

Characterization of Green Pigment from *Sauropus androgynus* Shoot Cultures

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ABSTRACT: The dark green leaves of *Sauropus androgynus* (Euphorbiaceae) have various nutritive values and are commonly used for human consumption as food, medicine, and natural dye substance in South-east Asia. Shoot cultures of this plant were established by adding various concentrations of kinetin (Kn) and benzyl adenine (BA) using nodal explants. The best results were recorded when Kn 0.1 mg/L was used with BA 1 mg/L ($BA_1Kn_{0.1}$). Spectrophotometric analysis showed two peaks of green pigment in shoot cultures, A pigment ($\lambda_{max} = 663.6 - 663.8$ nm, absorbance 0.1111) and B pigment ($\lambda_{max} = 611.3 - 613.9$ nm, absorbance 0.0390). Thin Layer Chromatography (TLC) analysis showed two green spots ($R_f Y = 0.31$ and $R_f Z = 0.25$) of shoot cultures on medium supplemented with $BA_1Kn_{0.1}$ for 10 days. Pigment profiles of shoot cultures were similar to their corresponding mother plants. Random Amplified Polymorphic DNA (RAPD) was used as a preliminary technique to evaluate the genetic similarity of the shoot cultures and their corresponding mother plants. It showed four similar DNA banding patterns to their leaves, ranging from 271-765 bp.

Keywords: green pigment; RAPD; *Sauropus androgynus*; shoot cultures; Spectrophotometry; Thin Layer Chromatography

1. Introduction

Sauropus androgynus (L.) Merr. (katuk) is a Southeast Asian indigenous plant widely cultivated for traditional medicinal purposes. The dark green leaves are effective for increasing human breast milk production and their leaves have the potential to become an antioxidant agent [1]. Green leafy plants have nutritive value and contain a high amount of vitamins, phyto-steroid, and phenolic compounds [1,2,3].

The leaves of *S. androgynus* are typically 2–6 cm long and 1.5–3 cm wide, the top and bottom of the leaves are dark green and lighter green, respectively. The leaf anatomy of these leaves shows epidermis, equal region of palisade, and spongy tissues with a dense amount of chlorophyll [4]. These leaves have total chlorophyll content of 1.658 mg/g leaf samples [5].

The antioxidant activity of chlorophyll from *S. androgynus* leaves have potential in the treatment of haemolytic anemia on female mice induced sodium nitrite [6]. Rajalakshmi and Banu [7] also reported a novel visible–light photodetector based on chlorophyll from *S. androgynus*–modified ZnO nanoparticles.

Fresh dark green leaves of *S. androgynus* from several different areas of East Java, Indonesia, showed different metabolic profiles and DNA banding patterns [8,9]. Rajalakshmi and Banu [10] also found that the concentration of chlorophyll may vary according to the different regions of plant habitat. It is desirable to develop plant tissue culture techniques to assist future studies on the production of chlorophyll to its propagation and genetic improvement. Since it was observed, that production is generally higher in differentiated plant tissues, i.e. shoots in medium because such cultures produced similar patterns of secondary metabolites as mother plants. Shoot cultures are relatively more stable in the production of secondary metabolites than cultures of undifferentiated cells, such as callus or suspension cultures [11,12,13].

S. androgynus shoot cultures had been cultivated in Murashige and Skoog's (MS) medium or Phillips and Collins (L2) medium supplemented with various concentrations of plant growth regulators, i.e. benzyl adenine (BA), kinetin (Kn), indole-3-acetic acid (IAA), 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), and indole-3-butyric acid (IBA) along with gibberellic acid (GA3) [11,14,15]. Eganathan & Parida [14] showed maximum number of shoots and shoot length was observed on the MS medium containing 1.0 mg/L BA and 0.1 mg/L Kn after 25 d. Frequent subculture of shoots did not show any morphological variation.

Our previous work used plant tissue culture techniques for obtaining similar green pigment profiles in the cultures with the profiles in the mother plants. Previous study of our work yielded green pigmented callus culture from *S. androgynus* leaves explants initiated on MS medium supplemented with 1 mg/L NAA and 0.5 mg/L BA. The callus induced on this medium was friable and green in color but the yield of green pigment was much lower than in the mother plants [16].

