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# Development of Photomyxotrophic Culture Protocol of Lemongrass Through Sucrose Concentration Increase and Light Intensity Decrease

**Desy Irmawati, Enni Suwarsi Rahayu***

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang, Kampus Sekaran, Gunungpati, Semarang, Indonesia 50229

*Corresponding Author: enni_sr@mail.unnes.ac.id

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**Abstract.** Lemongrass (*Cymbopogon nardus* (L.) Randle) plantlets are interesting to be used as tissue culture-based souvenir. The success of plantlet propagation through photomyxotrophic culture is influenced by several factors, including sucrose concentration and light intensity. The research aimed to analyze the effect of sucrose concentration and light intensity on the growth of lemongrass plantlets. This experimental research used randomized block factorial design with two factors, namely sucrose concentration (0 g/l, 10 g/l, 20 g/l, 30 g/l) and light intensity (2000 lux, 3000 lux, 4000 lux, 5000 lux). The variables observed were some parameters of growth and development of plantlet. The data were analyzed with two-way Analyses of Variance and Duncan Multiple Range Test. The results showed that sucrose concentration had significant effect on the number of shoots and leaves of plantlet. The light intensity and the interaction of both factors had also significant effect on the number of shoots and leaves. The most optimal treatments were sucrose of 20 g/l and light intensity of 3000 lux. The results of this study innovate the tissue culture technique using photomyxotrophic system in order to produce sterile lemongrass plantlets in large quantities.

**Keywords:** lemongrass; photomyxotrophic cultures; sucrose; light intensity

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## INTRODUCTION

Lemongrass (*Cymbopogon nardus* (L.) Randle) is a commercial essential oil-producing plants in Indonesia. Lemongrass essential oil contains aldehyde and alcohol compounds, namely citronella, citronellol, and geraniol which are used as fragrance agents in the manufacture of soaps and perfumes (Sulawatty, 2019). Lemongrass is one of the plants that have an important value in Essential Oil House (*Rumah Atsiri*) at Tawangmangu, Karanganyar Regency, Central of Java Province. Besides being used for essential oil source, lemongrass also be used as a component of unique tissue culture- based souvenir. This souvenir becomes an educational and iconic souvenir for visitors of the Essential Oil House. Naturally, lemongrass can be propagated vegetatively through separation of clumps. However, to be used as a tissue culture-based souvenir component, sterile plantlets are needed. The sterile plantlet can only be obtained through tissue culture propagation.

Plant tissue culture is a technique for growing plant cells, tissues, or organs aseptically in an artificial medium (Gaikwad *et al.*, 2017). Based on the type of energy source, tissue culture systems can be divided into three systems, there are heterotrophic, photomyxotrophic, and photoautotrophic. Plant tissues or organs grown in heterotrophic culture provide exogenous carbohydrates as the only energy source, otherwise in the autotrophic system they provide fully endogenous carbohydrates as a source of energy. Plant tissues or organs grown in the photomyxotrophic system provide endogenous and exogenous carbohydrates as source of energy (Rahayu, 2016; Gaikwad *et al.*, 2017). Photomyxotrophic cultures have advantages to produce better morphology and anatomy of plantlets and had high prospects than heterotrophic cultures (Gago *et al*., 2014; Rahayu, 2016).

Photomyxotrophic cultures provide exogenous carbohydrates in the form of sucrose derived from the culture medium. Sucrose in the medium plays an important role as source of energy and a carbon compound for the growth and development of explants (Ruan, 2012). The sucrose affects the

regulation of growth genes, so that the absence of sucrose can inhibit plant growth (Kunz *et al*., 2014). To increase plantlet viability, dependence on exogenous sucrose needs to be decreased by reducing the sucrose content in the culture medium. The reduction of sucrose will encourage plantlets to form carbon compound endogenously so that they more adaptive in the acclimatization environment (Rahayu, 2016).

