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The effect of sucrose concentration on growth and induction of somatic embryogenesis in *Catharanthus roseus* callus

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Abstract. *Herwinaldo DC, Solichatun, Anggarwulan E. 2018. The effect of sucrose concentration on growth and induction of somatic embryogenesis in* Catharanthus roseus *callus. Cell Biol Dev 2: 69-77.* The purpose of this study was to determine how sucrose concentration affected the growth and induction of somatic embryogenesis in Madagascar periwinkle callus cultures (*Catharanthus roseus* (L.) G.Don). This study employed a completely randomized design (CRD) with one treatment factor, namely the addition of sucrose to MS media, and five treatment levels, including 0 g/L, 10 g/L, 20 g/L, 30 g/L, and 40 g/L, each with five replicates. Data was collected in the form of qualitative data, such as the presence or absence of somatic embryos and callus color and texture, as well as quantitative data, such as callus fresh and dry weight. ANOVA was used in quantitative data analysis, followed by the DMRT test at the 5% level. Somatic embryos were not found in all treatments, according to the findings. Variations in sucrose concentration in callus culture significantly affected dry callus weight but not callus fresh weight. The higher the sucrose concentration, the greater the callus's dry weight.

Keywords: Callus, *Catharanthus roseus*, somatic embryogenesis, sucrose

INTRODUCTION

A quarter of all modern medicines are derived from active ingredients isolated and developed from plants. The problem is determining how to maintain the production level of these herbal medicines in the face of limited raw materials for herbal medicines because the majority of raw materials for herbal medicines are derived from the parent plant (Radji 2005).

Plants are totipotent, meaning they can reproduce not only from egg or sperm cells but also from root cells, leaves, stems, and other plant cells. The entire plant body can be regenerated if we use a plant cell. The tissue culture method allows for the indefinite propagation of plant clones. Tissue culture plant regeneration has been shown to produce the same chemicals as the parent plant (Radji 2005). When compared to traditional methods, plant tissue culture has several advantages. These benefits include: (i) bioactive compounds can be formed under controlled conditions and in a relatively short period with tissue culture technology; (ii) culture is free of microbial contamination; (iii) each cell can be propagated to produce certain secondary metabolites; controlled cell growth and metabolic processes can be regulated rationally; (iv) tissue culture is not dependent on environmental conditions such as geography, climate, and seasons; and (v) secondary metabolites can be produced continuously, (vi) does not require a large area (Fowler 1983; Sudirga 2002).

Organogenesis and somatic embryogenesis can be used to multiply culture in tissue culture. Somatic embryogenesis is the process by which somatic cells (haploid or diploid) develop to form new plants without gamete fusion during specific stages of embryonic development. Somatic embryogenesis has received much attention because the number of propagules is limitless and can be obtained in a shorter period. It can help plant breeding programs through genetic engineering (Purnamaningsih 2002).

Secondary metabolites can be produced in vitro using the tissue culture method. Plant cell suspension culture is considered an alternative method for obtaining secondary metabolites on a large scale, particularly alkaloids for pharmaceutical purposes (Radji 2005). Many important compounds are not synthesized in significant amounts in cell culture, according to Croteau et al. (2000), due to the expression of genes encoding alkaloid biosynthesis specific to specific tissues. According to Zhao et al. (2001), the ajmalicine content of compact callus suspension culture is higher than that of crumb callus because compact callus has a higher degree of cellular and tissue differentiation. Somatic embryos, like embryos derived from zygotes, have the potential to produce secondary metabolites because they have high cell differentiation capabilities.

Catharanthus roseus (L.) G.Don (Madagascar periwinkle) is a synonym for *Lochnera Rosea* Reichenb. Ex Endl. and the basionym *Vinca rosea* L., both of which are members of the Apocynaceae family. Madagascar periwinkle is used not only for traditional medicine but also by modern medical experts due to its substance content (Suryowinoto 1997). This plant is significant because it contains two antitumor alkaloid compounds, vinblastine, and vincristine, found in the leaves, and ajmalicine, found in the roots (El-Sayed and Verpoorte 2004). The *C. roseus* produces very complex indole alkaloids. Important

compounds like vinblastine and vincristine only produce a very small proportion (about 0.00025 percent of dry weight), making extraction and purification difficult and expensive (Hopkins 1999).

