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In vitro PLBs organogenesis of *Phalaenopsis* using different concentrations of HA9 and HA12 combination

Hasan Mehraj[1,2] **and Kazuhiko Shimasaki**[2]

[1]The United Graduate School of Agricultural Sciences, Ehime University, Ehime 790-8556, Japan
[2]Faculty of Agriculture, Kochi University, B200 Monobe, Nankoku, Kochi 783-8520, Japan

✉ For any information: ask.author@journalbinet.com, Available online: 14 March 2017.

ABSTRACT

The organogenesis of PLBs of Phalaenopsis 'Fmk02010' was studied to find out the effective treatment combination of HA9 and HA12. We used five different concentrations of HA9 and HA12 combinations and those were C0: 0, C1: 0.01, C2: 0.10, C3: 1.00 and C4: 10.00 mgL^{-1}. Explants were cultured in modified MS medium for 42 days. The highest numbers of PLBs (18.4/explant) were found from C2 which was statistically identical with C1 (15.20/explant). The PLBs formation rate was hundred percent in C1, C2 and C3. Numbers of PLBs were reduced in the higher concentrations (C3 and C4) which were lower than that of control while PLBs formation rate was reduced only in C4 (60.0%) than the control (86.7%). Numbers of shoots were also decreased with the increase of concentration. Maximum numbers of roots were found from C1 (1.0/explant) while rest of the treatments showed lower number of roots than that of control. The highest fresh weight was found in C2 (0.307 g) and lowest was in C4 (0.228 g); C4 was statistically identical with C3 (0.229 g).

Key Words: *Orchid, Hyaluronic Acid, Protocorm-like body and Formation rate*

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I. Introduction

Phalaenopsis is the most popular orchid genus in the floriculture market. It is very difficult to propagate vegetatively due to its monopodial epiphytic nature. Tissue culture technique has widely been used for the propagation of this orchid. Different techniques including PLB (Protocorm-Like Body) organogenesis (Sultana et al., 2015a; Sultana et al., 2015b; Nahar et al., 2012; Nahar et al., 2011; Niknejad et al., 2011) have been developed by researchers (Park et al., 2002 and Park et al., 2003; Tokuhara and Mii, 2001; Griesbach, 2002). PLBs can be used for the rapid and easy regeneration of the new plants. Use of plant growth regulators into culture media can play a vital role to increase the number of PLBs (Nahar et al., 2011). Hyaluronic acid (HA) is a polymer of disaccharides; composed of D-glucuronic acid and N-acetyl-D-glucosamine (Plate 01). HA is an additive for plant tissue culture that acts as a growth regulator (Kaewjampa et al., 2012). Exogenous HA function depends on its molecular

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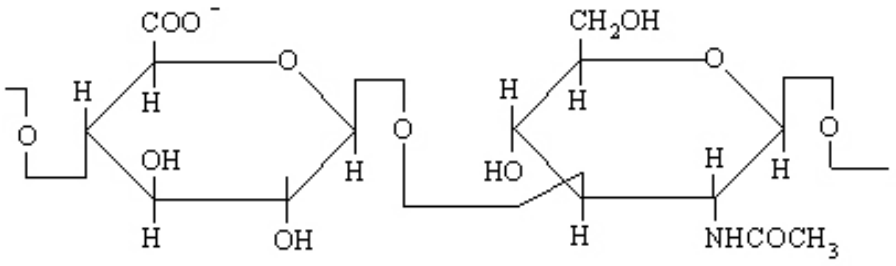
weight (Noble, 2002; Uthman et al, 2003; Hascall et al., 2004; Medina et al., 2006). In our previous study, we were found that addition of HA9 (Hyaluronic Acid with molecular weight $1.08x10^6$ Da) or HA12 (Hyaluronic Acid with molecular weight $1.2x10^6$ Da) in culture media increased PLBs of *Phalaenopsis* (Sultana et al., 2015a). Both HA9 and HA12 had the efficiency to improve PLB organogenesis in a certain concentration. It was hypothesized that combination application of both HA9 and HA12 will be more efficient for PLB organogenesis of *Phalaenopsis*. Concerning the hypothesis the study was conducted to find out the combined effect of the both HA9 and HA12 for more efficient PLBs organogenesis of *Phalaenopsis* 'Fmk02010'.