Some previous research showed that the sucrose decreasing in the medium had a positive effect on plantlet quality. Walnut plantlet grown in medium containing vermiculite and 15 g/l sucrose are lusher and the chlorophyll b and total chlorophyll significantly increased (Vahdati & Hassankhah, 2014). The combination treatment of zero or low levels of sucrose, increased ventilation and use of Indole Butyric Acid (9.8 µM) improve the quality and survival papaya plantlets (Perez *et al*., 2015). In banana rooting, the need of sucrose in culture medium decrease to 1% instead of 3% through the presence of CO2 enrichment. This treatment produced plantlets more vigorous and make them tolerate to the shock of acclimatization by enhancing photosynthesis (Emara *et al*., 2018).

Light controls plant growth and development indirectly because the light is needed for photosynthesis whose results play an important role for developing plant organs (Yuniardi, 2019). A multiscale system analysis using artificial intelligence technology suggests that the best condition for obtaining higher quality acclimatized plantlets is the combination of 2.3% sucrose and photon flux of 122–130 µmol $m^{-2}$ $s^{-1}$ (Gago *et al.,* 2014). The optimal light intensity during the date palm multiplication is 1000 lux (13.2 shoot buds per explant, 15.0% greening, 25.0% precocious rooting) (Meziani *et al*., 2016). The best shoot proliferation rates of *Haworthia* (65.57–81.01%) are achieved on media supplemented with either BA and NAA under a light intensity of 45 µmol $m^{-2}$ $s^{-1}$ or equivalent to 3300 lux (Chen *et al*., 2019).

Knowledge about the optimal sucrose concentration and light intensity is needed to increase photosynthesis capacity in photomyxotrophic cultures (Xiao *et al*., 2011). Therefore, it is necessary to conduct research to find a protocol of lemongrass micropropagation, especially the optimal combination of sucrose concentration and light intensity for the growth of plantlets. In particular, the study aimed to analyze the effect of sucrose concentration and light intensity on the growth of lemongrass plantlets. This study results are beneficial to develop an efficient and large-scale propagation of the lemongrass.

## METHODS

This research was conducted in October 2019 - November 2021 at Plant Tissue Culture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang.

### Materials and experimental design

The material plants were obtained from the Essential Oil House, Tawangmangu, Central of Java Province which were then grown at a shading house of Biology Department Universitas Negeri Semarang. The explants were stems with 3.0-3.5 cm long, 0.2-0.4 cm in diameter, and had a single node. The research was carried out experimentally by using randomized block factorial design with two factors, namely sucrose concentration and light intensity. Sucrose concentration consisted of four level, i.e. concentration of 0 g/l, 10 g/l, 20 g/l and 30 g/l, whereas light intensity consisted of four level, i.e. intensity of 2000 lux, 3000 lux, 4000 lux, and 5000 lux. The difference in light intensity was obtained by placing culture bottles under a white 17-watt light emitting diode (LED) at different distances. Each treatment combination was repeated three times.

### Media and explant preparation

The Murashige and Skoog (MS) medium supplemented with 0.5 ppm of NAA, 2 ppm of BA and sucrose according to the treatment levels were prepared by standard method. The media were sterilized at 121ºC and 1.15 psi for 20 minutes in an autoclave. The media were then kept in a sterile room for 4 days to control contamination

The explants were sterilized in a modified way from Sofian *et al.* (2018) by washing under running water, soaked in *dettol* for 15 minutes and rinsed three times with sterile water. Subsequently, they were soaked in fungicide and bactericide each for 40 minutes and rinsed three times using sterile water too. The explants were immersed in 20% sodium hypochlorite in laminar air flow for 20 minutes, soaked in 70% alcohol for 1 minute and then rinsed three times with sterile water. The sterile explants were planted in the culture medium in laminar air flow. According to the research design used, namely randomized block design, the culture bottles were placed randomly at each level of light intensity treatment. The culture bottles containing lemongrass

explants were stored in the incubation room at a temperature of 24-25 °C for 4 weeks.

**Data collection dan analyses**

The explant development was observed every day after planting. The percentage of explants growing shoots, shoot emergence time, number of shoots, number of leaves, and shoot morphology were measured in the 4th week after planting. Quantitative data were analyzed by two-way Analyses of Variance at a level of $p \leq 0.05$. Duncan Multiple Range Test (DMRT) at level of $p \leq 0.05$ was used to analyze the difference of the effect of treatments and their combinations.

