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Effect of two bio-polysaccharides on organogenesis of protocorm-like bodies in *Phalaenopsis* cultured *in vitro*

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ABSTRACT

Different types of bio-polysaccharide play a vital role in the growth of PLBs cultured in vitro. In this study, we investigated the potential impacts of two bio-polymers, hyaluronic acid (HA9) and sodium alginate (ALG) on the organogenesis of protocorm-like bodies (PLBs) in Phalaenopsis *under white LED lights. PLBs of Phalaenopsis 'Fmk02010' were explanted on modified MS medium with different concentrations of HA and (ALG). The highest average number of PLBs per explant (24.6) was recorded for ALG alone at a concentration of 0.01mg/L, and the fresh weight was also highest at the same concentration. The combination of 0.01mg/L ALG and 0.01mg/L HA also resulted in a large number of PLBs (23.8) and high fresh weight. As opposed to, the highest number of shoots /explant (3.6) was observed at the treatment of the combination of 1mg/L ALG and 10mg/L HA. This study shows that the application of ALG and HA alone, and in combination, at low concentrations, increased the average number of PLBs and the amount of fresh weight, but shoot formation was higher at a high concentration compared with control.*

Key Words*:* Phalaenopsis*, Protocorm*-like body (PLB), *Hyaluronic* acid (HA) *and Sodium alginate (ALG).*

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I. Introduction

Orchidaceae (commonly known as the orchid family) is a diverse and widespread family of flowering plants, with blooms that are often colorful and fragrant and it is known as the orchid family. Among orchids, the genus *Phalaenopsis* comprises the most beautiful flowers known as moth orchids. *Phalaenopsis* orchids are very popular in the flower trade and have been developed many artificial hybrids. Orchids Propagation is a complex process, that involves different changes such as environmental (structural and functional) and physiological changes, and may be influenced by

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internal and external signaling factors. Tissue culture techniques have been used for large-scale propagation of many orchid species and their hybrids (Arditti, 1993). In tissue culture, optimization of medium compositions has been an important approach in accelerating the micropropagation method and improving the quality of regenerated plantlets through culturing calluses, adventitious shoots, or protocorm-like bodies (PLBs) (Ichihashi, 1992; Chen et al., 2000; Park et al., 2002).

Plant growth regulators (PGRs) different types of organic compounds and nutrients that modify plant physiological processes. PGR, called bio-stimulants or bio-inhibitors, and its action is inside plant cells to stimulate or to inhibit specific enzymes or enzyme systems and help to regulate plant metabolism. Now a day, researchers are trying to use different organic or inorganic chemicals in plant tissue culture to understand their activity. In this study, we used two bio-polysaccharides: sodium alginate (ALG) and hyaluronic acid (HA). Meyer and Palmer (1934) reported a new polysaccharide isolated from bovine vitreous humor, contained a uronic acid and an aminosugar and named the polysaccharide hyaluronic acid from the hyaloids (vitreous) + uronic acid. Habiba et al. (2014) obtained a good result using HA on organogenesis of *Dendrobium* PLB under white LEDs.

Algenic acid is a polysaccharide distributed widely in the cell walls of brown algae. The acid is hydrophilic and forms a viscus gum when hydrated with a metal such as sodium and its salts are known as alginates. Algenic acid is also known as a marine biopolymer. ALG is used as a soil fertilizer for plants. Alginates can absorb water quickly, which makes them useful as an additive to dehydrated cells and tissues in plants. They help to raise the water-holding quality of soil and help the formation of crumb structure because alginic acid in seaweed combines with different metallic radicals in the soil to form a polymer with greatly increased molecular weight. Habiba et al. (2017) used alginate with modified MS medium in orchid micropropagation and observed a regulatory effect on *Dendrobium*. Single additon of HA with modified MS medium at the concentration 0.1mg/L proliferated PLB formation in *Phalaenopsis* (Kazi et al., 2015). The purpose of this study was to determine the effects of lower concentrations of single addition of HA and alginate and their combination treatment to determine the appropriate concentration of these two chemicals in the organogenesis of PLBs in *Phalaenopsis* cultured *in vitro*.

II. Materials and Methods

Explant Source

Protocorm-like bodies (PLBs) of Phalaenopsis 'Fmk02010' were proliferated in modified Murashige and Skoog medium (Shimasaki and Uemoto, 1990). After excision of PLBs, into singles, they were used for explants.

