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EFFECT OF PLANT GROWTH REGULATORS ON ORGANOGENESIS IN PROTOCORM-LIKE BODY (PLBS) OF *Cymbidium dayanum IN VITRO*

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ABSTRACT

The present study was conducted to investigate the effect of plant growth regulator on *in vitro* regulation of Protocom-like-bodies (PLBs) of *Cymbidium dayanum* without any phyto-hormone. PLBs of *Cymbidium dayanum* species were explanted on Modified Murashige and Skoog (Shimasaki, 1995) medium supplemented with three types of elicitors such as "Chitosan H", Marine Sweet (N-acethyl glucosamine) and "Hyaluronic acid " (HA) with various concentrations. New PLBs and shoots were successfully regenerated after the application of elicitor with modified MS medium. "Chitosan H" at 0.1 mg L^{-1} was the highest PLBs induction rate (93%) and shoots formation rate (79%) observed with medium containing "Chitosan H"at 1 mg L^{-1}. Medium containing "HA" at 1 mg L^{-1} was the high frequency of PLBs formation rate (100%) and shoots formation rate (93%) observed at medium containing 0.001 mg L^{-1} and 1 mg L^{-1} "HA". For induction of PLBs and shoots, "Marine Sweet" at 0.1 mg L^{-1} was most effective for maximum PLBs formation rate (87%) and shoot formation rate (67%). A comparative study of effective "Chitosan H", "Hyaluronic acid 9" and "Marine Sweet" found that "Hyaluronic acid" was the best plant growth regulators which enhance both PLBs and shoots formation.

Keywords: C*ymbidium dayanum*, plant growth regulators, PLBs, *in vitro*, chitosan, hyaluronic acid, NAG.

INTRODUCTION

Among the horticultural and floral crops, orchids are outstanding in many ways, like diverse shapes, forms and colours. Orchids are marketed both as plants and as cut flowers and their production has been increased in recent years (Tokuhara and Mii 1993, 2001; Chang and Chang 2000). Since the development of a method for non-symbiotic germination of orchid seeds by Knudson (1946), tissue culture techniques have been used for large scale propagation of a number of orchid species and their hybrids (Rao, 1977; Arditti and Ernst, 1993). Among orchids, the *Cymbidiums* have got a very high demand in cut flower trade. Regeneration of complete plantlets *in vitro* have been reported for a number of terrestrial *Cymbidium* species (Chung *et al*., 1985; Paek and yeung, 1991; Shimasaki and Uemoto, 1991).*Cymbidium dayanum* is important evergreen , epiphytic orchid which is valued for their attractive flowers. Plant should be grown in intermediate to warm areas with medium to bright light. There is however few report of *in vitro* regeneration of *Cymbidium dayanum*.

N-Acetylglucosamine (N-acetyl-D-glucosamine, or GlcNAc, or NAG) is a monosaccharide derivative of glucose. It is an amide between glucosamine and acetic acid. It has a molecular formula of $C_8H_{15}NO_6$, a molar mass of 221.21 g/mol, and it is significant in several biological systems. Polymerized with glucuronic acid it forms hyaluronan.

Chitosan is one of the most common natural polymers, comprising of glucosamine and N-acetylglucosamine is a polysaccharide obtained from N-deacetylation of chitin, which exhibits numerous interesting physiochemical and biological properties, such as biocompatibility, biodegradability and ability to accelerate wound healing. Chitosan used in many industries including waste treatment, membrane technology, pulp and paper, cosmetics, food industry, medical, biotechnology and agriculture (Imeri and Knorr 1998). In agriculture, there is a worldwide trend to use chitosan as an alternative compound because of its fungicidal effects and elicitation of defence mechanisms in many plant tissues. In orchid,chitosan sprayed on roots stimulated growth and enhanced resistance against fungi and viruses (Chandrkrachang, 2002). In orchid tissue culture, chitosan added to culture medium produced protocrom like body (PLB) cultures of a *Cymbidium* species (Shimasaki *et al*., 2003).