Environmental stress (Sukarman et al. 2000), as well as organogenic tissue culture methods (without going through embryogenesis) through acidification and addition of tryptophan (Pitoyo 2002), the addition of elicitor (Fitriyani et al. 1999), root transformation (Ciau-Uitz et al. 1994), and suspension culture with airlift bioreactor, have all been used to increase the content of secondary metabolites, particularly ajmalicine, in *C. roseus* (Esyanti and Muspiah 2006). The somatic embryogenesis method is rarely used in *C. roseus*, including efforts to increase its secondary metabolites (Junaid et al. 2007). According to Junaid et al. (2006), the formed somatic embryos have the potential to be used as raw materials for genetically modifying embryonic precursor cells to increase alkaloid content.

The effect of growth regulators on somatic embryogenesis in *C. roseus* callus and suspension cultures has been studied (Junaid et al. 2007), but the effect of sucrose variations on media has not been studied yet. The optimal sucrose concentration for somatic embryogenesis in other species has been determined, such as peanut (*Arachis hypogea* L.) by Srilestari (2005), which is 40 g/l, and neem (*Azadirachta indica* A. Juss) by Shrikhande et al. (1993). Sucrose is important in the process of somatic embryogenesis because it serves two functions: it provides energy and regulates osmotic concentration. The purpose of this research is to find out how sucrose concentration affects the initiation of somatic embryos.

The objectives of this study were as follows: (i) To investigate the effect of variations in sucrose concentration in MS media on the growth of callus culture of *C. roseus*. (ii) To investigate the effect of sucrose concentration variations in MS media on the induction of somatic embryogenesis in *C. roseus* callus culture.

MATERIALS AND METHODS

Research time and place

This study was carried out for six months in the Biology Sub-Lab, Central Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia.

Materials

Explant source plant material

The plant parts used as a source of explants were several pieces of hypocotyl derived from the seeds of the tapak dara *C. roseus* var. roseus (purple flower) that germinates. The seeds obtained were from the *C. roseus* plant grown in a greenhouse. The chemicals used include Murashige-Skoog (MS) media base.

Experimental design

The experimental design carried out in this study was a completely randomized design (CRD) with one treatment factor, namely the addition of sucrose with five concentration levels 0 g/L, 10 g/L, 20 g/L, 30 g/L, and 40 g/L) with five replicates.

Tool sterilization

The culture bottles and tools were washed with detergent, rinsed with water, and dried. After drying, aluminum foil was wrapped around the culture bottles and tube-shaped tools, while the paper was wrapped around the other tools (Petri dishes, scalpels, tweezers, spatulas, and pipettes). All tools and culture bottles were sterilized in an autoclave for one hour at 121°C and 1.5 atm.

Stock solution preparation

The MS media stock chemicals were weighed and then dissolved in 50 mL of distilled water in a beaker while stirring with a magnetic stirrer. Following the dissolution of the material, the volume is set to 100 mL, and the solution is placed in a stock bottle and labeled. After dissolving the Na_2EDTA solution, only the crushed Fe_2SO_4 was added to make a FeEDTA solution. Once the chemical has been dissolved, the volume is set to 100 mL and placed in a stock bottle before being labeled. All bottles containing stock solution are wrapped in aluminum foil and refrigerated.

Media preparation

Callus initiation media

The refrigerator is raided for stock solutions. A one-liter beaker is placed on a hot plate, and one-third of it is filled with distilled water. Afterward, each stock solution was added by the MS medium composition listed in Appendix 1. In a beaker, sucrose was added and stirred until completely dissolved. The distilled water is added until the ¾ beaker's capacity is reached. A pH meter is used to determine the acidity (pH) of the solution. If the pH of the solution does not reach the desired range of 5.6-5.8, it is adjusted by adding HCl if it is too high or KOH if it is too low. After the pH was optimized, up to 1 mg of a growth regulator (ZPT) in the form of 2,4-D (2,4-Diclorophenoxyacetic acid) was added to the solution. The media solution was stirred until all ingredients were dissolved, then agar and distilled water were added until a total volume of one liter was reached. The media is heated to boiling before being transferred to hot culture bottles. The culture bottles containing the media were then wrapped in aluminum foil and sterilized in an autoclave for 15 minutes at 121°C and 1.5 atm pressure (Junaid et al. 2007).

Treatment media

The preparation of the treatment media was almost the same as the callus initiation medium, but the PGR 2,4-D was replaced with NAA 1 mg/L and BA 1.5 mg/L (Junaid et al. 2007). Sucrose was added to the treatment medium according to the predetermined concentration.

Induction of callus formation

Explant sterilization

Surface sterilization of *C. roseus* seeds was accomplished by first washing the seeds with running

water, then immersing them in a 0.5 percent mercury chloride solution for 2 minutes, followed by another wash with distilled water.