## RESULTS AND DISCUSSION

**Percentage of explants growing shoots**

The data showed that most of the combinations of sucrose concentration and light intensity treatment resulted in 100% of explants growing shoots. There were only four treatment combinations grew shoots as much as 67% (Table 1).

**Table 1.** Effect of sucrose concentration and light intensity on the percentage of explant growing shoots

| Light intensity (lux) | Percentage of explants growing shoots (%) at sucrose concentration (g/l) | | | |
|---|---|---|---|---|
| | 0 | 10 | 20 | 30 |
| 2000 | 67 | 100 | 100 | 100 |
| 3000 | 100 | 100 | 100 | 67 |
| 4000 | 100 | 100 | 100 | 67 |
| 5000 | 100 | 100 | 67 | 100 |

Explant development occurs through cell division, cell expansion and cell differentiation (Taiz & Zeiger, 2017). The explant development begins from cell division in meristematic tissue. Consequently, the cells increase in number but their size is still small. Furthermore, the daughter cells enlarge through cell expansion caused by cell wall plasticity and water imbibition. When the new cell volume expands and equal to the parent cell, they will undergo differentiation to form shoots. Some cells will form prospective stems and leaves (Taiz & Zeiger, 2017). The 100% explant development can occur because the treatment of sucrose concentration and light intensity can support the process of cell division, cell elongation, and cell differentiation of explants optimally. Cell division, elongation and differentiation require considerable energy. The energy is obtained from sucrose in the culture medium. Consequently, the optimal available energy allows the explants develop better.

The light intensity determines the power or energy emitted by the light source. Light energy is needed by explants for photosynthesis. The result of photosynthesis in the form of glucose will be used for growth and development of explants in shoots development. The higher the light intensity, the more optimal the photosynthesis of explants (Singh & Patel, 2014). Glucose produced by photosynthesis is an energy source for cell division, cell differentiation, and a regulator of cell osmotic potential (Dijana *et al*., 2021). Hence, the availability of light intensity optimizes the photosynthesis of explants cells by providing energy for the growth of cultured explants.

**Shoot emergence time**

The analysis of variance showed that the concentration of sucrose had a significant effect, however the light intensity and their interactions did not have significant effect on shoot emergence time. Sucrose is a source of energy of the plant development; its concentration affects the availability of energy used for cell division, elongation and differentiation. When cell division, elongation and differentiation occur faster, it will affect the rate of shoot primordia forming (Taiz & Zeiger, 2017). Each explant requires sucrose at a certain concentration. The increase in sucrose up to a certain concentration increases the growth of explants. The light intensity did not affect the shoot emergence time because the light intensities given in this research provide relatively same energy for the photosynthesis process of lemongrass explants. The relatively same energy has a relatively equal effect on the lemongrass shoot emergence time.

The result of DMRT showed that sucrose concentration of 30 g/l was able to promote shoot emergence time of lemongrass fastest compared to others (Table 2). The sucrose at high concentrations (30 g/l) was able to accelerate the time of shoot emergence. The high sucrose

concentration increased the explant regeneration. Sitorus *et al.* (2011) explained that the media will be more concentrated because of a high concentration of sucrose. This situation causes the sucrose diffusion from medium to the cells of explant occur more quickly. As a result, the sucrose needs as a source of energy and carbon compound for the growth of explants are also met more quickly. The addition of sucrose in medium is still needed because in vitro cultured plants generally have a low rate of photosynthesis, so that the glucose resulted from photosynthesis does not meet the energy needs for explant growth (Gaikwad *et al.*, 2017).

Moreover, sucrose acts as a signal regulating meristem activity by accumulating the target of rapamycin (TOR) kinase. TOR kinase is the main sensory center that plays a role in promoting cell proliferation. The TOR kinase plays a role in phosphorylation and activation of transcription factors in the S phase of the cell cycle (Figueroa & Lunn, 2016). Therefore, explants developed in a medium without sucrose did not grow and develop optimally due to inhibition of cell division of explants. This is in line with the research of Ramirez *et al.* (2019) which concluded that the sucrose concentration of 30 g/l was able to increase in vitro shoot growth of *Bambusa vulgaris*.

