 25 1 month old plantlets. Reproduction of stock plants was done by subculturing the plants obtained from the nursery by transferring them to fresh medium to lengthen the life of the plants.

Explant culture: Explants from the lamina part of the plant that is mentioned earlier, were obtained and excised in size of 1 cm^2 each. Explants were cultured on sterilized Murashige and Skoogs (MS) medium containing different combinations of BAP, NAA and sucrose (0.5 mg L^{-1} BAP with 0.5 mg L^{-1} NAA and 20 g L^{-1} sucrose, 1.0 mg L^{-1} BAP with 0.5 mg L^{-1} NAA and 20 g L^{-1} sucrose, 1.0 mg L^{-1} BAP with 1.0 mg L^{-1} NAA and 20 g L^{-1} sucrose, 0.5 mg L^{-1} BAP with 0.5 mg L^{-1} NAA and 30 g L^{-1} sucrose, 1.0 mg L^{-1} BAP with 0.5 mg L^{-1} NAA and 30 g L^{-1} sucrose and 1.0 mg L^{-1} BAP with 1.0 mg L^{-1} NAA and 30 g L^{-1} sucrose), which later were labeled respectively as treatment I, II, III, IV, V and VI. Explants were cultured for ten weeks at temperature 25-28°C under 16 h of photoperiod with light intensity of 30 μmol m^{-2} sec^{-1} under 60-70% relative humidity in culture room. The treatments were laid in a completely randomized design (CRD) with 5 replications and six explants for each experimental unit.

Data collection

Number of shoots: Shoots in each flask were counted every week starting from week 5 of culturing. In this case, the term shoot was used to describe the developing buds and existence of shoots used for counting. For fear of contaminating the medium, explants were not taken out of the flask.

Number of roots: Numbers of roots were counted at the end of week 10 of culture. Only roots longer than 2 mm were counted as roots. To count the root numbers, plantlets were taken out of each flask.

Shoot height and root length: Height of shoots and the length of roots in each flask were measured at the end of week 10 of culture using a ruler. Plantlets were taken out of the flasks and shoot height and root length were measured in cm on plain white paper for better visuality.

Somaclonal variation of regenerated plants: Mother plant used in this analysis was obtained from a plantlet that had been grown in optimized MS medium added with 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 30 g L^{-1} sucrose. Leaf of explants in the size of 1 cm^2 from the parental plant (P) were excised and used as the explant to regenerate a daughter plant and cultured in the same composition of MS medium, which then labeled as S1 plantlet. The next three subculturing were carried out at 4 weeks interval, which then labeled as S2, S3 and S4. After 5 weeks of each culture, explants that had grown into plantlets were transferred into larger flask (1000 mL) for further elongation of shoots and roots in the same composition of MS medium used previously. Genomic DNA of *Cyclanthus bipartitus* was obtained from leaves of these plants. Ten leaves of each subculture group were randomly selected and bulked for DNA extraction. Somaclonal variation of regenerated plants at the DNA level was analyzed by using random amplified polymorphic DNA (RAPD) molecular markers. Ten arbitrary primers were screened for RAPD use. Primers were synthesized in 25 µL of polymerase chain reaction (PCR) reaction mixture containing 1 µL DNA template, 1 X PCR Buffer (Fermentas Inc., Hanover, MD, USA), 1 unit (U) of *Taq* DNA polymerase (Fermentas Inc., Hanover, MD, USA), 2 µL of $MgCl_2$, 1µM of each primer (Eurofins Genomics, Huntsville, Alabama, USA) and 200 µM dNTPs (Fermentas Inc., Hanover, MD, USA). DNA amplification was carried out in a thermocycler (Whatman, Maidstone, Kent, UK) for 35 cycles. Initial denaturation started at 94°C for 5 min, followed by denaturation at 94°C for 1 min. Initial extension started at 72°C for 1 min and final extension was done at 72°C for 5 min. PCR products were cooled at 4°C and were kept at the same temperature until further use. Following DNA amplification, PCR products were run on electrophoresis with 1.5% agarose gel. Gel electrophoresis was run for 1 h at 80 V and once done, the gel was stained using molecular grade ethidium bromide solution (1 µg mL^{-1}) for 20 min and destained in distilled water for 5 min. Gel image was captured on Gene Genius Gel documentation (Syngene, Frederick, Maryland, USA). Primers that produced score able bands were chosen to analyse polymorphism in regenerated plant DNA.