In order to increase green pigment yield in *S. androgynus* cultures before scaling up production of green pigment, this research used plant tissue culture techniques *in vitro*, by initiating shoot cultures formation from *S. androgynus* nodal explants cultivated in a medium supplemented with various concentrations of Kn and BA. The parameters examined were the growth index and green pigment profiles analyzed with spectrophotometry and thin layer chromatography (TLC), and genetic similarity of the shoot cultures and their corresponding mother plants evaluated by Random Amplified Polymorphic DNA (RAPD) method on internal transcribed spacer (ITS) regions.

This is the first report for the characterization of green pigment from *S. androgynus* shoot cultures. This tissue culture technique was supported by a molecular approach such as DNA

fingerprinting to characterize genotype of *S. androgynus* shoot cultures.

2. Materials and method

2.1. Sample preparation

Young shoots and leaves were obtained from juvenile plants of *S. androgynus* grown at a private garden in Gunung Anyar, East Surabaya, Indonesia (7° 14' 57" South, 112° 45' 3" East). Tender nodes were extracted as explants by cutting off the petioles of the young branches along within the leaves. All the samples were authenticated by the Center of Information and Development of Traditional Medicine (PIPOT), Faculty of Pharmacy, University of Surabaya, East Java, Indonesia. The leaves were light green, growing up to 3 leaves of shoots in branches, 1.5-3.5 cm length, 0.5-1.5 cm wide, had a smooth texture and they did not curl up. The nodal explants were green and had a smooth texture.

2.2. Culture medium

Murashige and Skoog (MS) medium was prepared and prior to addition of agar, the pH was adjusted to 5.8. The medium was supplemented with various concentrations of plant growth regulator, Kn, and BA with three combinations such as 0.1 mg/L BA and 0.01 mg/L Kn (BA_{0.1}Kn_{0.01}; M1), 0.5 mg/L BA and 0.05 mg/L Kn (BA_{0.5}Kn_{0.05}; M2), 1 mg/L BA and 0.1 mg/L Kn (BA₁Kn_{0.1}; M3).

2.3. Initiation and cultivation of shoot cultures

The nodes were first washed with running water without detergent for 1 hour, then followed by disinfecting with ethanol (70% v/v) and rinsing with sterile distilled water. The nodes in sterile distilled water were transferred to a laminar airflow cabinet and then surface disinfected with 1.75% of sodium hypochlorite for 3 minutes, then followed by rinsing three times with sterile distilled water.

Nodal explants were sectioned into square size (~1 cm²) and cultured in MS medium with different concentrations of BA and Kn. All cultures

were maintained to develop shoots in a culture room at 25 ± 2°C, humidity 45-55%, and under 8 watts/m² light intensity. The mean shoot height, shoot width and mean Growth Index based on fresh weight were recorded during the cultivation. The means comparison and statistical analysis were done using SPSS 16.0 software whereby the effects of plant growth regulator treatments on shoot induction were analyzed utilizing paired samples t-test at 95% confidence interval.

2.4. Green pigment extraction and analysis

S. androgynus leaves and shoot cultures were stored at -80°C before extraction. The samples were ground gently with chilled mortar and pestle since the pigments are heat sensitive and weighed out to 200 mg each in a test tube and then followed by adding 2 mL of methanol. The mixture was homogenized for 1 min with a vortex. The mixture was extracted 5 times by ultra-sonication followed by centrifugation for 1 min at 3000 rpm. The filtrate was transferred to a 10 mL volumetric flask with a filter paper. These clear green-colored supernatants were collected and ready for detection of pigments through absorbance spectra analysis by Shimadzu UV-1800 spectrophotometer. The pigment composition was analyzed by the use of TLC method based on the spot color and retardation factor (R_f) on silica gel 60 F254 TLC plates, with 10 mL mobile phase, comprised of 70% hexane and 30% acetone, from the initial orientation, data not shown. The green pigment was observed under visible light, UV light 254 nm, and 366 nm.