Plant Materials and Culture Conditions

PLBs of *Phalaenopsis*'Fmk02010' were proliferated in modified MS medium (For MS medium modification were added to ammonium nitrate 412.5 mg/L and potassium nitrate 950.0 mg/L,) with 2.2g/L of Phytagel™ (Sigma-Aldrich®, Tokyo, Japan) at the Laboratory of Vegetable and Floricultural Science, Faculty of Agriculture and Marine Science, Kochi University. We sheared single PLBs of *Phalaenopsis*'Fmk02010' to use as explants. The pH (5.5–5.8) of the medium was adjusted by using 1mM 2-(N-morpholino) ethanesulfonic acid sodium salt (MES-Na) before autoclaving. Hyaluronic acid (HA9, Shiseido, Japan) at concentrations of 0, 0.01, 0.1, 1, and 10 mg/L were added to the culture media alone and combined with sodium alginate (ULV-13; Kimica, Japan). Jars with plastic caps 250mL UM culture bottle AsOne, Japan containing 30mL of culture medium were used as culture vessels and autoclaved at 121°C for 15 min.

PLB Culture, Data Collection, and Data Analysis

Each 250mL culture bottle contained five PLBs (with three replications). PLBs were cultured for 42 days under white LED lights (Panasonic LED NNLK41509). The explants were cultured at 25°C with a 24h hour photoperiod. The number of PLBs and shoots were counted. The number of shoots and the fresh weight of the PLBs and shoots were measured. The average numbers and percentages were calculated as follows:

$$\text{Percentage of PLBs} = \frac{\text{Number of cultured explants with new PLBs}}{\text{Total number of cultured explants}} x100$$

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III. Results

The different concentrations of HA and ALG alone and combination in the modified MS medium under white LED lights and cultured for 42 days significantly affected the growth of the PLBs of *Phalaenopsis.* After 42 days of culture in the combined treatment, only the lower concentrations of HA and ALG raised the number of PLBs/explants, while the high concentrations raised the number of shoots significantly. After six weeks of culture, the highest number of PLBs of 24.6/explant (Table 01) and the highest fresh weight were obtained with the medium containing 0.01 mg/L ALG alone. For the HA alone treatments, the highest number of PLBs of 21.4/explant (Table 01) was recorded obtained with the medium containing 0.1 mg/L HA, which was significantly different from the higher concentration treatments. Notably, the fresh weight increased at the low concentrations, but, the average number of shoots increased at the high concentrations. The Highest concentration of 10mg/L HA resulted in the worst performance for PLB regeneration.

In the combination treatment (HA + ALG), under the comparatively low 0.01mg/L concentration of each, the number of PLBs and fresh weight increased, but at the higher concentrations of each the number of shoots increased and the number of PLBs decrease. The number of PLBs was lowest in the medium containing 10 mg /L HA and 10 mg/L ALG in combination (3.4 PLBs/explant). The highest number of shoots was observed for the medium containing 10 mg/L HA alone (4.1 shoots/explant) the second highest number of shoots (3.6/explant) was produced in the medium containing 10mg/L HA combined with 1mg/L ALG, and the third highest number of shoots (3.3/explant) was produced in the medium containing 10mg/L HA combined with 0.1 mg/L ALG.

Statistical analysis (Tukey HSD test) showed that a low concentration of ALG alone and of HA alone, and a low concentration of the combination treatment resulted in an increased number of PLBs and increased fresh weight. A comparatively high concentration of the combination of ALG and HA was effective increasing the number of shoots.

Table 01. Effect of HA and ALG on organogenesis of PLBs in *Phalaenopsis* 'Fmk02010'

`HA (mg/L)	ALG (mg/L)	Avg. No. of PLBs	Avg. No. of shoots	Fresh Weight (mg)
0	0	10.60b	1.1b	209b
0	0.01	24.6a	1.3ab	412a
0	0.1	21.3a	2.7a	364a
0	1	19.4a	3.1a	378a
0	10	08.40b	3.2a	340a
0.01	0	15.4a	0.7b	282b
0.01	0.01	23.8a	1.7ab	324a
0.01	0.1	14.60b	1.5b	223b
0.01	1	09.10b	2.7a	245b
0.01	10	08.50b	2.5a	252b
0.1	0	21.4a	1.1b	312ab
0.1	0.01	12.3b	2.2ab	293b
0.1	0.1	10.6ab	2.9ab	300ab
0.1	1	09.30b	3.2a	291b
0.1	10	07.41b	3.3a	280b
1	0	09.24b	1.9ab	219b
1	0.01	07.60b	2.1ab	221b
1	0.1	06.60b	1.4b	213b
1	1	06.50b	2.2ab	203b
1	10	05.12b	2.5a	172b
10	0	06.71b	1.5b	195b
10	0.01	06.43b	1.7b	276b
10	0.1	07.14b	3.3a	348a
10	1	06.22b	3.6a	329a
10	10	0 3.42b	2.5a	205b

