

Effect of sucrose and plant growth regulators on callogenesis and preliminary secondary metabolic of different explant *Myrmecodia tuberosa*

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Abstract. Sari YP, Kusumawati E, Saleh C, Kustiawan W, Sukartingsih. 2018. Effect of sucrose on callogenesis and preliminary secondary metabolic of different explant (*Myrmecodia tuberosa*). *Nusantara Bioscience* 10: 183-192. *Myrmecodia tuberosa* Jack is a medicinal plant that contains bioactive compounds, such as flavonoids, tannins, tocopherols, phenols, and an abundance of minerals, that are useful as antioxidants. With the constant increases in popularity of the medicinal plant, the *M. tuberosa* is threatened by extinction if over-exploitation continues. Thus, the effort to conserve this plant is vital. Tissue culture is an alternative method to conserve and produce active compounds that are similar to those of the native ant nest plant with callus. The addition of certain compounds such as sucrose can affect the secondary metabolite content through in vitro plant or callus. The aim of this research was to find the explant sources (cotyledon, stem, tuber, and root), determine the best growth regulator to produce the callus, and evaluate the optimum sucrose concentration to enhance secondary metabolite production of the callus. The results showed that callus was obtained from all explant sources and all growth regulators. The best callus that was marked by a friable green and yellowish green callus was provided by cotyledon with the growth regulator of 2 mg·L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg·L⁻¹ of kinetin. Calli treated with 30 g of sucrose resulted in the best secondary metabolites, containing alkaloids, phenols, flavonoids, saponins, and steroids.

Keywords: 2,4-D, 2,4-dichlorophenoxyacetic acid, callus, *Myrmecodia tuberosa*, kinetin, secondary metabolite, sucrose

INTRODUCTION

The ant nest plant (*Myrmecodia tuberosa*) is a herbal medicine that contains bioactive compounds, such as glycosides, vitamins, minerals, flavonoids, tocopherols, polyphenols, and tannins (Engida et al. 2013; Sanjaya et al. 2014; Sudiono et al. 2015), which are useful as antioxidants and anticancer compounds. Several researchers have shown that the ant nest plant inhibits the growth of various types of cancer cells, such as those in ovarian cancer (Hasanuddin et al. 2015), oral tongue squamous cell carcinoma (OTSCC) (Supriatno 2014), lung and colon cancer (Manoi 2008; Subroto and Saputro 2008), HeLa and MCM-B2 cell cancer (Soeksmanto et al. 2010). Another report showed that the ant nest plant has anti-microbial bioactivity and antioxidants (Prachayasittikul et al. 2008).

The popularity of the ant nest plant as a pharmaceutical plant is undoubtedly, causing many people to explore directly from nature. The over exploration of the *Myrmecodia* plant without cultivation might decrease the population. Thus, there is an urgent need to restore the natural population of this plant and use an alternative pharmaceutical source without taking it directly from nature. The tissue culture method is an alternative to

provide raw material to produce a medicinal compound to achieve great benefits in the pharmaceutical aspect (Radha et al. 2011) and to select genotypes that have considerable amounts of bioactive compound (Cantelmo et al. 2013). Generally, organ culture, cell suspension, and callus culture have been used to study secondary metabolite synthesis using the tissue culture method (George and Sherrington 1984; Smetanska 2008). Massive development through tissue culture indicates that it does not affect the active compound content from the plants.

To assess biomass accumulation and synthesis of secondary compounds in cultures, various strategies have been developed. Biomass accumulation and metabolite biosynthesis are two-stage events. The first stage is to control the parameters, including regulation the growth and multiplication of both cultured cells/organs and biomass accumulation, as well as parameters that related to biosynthesis of metabolites which are controlled in the next step. Further, to select high-producing cells or organ clones; optimize medium parameters such as suitable medium, salt, sugar, nitrogen, phosphate, and plant growth regulator levels; and physical factors such as temperature, illumination, light quality, medium pH, agitation, aeration, and environmental gas (e.g., oxygen, carbon dioxide, and ethylene) can be controlled in the

early stage of the culture process. Meanwhile, in the second stage of the culture process, elicitation, replenishment of nutrient and precursor feeding, permeabilization, and immobilization strategies that assist with the accumulation of metabolites can be applied. In addition, in line with stage-specific strategies, to produce huge number of biomass in accordance with an increase in the accumulation of secondary compounds can be also possible (Murthy et al. 2014).

The successful tissue culture depends on factors such as the medium, growth regulator, explant source, and environment. In addition, the successful tissue culture has been also affected by the types and concentration of the growth regulator added in the medium (Shi 2014). Auxin, cytokinin, and gibberellin are growth regulators usually used in tissue culture (Davies 2010; Coggins and Lovatt 2014; Erland et al. 2017). One of the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) which is a synthetic growth regulator, is beneficial to induce callus formation (Welsh and Mogeia 1991; Phua et al. 2016). Nevertheless, proper interaction between auxin and cytokinin should be considered to enhance callus growth.

