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Application of chitosan spraying on acclimatization success of tiger orchid (*Grammatophyllum scriptum*) plantlets

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Abstract. *Pitoyo A, Hani MR, Anggarwulan E. 2015. Application of chitosan spraying on acclimatization success of tiger orchid (*Grammatophyllum scriptum*) plantlets. Nusantara Bioscience 7: 185-191*. In Indonesia, tiger orchid or anggrek macan (*Grammatophyllum scriptum)* is one of the orchid species that have been mass propagated with *in vitro* culture technique. The successful determinant of *in vitro* culture after *planlet* formation is acclimatization. *Plantlet* have faced with absolutely different environmental condition between inside and outside bottle that potentially severe the survival of the *plantlet.* The objectives of this research were to know the effect of chitosan for success of chitosan spraying in acclimatization of plantlets of *G. scriptum* and to know the optimum concentration of chitosan that can help the successful of the acclimatization of plantlets *G. scriptum.* This research used a *Completely Randomized Design* (CRD) with four level concentrations of chitosan spraying, there were 0 g/L; 0,5 g/L; 0,75 g/L; 1 g/L. Every treatment underwent 15 repetitions. The data taken on this research were qualitative and quantitative data. Qualitative data was got from observation of morphology and anatomy then it was analyzed descriptively. The quantitative data was got from percentage of *plantlets* life, observation of shoot length, leaves length, leaves width, number of leaves, number of shoot, index and density of stomatal. The quantitative data was analyzed by *Analysis of Variance* (ANOVA), if there was a significant difference between treatment groups then followed by *Duncan's Multiple Range Test* (DMRT) at level significance of 5 %. The result of this observation indicated that the effect of chitosan was significant on several parameters including width leaf, *plantlets* height, and stomatal index while the length and number of leaves were not significantly different. The concentration of chitosan real effected on these ranged from 0.5-0.75 g/L. Chitosan concentration at above of 0,75 g/L showed a negative influence on the acclimatization successfully of *plantlets G. scriptum.*

Keywords: Acclimatization, chitosan, *Grammatophyllum scriptum*

INTRODUCTION

Tiger orchid or anggrek macan (*Grammatophyllum Scriptum* Bl.) is member or Orchidaceae, a large family of flowering plant that comprises more than 25.000 species (Dessler 2005; Fay and Chase 2009). Like other members of the orchid family, *G. scriptum* potentially emerging as world famous ornamental plant because a number of their flowers that many, around 25-50 per stalk, blooming time is quite long, and the habitus is well-built and strong. In nature, propagation of most orchid family is hamper by the fact that they dependency with symbionts for pollination success as well as germination. Morphology of the orchid flower such as the join of male and female reproductive organs in one structure of gynostemium have made them in a hard situation for self-pollination, Thus pollinator, and mostly insects are needed for pollination success. Furthermore, orchid seed as product of successful pollination and fertilization have very small in size, undeveloped embryo, and lacked endosperm as food reserve, this makes orchid seed have low preferences to germinate. The finding of in vitro technique by Knudson (1922) in germination of *Cymbidium* seed, have made orchid easily to mass propagated, and they become one of leading floricultural world commodity. According to Abbas et al. (2011) propagation method that is often used is the in vitro culture because it is an effective method to multiply orchids, because in a short time it can produce many plantlets.

The most limitation in the mass scale application of in vitro technology is high mortality during or the following laboratory to land transfer (Chandra et al. 2010). Morpho-physiological characteristic of in vitro plantlets may contribute to this phenomenon. As state by Pospíšilová et al. (1999), during in vitro culture, plantlets grow under very special environment in relatively air-tight cultivation vessels, e.g., air humidity is higher and irradiance lower than in conventional culture. In the field, irradiance is much higher and air humidity much lower than in the bottle. The plantlets may quickly wilt as water loss of their leaves is not restricted. Hazarika (2006) reported that abnormal symptoms have shown by plantlets after being removed from in vitro culture i.e. the thin epicuticular wax layer, the vascular tissue is not fully developed, and the malfunction of stomata. Fila et al. (1998) emphasized that water supply in such condition can be limiting because of low hydraulic conductivity of roots and root-stem connections. It is necessary to apply anti-transpiration compounds for successful acclimatization of in vitro plantlets.