Banding patterns were compiled as binary matrix and were subjected to cluster analysis. Jaccard's similarity coefficient was chosen as it portrays the relationships between all somaclones represented by similarity matrix. The similarity matrix was computed between any two columns of binary data using the GelAnalyzer software (GelAnalyzer.com). Cluster analysis was performed to construct a tree plot using the unweighted pair-group method with arithmetic averages (UPGMA)[13].

Statistical analysis: Analysis of variance (ANOVA) was used to analyze quantitative data using PROC GLM of SAS software version 9.1.3 (SAS Institute Inc., Cary, N. C., USA). In cases where significant differences were determined, Duncan's Multiple Range Test (DMRT) was used to compare the means at $p<0.05$[14].

RESULTS AND DISCUSSION

Explants growth in different combinations of BAP, NAA and sucrose: Data in Table 1 show that medium, which contained 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 30 g L^{-1} sucrose (V), produced the best output in terms of shoot number, shoot length, root number and root length. When compared to those in II, which had the same concentration of BAP and NAA but with 20 g L^{-1} of sucrose, there was no significant difference on the height of shoots produced. On the other hand, number of shoots produced by explants in V were significantly higher by 11.5% than those produced by explants in II. Similar effect was also observed on number of roots and roots length. Results show that increasing concentration of sucrose from 20-30 g L^{-1} promoted the production of shoots and roots, whereby the number of shoots had increased by 11.5%, number of roots by 22.8% and root length by 36.8%.

Increased NAA concentration from 0.5-1.0 mg L^{-1} significantly reduced the growth of plantlets at 1.0 mg L^{-1} BAP

Table 1: Numbers of shoots, shoot length, number of roots and root length affected by different combinations of concentrations of BAP, NAA and sucrose

Treatment	BAP (mg L^{-1})	NAA (mg L^{-1})	Sucrose (g L^{-1})	Number of shoots	Shoot length (cm)	Number of roots	Root length (cm)
I	0.5	0.5	20	13.76^{e}	0.34^{b}	0.43^{c}	0.24^{c}
II	1.0	0.5	20	49.22^{b}	3.66^{a}	2.54^{b}	0.57^{b}
III	1.0	1.0	20	18.92^{c}	0.33^{b}	0.56^{c}	0.27^{c}
IV	0.5	0.5	30	16.42^{d}	0.35^{b}	0.42^{c}	0.25^{c}
V	1.0	0.5	30	54.88^{a}	3.80^{a}	3.12^{a}	0.78^{a}
VI	1.0	1.0	30	20.82^{c}	0.37^{b}	0.34^{c}	0.27^{c}

Means followed by the same letter within column are not significantly different according to DMRT at $p<0.05$

(treatments II and III) as it reduced number of shoots by 61.5%, shoot length (91.0%), root number (78.0%)and root length (52.6%). This indicated that the increment of 0.5 mg L^{-1} NAA retarded proliferation of shoots and roots, leading to the reduction of number of shoots and roots produced.