On the other hand, Hyaluronic acid is a naturally occurring biopolymer, which serves important biological functions in bacteria and higher animals including humans. HA is comprised of linear, unbranching, polyanionic disaccharide units consisting of glucuronic acid (GlcUA) an N-acetyl glucosamine (GlcNAc) joined alternately by beta 1-3 and beta 1-4 glycosidic bonds. HA is a component of the extra cellular matrixes of some tissue, can modulate growth factor and cytokine secration, inhibit proteinase hydrolysis and influence several cellular functions such as adhesion, growth, migration, proliferation and differentiation. Hyaluronic acid has no antigenicity, and will not induce inflammatory or allergic reaction following implantation. Its degradation leads to no toxic to human body also. HA in including systemic resistance in cucumber, tomato and pepper (Kyungseok park *et al*., 2007). This is the first report demonstrating

HA as a growth regulator which increases PLBs, shoots and roots formation.

MATERIALS AND METHODS

Approximately 5 mm in length PLBs derived from meristem cultures of *Cymbidium dayanum* which proliferated in modified MS medium (Shimasaki, 1995) were served for explants. MS medium with 412.5 mg L^{-1} ammonium nitrate, 950 mg L^{-1} potassium nitrate, 20 g L^{-1} sucrose, and 2 g L^{-1} Phytagel (Sigma) was adjusted to pH 5.5- 5.8 before autoclaving. Chitosan H (Kimica Co., Japan) at concentrations of 0, 0.1, 1, and 10 mg L^{-1}, Hyaluronic acid (HA 9- Shiseido, Japan) and N-acethyl glucosamine (Yaizu Suisan, Japan) at concentrations of 0, .001, .01, 0.1, 1 and 10 were added to culture media before sterilization. 250 ml of UM culture bottles (As One, JAPAN) with plastic caps were used, each bottle receiving 30 ml of medium. Five explants were put in each culture vessel and three culture vessels were used for each treatment. All cultures were maintained at 25^0 C, a 16 h photoperiod with irradiance of 46 µmol m^{-2} for 40 days.

RESULTS

Influence of Hyaluronic acid (HA 9) on PLB formation

Application of HA 9 promoted new explants of PLBs, shoots and root formation. The highest percentage of PLB formation rate 100% was observed at 1 mg L^{-1} HA 9 with modified MS medium and highest percentage of shoot formation rate 93% indicated on the medium supplemented with 1 mg L^{-1} and 0.001 mg L^{-1} HA 9. The number of developing shoots that formed roots varied from 53% at medium containing 0.001 mg L^{-1} HA 9 within 30 days of culture (Table-1; Figure-1).

Influence of Chitosan H on PLB formation

The inclusion of Chitosan H at 0.1 mg L^{-1} in the media resulted in maxium parcentage of PLB 93% being produced, which was significantly higher than that in the control. The highest percentage of new developing shoots 79% and roots 50% was found with the addition of Chitosan H at 1 mg L^{-1} within 30 days of culture (Table-2; Figure-2).

Influence of marine sweet (NAG) on PLB formation

The media and concentration of NAG were studied for their effects on PLB, shoot and root formation from protocorms. The results showed that the highest percentage of PLB 87% and shoot formation rate 67% indicated on the medium supplemented with 0.1 mg L^{-1} NAG within 30 days of culture. But the number of developing shoots that formed roots maximum percentage 47% at medium containing 1 mg L^{-1} within 40 days of culture (Table-3; Figure-3).

DISCUSSIONS

The objective of the present investigation was to analyze the effects of various plant growth regulators on organogenesis in *Cymbidium dayanum*. Three plant growth regulators such as "Chitosan H", "Hyaluronic acid" and "Marine Sweet (NAG)" used for this experiment. The main aim of this study, which has now almost successfully been achieved that "Hyaluronic acid" act as a plant growth regulators which inducing PLBs, shoots and roots with very short duration of time.