Different letters in the same column indicate significant differences using Tukey HSD tests at the 5%level

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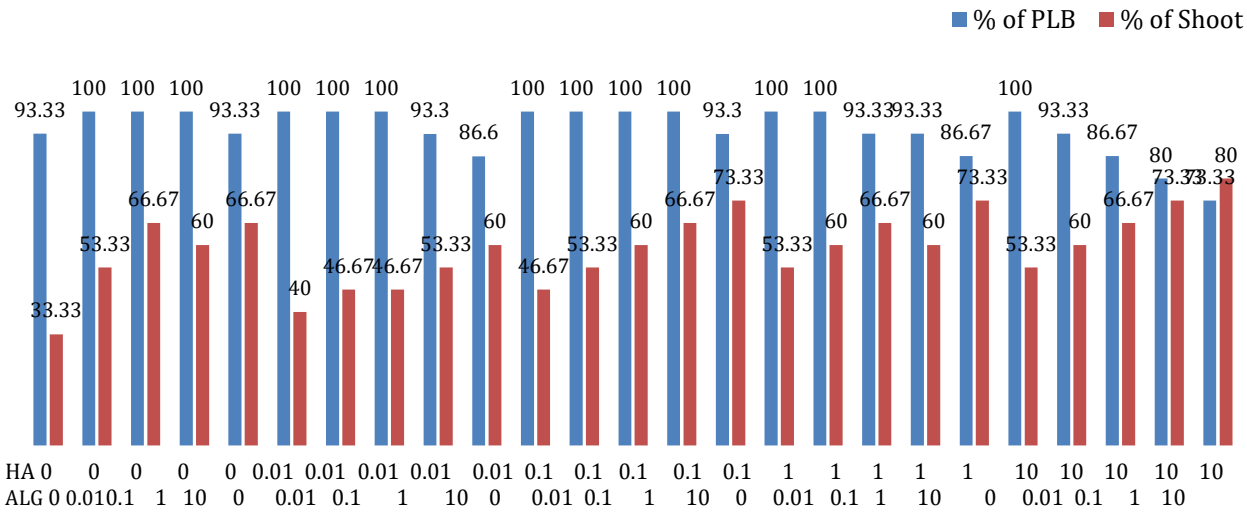

Figure 01. Effect of HA and ALG on PLB and shoot formation of *Phalaenopsis.*

The percentage of shoot and PLBs formation of *Phalaenopsis* 'Fmk02010' are shown in (Figure 01). PLB formation was 100% at all low concentration treatments of HA and ALG alone or in combination, and PLB formation for control was 86.7% (Figure 01). At high concentrations of both HA and ALG alone and in combination the PLB formation percentage decreased. Overall, the PLB formation percentage was higher in the medium containing low concentrations of HA and ALG alone and in combination. On the other hand, the shoot formation percentage is comparatively higher in high concentrations of HA and ALG alone and combination. After 42 days of culture, high concentrations of the combination treatments tended to produce shoots. The highest shoot formation 80% was found in the medium containing 10 mg/L HA and 1mg/L ALG (Figure 02).