In vitro seed germination

The sterile seeds were placed in a culture bottle with sterile tissue moistened with sterile distilled water. The bottle was then tightly wrapped in aluminum foil and kept in the dark place. In a laminar airflow, the germinated hypocotyl was removed from the culture bottle and cut into several pieces (Junaid et al. 2007).

Initiation of callus from hypocotyl

The hypocotyl was placed aseptically in a laminar airflow cabinet in a culture bottle containing MS medium containing 1 mg/L of 2,4-D. The culture bottles were then tightly sealed with aluminum foil and placed on a culture rack to incubate. Culture racks are sprayed with 70% alcohol at least once every three days to prevent contamination.

Callus planting on treatment media

Callus was transferred to the treatment medium aseptically in a laminar airflow cabinet using sterile tweezers from the callus initiation media. After inserting the callus, the culture bottles were tightly sealed with aluminum foil and incubated on the culture rack for one week. The callus formed after one week of planting is harvested.

Qualitative and quantitative tests on callus

Callus qualitative testing was performed, among other things, by observing callus morphology to determine whether embryogenic or non-embryogenic callus were formed. The callus' shape, color, and texture were all observed. During the incubation period, it was also observed on what day the embryogenic callus appeared. Weighing the overall weight of the callus, both wet and dry weights were used for quantitative testing.

The initial wet callus and the final callus fresh weight were weighed to determine the wet callus weight. The fresh weight of embryogenic callus was calculated by weighing the callus, culture bottles, media, and aluminum foil. The increase in callus fresh callus was calculated as the difference between the initial and final callus fresh weight. The callus dry weight was calculated by measuring the callus weight after it had been dried in a 50°C oven. Weight is measured every 24 hours until a constant weight is obtained.

Data analysis

The data obtained are both qualitative and quantitative. Qualitative data in the form of morphological data, such as callus color and texture, as well as the presence or absence of somatic embryos. Wet and dry callus weights were measured quantitatively. The qualitative data were analyzed descriptively, while the quantitative data were statistically analyzed using the ANOVA test and then the DMRT test at the 5% level.

RESULTS AND DISCUSSION

Callus growth on initiation media

The hypocotyl *C. roseus* explant was grown under aseptic conditions. Murashige-Skoog (MS) media was used because it contains a high mineral salt content and is widely used in in vitro culture (Lestari and Purwaningsih 1996). MS media was used as the initiation medium, along with an auxin hormone in the form of 2,4-dichlorophenoxyacetic acid (2,4-D).

After a week of being planted on initiation media, the callus on explants began to form. Because the cut end of the hypocotyl is in direct contact with the media and contains a transport bundle, callus forms. According to Gunawan (1998), cell autolysis causes callus formation on injured explants, and compounds produced by these damaged cells stimulate cell division in the next layer.

Because callus is a meristematic wound covering tissue, it will form from the wound of the explant or the edge of the explant slice, according to Suryowinoto (1996). This is also possible because there is one type of plant response to tissue or cell injury. According to Leon et al. (2001), injury to plant tissue or cells activates plant self-defense mechanisms both locally and systemically (in uninjured tissue) by changing the direction of metabolic pathways and inducing the expression of specific genes, but only in damaged tissues. Which will form an irregular cell structure, dedifferentiate, release stored compounds, and lose a significant amount of water. A callus will form from this irregular cell structure. The callus is an undifferentiated mass of cells.

Because callus histologically originates from the division of many parenchyma cells around the transport bundle except the xylem, it can appear around the carrier bundle. The callus that forms on the hypocotyl incision is caused by faster explants peripheral cells dividing faster due to increased oxygen availability, CO_2 release, and the presence of light (Gunawan 1988).

The use of 2,4-D hormone aids in the formation of callus on explants. According to Agustina (2003), 2,4-D can increase osmotic pressure, protein synthesis, cell wall permeability to water, and soften cell walls, followed by a decrease in cell wall pressure that allows water to enter the cell, resulting in a decrease in cell wall pressure. Over time, cell volume increases, and the callus expands. Protein synthesis stimulated by 2,4-D will also promote growth. Because it grows and absorbs water from its surroundings, the callus expands in size over time.

The formed callus was then subcultured several times in the same media composition to obtain a sufficient number of the callus to be subcultured into the treatment medium. Callus that has grown on a medium must be moved regularly within a certain time frame. A long culture period in fixed media depletes nutrients and water. Running out of water can happen not only because of growth but also because the media occasionally evaporates water. In addition to running out of nutrients, the cells in the callus secrete compounds from metabolism that inhibit callus growth, so the resulting callus must be subcultured to maintain life and continuous propagation (Gunawan 1988).