Past researchers have stated that the highest weight of callus was obtained from 1.00 mg·L⁻¹ of BA in combination with 0.99 mg·L⁻¹ of 2,4-D in *Solanum khasianum* (Tambe 2013) and 3.0 mg·L⁻¹ of 2,4-D in *Gardenia latifolia* (Reddy and Saritha 2012). Meanwhile, Sari and Kusuma (2015) revealed that an ant nest plant callus was formed in solid and liquid Murashige and Skoog (MS) medium with 2,4-D (2 mg·L⁻¹) and kinetin (2 mg·L⁻¹). In addition, a high quality of callus can be produced using specific nutrients and manipulate nutrient components in the culture medium (e.g., carbon source, nitrogen, and phosphate) to induce cell growth for effectiveness of secondary metabolite production (Tapia et al. 2001).

Sucrose has a positive effect on the production of secondary metabolites both in cell and organ cultures (Paiva and Janick 1982; Fowler 1983). The effect of sucrose concentration (i.e. 20, 30, 40 and 60 g L⁻¹) was investigated in suspension cultures of *Panax notoginseng* for production of ginseng saponin (secondary metabolite) and polysaccharide (primary metabolite). In addition, a sugar feeding strategy was formulated to enhance the saponin accumulation by *P. notoginseng* cells (Zhang et al. 1996). Sucrose concentration (5, 7, and 9% w/v) showed increased accumulation of phenols, flavonoids, chlorogenic acid, and total hypericin in adventitious root cultures of *Hypericum perforatum* L (Cui et al. 2010). Sucrose levels below 6-9% did not significantly alter the specific alkaloid content, but biomass production by *Solanum aviculare* hairy roots was maximum and about 60% higher than in 3% sucrose medium when the initial sucrose concentration was 4-6% (Yu et al. 1996). The suitable medium for both biomass Brahmi (*Bacopa monnieri*) (6.31 ± 0.12 g fresh weight and 250 ± 5.00 mg dry weight) and bacoside A accumulation (13.09 mg g⁻¹ dry wt) found in MS medium supplemented with 2% sucrose and pH set at 4.5 (Naik et al. 2010).

Based on the information above, the purpose of this research was to determine the optimum combination of growth regulators (2,4-D: 0.5, 1, 1.5, and 2 mg·L⁻¹ and kinetin 2 mg·L⁻¹) and explant source (cotyledon, stem, tuber, and root) on callus production in the ant nest plant using an in vitro method. The research was also aimed to evaluate the optimum sucrose concentration to enhance secondary metabolite production from the callus.

MATERIALS AND METHODS

Plant materials

Seeds of the ant nest plant (*Myrmecodia tuberosa* L. family Rubiaceae) were provided from the ripe fruit, which were collected from Antutan village, Tanjung Palas regency, Bulungan district, North Borneo. The seeds collection has been granted and permitted by the head of local authority (reference 025/RT-004/ATT/XI/2017)

Sample preparation

Seeds were sterilized using 70% alcohol for 1 minute, followed by 30%, 20%, and 10% sodium hypochlorite (v/v) for 10 minutes each and washed three times for 5 minutes in sterile conditions. For germination, seeds were placed in MS medium and agar (7.5 g L⁻¹) with 30 g L⁻¹ sucrose (Murashige and Skoog 1962).

Seed germination

Sterile seeds of the ant nest plant were grown in glass bottles, containing MS0 (basal medium without plant growth regulators), incubated in a culture room with a light intensity of ±1000-2000 Lux with a temperature of 20°C-25°C. After one month, the seedling plant was used as an explant source, which was grown in a callus formation medium. The cotyledon, hypocotyl, tuber, and root were used as explant sources.

Callus formation

Cotyledon from a month-old plant was prepared to use as an explant source. The explant was grown in MS with a growth regulator combination of 2,4-D (0.5, 1, 1.5, and 2 mg·L⁻¹) and kinetin (2 mg·L⁻¹), incubated in a tissue culture room with a light intensity of ±1000-2000 lux with a temperature of 20°C-25°C for 10 weeks. All growth regulation concentration was selected as described in previous experiment (Sari and Kusuma 2015). All treatment was done in triplicates.

Sucrose treatment

The best callus from induction was used in sucrose treatment. The 0.5 g callus was weighed and placed in the MS medium that was added with different concentrations of sucrose (30, 60, 90, and 120 g). After 8 weeks, secondary metabolite content was determined from each callus.

Secondary metabolite extraction

The callus was extracted using ethanol as a solvent for 48 h. The extraction process continued until the extraction solution become clear (\pm 48 h) followed by filtration using Whatman paper (Whatman 2; Sigma-Aldrich, Germany). After filtration, a rotary evaporator was used to evaporate the remaining solvent. The extracted ethanol was then collected to be used as a sample extract in the phytochemical test to determine the phytochemical contents in the ant nest plant extract.