Previous researchers reported about the usefulness of chitosan as anti-transpiration agent. Chitosan is deacetylation of chitin that primarily composed of glucosamine and N-acetyl glucosamine residues with a 1,4-

b-linkage. This material derived from exoskeleton of *Crustacean* and another invertebrate. The application of chitosan is widely applied in many areas such as biomedicine, membranes, drug delivery systems, hydrogels, water treatment, food packaging, etc. (Honarkar and Barikani 2009). In agricultural and plant research chitosan was used as coating material such as fruit and vegetable coating as reviewed by Duan and Zhang (2013). According to Bittelli et al. (2001), chitosan can reduce water loss in pepper (*Capsicum* sp.) for as much as 26-43%. In tissue culture, medium with addition of chitosan shows positive result in growth of in vitro products (Sopalun et al. 2010). Spraying with chitosan has been shown to reduce significantly the severity of leaf spot disease in orchids. In *Dendrobium*, this applied technique of chitosan also has positive impact on open florets and length of the inflorescences (Uthairatanakij et al. 2007).

In order to increase growth and survival rate in plantlets at the acclimatization stage, the objectives of this research were to know the effect of chitosan spraying in acclimatization success of *G. scriptum* plantlets and to know the optimum concentration of chitosan that can help the *G. scriptum* plantlets to be successful during acclimatization.

MATERIALS AND METHODS

Plant materials and planting medium

Plant materials used in this research are sterile plantlets derived from in vitro germination of *Grammatophyllum scriptum* seeds. The Plantlets of *G. scriptum* were harvested and collected from Tissue Culture Laboratory Facilities of Faculty of Mathematics and Natural Science, Sebelas Maret University, Surakarta, Indonesia. The planting medium used in this study was *Sphagnum flexuosum* moss fixed in 10 mm plastic pots.

Spraying agents

Several level concentration of spraying solutions (w/v) were made by diluting each gram (0, 0.5 g, 0.75 g, and 1 g) of chitosan powders by small amount (10 ml of 1% Acetic acid and glycerol) before completely dissolve in 1L distilled water. This formulation for makes a chitosan spraying in concentration of 0, 0.5 g/L, 0.75 g/L and 1 g/L respectively.

Design experiment and procedures

This study uses a completely randomized design (CRD) with 15 repetitions in each treatment group. The treatment consists of 4 levels: 0 g/L; 0,5 g/L; 0,75 g/L and 1 g/L. Selection of plantlets is done by gently removing them from the bottle and washing them with water, and then plantlets are selected according to the criteria of Andriyani (2010). According to Andriyani (2010), the characteristics of good quality plantlets for acclimatized are it looks healthy with no fungus, similar size, fresh green leaves without yellow leave, it grows normally, new shoots begin to appear and a small portion of shoot has grown and it has 3-4 fibrous roots.

Growing mediums for acclimatization is *S. flexuosum* moss that is washed with water and sterilized within autoclave. The sterilized moss is then placed in plastic pot and each plantlet was fixed on them. Each chitosan solution is then sprayed onto the plantlets every two days with 5 mL or 5 times of spraying.

Variable observation used is the percentage of life expectancy, the number of shoots, plant height, leaf number, length and width of leaves, epidermis thickness, epicuticular thickness, stomatal indexes, and stomata density, leaf color and morphology (leaf strand including general leaves shape, leaf tip, leaf edges, leaf bone). This variable is calculated at 56 days after planting (DAP).

Anatomical observation

Anatomical data including leaf stomatal index, leaf epidermis and epicuticular wax thickness was observed by digital microscope Nikon ND 600. The image was subject for analysis using imageJ for quantification. Microscopic slide for epidermis and its epicuticular wax were prepared by section paraffin method (Ruzin 1999). The sample was fixed by FAA (Formaldehyde, Alcohol, Glacial Acetic Acid), and dehydrated using serial alcohol concentration of (70%, 80%, 95%, absolute alcohol), and paraffin used as embedding materials. Section of 10 um slide was performed by microtome and staining using safranin in 70% alcohol.

Imprints of epidermal cells and stomata were taken from fully expanded, mature. Imprints were made by coating a 25-mm2 area at the base and 1 cm from the leaf edge with clear nail varnish, covering with ‘sellotape’, applying pressure and replacing onto a glass microscope slide. Images of each slide were captured at 100 magnifications using a digital camera microscope Nikon ND 600.

Data analysis

Qualitative data include color, leaf morphology and plantlets texture which were analyzed descriptively. The quantitative data in the form of the number of shoots, plant height, number of leaves, leaf dimension, epidermal and epicuticular leaf thickness, stomatal index and stomata density were analyzed by Analysis of Variance (ANOVA) with a 95% of confidence level. Quantitative data analyzed descriptively is the percentage of life expectancy. If there is a real difference then it will be analyzed using Duncan's Multiple Range Test (DMRT) at the level of 5%.