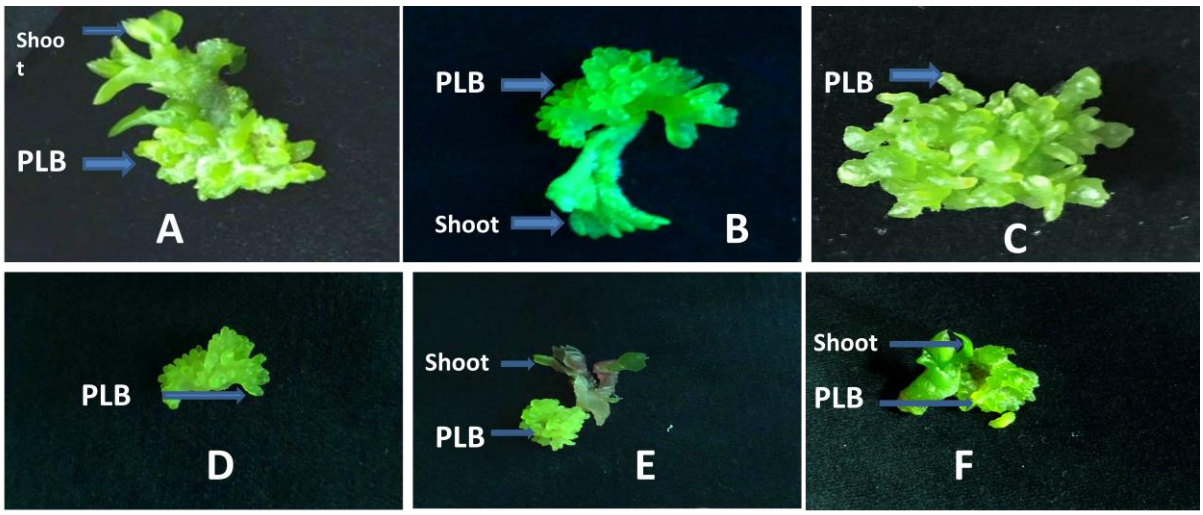

Figure 02. Effect of HA and ALG on organogenesis in PLB cultures of *Phalaenopsis* 'Fmk02010'.
A: HA 0.1mg/L + ALG 1mg/L; B: HA 0.1mg/L+ ALG 0.1mg/L; C: ALG 0.01 mg/L; D: HA 0.01mg/L+ ALG 0.01mg/L; E: HA 10mg/L + ALG 10 mg/L; F: HA 10mg/L+ ALG 1mg/L.

IV. Discussion

Different types of bio-polysaccharide growth chemicals are used for *in vitro* regeneration. Hyaluronic acid (HA) has recently introduced growth chemicals in plant tissue culture. In orchid tissue culture (Nahar et al., 2012) used HA as a plant growth regulator in *Cymbidium dayanum*. In this study, among the treatments with HA and ALG alone and in combination (HA and ALG), the treatment with HA alone, at the low concentration of 0.01mg/L resulted in an increased average number of PLBs (21.4/ explant). HA induces PLB and shoot formation in *Cymbidium* orchid plants (Nahar et al., 2014). Minimizes the acclimation time of cells on the material surface, and then cells rapidly enter the normal cell cycle (Milelle, et al.. 2002) and when added to modified MS media in low concentrations, HA acts as a plant growth regulator to induce PLBs formation (Alam et al., 2020). Low concentration of

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hyaluronic acid induced PLBs formation very rapidly in *Phalaenopsis*. (Kazi et al., 2015). The second highest number of PLBs (23.8/explant) in this study was found with a low concentration of a combination treatment (0.01mg/L HA + 0.01mg/L ALG). Low concentrations of HA and ALG alone and in a combined treatment on modified MS medium significantly affect the growth of *Dendrobium kingianum* PLBs (Habiba et al., 2014). In this study, HA neither exhibited antigenicity nor induced an inflammatory or allergic reaction. Among the combination of treatments (HA and ALG), the highest number of PLBs (23.8/explant) was found at a lower concentration (0.01mg/L HA and 0.01mg/L ALG). HA has been used to induce systemic resistance to cucumber mosaic virus in pepper, *Pseudomonas syringaepv*. Tomato (tomato speck disease), *Xanthomonas axonopodispv*. *vesicatoria* (tomato spot disease), *Pseudomonas syringaepv*. *lachrymans* (cucumber angular leaf spot) and *Colletotrichum orbiculare* (cucumber anthracnose) (Park et al., 2008). In the present study, higher concentration (10mg/L) of HA alone and a higher concentration of a combination treatment (HA 10mg/L and ALG 1mg/L) produced the maximum number of shoots. The Number of shoot and formation percentage of *D. kingianum* PLBs increases comparatively high concentrations (Habiba et al., 2014). This study proved that very low concentrations of sodium alginate produced the maximum number of PLBs.

V. Conclusion

The result of this research showed that a very low concentration of 0.01mg/L ALG with modified MS medium induced the highest number of PLBs. This research also showed that low concentrations of HA and ALG added to modified MS media acts as a plant growth regulator to induce PLBs and shoot formation needs a combination of high concentration.

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