Phytochemical test

To detect the presence of possible phytochemicals in the sample extract, some phytochemicals, such as alkaloids, flavonoids, phenols, saponins, and steroids/triterpenoids, were tested, following the methods of Pal et al. (2012). For the alkaloid test (Mayer's test), 1 mL of Wagner's reagent was added to the extracted sample. The formation of a white precipitate indicates a positive test. In the flavonoid test (Shinoda's test), 5 mL of extract and 1 mL of concentrated hydrochloric acid and magnesium ribbon were mixed and shaken. The appearance of a pink-red color indicates the presence of flavonoids. For the phenolic test, the extracted sample was mixed with a few drops of 1% solution of FeCl_3 . A blue-green or black coloration indicates the presence of phenol. In the saponin test (foam test), the extracted sample was added to 10 mL of distilled water, cooled, air dried, and shaken vigorously for 10 s. The appearance of stable foam (1-3 height) indicates the presence of saponin. In the steroid/triterpenoid test (Liebermann-Burchard test), CH_3COOH glacial and absolute H_2SO_4 were added to the sample extract with a ratio of 20:1. The appearance of a blue or green color indicates the presence of steroids, and the appearance of a red or brown color indicates the presence of triterpenoids.

Data analysis

The results were presented as descriptive data. Callus induction was observed at the first growth of the callus along with the percentage of callus growth, callus morphology (texture and color), and callus growth

intensity. For the phytochemical test, the presence or absence of phytochemicals such as alkaloids, flavonoids, phenols, saponins, triterpenoids, and steroids in the extract was indicated as positive (presence) or negative (absence). Weight of callus which were obtained after various concentration of sucrose treatment were analysed by using ANOVA (SPSS 22, Inc., USA), followed by Tukey's post hoc test to evaluate any significant differences at $p < 0.05$.

RESULTS AND DISCUSSION

Seed germination

The seeds of the ant nest plant that were grown in MS0 started to grow on day 3, reaching full growth as a plant at day 30 (Figure 1).

Seed germination is a mechanism related to the alteration of both morphological and physiological aspects, which is caused by embryo activation. The process of embryo expansion and elongation is a process that is initiated by absorbing water by the seed before germination. The germination process is terminated when the radicle has completely grown out to cover the seed layers (Hermann et al. 2007).

Seed germination of the ant nest plant using tissue culture was affected by two types of factors: internal and external factors. The internal factors include the endosperm (food stores) and the level of seed maturity. In agreement with the findings by Miransari and Smith (2009), for the seed to germinate, the availability of starches, proteins, lipids, and nutrients must be completed in the seed embryo through the activity of specific enzymes and pathways. Müller et al. (2013) added that the activity of the enzyme pectin methylesterases is also influenced in seed germination. Further, the homogalacturonans of the wall, a methyl esterified, are facilitated by the enzyme that affects the cell-wall porosity and elasticity, causing cell growth and water uptake. In addition, the expansion of the cell wall of the radicle and the tissues occurs during the process of seed germination.

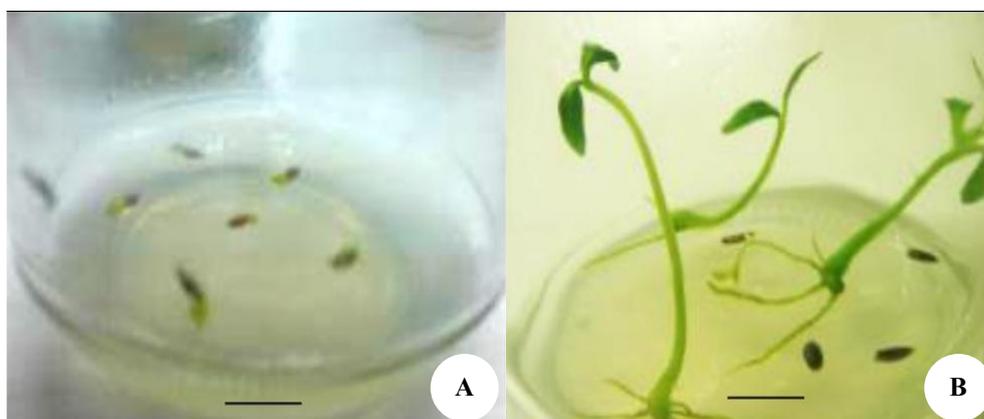


Figure 1. Seed germination of the ant nest plant (*Myrmecodia tuberosa*) in MS0 medium. A. 3 days, B. 30 days. Bar = 1 cm

Besides internal factors, seed germination is also affected by external factors, such as plant growth regulators, light, and medium composition. The successful seed germination of the ant nest plant in a brief time is influenced by the MS medium composition, which contains several amino acids that might boost seed germination. Waes and Debergh (1986) reported that the addition of amino acids, such as serine, glutamic acid, peptone, hydrolysate casein, and yeast extract, in the medium induce seed germination. In comparison with other salt formation, MS inorganic salts have high contents of nitrate, potassium, and ammonium. The MS medium also contains high macro and microelements and inorganic salts that are sufficient to support the optimum growth of the plant. In addition, the concentration and quality of nitrogen in the MS medium contribute to the prolific growth obtained with plant-derived seed types that are incubated in this medium. Thus, the nitrogen supplied in the medium is in inorganic form as

the anion NO_3 or the cation NH_4 . According to Malmgren (1996), the ammoniated form of nitrogen is more beneficial than the nitrate form, which is proved by the fastest growth of the *Dactylorhiza* species seedlings at $50\text{-}100\text{ mg}\cdot\text{L}^{-1}$ NH_4NO_3 . Similar results were found in past research, which revealed that an efficient concentration of organic and inorganic nitrogen sources can be used to promote the growth of explants (Chen and Chang 2002). The 30-day old ant nest plant was used as an explant source to be grown in the initial medium treatment.