Bacterially produced hyaluronic acid could induce systemic disease protection in plants (Kyungseok park *et al*., 2007). HA is comprised of linear, unbranching, polyionic disaccharide units consisting of glucuronic acid (GlcUA) an N-acetyl glucosamine (Glc-NAc) joined alternately by beta 1-3 and beta 1-4 glycosidic bonds. Bacterial fermentation methods for large -scale economic production of HA have been well standardized as HA is of used in medical and cosmetic industry extensively (Akasaka *et al*., 1998; Hasagawa *et al*., 1999). Application of HA resulted in significant formation of PLB, Shoot and Root, compared to the control phase and this new PLB formation occurred even at the short time such as after 10 days of application. Within 30 days of culture PLB formation rate 100% was observed at 1 mg L^{-1} HA 9 and highest percentage of shoot formation rate 93% indicated on the medium supplemented with 1 mg L^{-1} and 0.001 mg L^{-1} HA 9 and root formation varied from 53% at medium containing 0.001 mg L^{-1} and 0.01 mg L^{-1} HA 9 (Table-1).

Table-1. Effects of HA-9 on the organogenesis responses from PLB of *Cymbidium dayanum*.

HA 9 (mgL^{-1})	PLB			Shoot			Root		
	No./explants	Rate[a] (%)	FW (mg)	No./Shoots	Rate[b] (%)	Length (mm)	No./Roots	Rate[c] (%)	Length (mm)
Control	2.0 ± 0.4	80	173.5	1.1 ± 0.6	47	3.4	0.4 ± 0.2	33	8.0
0.001	1.7 ± 0.6	60	129.5	2.2 ± 0.3	93	12.0	0.8 ± 0.3	53	14.4
0.01	2.1 ± 0.3	93	135.6	2.0 ± 0.6	73	11.5	0.5 ± 0.2	47	15.0
0.1	2.3 ± 0.4	93	96.5	1.4 ± 0.5	53	6.9	0.5 ± 0.3	40	7.7
1	2.5 ± 0.3	100	171.2	3.0 ± 0.4	93	10.4	0.8 ± 0.3	47	13.6
10	1.9 ± 0.4	80	186.0	2.9 ± 0.9	60	5.8	0.6 ± 0.3	47	9.0

Each value represents mean ± SE with 15 PLB samples. PLB, shoot and root (Rate [abc)] formation is calculated only growing of green PLB.