2.5. DNA banding pattern analysis

Total DNA was isolated from the leaves and shoot culture samples of *S. androgynus* using NucleoSpin® Plant II, according to the manufacturer's instructions.

The primers used for amplification of the ITS regions were ITS-5 (5'-TAGAGGAAGGAGAAGTCGTAACAA-3') and ITS-4 (5'-CCGCCTGACCTGGGG TCGC-3')[9]. The amplification profile consisted of 95°C for

2 min, cycled 35 times at 95°C for 30 s, 57°C for 1 min and 71°C for 2 min, with a final extension at 71°C for 5 min in a T100 PCR thermal cycler. The ITS region on DNA was then amplified by the RAPD primers (OPD-11: 5'-AGCGCCATTG-3'). The amplification profile consisted of 94°C for 5 min, cycled 30 times at 94°C for 1 min, 35 °C for 2 min and 72°C for 1 min, with a final extension at 71°C for 5 min in a T100 PCR thermal cycler. The PCR products were visualized in a 1.5 % agarose gel electrophoresis under ultraviolet light to check the presence of amplified bands, using a gel documentation system.

3. Results and discussion

The utilization of plant shoot cultures for the production of natural compounds has gained increasing attention over the past decades. Chlorophyll, as photosensitive light-harvesting pigment, is one of the best candidates of the natural compounds for several biological activities. Chlorophylls could act as an important antioxidant found in foods to prevent oxidative DNA damage and lipid peroxidation [17]. The dark green leaves of *S. androgynus* showed attenuation of cyclooxygenase and induced nitric oxide synthase and antioxidant properties [18]. It also provides a rich source of chlorophyll which possesses antioxidant potentials to protect against toxicities induced by sodium nitrate [10] and the anti-anemia effect on female mice induced sodium nitrite [6].

The production of chlorophyll in the plant could be influenced by an interaction of environmental factors with biochemical, physiological, and genetic characteristics of plant, such as the influence of environmental factors on chlorophyll production by *Festuca arundinacea* [20] and *Salvia chamelaeagnea* (Lamiaceae) [21]. Plant cell and tissue culture strategies are an attractive alternative to the use of whole plants for the production of pigments and have been the subject of extended research in the last decades. The ability to control both

physical and chemical conditions allows the establishment of methodologies that result in increased production of plant metabolites [22]. Therefore, the present study investigates the effects of various concentrations of the plant growth regulator on shoot cultures induction from nodal explants for obtaining green pigment which is similar to corresponding mother plants.

Different concentrations of growth regulators, Kn and BA were tested in order to obtain the best shoot formation. Initiation of shoots was observed on the medium containing several concentrations of Kn and BA studied adapted from Eganathan & Parida [14]. Shoot cultures cultivated in various media, such as BA_{0.1}Kn_{0.01} (M1), BA_{0.5}Kn_{0.05} (M2) and BA₁Kn_{0.1} (M3) could stimulate the production of green pigment after 5 days of culture. The degree of shoot formation was found to be the highest if nodal explants were cultivated in BA₁Kn_{0.1} medium (M3), while the production of green shoot cultures was maintained until 10 days of culture. Growth of shoot cultures on a fresh weight basis (Figure 1 and 2) was best promoted by BA₁Kn_{0.1} (M3). Similar reports were showed by Eganathan and Parida [14] while shoot induction was observed in 1.0 mg/L BA and 0.1 mg/L Kn and more number of shoots were achieved in shoot induction medium. In contrast, the degree of shoot formed from the nodal explants cultured in another media, M1 & M2, was evidently lower. Most of the cultures turned brown, while browning in tissue culture indicated the release of phenolic compounds, which are toxic to the cultures by inhibition of the cultures growth [23,24].