Notably, the low concentration of BAP and NAA (0.5 mg L^{-1}, respectively) was shown to be insufficient to promote good development of plantlets. Yet, increasing sucrose from 20-30 g L^{-1} significantly enhanced the generation of shoots which had resulted an increase in shoot number by 19.3% (13.76-16.42 shoots/plantlet). Consequently, when BAP concentration was increased from 0.5-1.0 mg L^{-1} in both treatments II and IV, number of shoots and roots and length of shoots and roots increased markedly. Parallel with this result, a study by Yildiz *et al.*[15] on beetroot (line ELK 345) using leaf explants produced a result showing that increasing BAP concentration from 0.5-1.0 mg L^{-1} (with similar NAA concentration) significantly increased number of shoots produced/explant, from 1.7-6.1 shoots. The development of explants in medium supplemented with 1.0 mg L^{-1} of BAP and 1.0 mg L^{-1} of NAA was inhibited when compared to other treatments. Alongside, increasing sucrose concentration in the medium also did not produce significant difference in numbers of generated shoots and roots and the length of shoots and roots, suggesting that root generation was induced by high ratio of auxin/cytokinin ratio, whereas shoot production was promoted by low ratio of auxin/cytokinin. Consistent with the results of earlier studies[3], this study results clearly report the synergistic effect of the cytokinin-auxin combination on the adventitious shoot and root multiplication from leaf explants of *Cyclanthus bipartitus*.

Su *et al.*[16], claimed that a cross talk might have happened during an *in vitro* organogenesis. To date, such molecular interaction in an *in vitro* environment between auxin and cytokinin and the effects they have on meristem formation still remains mostly unknown. The auxin in root meristem which induces merismetic cell division while cytokinin has a role to promote the cell to switch to the differentiated stage from the merismetic, through auxin signaling inhibition. On the contrary, stem cell proliferation was promoted by cytokinin in the shoot meristem and stem cell differentiation was inhibited. Also in shoot meristem, organ primordium initiation is triggered by auxin through cytokinin biosynthesis repression. The key regulation of cell differentiation and maintenance is the antagonistic interactions between auxin-cytokinin in both shoot and root meristems[16].

Responses towards sucrose at suitable concentration (30 g L^{-1}) as observed here may be related to its hydrolysis, which causes the endogenous content of glucose and fructose of cultured tissues to increase. These reducing sugars will then increase the osmotic potential in cultured tissues and positively influence organogenesis by speeding up cell division and consequently leading to an increase of explants growth. Similar findings were also obtained by several studies supporting the results that 3% of sucrose concentrations produced maximum shoot numbers as well as favorable root length in valuable medicinal herb *Eclipta alba*[17] and cork oak[18].

Results obtained in this experiment showed that rooting ability was characterized by specific auxin and cytokinin levels. The optimum concentrations for BAP, NAA and sucrose were found to be 1.0, 0.5 and 30 g L^{-1}, respectively.

Somaclonal variation: In evaluating the DNA sequence variation in plantlets, a total of 10 arbitrary 10-mer primers were screened for reproducible bands and out of the 10 primers chosen and test, only six were selected for further RAPD analysis. The remaining four oligo primers either did not produce any bands or produced bands without polymorphisms. Each primer generated a unique set of amplification products ranging in size from 300 bp by primer OPE-13 and 2100 bp by primer OPE-19. The number of scored bands produced by each primer varied from four in primer OPE-19 to eight in primer OPE-15. Result shows that the six primers used in this analysis yielded 26 score-able bands with an average of 4.3 score able bands/primer used (Table 2). Among the 26 bands scored from these six primers, 18 were polymorphic and 8 were monomorphic, which gave 69.2% of polymorphism frequency. RAPD analysis of *Cyclanthus bipartitus* plantlets revealed a variation in the electrophoresis

Table 2: Primers used in RAPD analysis of genetic stability in *Cyclanthus bipartitus* regenerated plants and number of score able bands produced by each primer

Primer	Sequence (5'-3')	Number of bands	Polymorphic bands	Polymorphism (%)	Band mol. weight range (bp)
OPE-01	CCCAAGGTCC	4	2	50.0	360-1650
OPE-05	TCAGGGAGGT	7	5	71.4	450-1300
OPE-08	TCACCACGGT	6	4	66.7	410-1950
OPE-13	CCCGATTCGG	5	3	60.0	300-1500
OPE-15	ACGCACAACC	8	3	37.5	250-1900
OPE-19	ACGGCGTATG	4	1	25.0	500-2100
Total band scored		26	18	69.2	300-2100