Because new media keeps the callus in an exponential phase, subculture also serves to multiply it.

Growth and development of callus on treatment media

Morphology (color and texture) of callus on treatment media

The initiated callus was then transferred to the treatment medium, which was MS media supplemented with NAA and BA hormones. The color and texture of the callus were observed after a week. Table 1 displays callus color and texture data, while Figure 1 displays callus morphology photos.

Some calluses were white when they were first initiated, some that turned yellowish, and some that had browning to dark brown. Light yellow callus develops when the callus begins in an area exposed to light, causing the callus to become pigmented (yellow) (Sudirga 2002). Callus that is still white and yellow or has not browned is thought to have not experienced senescence or aging. This is most likely due to the BA content of the treatment media, which slows the aging process. Wattimena (1998) describes BA as a cytokinin that slows the process of cell senescence by inhibiting the overhaul of chlorophyll grains and proteins in cells.

Table 1. Color and texture of *C. roseus* callus on treatment media

Treatment media	Callus morphology	
	Color	Texture
A1	Dark brown	Compact
A2	Brownish-yellow	Compact
A3	Brown	Compact
A4	Yellow	Compact
A5	Yellow	Compact
B1	Dark brown	Compact
B2	Clear white	Compact
B3	Yellow	Compact
B4	Clear yellow	Compact
B5	Brown	Compact
C1	Dark brown	Crumb
C2	Clear yellow	Crumb
C3	Clear yellow	Compact
C4	Clear yellow	Compact
C5	Brownish-yellow	Compact
D1	Cloudy yellow	Crumb
D2	Yellowish white, slightly brown	Crumb
D3	Yellow	Crumb
D4	Yellow	Crumb
D5	Brown	Crumb
E1	Yellowish white	Crumb
E2	Yellowish white	Crumb
E3	Yellow	Crumb
E4	Yellowish white	Crumb
E5	Yellowish white	Crumb

Notes: A1, A2, A3, A4, A5: callus on media with the addition of sucrose of 0 g/L repetitions of 1, 2, 3, 4, and 5; B1, B2, B3, B4, B5: callus on media with the addition of sucrose of 10 g/L repetitions of 1, 2, 3, 4, and 5; C1, C2, C3, C4, C5: callus on media with the addition of sucrose of 20 g/L repetitions of 1, 2, 3, 4, and 5; D1, D2, D3, D4, D5: callus on media with the addition of sucrose of 30 g/L repetitions of 1, 2, 3, 4, and 5; E1, E2, E3, E4, E5: callus on media with the addition of sucrose of 40 g/L repetitions of 1, 2, 3, 4, and 5

The brown color of the callus may be due to natural aging symptoms and the production of phenolic compounds in response to tissue injury caused by cutting. Some phenolic compounds are auxin protectors and antioxidants that act as inhibitors of the peroxidase enzyme-catalyzed IAA oxidation process. Generally, phenolics are extremely labile and easily oxidized to phytotoxic compounds (Debergh and Zimmerman 1991). This can be avoided by introducing activated charcoal into the media (Dodds and Roberts 1995).

Figure 1 shows that brownish callus was still obtained in the sucrose treatments of 0 g/L to 30 g/L, while callus grown at a sucrose concentration of 40 g/L was still fresh. The difference in callus color is caused by the age of the hypocotyl used. Callus that has browned is most likely the result of hypocotyl fragments near the root (older age), whereas callus that is still young is the result of hypocotyl fragments near the shoot's tip (younger age).

Observation revealed that some callus textures were compact while others were crumbly. A compact callus has a tight, dense, and difficult to separate cell structure, larger vacuoles, and a thicker polysaccharide wall. This large vacuole allows the callus to store water in the cell, increasing its water content. According to Zhao et al. (2001), compact callus is formed by NAA, which does not induce the synthesis of cellulase and pectinase enzymes, which have lytic activity on the middle lamella and provide a compact structure so that the bonds between cells are not tenuous.

Callus crumbs have a loose arrangement of easily separated cells that are meristematic and actively dividing (Street 1993). Steves and Sussex (1994) mentioned that cells with crumb textures are irregular and relatively small in size, with a large nucleus and thick cytoplasm. The presence of endogenous auxin hormone, produced internally by explants that have grown to form the callus, also stimulates the formation of callus with a crumb texture.