Growth of shoot cultures was best promoted by BA₁Kn_{0.1} medium (M3), while this medium could promote shoots of about 0.100 cm in height and 0.160 cm in width after 14 days (Table 1). Growth of shoot cultures on fresh weight basis (Figure 2) was best promoted by BA₁Kn_{0.1} (M3). The growth curve of shoot cultures indicated that the growth rate of cells was initially slow during the first 9 days. However, after 10 days (exponential phase) the cultures showed a marked increase in growth

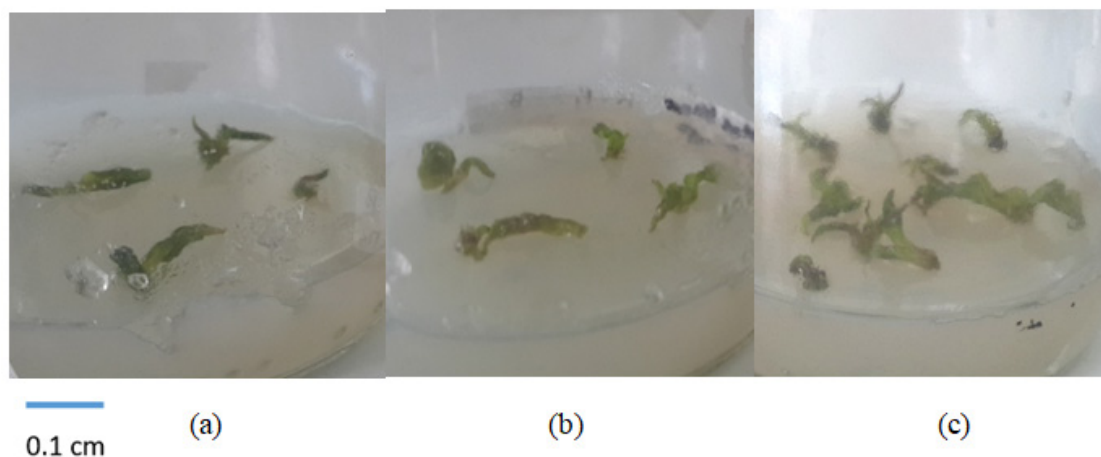


Figure 1. Six-week-old shoots cultures induced from nodal explants of *Sauropus androgynus* on various combinations of Kinetin (Kn) and Benzyl adenine (BA)
 (a) Shoots formation on MS + 0.1 mg/L BA and 0.01 mg/L Kn ($BA_{0.1}Kn_{0.01}$; M1) medium
 (b) Shoots formation on MS + 0.5 mg/L BA and 0.05 mg/L Kn ($BA_{0.5}Kn_{0.05}$; M2) medium
 (c) Shoots formation on MS + 1 mg/L BA and 0.1 mg/L Kn ($BA_1Kn_{0.1}$; M3) medium