Callus induction

Callus induction of cotyledon, stem, tuber, and root explants of the ant nest plant was affected by the combination of growth regulators 2.4-D and kinetin (Table 1).

Table 1. Effects of 2.4-D and kinetin composition on the callus induction of the ant nest plant (*Myrmecodia tuberosa* Jack) from the cotyledon, stem, tuber, and root explants at week 10

Explant	Growth regulator		Percentage of callus formation (%)	Callus formation time (week)	Callus morphology		Callus intensity
	2.4-D $\text{mg}\cdot\text{L}^{-1}$	Kinetin $\text{mg}\cdot\text{L}^{-1}$			Color	Texture	
Cotyledon	0.5	0.0	100	2	Yellow	Friable	++
	1.0	0.0	100	2	Yellow	Friable	++
	1.5	0.0	100	2	Yellowish green	Friable	++
	2.0	0.0	100	2	yellowish green	Friable	++
	0.5	2.0	100	2	Yellowish green	Friable	+++
	1.0	2.0	100	2	Yellowish green	Friable	+++
	1.5	2.0	100	2	Yellowish green	Friable	+++
	2.0	2.0	100	2	Green-yellowish green	Friable	+++
Stem	0.5	0.0	100	2	Yellowish green	Friable	++
	1.0	0.0	100	2	Yellow	Friable	++
	1.5	0.0	100	2	Yellowish green	Friable	++
	2.0	0.0	100	2	Yellow	Friable	++
	0.5	2.0	100	2	Yellow	Friable	+++
	1.0	2.0	100	2	Yellowish green	Friable	+++
	1.5	2.0	100	2	Yellowish green	Friable	+++
	2.0	2.0	100	2	Yellowish green	Friable	+++
Tuber	0.5	0.0	100	2	Yellowish green	Friable	++
	1.0	0.0	100	2	Yellowish green	Friable	++
	1.5	0.0	100	2	Yellowish green	Friable	++
	2.0	0.0	100	2	Yellow	Friable	++
	0.5	2.0	100	2	Yellowish green	Friable	+++
	1.0	2.0	100	2	Yellow	Friable	+++
	1.5	2.0	100	2	Yellow	Friable	+++
	2.0	2.0	100	2	Yellowish green	Friable	+++
Root	0.5	0.0	100	2	Yellowish green	Friable	+
	1.0	0.0	100	2	Yellowish green	Friable	+
	1.5	0.0	100	2	Yellowish green	Friable	++
	2.0	0.0	100	2	Yellowish green	Friable	++
	0.5	2.0	100	2	Yellowish green	Friable	+++
	1.0	2.0	100	2	Yellowish green	Friable	+++
	1.5	2.0	100	2	Yellowish green	Friable	+++
	2.0	2.0	100	2	Yellowish green	Friable	+++

Note: + = low, ++ = intermediate, +++ = high. Three explants were used for each PGRs combination

All treatment combinations of growth regulators resulted in calli with a percentage of 100%. A callus is a collective form of disorganised cell masses. The formation of callus via growth and accumulation of cells has a relationship with wounding. Further, a single differentiated cell and other totipotent cells could produce a callus that can regenerate the whole plant body (Nagata and Takebe 1971). The callus is formed at 2 weeks of age, marked by explant swelling and callus formation in the edge, slice, or lesion of the explant. The callus is fully formed at the leaf surface after a month. This is related to the uptake of nutrition in the medium by the explant. The absorption of nutrients is even better with direct contact between the medium and explant.

This result was in line with previous research performed by Morini et al. (2000), which revealed that the callus formation of quince leaves was found only in the abaxial surface part. The appearance of the callus on the injured part might be because of the excitement of the tissue on the explant to cover the wound. Explant response on the treatment medium is started with explant swelling or elongation. The size of the explant becomes bigger from the beginning, and the callus begins to form on part of the injured explant. The combination of auxin (2,4-D) and cytokinin (kinetin) as growth regulators on the MS medium can induce callus formation, both in single and combination treatments. The callus began to appear on the edge of the explants and on the wounded parts and continued to grow until the end of the observation at 10 weeks after planting. In addition, Stobbe et al. (2002) revealed that the wound-induced calli regenerate new organs or new tissues, suggesting that they are highly pluripotent.