Table 3: Summary of amplification products produced by primer OPE-01

Band size (bp)	Mother plant	Subculture 1	Subculture 2	Subculture 3	Subculture 4
360	1	0	1	1	0
570	1	1	1	0	0
910	1	1	1	1	1
1650	1	1	1	1	1

Score 1: Present, Score 0: Absent

Table 4: Summary of amplification products produced by primer OPE-05

Band size (bp)	Mother plant	Subculture 1	Subculture 2	Subculture 3	Subculture 4
450	1	0	1	1	0
490	1	0	1	0	1
550	1	1	1	1	1
700	1	1	0	1	1
850	1	1	0	1	1
1100	1	1	1	1	1
1300	0	0	1	0	1

Score 1: Present, Score 0: Absent

Table 5: Summary of amplification products produced by primer OPE-08

Band size (bp)	Mother plant	Subculture 1	Subculture 2	Subculture 3	Subculture 4
410	1	1	1	0	0
490	1	1	1	1	1
550	1	0	1	0	1
700	1	0	1	1	1
850	1	1	1	1	1
1950	0	1	1	1	1

Score 1: Present, Score 0: Absent

Table 6: Summary of amplification products produced by primer OPE-13

Band size (bp)	Mother plant	Subculture 1	Subculture 2	Subculture 3	Subculture 4
300	0	1	0	0	0
550	1	1	1	1	1
750	0	1	1	0	1
1350	1	1	1	1	1
1500	1	1	0	1	1

Score 1: Present, Score 0: Absent

patterns, which indicated the genetic variability of the subcultured plantlets. However, there was no morphological variation observed in *Cyclanthus bipartitus* plantlets at the end of the culture.

Results shown in Table 3-8 showed that the polymorphic characteristics of the four generations produced. The results showed that, *Cyclanthus bipartitus* plantlets had different level of DNA sequence variation on different stage of subculture. This indicates that the length of time of explants in the culture did not directly affect the induction of somaclonal variation, as it showed no particular behavior.

The six primers used in RAPD analysis showed polymorphism within and between all the somaclones from regenerated plants. However, these primers did not produce specific patterns that could further present the degree of difference between P and S1, S2, S3 and S4. Banding patterns produced by each primer were highly variable and most amplified bands were polymorphic, which was calculated to be 69.2% polymorphism, indicating genetic variation amongst all the somaclones.

Data exhibited in Table 9 show that the mean genetic similarity among the somaclones derived from parent (P) is

Table 7: Summary of amplification products produced by primer OPE-15

Band size (bp)	Mother plant	Subculture 1	Subculture 2	Subculture 3	Subculture 4
250	1	0	1	0	0
450	1	1	0	0	0
500	1	1	1	1	1
700	1	1	1	1	1
850	0	0	0	0	1
1100	1	1	1	1	1
1300	1	1	1	1	1
1900	1	1	1	1	1

Score 1: Present, Score 0: Absent

Table 8: Summary of amplification products produced by primer OPE-19

Band size (bp)	Mother plant	Subculture 1	Subculture 2	Subculture 3	Subculture 4
300	1	1	1	1	1
750	1	1	1	1	1
1500	1	1	1	1	1
2100	1	0	1	1	0

Score 1: Present, Score 0: Absent

Table 9: Summary of similarity matrix using Jaccard's coefficient

	p	S1	S2	S3	S4
P	1.00	0.69	0.78	0.77	0.67
S1		1.00	0.61	0.69	0.70
S2			1.00	0.68	0.69
S3				1.00	0.72
S4					1.00

P: Mother plant, S1: Subculture 1, S2: Subculture 2, S3: Subculture 3 and S4: Subculture 4