RESULTS AND DISCUSSION

Results of this study were divided into three data sets, namely the percentage of life expectancy, vegetative growth, and the anatomical and morphological data. The vegetative growth parameters including the number of shoots, shoot height, leaf number, and leaf dimension. The anatomical and morphological parameters including were epidermises, cuticle, and stomata. Observed leaf morphologies were color and leaf shape.

Percentage of life expectancy

On the results of this study, each concentration shows quite a high percentage of life expectancy (Table 1). Percentage of life expectancy in two treatments shows less than 100% due to root rot and some sample gone dried. Decaying in root may occur because of the plantlets cannot absorb water efficiently via transpiration stream and an undeveloped vascular connection between root and shoot, thus moss substrate in a situation of prolonged moisture that fungi and other microbes are readily growing well. Conversely, another lethal symptom is shoot plantlets become dry due to the ability of the tissue to absorb water are still low and the occurrence of high transpiration resulting plantlets to dry. Thus, the two causalities of mortality are the common attribute of in vitro products. This result also indicates how chitosan spraying in a concentration below 1 g/L can reduce the risk of death.

Vegetative growth

Vegetative growth was measured and observed by several parameters, among which are the increase of plant height, length and width of the leaf, and the number of new leaves and shoots. Table 2 shows the vegetative growths of plantlets on 56 DAP. Table 2 shows the application of chitosan spraying give significant effects on some growth parameters such as the width of the leaf and stem height but give no different influence on the length and number of leaves.

Plant height

In this study, the highest increase of stem height was shown in chitosan concentration of 0.5 g/L at 1.2 cm. On increasing chitosan concentration, it is shown a declining result of stem height. The increase of stem height was allegedly under the influence of photosynthesis. According to Barka et al. (2004) the addition of chitosan derivative i.e. chitogel can increase O_2 production twofold and the CO^2 fixation is increased by 1.5 times than normal, indicating that chitosan derivative can increase photosynthesis. Photosynthesis result is used to increase plant growth.

The length and width of leaf

On 56 DAP observation; the length and width of leaves were increased. The observation showed that chitosan concentration of 0.75 g/L resulted in the highest increase of leaf length by 0.5 cm. Moreover, chitosan concentration of 0 g/L resulted higher length of leaf than with a concentration of 0.5 g/L. An increased the width of leaves at a concentration of 0 g/L showed the highest increase compared to plants sprayed by chitosan. On plants which are sprayed by chitosan 0.5 g/L, the result was the greatest increase width of leaf by 1.7 mm.

The number of leaves

The highest increase in the number of leaves is indicated in the treatment with chitosan concentration of 0 g/L and the chitosan concentration of 1 g/L that is equal to 1.5 new leaves, while the lowest leaf increase is shown in the chitosan concentration of 0.5 g/L that is as much as 1.1new leaves. In this study, the increase number leaves show that the higher the concentrations of chitosan the higher the number of leaves produced, except that the treatment of 0 g/L and 1 g/L produces the same increase for as much as 1.5. In a study using chitosan concentration, this concentration is allegedly able to increase the yield of photosynthesis and can increase the number of leaves. Plantlets that were not sprayed with chitosan can increase the number of leaves equal to those produced by plantlets sprayed with a concentration of 1 g/L. It is believed the effect of granting the hood can help reduce transpiration excess of plantlets unsprayed by chitosan so that plantlets grow well.

New emerging shoots

In this study, both in the control and treatment, the emerging of new shoots does not happen until 56th DAP. It is possible due to the dominance of apical shoots that inhibits the growth of lateral shoots since the apical meristem is still active and this activeness will form new leaves that can inhibit the formation of side shoots. According to Sustetyoadi (2004) the occurrence of apical dominance is caused by auxin that is diffused from tip to lower of the shoot (polar) and is deposited on the lateral shoots. Moreover, this inhibits the growth of lateral shoots. This high concentration of auxin will inhibit the growth of lateral shoots near the top. Apical bud is the place to produce auxin, if the apical bud (shoot tips) is trimmed then the production of auxin will be stopped and side shoots can grow. In accordance with the results of research of Esrita (2012) that the greater the length of apical buds of soybean (*Glycine max* (L). Merrill) was trimmed, the more the number of branches of plants produced.