In 2,4-D without kinetin, callus growth response in all explants (cotyledon, stem, tuber, and root) with a small callus needed extended time to grow bigger. This suggests that the ant nest plant callus cannot grow optimally with a single auxin administration without the addition of a growth regulator from the cytokine group. According to Kala et al. (2014), the growth regulators cannot induce a callus from *C. parviflorum* leaf explants. However, the combinations of growth regulators resulted in optimum callus production. Callus formation is strongly influenced by the type and concentration of the growth regulator. Zulfiqar et al. (2009) revealed that the growth and morphogenesis of plants in vitro are controlled by the balance and interaction of the growth regulator that was absorbed from the media. Auxins play a role in stimulating the growth of explant cells; thus, auxin tends to form a callus that begins from cell division in the meristematic area. At the beginning of the growth response, auxin triggered the elongation of cells through loosening the cellulosic cell wall. This elongation of the cell was due to the response of 2,4-D, but the cell cannot divide rapidly because there was no addition of kinetin. The combination of the growth regulators, 2,4-D, and kinetin, in all explant sources (cotyledons, stems, tubers, and roots) resulted in a larger callus size compared to only 2,4-D. In line with past research (Rout et al. 2000), the combination of 2,4-D and

kinetin resulted in the largest callus of *Cephaelis ipecacuanha* and *Melaleuca alternifolia* (Kiong et al. 2007). Stella and Braga (2002) used a combination of auxin (picloram) and cytokinin (kinetin) in the callus of *Rudgea jasminoides*. The best callus was found through the addition of $2 \text{ mg}\cdot\text{L}^{-1}$ of 2,4-D and $2 \text{ mg}\cdot\text{L}^{-1}$ of kinetin in all explants that were used. In addition to the larger callus size, the resulting callus had a more intensive green color with friable callus texture. The large callus size might be due to the corresponding concentration of growth regulators, causing rapid cell division in the ant nest explants. The callus can be provided with two plant hormones, auxin, and cytokinin in a balanced condition. Pande et al. (2015) also got highest callus induction frequency on MS media supplemented with 1.5 mg L^{-1} 2, 4-Dichlorophenoxyacetic acid, and 1.5 mg L^{-1} of Benzyl amino purine.

Callus color

The results of the observation for 10 weeks after planting found that the combination of growth regulator 2,4-D and kinetin on the MS medium induced the formation of a callus on various explants from the ant nest plants (Figure 2). The color of the calli at all treatments ranged from yellow and yellowish green to green. The resulting color variations might be due to the diverse types of growth regulators, the difference in growth regulator concentration, and the type of explant. Compared to only auxin, a combination of auxin and cytokinin resulted in a callus color that was more green, caused by cytokinin, which tends to promote chlorophyll formation (Edwin F George et al. 2008). According to Afshari et al. (2011), various callus color conditions could be caused by the pigmentation, the influence of light, and the plant parts used as the source explant.

Based on the callus color (Figure 2), the addition of $2 \text{ mg}\cdot\text{L}^{-1}$ of 2,4-D and $2 \text{ mg}\cdot\text{L}^{-1}$ of kinetin resulted in assorted colors from pale green and yellowish green to green. The green color of the callus of the ant nest plant was because of the chlorophyll content. The chlorophyll was formed in the callus due to the addition of the growth regulator 2,4-D and kinetin. Kinetin from the cytokinin group is involved in the chlorophyll formation in the callus along with the existence of light. This finding was in line with previous research by Leupin et al. (2000), which reported that the color change in callus to green was because of chlorophyll formation.

Meanwhile, the yellow to yellowish green callus (Table 1) was found because of the kinetin concentrations added to the media, which had a lower concentration than 2,4-D. Kinetin as a cytokinin stimulated chlorophyll formation, whereas auxin can be an inhibitor. According to George and Sherrington (1984), the decrease in the formation of chlorophyll with 2,4-D was found in the culture of peas and potatoes. In addition, the yellow color callus was also caused by the chlorophyll degradation process, lack of kinetin, and low kinetin concentration. In addition, kinetin plays a role in the formation of chlorophyll, causing the green color to appear.