d-Glucuronic acid N-acetyl-D-glucosamine

Plate 01. Chemical structure of hyaluronic acid

II. Materials and Methods

Firstly, we multiplied PLBs of *Phalaenopsis* 'Fmk02010' in the modified MS medium (Shimasaki and Uemoto, 1990) at the Lab of Vegetable and Floricultural Science, Kochi University, Japan; and then excised into single PLB to use as explant. Extra addition of two major salts ammonium nitrate (412.5 mgL^{-1}) and potassium nitrate (950.0 mgL^{-1}) to the MS medium was done for the modification. We used sucrose (20.0 gL^{-1}) and Phytagel (Sigma) (2 gL^{-1}) to the modified MS medium. The pH was adjusted at 5.5- 5.8 through the addition of 1.0 mM MES-Na (2-(N-Morpholino) Ethanesulfonic Acid Sodium) salts prior to autoclave. The media was autoclaved at 121°C for 15 min with 147.1 KPa pressure. We used 30 ml of culture media in a 250 ml culture bottle (UM culture bottle: AsOne, Japan) that had plastic cap. We used five different HA9 and HA12 (Shiseido, Japan) combinations (viz. C0: 0.00 i.e., control; C1: 0.01; C2: 0.10; C3: 1.00 and C4: 10.00 mgL^{-1}). We put five single PLBs in each bottle and three bottles were used for each treatment combination. The numbers of PLBs, the numbers of shoots and number of root were counted whereas fresh weight of PLBs was measured after 42 days of culture. We considered the experiment as a completely randomized design with 3 replications. Each replication contained 5 explants (i.e., PLBs). Explants were cultured at 25±2C and 16 hr photoperiod with irradiance of 54 $\mu mol/m^{-2}s^{-1}$ for 42 days. We used following formulas to calculate average number and percentage.

A. Average number = Number of cultured explants with new PLBs or shoot/Total number of cultured explants
B. Percentage of PLB/shoot/root formation (%) = (Number of cultured explants with new PLBs or shoot/Total number of cultured explants) x 100

Data are presented as the mean ± standard error (SE). Significant differences among the treatments were determined by Tukey's HSD test ($P< 0.05$).

III. Results

Number and formation rate of PLBs

We found a significant variation among the different concentrations of HA9 and HA12 combination. The maximum number of PLBs per explant (18.40) was found from C2 which was statistically identical with C1 (15.20). The lowest number of PLBs (10.53/explant) was found from C4 which was statistically similar with C3 (Table 01). The numbers of PLBs in C3 and C4 were lower than the C0. Hundred percentage of the explants regenerated PLBs for the C1, C2 and C3 whereas lowest (60%) was found from C4 (Figure 01). The number of PLBs and their formation rate in C4 was (10.53/explant

and 60% respectively) lower than the control (13.60/explnat and 86.7% respectively) (Table 01 and Figure 01).

Number and formation rate of shoot and root: The highest number of shoot (3.60/explant) was recorded in control and the lowest (C4: 0.47/explant) was in the culture media treated with 10.00 mgL^{-1} of HA combination. The maximum number of root (1.00/explant) was recorded in C1 which was statistically identical with C0 and C2 (Table 01). The highest and lowest percentage of shoot formation was found from C0 (93.3%) and C4 (33.3%), respectively. On the other hand, the highest and lowest percentage of root formation was observed in C1 (80.0%) and C4 (26.7%), respectively. Both shoot and root formation rate were lowest in C4. The percentage of root and shoot formation was equal in C1 (Figure 01).

Total fresh weight: The highest fresh weight of PLBs also was found in C2 (0.307 g) and lowest was found in C4 (0.228 g). C4 was statistically identical with C3 (0.229) (Table 01).

Table 01. *In vitro* PLBs organogenesis of *Phalaenopsis* using different concentrations of HA9 and HA12 combination

Concentrations of combined HA9 and HA12 (mgL^{-1})	Number of			Total fresh weight (g) ± SE
	PLBs ± SE	shoot ± SE	root ± SE	
C0 (0.00)	13.60^{bc} ± 1.66	3.60^{a} ± 0.35	0.87^{a} ± 0.19	0.245^{c} ± 0.03
C1 (0.01)	15.20^{ab} ± 1.38	2.27^{b} ± 0.34	1.00^{a} ± 0.17	0.254^{b} ± 0.02
C2 (0.10)	18.40^{a} ± 1.09	1.20^{bc} ± 0.31	0.53^{ab} ± 0.17	0.307^{a} ± 0.01
C3 (1.00)	13.00^{bc} ± 2.24	1.33^{bc} ± 0.33	0.33^{b} ± 0.13	0.229^{d} ± 0.03
C4 (10.00)	10.53^{c} ± 2.32	0.47^{c} ± 0.19	0.27^{b} ± 0.12	0.228^{d} ±0.01

Superscript letters in the each column denoted the mean separation by Tukey's HSD test at 5% level of significance