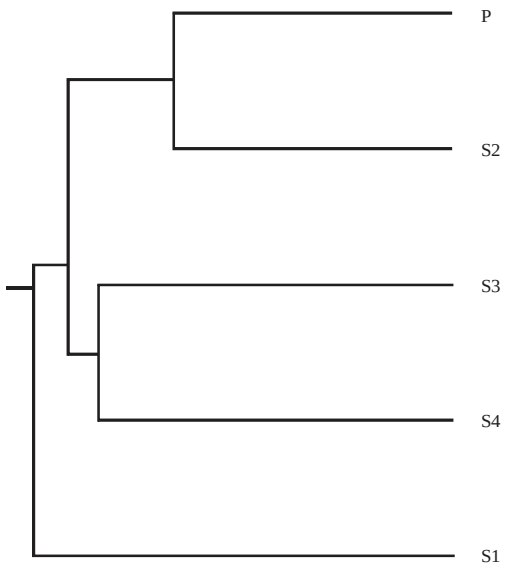

Fig. 1: Dendogram constructed using UPGMA (unweighted pair-group method with arithmetic averages, which represents relationships of similarity among parental plant and somaclones). P represents the parental plant, S2 is for the second subculture plantlets, S3 is for the third subculture plantlets and S4 is for the fourth subculture plantlets

68.08% (0.606-0.724). This shows that the somaclones are similar for more than half part of the plant genome. This may be due to the fact that the parental plant had undergone many cycles of subculturing before being selected as parental plant for RAPD analysis. The greatest similarity (78.13%) was recorded between P and S2 somaclones followed by similarity indices of 76.67% between P and S3 somaclones. S4 somaclones had the least genetic similarity with the parental genotype (P) and it can be concluded that somaclones S4 differs the most from the parent (P) compared to the other somaclones. This provides evidence that maximum changes in genetic in this clone had occurred in the course of its micropropagation stage.

A dendrogram of four somaclones (S1, S2, S3 and S4) and their parent (P) (Fig. 1) was constructed based on the genetic similarity matrix generated from Table 9. Somaclones S3 and S4 was found out to be the most dissimilar pair, compared to P and S2 somaclones. S1 somaclones was the most divergent from all the somaclones. Clustering, as depicted in the dendrogram demonstrated the induction of variability at the DNA level through somaclonal variation.

Although the source of somaclonal variations cannot be determined at this point, it could have happened during the callus stage, in which DNA sequence could have been altered in the presence of plant growth regulator such as BAP and NAA. Such differences might in part be attributed to the influence of the micro environment on cellular behavior, which was affected by the addition of plant growth regulators present in culture media[19,20].

RAPD technique can potentially detect a single base pair change, which can be mutation, deletion or insertion in DNA sequence. Results from this study also indicated that some

mutations such as deletion or insertion might have occurred in the amplified region and that base changes might have induced the alteration of primer binding sites. It appeared that the changes at genetic level were abrupt and could not be reproduced successfully at similar tissue culture condition. In general, *in vitro* conditions can be extremely stressful on plant cells and may set in motion highly mutagenic processes during explant establishment, callus induction, embryo development or plant regeneration[8].

CONCLUSION

Optimum concentrations of BAP, NAA and sucrose for shoot and root development was determined to be 1.0 mg L^{-1} of BAP, 0.5% of NAA and 30 g L^{-1} of sucrose as compared to other treatments used. 69.2% polymorphism was detected in regenerated plants indicating genetic variation had occurred amongst somaclones. Data and results obtained from this study could be used for subsequent work of propagating this plant *in vitro*.

SIGNIFICANCE STATEMENTS

This paper reports results of a study on micropropagation and assessment of genetic variability of *Cyclanthus bipartitus* plantlets generated from the culture. Results generated here help plant propagator to possibly use micropropagation technique to propagate *Cyclanthus bipartitus.* The results presented give indication on degree of somaclonal variability amongst the plantlets produced which is dependent on number of subculturing performed.

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