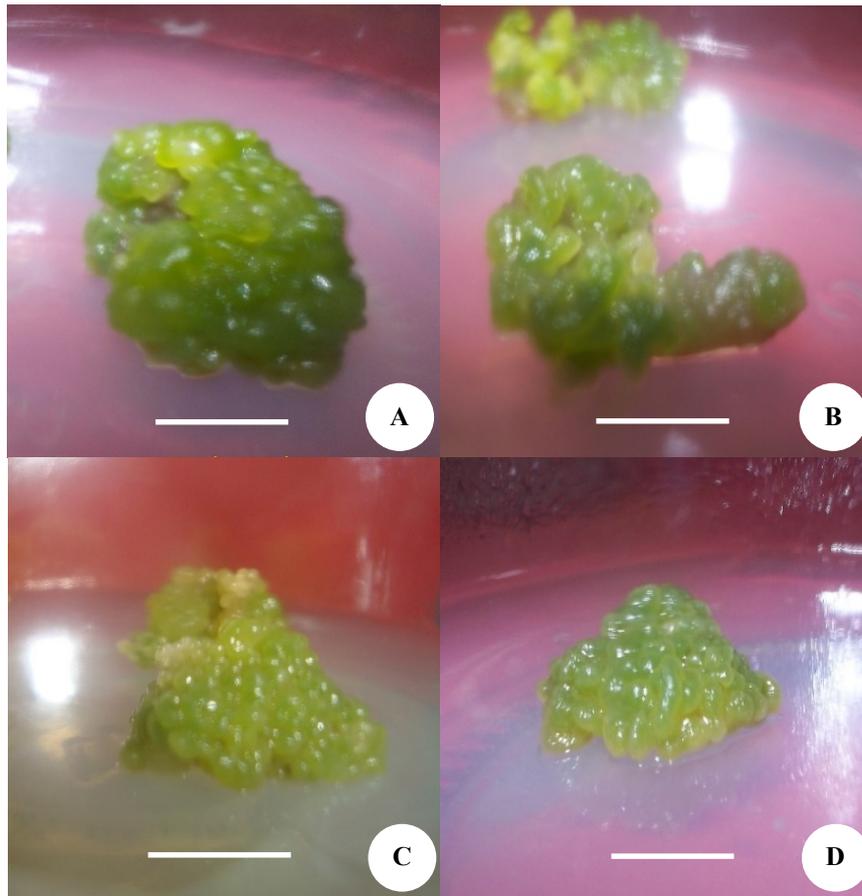


Figure 2. Optimum calli at a concentration of $2 \text{ mg}\cdot\text{L}^{-1}$ of 2.4-D and $2 \text{ mg}\cdot\text{L}^{-1}$ of kinetin at 10 weeks after planting in explant. A. Cotyledon, B. Stem, C. Tuber, D. Root. Bar = 1 cm

Callus texture

Table 1 shows friable callus was found in calli with all treatments. The friable callus was formed through the growth of cells at a small size and loose cell interaction that was affected by the occurrence of auxin. Previous researchers reported that 2.4-D stimulated cell elongation by increasing of plasticity of the cell wall to become loose, causing water to easily flow to the inner cell by osmosis, causing the cell to become elongated (Robbiani et al. 2010). Thus, friable calli contain much water because the wall cell has not reached lignification yet, and the group of cells can be easily separated from the others. The callus texture from the explant can be distinguished as friable and non-friable. The non-friable callus has compact and tight cells that are difficult to separate. In contrast, a friable callus from an explant has loose cell interaction that is easily detached using tweezers.

The addition of growth regulator with a combination of 2.4-D and kinetin on various explants formed a friable texture in the callus (Figure 2). The friable callus was also obtained in a study conducted by Sari and Kusuma (2015), which reported that the friable callus was also obtained on ant nest cotyledons planted in solid and liquid MS media.

In line with Chakraborty et al. (2013), the MS medium with 2.4-D ($0.5 \text{ mg}\cdot\text{L}^{-1}$) and kinetin ($0.2 \text{ mg}\cdot\text{L}^{-1}$) resulted in the best explant *Withania somnifera* (L.) for callusing with soft, friable, and greenish white features. The addition of growth regulators in the medium caused the callus cells to be active in cell division and cell enlargement, raised the osmotic pressure, and increased protein synthesis.

The current visual results indicated that the appearance of the callus with only the 2.4-D and with the 2.4-D and kinetin combination produced a friable callus and formed nodules (globular). This indicated that the callus can be further grown into shoots or plantlets. Purnamaningsih (2016) stated that the callus structure usually describes regeneration potency to form buds and roots. The friable callus has a higher ability to form buds than the compact callus. In this case, the growth regulator increased callus regeneration. In addition, the growth of the callus was also greatly affected by the nutrient balance.

Sucrose treatment

The weight gain of the callus was significantly affected by different concentrations of sucrose. The average weight gain of the callus is shown in Table 2.

Table 2. Callus morphology and average of weights of the callus of the ant nest plant (*Myrmecodia tuberosa*) treated with different concentrations of sucrose for 8 weeks

Sucrose (g)	Callus morphology		Mean weight of callus (g)
	Color	Texture	
30	Green	Friable	2.12 ± 0.40 ^d
60	Green-yellowish green	Friable	1.60 ± 0.18 ^c
90	Yellowish green	Friable	1.15 ± 0.28 ^b
120	Yellowish green	Friable	0.68 ± 0.69 ^a

Note: Different letters (a, b, c) indicate significantly different means for different treatments at $p < 0.05$ using Tukey's test

Callus morphology

Callogenesis is the initial response, characterized by the formation of the callus, which starts from the edge of the explant (wounded part) at the top and bottom that has direct contact with the medium. The callus is formed faster on the part that has direct contact with the media. This is probably related to the process of nutrient uptake in the medium by the explant. The appearance of the callus on the wounded part might be caused by the excitement of the tissue on the explant to cover the wound. George and Sherrington (1984) stated that the cell division that leads to the callus formation occurs from the injuries and both the natural and artificial hormone supply from the outside into the explant.

Light is an external factor that influences callus formation. The color change that exists in the callus was because of pigment, nutrients, and environmental factors, such as light (Evans et al. 2003). George and Sherrington (1984) stated that white light could induce callus formation and organogenesis in the plant tissue. A callus that has yellowish green and green color was formed with the addition of kinetin. The green color was because of chlorophyll, resulting from the 2.4 D interaction with kinetin, mainly because kinetin (cytokinin) has a function in the formation of chlorophyll in the callus and due to environmental factors, such as exposure to light. Leupin et al. (2000) claimed that the color change in the callus from white to green was due to chlorophyll formation.