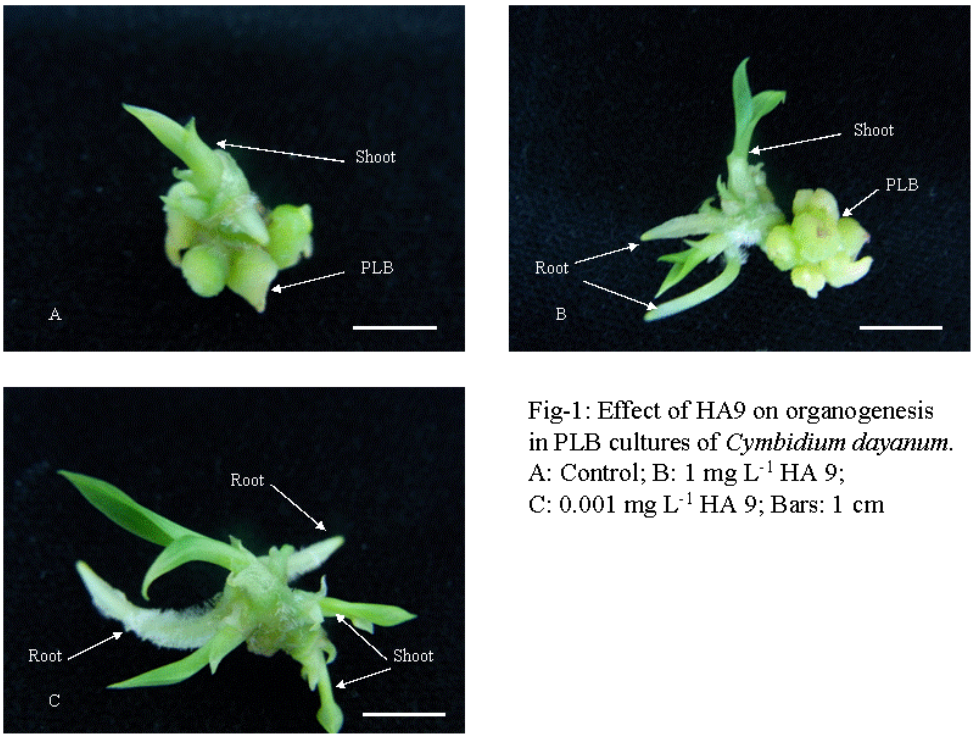

Fig-1: Effect of HA9 on organogenesis in PLB cultures of *Cymbidium dayanum*. A: Control; B: 1 mg L^{-1} HA 9; C: 0.001 mg L^{-1} HA 9; Bars: 1 cm

The ability of chitosan to stimulate plant growth *in vitro* has been shown before in the grape vine, *Vitis vinifera* L. (Ait Barka *et al*., 2004) and in the orchid, *D. phalaenopsis* (Nge *et al*., 2006). It was also shown to improve the potato micro plant quality *in vitro* and to increase the yield and seed quality of minitubers (Kowalski *et al*., 2006). The use of chitosan as a plant growth stimulator is of interest as it is a widely available, cheap and generally viewed as a safe material for humans and the environment. Chitosan is an appropriate growth stimulator in orchid micro propagation (Nge *et al*., 2006). It has been reported that chitosan increased the growth rates of roots and shoots of daikon radish (Raphanus sativas L.) (Tsugita *et al*., 1993). Plant responses to each chitosan are seemingly variable from species to species, although the appropriate chitosan type and concentration for each plant stimulating activity can be identified, and this seems to broadly hold across the responses seen within orchids too (Uthairatanakij *et al*., 2007). In this study, within 30 days of culture 93% of PLB produced with the inclusion of media containing Chitosan H at 0.1 mg L^{-1} and 79% shoots and 50% roots was found with the addition of Chitosan H at 1 mg L^{-1} (Table-2).

Table-2. Effects of Chitosan H on the organogenesis responses from PLB of *Cymbidium dayanum*.

Chitosan H (mgL^{-1})	**PLB**			**Shoot**			**Root**		
	No./explants	**Rate**[a] (%)	**FW** (mg)	**No./Shoots**	**Rate**[b] (%)	**Length** (mm)	**No./Roots**	**Rate**[c] (%)	**Length** (mm)
Control	1.6 ± 0.7	57	58.0	0.7 ± 0.2	64	5.0	0.6 ± 0.4	43	4.6
0.1	2.7 ± 0.5	93	83.8	0.6 ± 0.4	40	6.5	0.3 ± 0.5	20	10.0
1	2.1 ± 0.6	71	126.2	1.0 ± 0.2	79	7.6	1.1 ± 0.4	50	14.3
10	1.7 ± 0.4	64	60.57	0.9 ± 0.3	64	3.8	0.6 ± 0.3	43	4.9

Each value represents mean ± SE with 15 PLB samples. PLB, shoot and root (Rate [abc)] formation is calculated only growing of green PLB.