A somatic embryo in the callus

For a week, the callus was examined every day to determine the presence or absence of somatic embryos. There was no embryogenic callus in any of the treatments. Somatic embryos can be formed directly in the mesophyll of endogenous explants, according to Trisnawati et al.'s (1999) research on Allium sativum L, but they can be degraded back into a callus. Only somatic embryos (exogenous) formed on the callus surface could be observed.

The type of explant, the developmental stage, and the interaction between the explant and the growth medium are the parameters that determine the success of somatic embryogenesis (Debergh and Zimmerman 1991). Different types of explants require different times in the auxin-containing induction medium of somatic embryos. This is because the role of exogenous auxin in the induction of somatic embryos depends on the type of explants used in the study. Explants derived from petioles, hypocotyls, and cells isolated from suspension cultures in carrot (*Daucus carota* L.) required 1, 2, and 7 days of auxin administration

in the initiation medium before becoming competent to undergo incubation embryogenesis after being transferred to auxin-free media (Zimmerman 1993). This study found no somatic embryos, possibly because the callus had been in the initiation medium for too long. Initially, the goal of initiating callus in a medium containing 2,4-D for a long enough period was to obtain enough callus to subculture in the treatment medium. Still, this step was ineffective in growing somatic embryos. Junaid et al. (2007) discovered that the effective time for *C. roseus* hypocotyl callus culture in the initiation medium was approximately one month before being transferred to the treatment medium.

The explants used may differ depending on the type of explant source plant and the explant's developmental stage. Using meristematic explants generally results in a higher success rate in somatic embryo formation. Explants in the form of the axis of young and adult zygotic embryos, cotyledons, buds, epicotyl, and hypocotyl can be used (Purnamaningsih 2002). There are also differences in the hypocotyl cells' meristematic abilities. Cells near the shoot tip were younger and more meristematic than older cells near the root.

Explants will also interact well with the growth medium and produce somatic embryos if the media contains enough substances required to induce somatic embryogenesis. Interactions include nutrients in the media, particularly nitrogen sources, which are the main factor that stimulates morphogenesis in vitro culture and will also indirectly affect culture pH, carbon source (sugar), and growth regulators (Purnamaningsih 2002).

Junaid et al. (2007) found that the same treatment medium was very suitable and effective in producing embryogenic callus from hypocotyl *C. roseus* explants. When the media was applied to the same explants in this study, it produced only non-embryogenic callus. This is possible because the hypocotyl used is too old, resulting in differences in meristematic abilities compared to younger tissue and variations in the level of endogenous growth regulators in the hypocotyl used. Young tissue in the hypocotyl can certainly divide faster than adult tissue. The length of the hypocotyl indicates the age of the plant.