Callus growth

The largest mean (2.12 g) of callus weight of the ant nest plant was found in sucrose-treated medium at 30 g L⁻¹ (Table 2). However, the sucrose-treated medium leads to reduced callus weight. In the treatment of 60 g L⁻¹, sucrose decreased the weight of the callus to 1.60 g. This was due to the increasing concentration of medium that inhibited the absorption of water and minerals. These inhibitory effects were found in the 90 and 120 g L⁻¹ sucrose-treated medium, resulting in a mean weight of callus of 1.15 and 0.68 g. Based on the current results, it can be estimated that the sucrose-treated medium above 30 g L⁻¹ can inhibit the growth of the *M. tuberosa* callus. Lindsey and Yeoman (1983) stated that the inhibition of growth in cultures that

produced secondary metabolites is probably due to a competition between primary metabolism and secondary metabolism for the same substance. The *M. tuberosa* plant is a medicinal plant for which the callus formation in the tissue culture also has the same secondary metabolite content with the original plant in nature.

The current results found that the callus color ranged from green to yellowish green. The callus color indicates the presence of chlorophyll in the tissues. The green callus was present in MS-treated medium with a sucrose concentration of 30 and 60 g L⁻¹. The greener color callus indicates more chlorophyll content, which can support the growth of the callus. The yellowish green callus was also found in the sucrose treatment with different concentrations: 90 and 120 g L⁻¹. The resulting color variation was caused by the difference in sucrose concentration in each treatment. This finding is in agreement with that of George and Sherrington (1984) in which sucrose in the tissue culture media can inhibit chlorophyll synthesis with varying levels of inhibition, depending on the tissue and plant species. The accumulation of sucrose in cells can also inhibit the process of photosynthesis because of the accumulation of sucrose in the cell, causing the demand of sugar in the cell to be fulfilled. As a result, the cells inhibit photosynthesis and the formation of chlorophyll.

Growth in tissue culture can be characterized by the increase of the wet weight of the callus. Physiologically, the weight of the wet callus consists of water and carbohydrates that have a relationship with sucrose in the culture medium as a carbon source and osmotic regulator, which is critical for embryoid and callus formation (Last and Brettell 1990). Sucrose can be rapidly hydrolyzed to form glucose and fructose, increasing the osmolality of the medium. In this study, the sucrose-treated medium entered the plant cell through diffusion and osmosis processes. Shahnewaz and Bari (2004) revealed that the effect of sucrose concentration influences the callus induction frequency that might be due to its contribution to the osmotic potential of the medium instead of its utilization as a carbon source.

In cell metabolism, glucose and fructose enter into the glycolysis and Krebs cycle to form ATP to be used for callus growth. Perry et al. (1987) revealed the change in solute content (e.g. carbon source) could cause various change in turgor and osmotic turgor. In tissue culture, turgor pressure might cause elongation and magnification of callus cells. Furthermore, turgor pressure might be different in each cell, and the cell growth response to the addition of carbohydrates also varies for each species.

Phytochemical test

The phytochemical test results of the callus of the ant nest plant with different concentrations of sucrose are shown in Table 3.

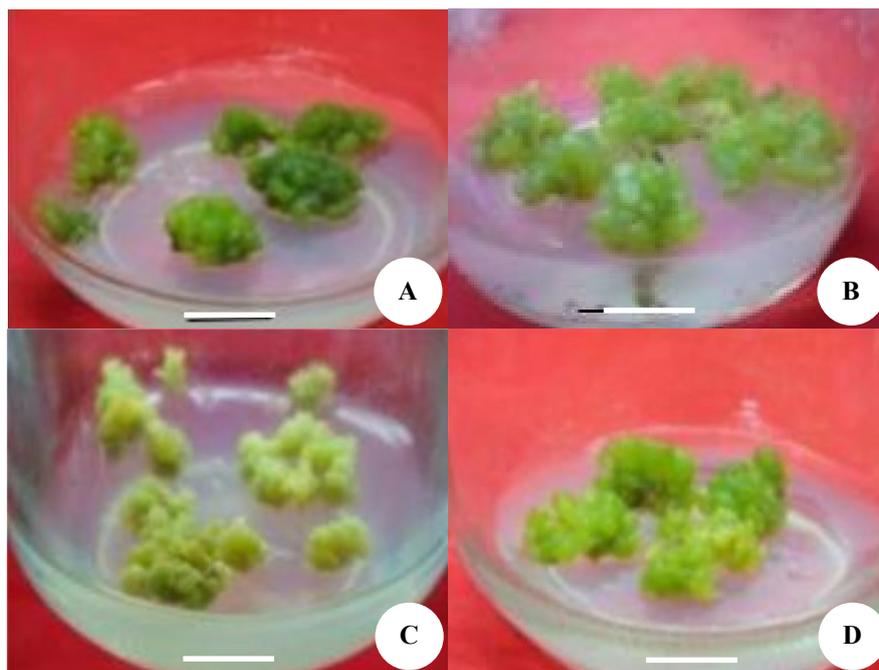


Figure 3. Callus of the ant nest plant (*Myrmecodia tuberosa*) with different concentrations of sucrose. A. 30 g·L⁻¹, B. 60 g·L⁻¹, C. 90 g·L⁻¹, D. 120 g·L⁻¹ for 8 weeks. Bar = 1 cm