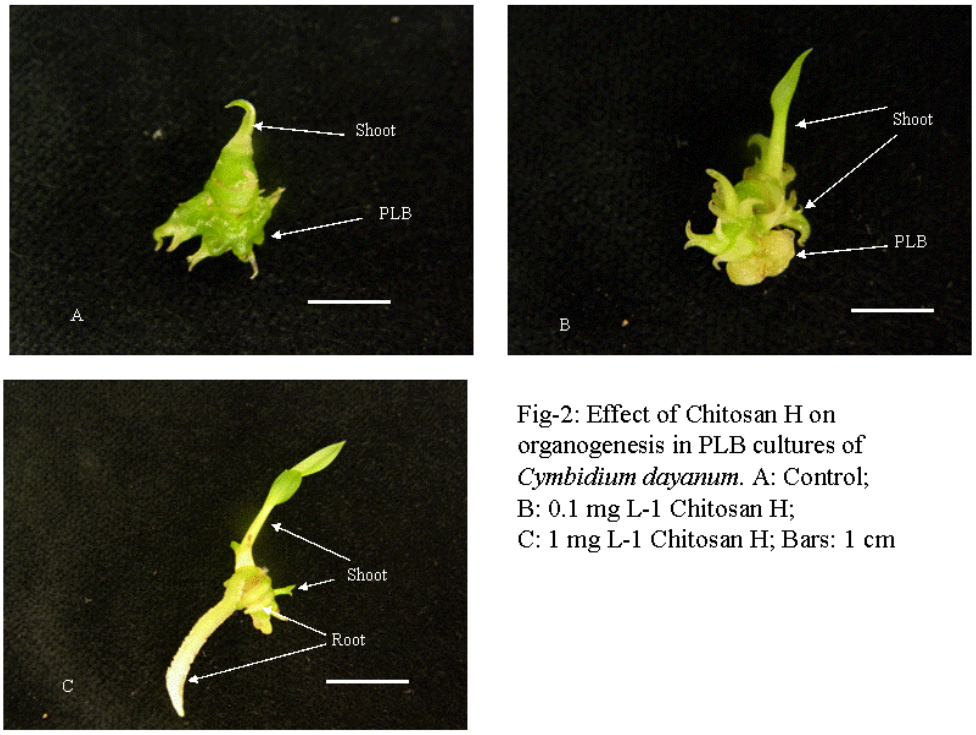

Fig-2: Effect of Chitosan H on organogenesis in PLB cultures of *Cymbidium dayanum*. A: Control; B: 0.1 mg L-1 Chitosan H; C: 1 mg L-1 Chitosan H; Bars: 1 cm

On the other hand, Marine Sweet or N-acetyl-D-Glucosamine (GlcNAc) is a monosaccharide derivative of glucose. It is released by the action of O-GlcNAcase, in mammalian systems from proteins that have been post-translationally modified with O-GlcNAc. Levels of O-GlcNAcylation proteins from Alzheimer's disease brain extracts are decreased as compared to that in controls, suggesting that release of GlcNAc may contribute to pathogenesis. In E. coli, GlcNAc induces the expression of multidrug exporter genes, indicating that this sugar can alter gene expression. GlcNAc is also the monomeric unit of chitin, which is found in fungi and many invertebrates, including crustaceans, insects, and nematodes. For this reason, chemicals that inhibit the incorporation of GlcNAc into chitin are cytotoxic to these organisms (Liu, F., Iqbal, K., Grundke-Iqbal, I., *et al*., 2004). In this study, after being cultured on the PLBs, within 30 days new PLB and shoot developed but Root didn't developed. The maximum percentage of the new PLB formation rate 87% and shoot formation rate 67% indicated on the medium supplemented with 0.1 mg L^{-1} marine sweet and the maximum root formation rate 47% observed at medium containing 1 mg L^{-1} marine sweet (Table-3) after 40 days of culture.