**Number of Shoots and Leaves**

The results of two-way Analyses of Variance showed that sucrose concentration had significant effect on the number of shoots and leaves. Leaves develop from leaf primordia. Primordial leaves will continue to grow and increase in their cell size until they reach a certain size due to cell division and elongation. The primordial stage of leaf development begins with periclinal cell division in tunica and corpus which produces a mass of cells that protrude outward. Periclinal division followed by cell expansion will form leaf supports. Then it undergoes differentiation to form the epidermis and its derivatives, mesophyll, and leaf carrier bundles to form leaf blades. Differential activity in leaf meristems causes the formation of different leaf morphology (Raven *et al.*, 2012). The increase of leaves number was influenced by the number of nodes and the number of leaf primordia. The more nodes, the more leaves. This is because the nodes are place where the leaves attach to the stem (Kunz *et al*., 2014).

The concentration of sucrose affects the availability of energy and carbon compound for division and differentiation of explant cells. Sufficient energy will increase cell division, elongation and differentiation, and then they will develop to shoot primordia. The more primordia formed, the more the number of shoots and the number of leaves. The DMRT showed that the sucrose concentration of 10 g/l produced the highest number of shoots and leaves. This indicated that the concentration sucrose 10g/l is optimal for the growth of shoots and leaves of lemongrass (Table 2).

**Table 2.** The effect of sucrose concentration on shoot emergence time, shoots number, and leaves number of lemongrass

| Sucrose concentration (g/l) | Shoots emergence time (days) | Shoot number | Leaves number |
|---|---|---|---|
| 0 | 15.12[a] | 1.83 [c] | 7.00 [c] |
| 10 | 13.37[a] | 21.66 [a] | 98.00 [a] |
| 20 | 13.68[a] | 2.33 [c] | 3.66 [c] |
| 30 | 9.51[b] | 18.58 [b] | 82.41 [b] |

Note: The numbers followed by the same letter did not significantly different based on the results of the 0.05% DMRT

Explants grown on media without sucrose produced the least number of shoots. This result showed that endogenous carbohydrates from photosynthesis not enough to meet the needs for the growth of explants therefore explants is still required sucrose from medium to increase their growth. In addition, the availability of sucrose affects the regulation of genes required for the G1/S phase transition (Kunz *et al*., 2014). Sucrose deficiency in culture media causes explants to slow down their cell cycle or go to the G0 stage so that cell division is slow or cannot occur. Dijana *et al.* (2021) explained that the addition of sucrose concentration in the media could accelerate the growth of explants. The more energy provided, the faster cell division and differentiation so that the growth of explants also accelerates.

However, the addition of high concentrations

of sucrose can cause inhibition of culture growth due to a decrease in the osmotic potential of the medium which causes the nutrient flow to run slowly (Roostika *et al*., 2017). The results of this study are in line with the research of Triyastuti *et al.* (2018) that the sucrose concentration of 20 g/l was able to produce the highest shoots, the widest leaves, and to encourage the emergence of faster shoots in chrysanthemum plantlets.

The analysis of variance showed that intensity of light has a significant effect on the number of shoots and leaves of lemongrass. Light affects photosynthesis and morphogenesis processes. The need of light in tissue culture is adjusted to the type culture and characteristics of plant (Wang *et al.*, 2016). Plants' response to quality or quantity of light occurs on transcription of genes that are directly involved in cell wall development (Batista *et al*, 2018).