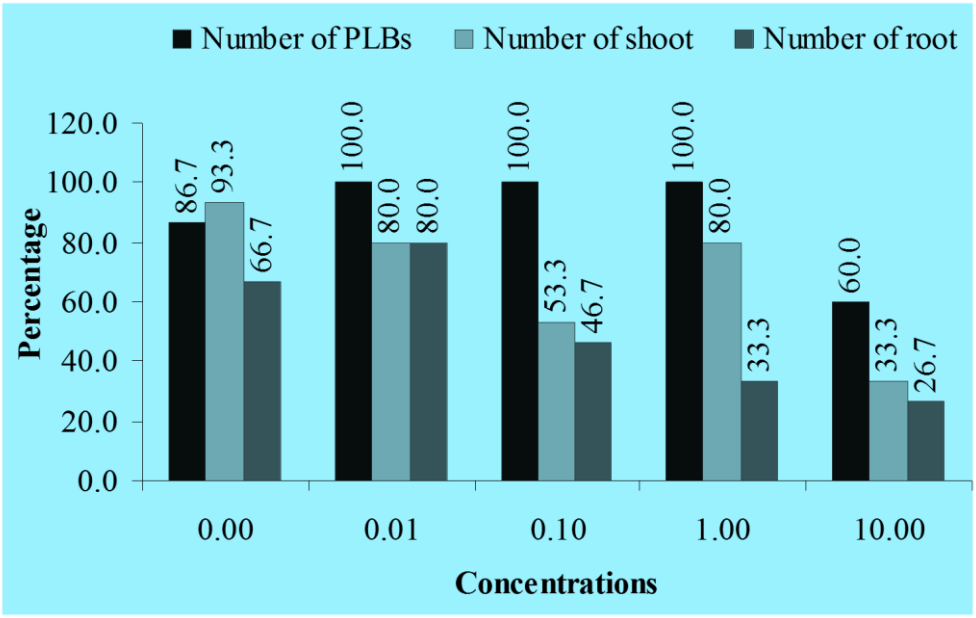


Figure 01. PLBs, shoot and root formation rate of *Phalaenopsis* 'Fmk02010' to the different concentrations of HA9 and H12 combination
C0: 0.00 i.e., control; C1: 0.01; C2: 0.10; C3: 1.00 and C4: 10.00 mgL^{-1}

IV. Discussion

HA is a polysaccharide, an abiotic elicitor that enhances secondary metabolite production in plant tissue culture (Zhou and Wu, 2006). HA has several biological functions including cell proliferation and differentiation, and gene expression (Kogan et al., 2007). HA9 and HA12 is the product name of Sodium bio hyaluronic acid having the molecular weight 0.8～1.17×10^{6} Da and 1.1～1.6×10^{6} Da respectively. Recently, the names have been changed to HA9N and HA12N respectively (Shiseido,

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Japan). HA in culture media have also been shown to improve in vitro organogenesis of (Nahar et al., 2011; Kaewjampa et al., 2012; Teixeira da Silva et al., 2013) and *Phalaenopsis* (Sultana et al. 2015a) orchids. The highest number of PLBs was recorded in *Cymbidium* Waltz 'Idol' (12.4 PLBs/explant) on medium containing 0.1 mgL^{-1} HA9 (Kaewjampa et al., 2012) while in *Cymbidium dayanum* (2.5 PLBs/explant) on medium containing 1.0 mgL^{-1} HA9 (Nahar et al., 2011). Teixeira da Silva et al. (2013) studied on the HA9 concentration using different LED in *Cymbidium finlaysonianum* and they used two different HA9 concentrations (0.1 and 1.0 mgL^{-1}). Their result showed that *Cymbidium finlaysonianum* produced highest number of PLBs at 1.0 mgL^{-1} HA9 under each LED light. Different concentrations of both HA9 and HA12 were studied on PLBs organogenesis of *Phalaenopsis* and found that HA12 was more effective than HA9 (Sultana et al., 2015a). The results of our study showed that 0.1 mgL^{-1} combinations produced highest number of PLBs and the numbers of PLBs were 18.40/explant. The results of our previous study showed that both HA9 (18.2/explant) and HA12 (23.3/explant) produced the highest number of PLBs on 0.1 mgL^{-1} among the concentration (Sultana et al., 2015a). The current study showed that maximum number of shoot was found from control (3.60/explant) and the number of shoot was reduced with the increase of the concentration. Sultana et al. (2015a) found the maximum number of shoot in 0.01 mgL^{-1} HA9 (14.8/explant) and 0.1 mgL^{-1} HA12 (15.1/explant) in *Phalaenopsis*. Individual treatment of either HA9 or HA12 produced more number of shoot than their combination. Use of the HA9 and HA12 combination drastically reduced the number o shoots of *Phalaenopsis*. We found maximum 80% shoot formation rate in both 0.01 and 1.0 mgL^{-1} combinations. Kaewjampa et al. (2012) found 80% shoot formation rate in 0.1 and 1.0 mgL^{-1} HA9. Our current study showed the maximum number of root (1.00/explant) and their formation rate (80%) in 0.01 mgL^{-1} concentration. Both the number and formation rate of root was decreased with the increase of their concentrations. We found maximum fresh weight from C2 (0.307 g); whereas in our previous study best performance regarding fresh weight was found from 0.1 mgL^{-1} both in HA9 (0.291 g) and HA12 (0.596 g) (Sultana et al., 2015a). This contrast confirmed that HA12 is better than not only HA9 but also the combinations of both HA9 and HA12 performed.

V. Conclusion

Use of 0.1 mgL^{-1} HA9 and HA12 combination in the culture media was found as the best between the combinations used in the current study. From the results of the current and our previous study, it can be suggested to use HA12 and avoid HA9 for the in vitro PLB organogenesis of *Phalaenopsis*.

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Conflict of Interest

Authors declared that there is no conflict of interest.

Author Contribution

Hasan Mehraj planned, designed and conducted the experiment. He was also responsible for data collection, compilation and analysis. He wrote the manuscript as well. Kazuhiko Shimasaki supervised the experiment.

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