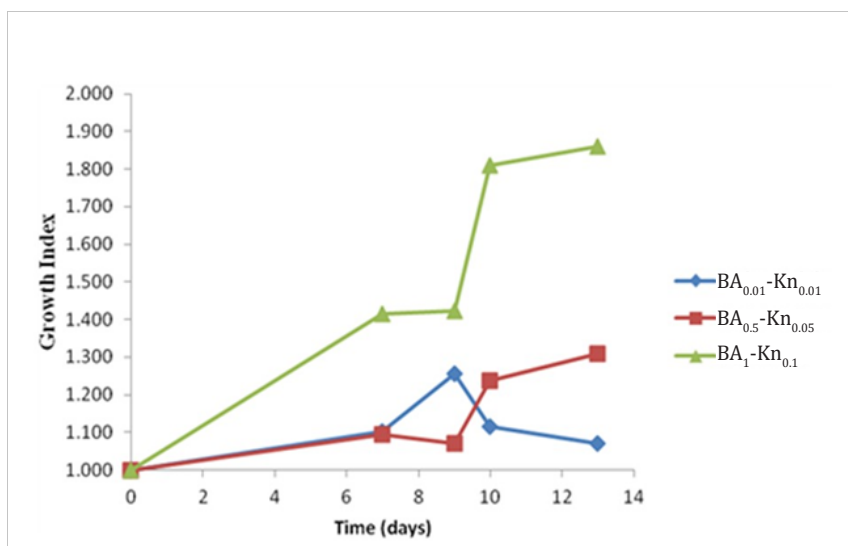


Figure 2. Growth index of shoot cultures of *Sauropus androgynus* in MS medium supplemented with various combinations of Kinetin (Kn) and Benzyl adenine (BA): 0.1 mg/L BA and 0.01 mg/L Kn ($BA_{0.1}Kn_{0.01}$; M1); 0.5 mg/L BA and 0.05 mg/L Kn ($BA_{0.5}Kn_{0.05}$; M2); 1 mg/L BA and 0.1 mg/L Kn ($BA_1Kn_{0.1}$; M3).
 Values are mean of 3 replicates from 6-week-old shoot cultures

potential and accumulated a large amount of fresh weight. The rate of growth was stable for 13 days (stationary phase) followed by a gradual reduction in cell density. Mean comparison showed an average growth index of shoots significantly varied with $BA_1Kn_{0.1}$ (M3) (Table 1). The higher concentration of BA promotes the development of shoot cultures and increases the number of shoots [25,26].

Green pigment estimation was done in the

fresh green leaves samples and shoot cultures of *S. androgynus* in $BA_1Kn_{0.1}$ medium extracted with methanol. The absorbancy readings of green pigment extracts were measured at interval wavelengths between 550 and 750 nm (Figure 3). Chlorophyll a possesses a green-blue color, and chlorophyll b possesses a green-yellow color, accordingly to Inanç [27] a green leaf absorbs blue light (mostly at 430 nm) and red light (mostly at 660 nm) regions of the visible spectrum.

Table 1. Effect of various concentrations of kinetin (Kn) and benzyl adenine (BA) on shoot regeneration from nodal explant of *Sauropus androgynus* on MS medium

| Hormonal treatment | Average of shoot height (cm) (Mean ± SD) | Average of shoot width (cm) (Means ± SD) | Average of growth index based on fresh weight (Mean ± SD) |
|--------------------------------------|---|--|--|
| BA _{0.1} Kn _{0.01} | 0.033 ± 0.0577 | 0.067 ± 0.0577 | 1.069 ± 0.0900 |
| BA _{0.5} Kn _{0.05} | 0.075 ± 0.0500 | 0.150 ± 0.0577 | 1.310 ± 0.2460 |
| BA ₁ Kn _{0.1} | 0.100 ± 0.0707 | 0.160 ± 0.1342 | 1.890* ± 0.4500 |

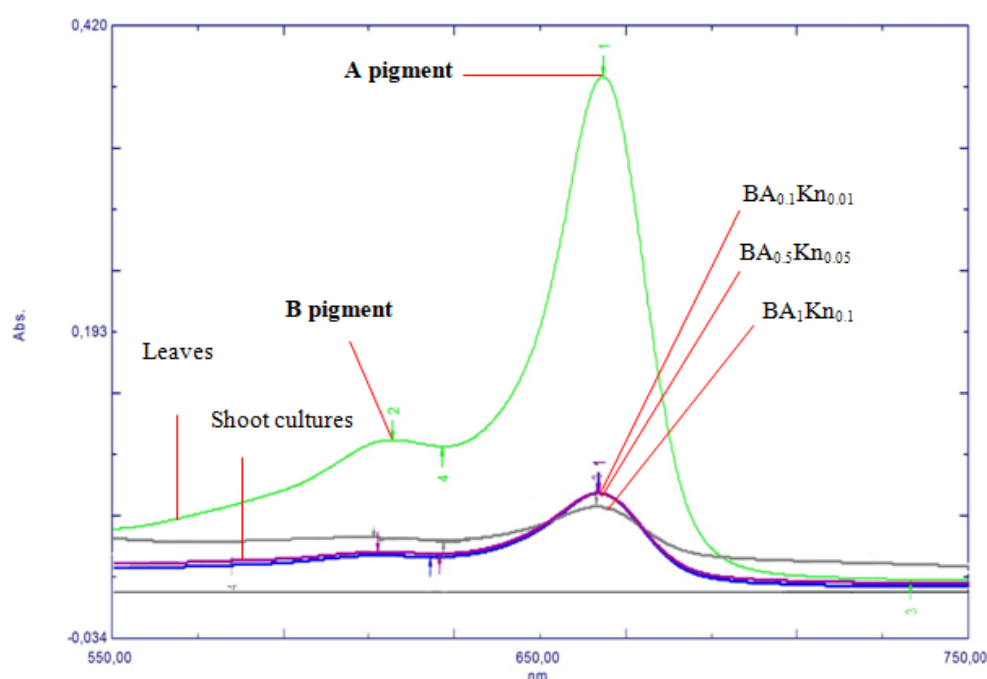
Each value represents mean ± SD from 6-week-old shoot cultures in three repeated experiments