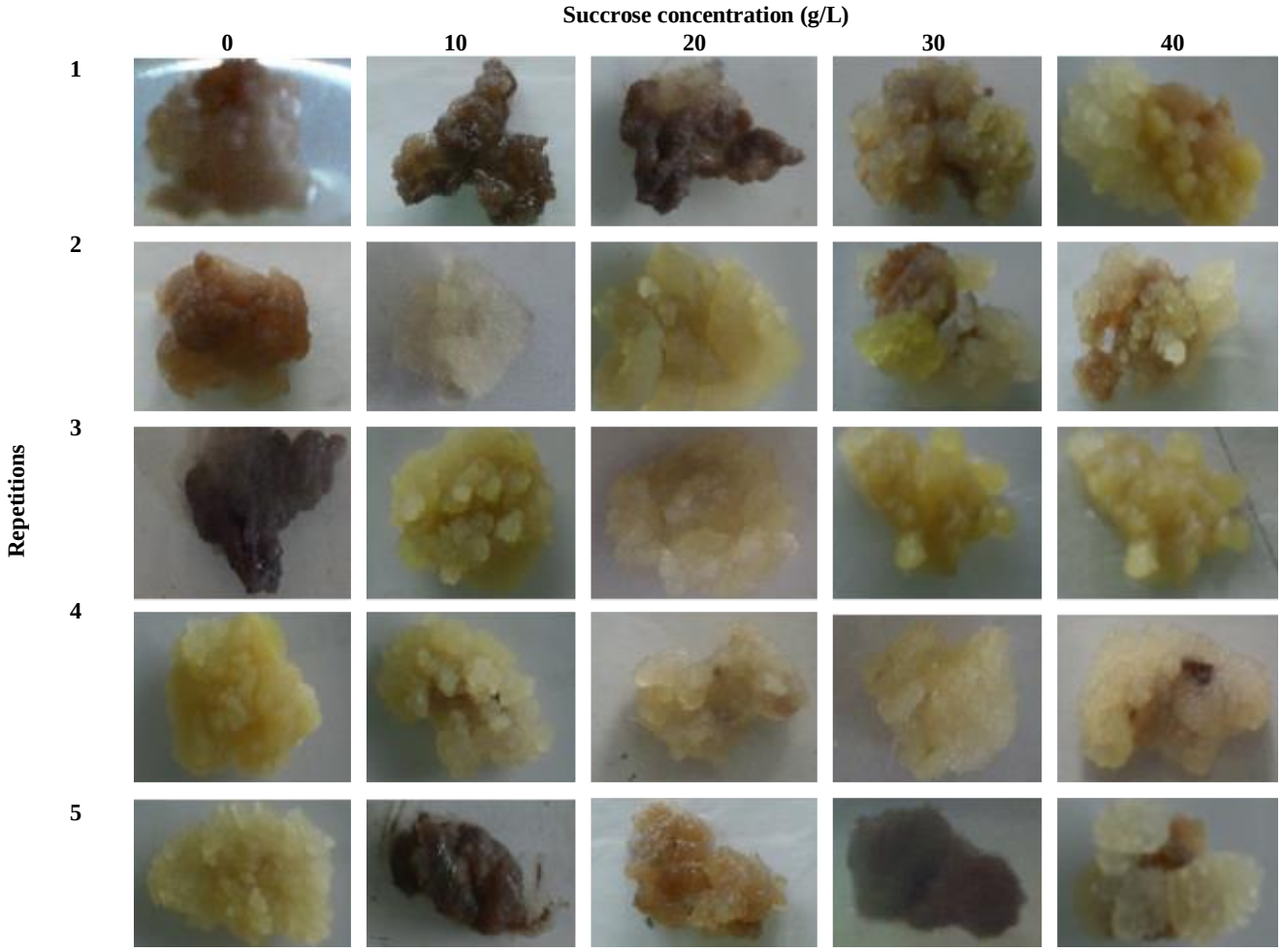

Figure 1. Displays callus morphology

When compared to other methods such as suspension culture, in vitro culture, particularly somatic embryogenesis, has the potential to produce secondary metabolites more effectively. Culture methods commonly used to produce secondary metabolites in large quantities have encountered several challenges, one of which is that important medicinal compounds such as vinblastine, catharanthine, ajmalicine, and serpentine are produced at extremely low levels in *C. roseus* cell culture. Many factors contribute to this, but the lack of cellular and tissue differentiation in cell suspension cultures is thought to be the most important (Moreno et al. 1996 in; Zhao et al. 2001). Biosynthetic products are influenced by molecular, cellular, and organ differentiation (Heble 1996). Embryogenesis is one type of organized development, according to Torres (1989). This is demonstrated by the ability of embryos formed in vitro to develop into complete plantlets through stages similar to normal embryogenesis. Because of its high level of cell differentiation, somatic embryogenesis has the potential to produce secondary metabolites, particularly drug compounds, making it more effective than other culture methods.

Callus fresh weight

The fresh weight of the plant can be used to determine the growth of cell volume and mass (Salisbury and Ross 1995). Cell elongation will occur, followed by cell enlargement and an increase in fresh weight. The increase in the fresh weight of the callus indicates that the cell growth process has occurred.

The fresh weight of the explant culture was calculated by weighing the callus, along with the bottle and aluminum foil cap, before and after subculture into the treatment medium and then calculating the difference to obtain the value of the callus's fresh weight gain. Fresh weight can be used to assess the growth of explant culture results, but it cannot be used as a standard because fresh weight is affected by cell water content, plant metabolism, and plant moisture conditions (Sitompul and Guritno 1995). Table 2 and Figure 2 display the average data for callus fresh weight gain.

Because the fresh weight of the callus in the treatment media decreased over time, all values from the average increase in fresh weight were negative. The ANOVA test results showed that the results were not significantly different. This means that the treatment of varying sucrose concentrations did not affect the increase in fresh weight of *C. roseus* callus.

Because callus fresh weight measurement is still highly dependent on callus water content, the decrease in fresh callus weight is most likely due to a decrease in callus water content. The difference in fresh weight loss is thought to be due to the tissue's ability to store water and different nutrients, specifically the ability to carry out diffusion, osmosis, and cell turgor pressure regulation (Sriyanti 2000). The accumulation of secondary metabolites that are toxic to cells, as well as the occurrence of lysis, contribute to the decrease in fresh weight (cell death).