Table 1. Percentage of life expectancy on 56th DAP observations

Chitosan treatment	Percentage of life expectancy
0 g/L	93,33%
0,5 g/L	100%
0,75 g/L	100%
1 g/L	86,67%

Table 2. The average growth of vegetative plantlets of *G. scriptum* when acclimatized until the 56th DAP observations

Chitosan	$\bar{X}$ Vegetative growth on 56th DAP			
	Plant height (cm) ± SD	Leaf width (mm) ± SD	number of leaves ± SD	leaf length (cm) ± SD
K0	$0,6^{a} \pm 0,4$	$2,0^{b} \pm 1,1$	$1,5^{a} \pm 1,3$	$0,3^{a} \pm 0,4$
K0,5	$1,2^{b} \pm 0,5$	$1,7^{b} \pm 0,9$	$1,1^{a} \pm 1,1$	$0,2^{a} \pm 0,3$
K0,75	$0,8^{ab} \pm 0,8$	$0,9^{a} \pm 0,5$	$1,3^{a} \pm 1,2$	$0,5^{a} \pm 0,7$
K1	$0,4^{a} \pm 0,7$	$0,7^{a} \pm 0,9$	$1,5^{a} \pm 1,4$	$0,3^{a} \pm 0,4$

Note: number followed by the same letter shows no significant different on DMRT 5%. $\bar{X}$: Average, SD: Standard Deviation, DAP: Days After Planting

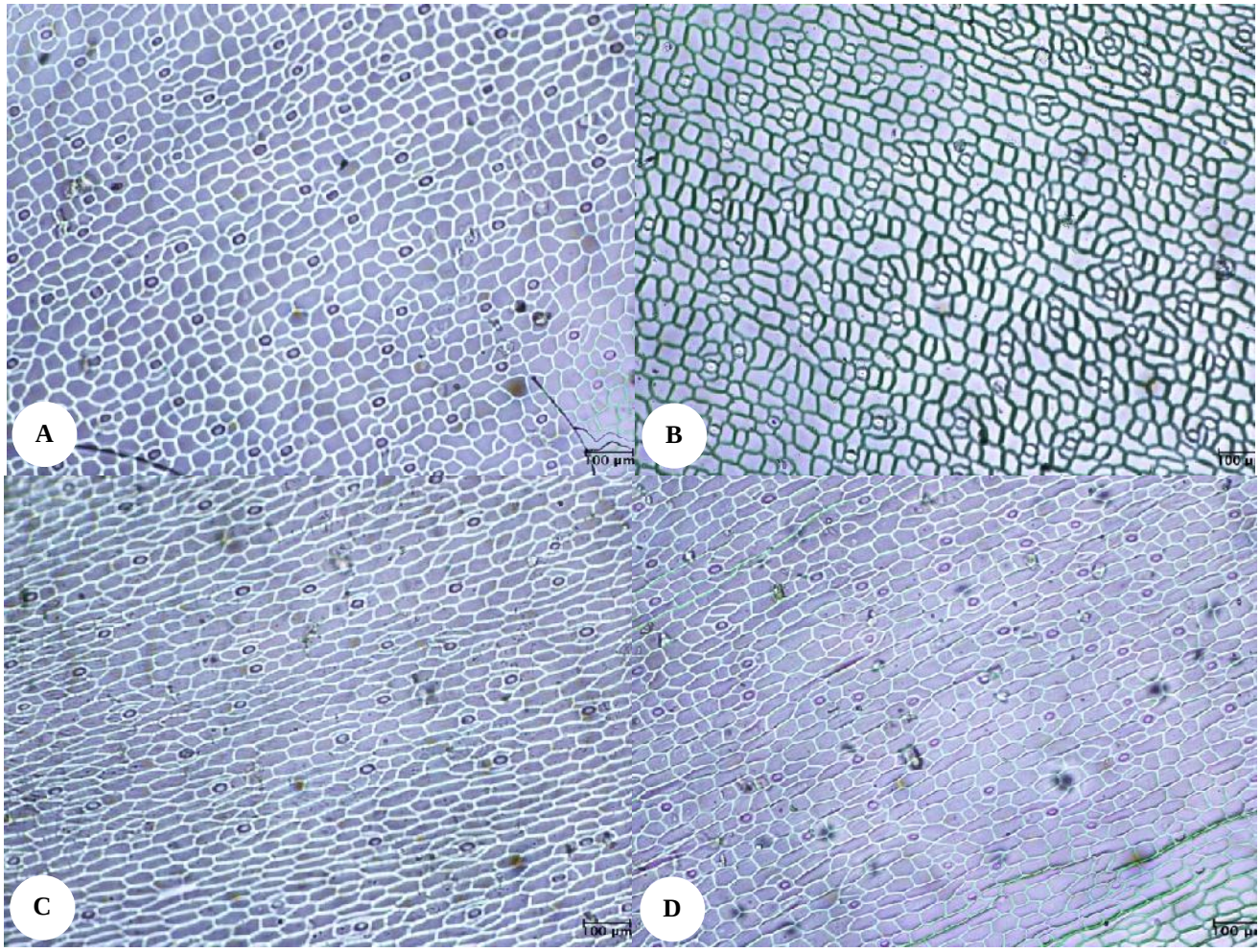

Figure 1. Surface cross sections of abaxial side of *G. scriptum* leaf on 56th DAP with chitosan spraying treatment variations. A. 0 g/L; B. 0.5 g/L; C. 0.75 g/L; D. 1 g/L. Optical magnification of 100x