*significantly different at P<0.05

BA_{0.1}Kn_{0.01} = 0.1 mg/L BA and 0.01 mg/L Kn

BA_{0.5}Kn_{0.05} = 0.5 mg/L BA and 0.05 mg/L Kn

BA₁Kn_{0.1} = 1 mg/L BA and 0.1 mg/L Kn

**Figure 3.** Spectra profiles of green pigments of *Sauropus androgynus* in leaves and 6-week-old shoot cultures

BA_{0.1}Kn_{0.01} = 0.1 mg/L BA and 0.01 mg/L Kn

BA_{0.5}Kn_{0.05} = 0.5 mg/L BA and 0.05 mg/L Kn

BA₁Kn_{0.1} = 1 mg/L BA and 0.1 mg/L Kn

The spectra profile of shoot cultures is similar to the profile of fresh leaves (Figure 3). Each spectra had two main peaks annotated as A pigment (fresh leaves λ = 664.5 to 664.7 nm; shoot cultures λ = 663.6 to 663.8 nm) and B pigment (fresh leaves λ = 615.2 to 616.3 nm; shoot cultures λ = 611.3 to 613.9 nm). According to Hosikian *et al.* [28], chlorophyll *a* absorbs light at wavelengths between 660-665 nm, while chlorophyll *b* absorbs light at wavelengths

between 642-652 nm.

The absorbance value of green pigment in shoot cultures of *S. androgynus* is lower than fresh leaves. The absorbance value of A pigment in both extracts was higher than the value of B pigment (shoot cultures, absorbance value of A pigment = 0.1111; B pigment = 0.0390). The ratio of chlorophyll *a* to chlorophyll *b* in higher plants is approximately 3:1 [29, 30].

TLC analysis of fresh leaves and shoot cultures

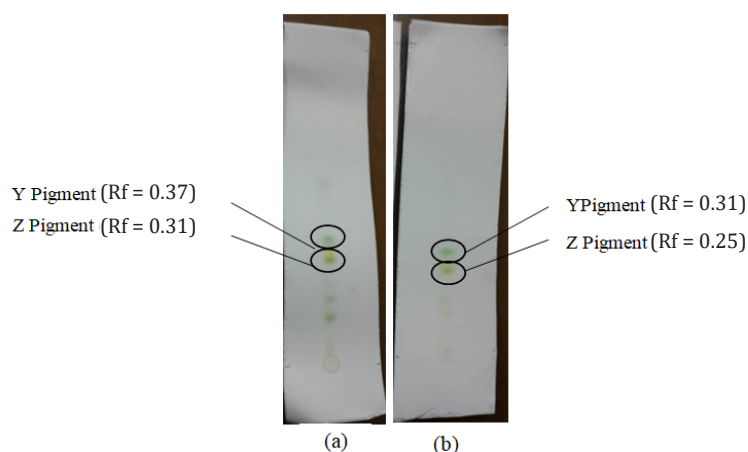


Figure 4. Chromatogram profiles of leaves extract with RF Y= 0.37, Rf Z= 0.31 (a); and shoot cultures of *Sauropus androgynus* with Rf Y= 0.31, Rf Z= 0.25 (b) in visible light