**Table 3.** The effect of light intensity on the shoots and leaves number of lemongrass

| Light intensity (lux) | Shoots number | Leaves number |
|---|---|---|
| 2000 | 10.66 [b] | 36.33 [b] |
| 3000 | 15.33 [a] | 59.33 [a] |
| 4000 | 12.91 [ab] | 41.75 [b] |
| 5000 | 5.05 [c] | 23.25 [c] |

Note: The numbers followed by the same letter mean did not significantly different based on 0.05% DMRT

The results of DMRT showed that light intensity of 3000 lux produced the highest number of shoots and number of leaves (Table 3). This result indicated that the light intensity of 3000 lux was optimal for the growth of lemongrass shoots and leaves. This study was in line with Gurav *et al.* (2019) which conclude that the highest number of shoots of *Clerodendrum serratum* L. multiplication provide at 3000 lux lighting treatment. In the same genus of lemongrass, *Cymbopogon schoenathus*, plantlet also grows well at 3000 lux lighting (Abdelsalam *et al.*, 2017). Meziani *et al.* (2016) stated that low light intensity was able to increase the growth of *Phoenix dactylifera* explants, while at high light intensity it decreased shoot proliferation.

The analysis of variance showed that the interaction of sucrose concentration and light intensity has a significant effect on the number of shoots and leaves of lemongrass. The DMRT showed that the interaction of sucrose concentration of 20 g/l and 3000 lux light intensity resulted in the highest number of shoots and number of leaves. Based on these results, sucrose 20 g/l and light intensity 3000 lux were the best treatment for shoot and leaf induction of lemongrass.

**Plantlet Morphology**

The morphological observations of lemongrass plantlets showed that leaf color varied between treatment levels. There were some leaf colors of lemongrass plantlets, namely fresh green, yellowish green, whitish green, and brownish. The yellowish green and whitish green color found at the treatment of light 2000 lux and sucrose 0 g/l (Fig. 1a), and light 4000 lux and sucrose 0 g/l (Fig. 1b). The normal leaf color or fresh green was obtained by treatment of light intensity of 4000 lux with sucrose 10 g/l (Fig. 1c).

The green color of leaves is caused by the presence of chlorophyll pigments in the chloroplasts. In leaves, chloroplasts are abundant in palisade tissue and spongy tissue. The chloroplasts are derived from proplastids found in meristem cells. In the dark, the proplastids will differentiate into etioplasts. When exposed to light, etioplasts will differentiate into chloroplasts by converting protochlorophyll into chlorophyll (Taiz & Zeiger, 2017). These results indicate that without sucrose the plantlet can't produce chloroplast (Fig. 1a, Fig. 1b). The ability in chlorophyll forming normally occurs in 10% sucrose treatment (Fig. 1c).

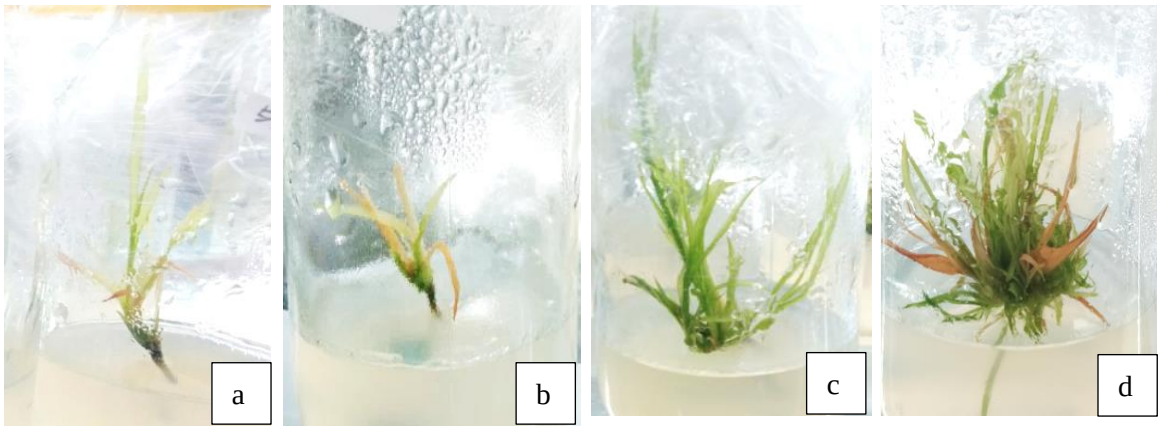


**Figure 1.** Morphology of lemongrass plantlets at some combination of treatment levels a) light 2000 lux and sucrose 0 g/l, b) light 4000 lux and sucrose 0 g/l, c) light 4000 lux and sucrose 10 g/l, d) light 3000 lux and sucrose 20 g/l