Index of stomata

The following preparation is the printed abaxial surface. The preparation is used to calculate the stomatal index and density. In this study, the number of stomata and epidermis at the upper side and bottom side of the leaf are calculated. Leaf samples used are the leaves that are still in the bottle (Pre) and leaves that have been sprayed with chitosan (K) for 56 days. Chitosan concentration used is 0 g/L; 0.5 g/L; 0.75 g/L, 1 g/L.

As shown in figure 1 the type of the stomata is mostly tetracytic which characterized by two guard cells surround by four epidermal cells. This type is common for some cymbidium genera as report by Yukawa and Stern (2002) that previously Dessler (1993) include *Grammatophyllum* as close relative among this genus. Comparing of the four abaxial images we know that sample with none (Figure 1A) and low (Figure 1B) concentration of chitosan spraying have rectangular and semi isodiametric shape. This is contrast with 2 other treatments with chitosan in higher concentration (0.75 g/L and 1 g/L) that show slimmer in shape with higher ratio of length: width dimension. This image confirms the previous result of leaf width measurement that the leaf of non-chitosan spraying or leaf with low chitosan spraying is wider than others.

Table 3. The average of stomatal index and density on 56 DAP on *G. scriptum* plant

Treatment of chitosan concentration	56th DAP Stomatal index ± SD	Average of length + width (µm)
Pre upper side	$3.65^{a} \pm 2.9$	1437.5 + 1025
K0 upper side	$5.59^{abc} \pm 1.2$	1437.5 + 1025
K0,5 upper side	$11.04^{e} \pm 2.1$	1437.5 + 1025
K0,75 upper side	$8.18^{cde} \pm 1.2$	1437.5 + 1025
K 1 upper side	$4.22^{ab} \pm 0.28$	1437.5 + 1025
Pre bottom side	$7.03^{abcd} \pm 2.6$	1437.5 + 1025
K0 bottom side	$10.61^{de} \pm 2.3$	1437.5 + 1025
K0,5 bottom side	$10.31^{de} \pm 2.9$	1437.5 + 1025
K0,75 bottom side	$11.44^{e} \pm 1.6$	1437.5 + 1025
K1 bottom side	$7.83^{bcde} \pm 0.68$	1437.5 + 1025

Note: a number followed by the same letter in the column showed no significant difference in DMRT5%. Pre: the leaves that are still in the bottle. K0; 0.5; 0.75; 1: chitosan concentration 0 g/L, 0,5 g/L, 0,75 g/L, 1 g/L. upper side and bottom side: the upper and the bottom leaf surface. DAP: Days After Planting.

Table 3 shows the results of the activity of chitosan spraying that give significantly different on the stomatal index. Plantlets were sprayed either chitosan or not, show increased on the stomatal index when compared with plantlets that are just removing from bottles. The optimum concentration of chitosan spraying is varying between the upper side and lower side. In the upper side or adaxial leaf surface, 0.5 g/L chitosan spraying give maximum stomatal index. However, application of spraying in the bottom side or abaxial leaf surface gives maximum stomatal index in 0.75 g/L chitosan. Increasing concentrations of chitosan above both optimum concentrations give negative impact on the index.

According to the fact that we calculate stomata parameter in newly developed leaf, the different between the treatments is assume due to indirect effect from previous developed mature leaf. As we know, stomata grow and develop from immature meristemoid of epidermal cell, so they sensitive to environmental condition. Internal physiological carbon budged due to photosynthesis-respiration activity and inorganic gas CO2 in environmental surrounding would response by plant in the formation of stomata. Low concentration of chitosan spraying to the surface of the leaf would bottle neck the excessive water loss in early acclimatization day, this low concentration of layering would also allowed CO2 to diffuse to internal leaf. But, increasing chitosan concentration may negatively impacted because CO2 hard to penetrate the layering barrier. According to Iriti et al. (2009) the anti transpirant characteristic of chitosan not only attribute of their layered film formation but also the fact that it can elicited some substance that can communicated with internal ABA (abcisic acid) for stomatal aperture closure. In addition, Ordog et al. (2011) reported that chitosan can elicit immune response that reduces photosynthetic electron transport and ion channel activity in the guard cells of *Vicia*, this situation can trigger stomata closure. Thus, this may lead in reduction of stomata in next new developing leaf although the mechanism still far to confirmed. This assumption might analog to the case of reduction of stomatal index because of the long-term reduction in stomatal conductance through regulation its aperture. Over long term chronological events this lead the consequence of arresting the development of stomata from initial meristemoid cells (Haworth et al. 2010).