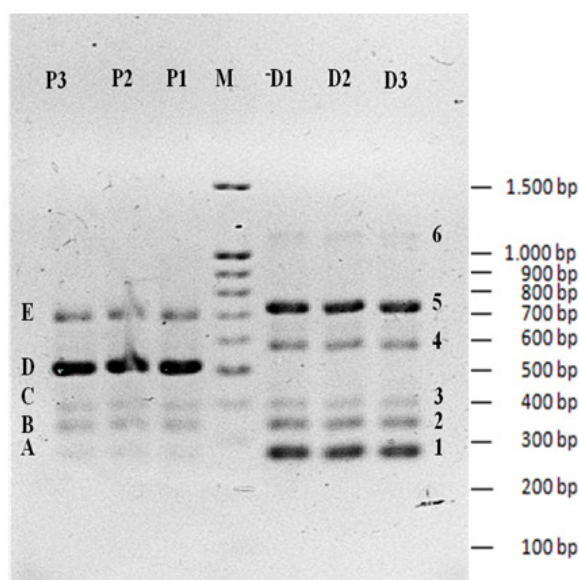


Figure 5. PCR-RAPD on DNA of leaves and shoot cultures of *Sauropus androgynus*
M = Marker 100 bp ladder, D1 = leaves 1, D2 = leaves 2, D3 = leaves 3, P1 = shoot culture 1,
P2 = shoot culture 2, P3 = shoot culture 3

of *S. androgynus* extract (Figure 4), showed two green spots corresponding to Y pigment and Z pigment, with Rf values from fresh leaves were 0.37 and 0.31, in Y and Z pigment respectively, and the Rf values from shoot cultures were 0.31 and 0.25, in Y and Z pigment respectively.

Chromatogram profiles of fresh leaves and shoot cultures showed a similar pattern. Consistently, in a study in *Cassia siamea*, the chlorophyll in micropropagated plantlets was similar to the mother plants. In these plant tissue cultures, the percentage of chlorophyll content has increased. This may be due to the balanced

nutrient level and plant growth regulators in the medium [31].

Rf value of Y pigment was higher than Z pigment. This pattern showed that Y pigment was a slightly more non-polar pigment than Z pigment. More polar compounds will bound to the polar silica gel and travel short distances on the plate, while non-polar substances will diffuse into the solvent and travel large distances on the plate.

The spectra and chromatogram profiles of fresh leaves and shoot cultures of *S. androgynus* cultivated in BA₁Kn_{0.1} medium demonstrated that

pigment profiles of shoot cultures were similar to their corresponding mother plants.

DNA banding patterns of leaves and shoot cultures (Figure 5) showed four similar banding patterns ranging from 271 to 765 bp, while the primer used in this study produced polymorphic bands in leaves and shoot cultures. The different DNA band of shoot cultures on approximately 500 bp is assumed as a variation in *in vitro* cultures. Our previous work showed that callus cultures of *S. androgynus* had three similar banding patterns to its mother plant and one band that was detected only in fresh leaves [16].

This type of variation can be influenced by alterations in plant growth regulator concentrations and their ratio, *in vitro* stress due to unnatural conditions, nutritional conditions, and altered diurnal rhythm [33]. Lakshmanan *et al.* [32] reported that the use of cytokinins, especially the exposure to BA coupled with altered diurnal rhythm and continuous availability of high levels of nutrients have been noted to induce alterations in morphology, but these morphological changes, were found unassociated with the genetic change.

4. Conclusion

Shoot cultures of *S. androgynus* cultivated in medium supplemented with 1 mg/L BA and 0.1 mg/L Kn yielded two green pigments, Y and Z, which have similar spectra and chromatogram profiles with their corresponding mother plants. Similar DNA banding patterns of leaves and shoot cultures were showed, ranging from 271-765 bp. In conclusion, the shoot culture of *S. androgynus* can produce green pigments that are similar to their corresponding mother plants. This result can be useful for the combination of molecular genetic and chemical analysis that eventually should permit the application of any marker in the selection of high chlorophyll-producing *S. androgynus* shoot cultures.

Further studies on the production of green pigment in shoot cultures of *S. androgynus* may

contribute valuable profits in many industries, especially in the pharmaceutical, cosmetics, or food industries.

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