The yellowish green and whitish green leaves might happen because of chlorosis; whereas the brown leaf color indicates the leaves are aging (Fig. 1d). The percentage of chlorosis in each treatment varied. Treatments with sucrose concentrations of 0 g/l and 30 g/l interacted with light intensity of 2000 lux, 3000lux, 4000 lux, and 5000 lux showed the highest percentage of chlorosis. The results showed that the interaction of 0 g/l and 30 g/l sucrose and all level of light intensity resulted in 100% chlorosis percentage (Table 4). This is caused by the sucrose concentration of 0 g/l does not provide the energy needed by plantlets for chlorophyll synthesis. Otherwise, the sucrose concentration of 30 g/l decreased the osmotic potential of the medium. As a consequence, nutrient flow from medium to explants is reduced and the synthesis of chlorophyll was inhibited (Taiz & Zeiger, 2017).

**Table 4.** Percentage of chlorosis of lemongress leaves due to the sucrose concentration and light intensity

| Light intensity (lux) | Percentage of chlorosis (%) at sucrose concentrations (g/l) | | | |
|---|---|---|---|---|
| | 0 | 10 | 20 | 30 |
| 2000 | 100.00 | 19.05 | 16.50 | 100.00 |
| 3000 | 100.00 | 10.65 | 9.98 | 100.00 |
| 4000 | 100.00 | 18.00 | 14.45 | 100.00 |
| 5000 | 100.00 | 8.33 | 3.84 | 100.00 |

The result of this study was in line with Ramirez *et al.* (2019) which concludes that the chlorophyll content in bamboo leaves is influenced by the concentration of sucrose; at a concentration of 30 g/l showed a low value of chlorophyll content. Regueira *et al.* (2018) added that both concentration and duration of exposure to sugar resulted in a decrease in chlorophyll content. Increasing the concentration of sucrose reduces the levels of chlorophyll a and b which affect the physiological response of plants (Martins *et al.*, 2016). A decrease in cell osmotic potential due to an increase in sucrose concentration will affect chlorophyll levels, change leaf color, and decrease shoot growth (Sigh & Patel, 2014).

The development of lemongrass micropropagation protocol by decreasing sucrose concentration and increasing light intensity has never been done. The micropropagation research that has been carried out so far using a standard sucrose concentration of 30 g/l with a standard light intensity of 1000 lux. Through this technique, the lack vigor plantlets are produced. In this study, sucrose concentration was studied to be reduced so that plantlets were encouraged to utilize endogenous carbon compounds from photosynthesis. In order to optimize photosynthesis, the effect of light intensity higher than 1000 lux is also studied. Therefore, theoretically the research results are useful as a basis for developing photomyxotrophic in vitro culture techniques of lemongrass and other related plants. Practically, the results of this study are useful as a basis for developing lemongrass propagation techniques in order to produce sterile lemongrass plantlets efficiently on a large scale. Sterile lemongrass plantlets can be used as the main component in making educational and unique tissue culture-based souvenirs.

## CONCLUSION

Increased light intensity, decreased sucrose concentration, and interactions of both had a significant effect on the growth of lemongrass

plantlets. The light intensity of 3000 lux resulted in the highest number of shoots and the number of leaves. A sucrose concentration of 30 g/l resulted in the fastest shoot emergence time, while a concentration of 20 g/l resulted in the highest number of shoots and number of leaves. The interaction of light intensity 3000 lux and sucrose 20 g/l resulted in the highest number of shoots and number of leaves. The best protocol of photomyxotrophic growth of lemongrass plantlets were a sucrose concentration of 20 g/l and a light intensity of 3000 lux. Based on the results, it is suggested to carry out further research about the effect of $CO_2$ enrichment on photosynthetic intensity. The research is expected to increase the effectiveness of photomyxotrophic tissue culture techniques which can produce more vigor lemongrass plantlets.

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