The epidermis and cuticle

Epidermis and its epicuticular wax properties are important barrier for plant protection from severe environment particularly during drought. Table 4 shows the thickness of epidermis and cuticle that is captured by MD 600 Nikon Digital Microscope and measured by image processing software imageJ.

Table 4 shows the thickness of the epidermis and the cuticle of data visualized in Figure 2. Treatment of chitosan at various concentration levels shows the same epidermal thickness, i.e., 20 µm. However, there are differences in the thickness of the cuticle at a concentration of chitosan 1 g/L i.e. 20 µm if compared to other treatment in a concentration below this level i.e. 10µm. According to Iriti et al. (2009) differences in the thickness of the cuticle shows anti-transpirant characteristic on chitosan. This study is supported by the statement of Courtois and Lafitte (1999) that stated that the plants resistant to drought can be identified by the thickness of the cuticle layer. Thick cuticle on plantlets showed the plantlets are resistant to drought. Also, Kosma et al. (2009) have found that the model species, Arabidopsis show enhancement of cuticle layer biosynthesis during water deficit. This finding indicates that excessive application of chitosan may signal the plant to more defensive by developing protective structure.

Plantlets morphological observation

Change in leaves color

Plantlet was facing with different light quality between inside storage room and acclimatization site in the greenhouse. In the storage, room irradiance has tight control with an illumination from the lamps, this contrast with the irradiance outside the storage room or greenhouse that more light intensities are coming to the plantlets. This situation may lead to a shift of the color of the leaves that turn from yellow-greenish to bright green and dark green. Based on the results in Table 5, leaves of plantlets show a change in their color. After removing from the bottle, the leaves of plantlets are light green and after being acclimatized, the leaves become more dark green. Based on the Royal Horticulture Society Color Chart (RHS Color Chart), plantlets changes from RHS 144A into RHS 143A. Change in leaf color may attribute of the pigment composition in the leaf. This phenomenon is same as plants in the situation between the shaded area and open area.

Table 4. The thickness of epidermis and cuticle of *G. scriptum* at various levels of chitosan concentration

Treatment	Epidermis thickness (µm)	Cuticle thickness (µm)
Inside the bottle	20	10
Chitosan 0 g/L	20	10
Chitosan 0,5 g/L	20	10
Chitosan 0,75 g/L	20	10
Chitosan 1 g/L	20	20

Table 5. Leaf color change before and after chitosan spraying on 56 DAP

Description	Plantlet on 56th DAP
Plantlets removed from the bottle	RHS 144A
Plantlets on 56th DAP	RHS 143A

Note: RHS 144A The color changes observed with Color Charts Royal Horticultural Society (RHS Color Charts). The plantlets color strengthening may be caused by the chloroplast.