Table-3. Effects of Marine Sweet (NAG) on the organogenesis responses from PLB of *Cymbidium dayanum.*

Marine sweet (mgL^{-1})	**PLB**			**Shoot**			**Root**		
	No./explants	**Rate**[a] (%)	**FW** (mg)	**No./Shoots**	**Rate**[b] (%)	**Length** (mm)	**No./Roots**	**Rate**[c] (%)	**Length** (mm)
Control	0.9 ± 0.4	47	62.4	0.4 ± 0.4	27	5.5	0.3 ± 0.3	20	8.3
0.001	1.9 ± 0.6	67	122.8	1.1 ± 0.6	47	6.3	0.7 ± 0.4	40	10.2
0.01	1.4 ± 0.6	53	108.8	1.9 ± 0.8	53	5.2	0.9 ± 0.6	33	10.6
0.1	2.7 ± 0.5	87	168.7	2.1 ± 0.7	67	4.1	0.5 ± 0.4	33	7.2
1	2.2 ± 0.5	80	154.4	1.5 ± 0.5	60	4.2	0.9 ± 0.4	47	10.0
10	1.9 ± 0.4	80	103.3	0.7 ± 0.4	40	5.3	0.5 ± 0.4	33	11.2

Each value represents mean ± SE with 15 PLB samples. PLB, shoot and root (Rate [abc)] formation is calculated only growing of green PLB.

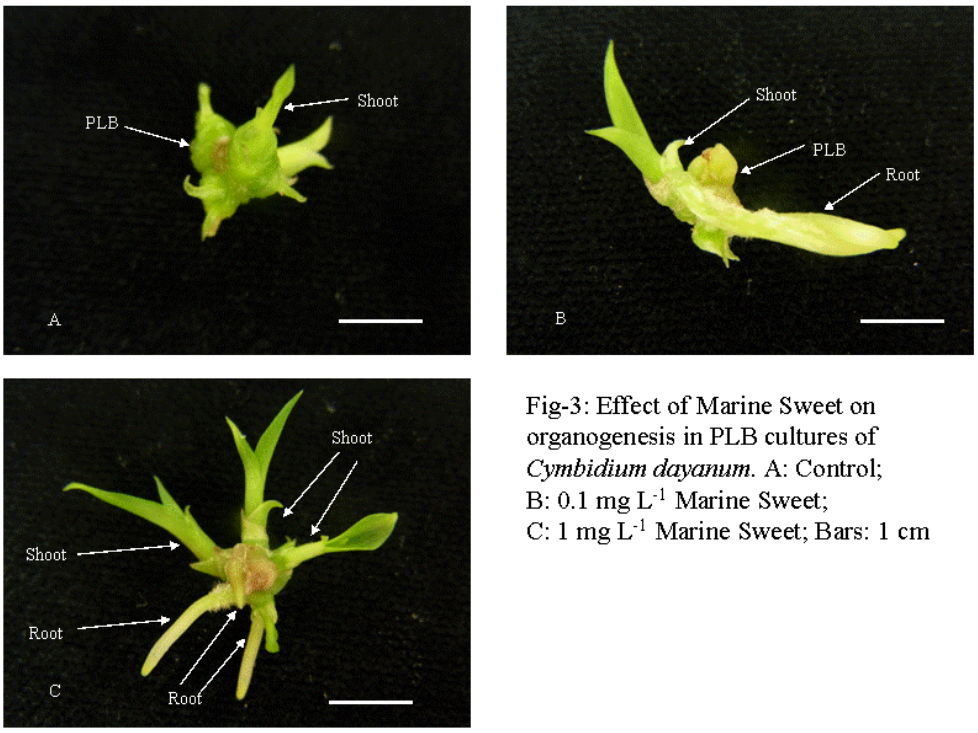

Fig-3: Effect of Marine Sweet on organogenesis in PLB cultures of *Cymbidium dayanum*. A: Control; B: 0.1 mg L^{-1} Marine Sweet; C: 1 mg L^{-1} Marine Sweet; Bars: 1 cm

CONCLUSIONS

In this study, we confirmed that "Hyaluronic acid " can be use as a plant growth regulator for orchid production or in tissue culture. It increases the formation of PLBs, shoots and roots with very short duration of time.By the application of three elicitors in *Cymbidium dayanum*, hyaluronic acid was the best plant growth regulator.

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