Table 3. Phytochemical screening test of the ant nest plant callus with different sucrose concentrations

Sucrose (g)	Flavonoid	Phenol	Alkaloid	Saponin	Steroid	Triterpenoid
30	++	++	++	++	++	-
60	+	+	+	+	+	-
90	+	+	+	+	+	-
120	+	+	+	+	+	-

The phytochemical test on the ant nest plant calli treated with different sucrose concentrations found that metabolite content was positive from each treatment, which contained flavonoids, phenols, alkaloids, and steroids, whereas a negative result was found for triterpenoid. The best sucrose treatment was obtained with the addition of 30 g, resulting in a more concentrated test color than the others. There was no change in metabolite content between the ant nest plant callus from the tissue culture and from nature. Sari et al. (2017) stated that all parts of the ant nest plant (tuber, stem, and leaves) from nature contained phenols, flavonoids, saponins, and alkaloids but not triterpenoid. According to Fowler (1983), the tissue culture method can be used to produce chemical compounds that are usually derived from the native plant as well as new compounds synthesis, which is not produced by the plant. The secondary metabolites synthesized from the explant with the tissue culture depend on the culture condition. The proper culture produces secondary metabolites that are similar to those of the native plant. Based on the utilization aspect, ant nest plants have various chemical compounds. The chemical compounds from this plant might have a role in the activity of pathogenic resistance, allelopathy, and

body defense against pest attacks. The compounds are utilized by the plants as a self-defense system, while for humans, they are used as an active ingredient in medicine. According to Subroto and Saputro (2008), ant nest plants are rich in tocopherol antioxidants (vitamin E) and some important minerals (calcium) for the body.

Koes et al. (2005) stated that flavonoids can be synthesized in all parts of the plant that have a pivotal role in providing color, fragrance, and taste to the fruits, flowers, and seeds, which makes them attractants for insects, birds, or mammals and aids in pollen or seed transmission. In addition, Blount et al. (1992) stated that flavonoids that are very important in plant resistance against pathogenic bacteria and fungi also have antipathogenic properties that can be non-specific and result, in part, from their antioxidative properties. Moreover, Beckman (2000) added that flavonoids affect the tightening of the plant structures and tissues by stimulating auxin (IAA) activity, which promotes the differentiation of tissues and promotes of callus and tylose formation and the closure of the vascular system to protect against pathogen infection.

Subroto and Saputro (2008) claimed that the function of flavonoids as an antiviral, including for the HIV virus (AIDS) and herpes virus, has been widely published. Moreover, flavonoids are also reported to play a pivotal role in the prevention and treatment of several diseases, such as cancer, asthma, cataracts, diabetes, gout, rheumatism, haemorrhoids, and periodontitis (inflammation of the connective tissue of the tooth root), protecting the cell structure, having a synergistic relationship with vitamin C, and are anti-inflammatory and prevent bone loss.

Flavonoids and phenolic acids are major groups of secondary metabolites and bioactive compounds in plants (Kim et al. 2003). Both have a function as reducing agents, free radical scavengers, and quenchers of singlet oxygen formation. In addition, flavonoids and phenolic acid components play important roles in the control of cancer and other human diseases (Ghasemzadeh and Ghasemzadeh 2011). Meanwhile, phenolic compounds that are important for plant growth and reproduction are formed as a response to environmental factors, for example, light and chilling, pollution, etc.) and to defend wounded plants (Kefeli et al. 2003).

Besides flavonoids and phenols, alkaloids have certain pharmacological activity. Some of them are toxic to humans, but many also can be used in the medicinal field, such as to raise blood pressure, reduce pain, and fight microbial infections. In addition, alkaloids for plants, among others, function as toxic substances to fight insects or plant-eating animals (Subroto and Saputro 2008).

Another secondary metabolite, namely, saponin, a glycoside of triterpenes and steroids, is important for pharmaceuticals. Moreover, the underexplored biodiversity of plant saponins is likely to prove to be a vital resource for future drug discovery. Saponin is also one of the most numerous and diverse groups of natural plant products that serve a range of ecological roles, including plant defense mechanisms against disease and herbivores and possibly as allelopathy agents in competitive interactions between plants (Makkar et al. 2007).

In summary, this study indicates that $2 \text{ mg}\cdot\text{L}^{-1}$ of 2.4-D and $2 \text{ mg}\cdot\text{L}^{-1}$ of kinetin can potentially be used to obtain the best callus from the cotyledon explant source. The study has also shown that alkaloid, flavonoid, phenol, saponin, and steroid are found in the secondary metabolites of the ant nest plant callus. Further research needs to be conducted to examine antioxidant profiles, antimicrobial properties and toxicity level of ant nest plant callus extract.

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