Table 2. The average fresh weight gain of *C. roseus* callus (g) after incubation for a week on the treatment media.

Sucrose concentration (g/L)	**0**	**10**	**20**	**30**	**40**
Average fresh weight gain of callus (g)	- 0.285[a]	- 0.439[a]	- 0.615[a]	- 0.295[a]	- 0.670[a]

Note: numbers followed by the same superscript letter in the same line show no significant difference in the 5% DMRT test

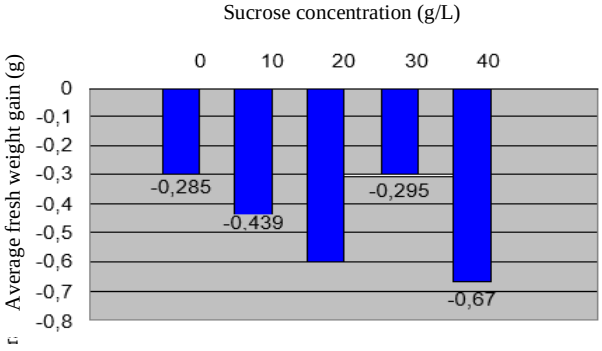

Figure 2. Average fresh weight gain of *C. roseus* callus

The decrease in fresh callus weight may also be due to the callus having endogenous auxin hormone, causing the hormone content in the media to become too high. Auxin's role is to change the osmotic pressure in the cell, which affects the biochemical processes in the cell (Wattimena et al. 1992). The high concentration of auxin reduces osmotic pressure, which results in the release of water from the cell (Palupi et al. 2004). The difference in fresh weight loss between treatments was most likely due to differences in callus endogenous auxin levels. Endogenous auxins contribute to increased cell permeability to water. Because the callus was formed from hypocotyls of varying ages, the endogenous auxin level in each callus varied. As a result, the level of cell permeability to water in each callus varies (Abidin 1990).

Dry weight

Plant products are typically expressed in terms of total dry matter rather than fresh weight. The plant's total dry weight manifests the processes and events during plant growth (Sitompul and Guritno 1995). Unlike fresh weight, dry weight provides an acceptable estimate of a culture's metabolic activity (Dodds and Roberts 1995).

According to Gardner et al. (1991), fresh weight measurements are less representative of growth parameters because the numbers fluctuate depending on the humidity conditions of the plant. Because the dry weight of the callus was measured from a constant weight, the number of measurements on the dry weight of the callus did not vary. Dry weight was obtained by baking the callus harvested at the end of the treatment until the weight was constant. According to Sitompul and Guritno (1995), drying the material aims to stop metabolic activity in the material, as

opposed to a fresh weight measurement, which is still influenced by metabolic activities such as transpiration, causing difficulties in measuring and gaining constant weight. The amount of biomass produced highly depends on the rate at which these cells divide and multiply, which can be influenced by the medium's composition (Wattimena et al. 1992).

The ANOVA statistical test on dry callus weight revealed that sucrose administration at various concentrations significantly affected callus dry weight in each treatment. Table 3 shows that the highest average callus weight was obtained with media containing 40 g/L sucrose, which was 0.037 g, significantly different from all treatments. Because there was no decrease in dry callus weight in this study, the results were not optimal.

The media used in plant tissue culture is critical to its success. Plant tissue culture media not only supply macro and micronutrients but also carbohydrates in the form of sugar. This sugar is a carbon source that plants use to replace the carbon they get from the atmosphere in the form of CO_2, which is a component of photosynthesis. Sucrose is an important carbon source used as a building block for cells, according to George and Sherrington (1984). With enough sucrose, cell division, cell enlargement, and further cell differentiation can occur normally. Sucrose provides energy and carbon for callus growth as well as cell constituents. The abundant availability of sucrose enables the occurrence of sufficient energy and essential materials for biomass growth and formation.

Sucrose serves several functions, including being a carbon and energy source, regulating osmotic pressure, a key factor in membrane stabilization, acting as a stress protector, and a signaling molecule (Tomaz et al. 2001; Lipavska and Konradova 2004). Meanwhile, Iraqi and Tremblay (2001) attribute the importance of sucrose in the media to the following factors: (i) sucrose is hydrolyzed by the enzymes invertase and sucrose synthase into hexose, namely glucose and fructose, which can be directly utilized by plants; (ii) the result of sucrose hydrolysis increases the osmotic concentration of the medium; and (iii) sucrose acts as a signal for storage protein synthesis.