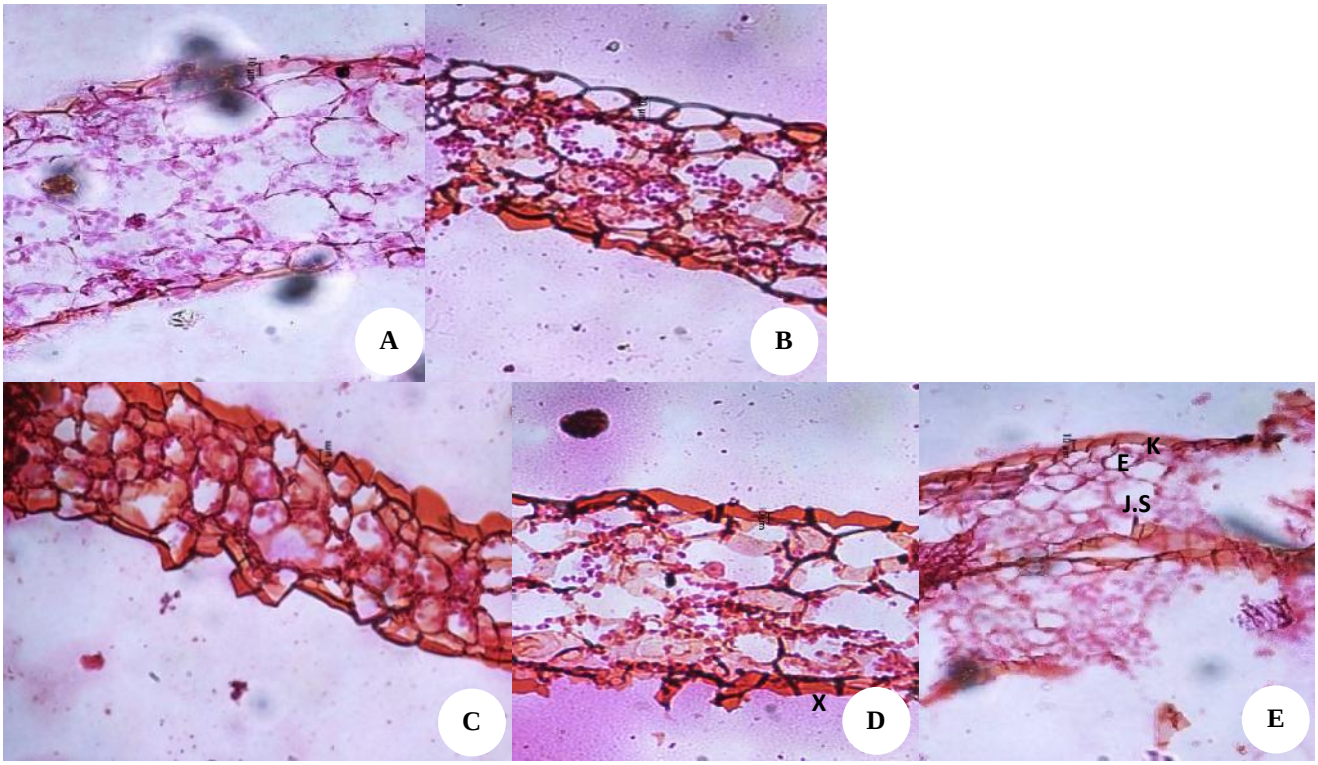

Figure 2. Leaf Transverse Cross-section on some chitosan treatment variations. A. 0 g/L. B. chitosan concentration of 0.5 g/L. C. The chitosan concentration of 0.75 g/L. D. inside the bottle. E. 1 g/L. Explanation: K: cuticle, JS: sponge or spongy tissue, X: The chloroplasts, E: epidermis

Figure 3. Morphology of leaves before and after acclimatization. A. The leaves condition in the bottle (all close); B. Leaves conditions outside the bottle (some leaves open)

Plantlets leaf morphology

In general, as show in Figure 3, morphology of the plantlets after removing from the bottle is quite different with the plantlet after 56th DAP especially in leaf morphology. The leaf morphology of plantlet before acclimatization is slender and position of the two lamina side is sticking each other. Conversely, plantlet of 56th DAP has unfolded lamina so it looks wider than before. Shifting in leaves shape of plantlets during acclimatization are due to the change their environmental surrounding. Leaves must maximize their surface area as irradiance harvesting panel to drive photosynthetic machinery. This

condition is not necessary if they life inside bottle as organic carbons supply by sucrose in medium. We must state that, the changes in leaf shape are occurred in new developing leaves as mature leaf in condition fully differentiated.

Effect of chitosan spraying

In the study conducted by Barka et al. (2004), the addition of chitogel, namely chitosan derivative, on plantlets of wine can increase O_2 production two-fold and CO_2 fixation increased by 1.5 times, indicating that chitosan derivative can increase photosynthesis. The results of photosynthesis can be used for the growth and development of these plantlets. This proves that chitosan 0.5 g/L and 0.75 g/L can increase the number of indexes of stomata (Table 3). The increase of stomatal index at a concentration of 0.5 g/L and 0.75 g/L may influence an increase in some vegetative parameters at the same concentrations of chitosan. The highest increase in plant height and leaf width is shown in concentrations of chitosan 0.5 g/L, while the highest leaf length achieved is shown in the spraying of chitosan 0.75 g/L, the higher the number of stomata the higher the vegetative growth of the plants.

In conclusion, the chitosan effect on acclimatization of *G. scriptum* shows that the chitosan spraying gives significantly different effect on some growth parameters such as the width of the leaf and stem height, and stomatal index. Chitosan does not give a significantly different effect on the length and number of leaves. The effect of chitosan on anatomy shows significant differences in the leaf cuticle. The concentration of chitosan that gives real effect on some parameter extends from 0.5 g/L to 1 g/L, but in this study, the chitosan concentration above 0.75 g/L gives a negative influence on the success of *G. scriptum* plantlets.