The increase in dry callus weight was accompanied by an increase in sucrose concentration, as shown in Figure 3. The increase in sucrose concentration is thought to cause the formation of metabolites that promote callus division and growth. Sucrose in the media stimulates callus cell division (Suskendriyati et al. 2004).

Table 3. *Catharanthus roseus* callus dry weight (g) after incubation for a week on treatment media

Sucrose concentration (g/L)	0	10	20	30	40
Callus dry weight (g)	0.015a	0.017a	0.024ab	0.033bc	0.037c

Notes: numbers followed by the same superscript letters in the same line show no significant difference in the 5% DMRT test

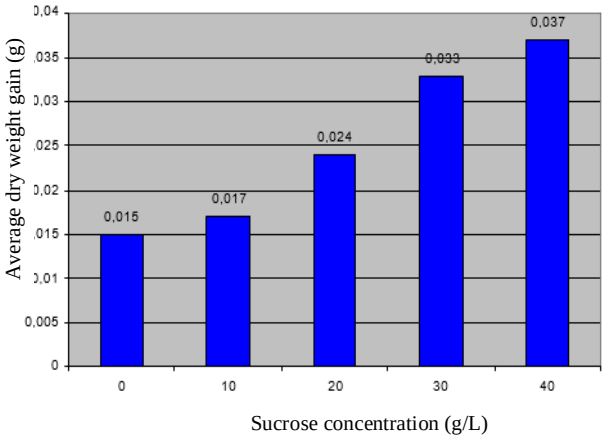

Figure 3. The average dry weight of *Catharanthus roseus* callus (g)

According to Sitompul and Guritno (1995), some sucrose transported into cells is metabolized to produce energy and carbon. Others are converted into essential materials such as cell wall materials, proteins, and other growth materials. Sucrose will be hydrolyzed into glucose and fructose in the plant body. Both are essential for cell growth. Cells will use phosphorylated glucose and fructose for further metabolism, according to Strum (1999). Glucose can enter glycolysis and the Krebs cycle to produce ATP and NADH, which are used to fuel growth. Fructose also plays an important antioxidant role in membrane stabilization (van den Endel and Vallumu 2009). In addition to producing energy, Sucrose metabolism also provides a carbon skeleton, among other things, which can be used to produce other essential products in plants, such as secondary metabolites (Salisbury and Ross 1995).

Media containing a high sucrose concentration will be more concentrated than media containing no sucrose. Because a medium with a high concentration contains many molecules, the diffusion movement is directed toward a location with a scarcity of molecules or a low concentration. This condition causes cells in explant tissue grown on media containing the highest amount of sucrose, namely 40 g/L, to receive nutrients more quickly. Furthermore, sucrose is hydrolyzed into glucose and fructose when sterilized at the proper temperature. Glucose provides energy for cells to grow and develop to form new cells. This results in the highest dry weight of 0.037 g in the treatment with the highest sucrose concentration. The lowest callus dry weight was obtained on media without sucrose, which was 0.015 g. The callus did not receive enough material to form biomass in this medium because the number of carbohydrates (sucrose) that served as a source of energy and carbon was very low, if not non-existent.

The fresh and callus dry weight measurements yielded different results in the study. The fresh weight measurement revealed a decrease in callus growth, whereas adding sucrose to the dry weight measurement increased as the sucrose concentration increased. The callus's fresh weight is still heavily influenced by its water content. The ability of a callus to absorb and store water is influenced by

its texture. According to Abidin (1990), cells in the outer layer that are in contact with the media absorb water more easily than cells in the inner layer. Because of the uneven callus texture, not all callus cells, particularly those on the inside, were able to touch the media. As a result, the callus' ability to absorb and store water differs. Callus cells with larger vacuoles store more water than callus cells with small vacuoles. In this study, a decrease in fresh weight does not imply a decrease in growth because dry weight is the more appropriate parameter to indicate an increase or decrease in growth. Only a decrease in water content, not a decrease in growth, affected the decrease in fresh weight. The callus's higher dry weight and the increasing sucrose concentration indicated that the callus was still growing. This is thought to cause the pattern difference between callus's wet and dry weight.

Based on the research, it could be concluded that: (i) Sucrose administration at various concentrations (0; 10; 20; 30; 40 g/L) had a significant effect on dry callus weight but did not affect fresh weight. The *C. roseus* callus (ii) Sucrose administration at various concentrations (0; 10; 20; 30; 40 g/L) had no effect on the formation of *C. roseus* callus embryos.

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