REFERENCES

Abbas B, Listyorini FH, Amriati B. 2011. In vitro seed germination and plantlet development of *Grammatophyllum scriptum* Lindl. (Orchidaceae). Intl Res J Plant Sci 2 (5): 154-159.

Andriyani LA, Buhaira, Nancy. 2010. The effect of concentration and frequency of spraying foliar fertilizer on the growth of *Dendrobium* sp. orchid plantlets at the stage of acclimatization. J Agronomi 10 (1): 51-54. [Indonesian]

Barka EA, Eullaffroy P, Clement C, Vernet G. 2004. Chitosan improves development, and protects *Vitis vinifera* L. against *Botrytis cinerea*. Plant Cell Rep 22: 608-614.

Bittelli M, Flury M, Campbell G, Nichils EJ. 2001. Reduction of transpiration through foliar application of chitosan. J Agric For Meteorol 107: 167-175.

Chandra S, R Bandopadhyay, V Kumar, R Chandra. 2010. Acclimatization of tissue cultured plantlets: from laboratory to land. Biotechnol Lett 32:1199-1205.

Courtois B, Lafitte R. 1999. Improving Rice For Drought-Prone Upland Envinronments. In: Ito-O'Toole J, Hardy B (eds.) Genetic Improvements. International Rice Research Institute, Los Banos.

Dessler R. 2005. How many orchid species? Selbyana 26 (1): 155-158

Duan JL, Zhang SY. 2013. Application of chitosan based coating in fruit and vegetable preservation: A review. J Food Process Technol 4: 5 DOI: 10.4172/2157-7110.1000227

Esrita. 2012. Influence of apical shoots pruning on growth and yield of soybean (*Glycine max* (L). Merrill). Program of Agroecotechnology, Faculty of Agriculture, University of Jambi, Jambi. [Indonesian]

Fay MF, Chase MW. 2009. Orchid biology: from Linnaeus via Darwin to the 21st century. Ann Bot 104 (3): 359-364.

Haworth M, Heath J, McElwain JC. 2010. Differences in the response sensitivity of stomatal index to atmospheric CO2 among four genera of Cupressaceae conifers. Ann Bot 105 (3): 411-418.

Hazarika BN. 2006. Morpho-physiological disorders in in vitro culture of plants. Scientia Horticulturae 108 (2): 105-120.

Honarkar H, Barikani M. 2009. Applications of biopolymers I: chitosan. Monatsh Chem 140:1403-1420.

Iriti M, Picchi V, Rossoni M, Gomarasca S, Ludwi N, Gargan M, Faoro F. 2009. Chitosan antitranspirant activity is due to abscisic acid-dependent Stomatal Closure. J Environ Exp Bot 66: 493-500.

Knudson, L. 1922. Nonsymbiotic germination of orchid. Bot Gaz 26 (1): 1-25.

Kosma DK, Bourdenx B, Bernard A, Parsons EP, Lü S, Joubès J, Jenks MA. 2009. The impact of water deficiency on leaf cuticle lipids of arabidopsis. Plant Physiol 151 (4): 1918-1929.

Ördög A, Wodala B, Hideg E, Ayaydin F, Deák Z, Horváth F. 2011. Chitosan elicited immune response reduces photosynthetic electron transport and ion channel activity in the guard cells of Vicia. Acta Biologica Szegediensis 55 (1):135-138

Pospíšilová J, Tichá I, Kadleček P, Haisel D, Plzáková S. 1999. Acclimatization of micropropagated plants to ex vitro conditions. Biologia Plantarum 42 (4): 481-497

Ruzin SE. 1999.Plant Microtechnique and Microscopy. Oxford University Press, Oxford.

Sopalun K, Thammasiri K, Ishikawa K. 2010. Effects of chitosan as the growth stimulator for *Grammatophyllum speciosum* in vitro culture. World Acad Sci Eng Technol 71: 449-451.

Sustetyoadi S. 2004. Plant Anatomy. UM Press, Malang. [Indonesian]

Uthairatanakij A, Teixeira da Silva JA, Obsuwan K. 2007. Chitosan for improving orchid production and quality. Orchid Sci Biotechnol 1 (1): 1-5.

Yukawa T, Stern WL. 2002. Comparative vegetative anatomy and systematics of *Cymbidium* (Cymbidieae: Orchidaceae). Bot J Linn Soc 138 (4): 383-419.