

Combination Treatment of 6-Benzylaminopurine (BA) and Hyaluronic Acid (HA) on PLB Culture of *Cymbidium* Spp. *In vitro*

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Abstract: Plant growth regulators (PGRs) function as chemical messengers for intercellular communication of plant Hyaluronic acid (HA) is a naturally occurring biopolymer, which serves important biological functions in bacteria and higher animals including humans. PGRs such as 6-benzylaminopurine (BA) is a first-generation synthetic cytokinin and widely used in orchid tissue culture. The objectives of this study is to assess the combination effect of HA and BA on PLB and shoot formation of *Cymbidium insigne* and *Cymbidium finlaysonianum* *in vitro*. Because of the combinations, concentrations and the ratio of plant growth regulators (PGRs) are critically important for *in vitro* culture techniques. Results of this study revealed that, among all the concentrations and combinations, 0.1 mg/l BA+0.1 mg/l HA were found to be the most effective combination concentration for PLB, shoot formation and maximum fresh weight of PLBs of *Cymbidium insigne*. In *C. finlaysonianum* 0.1 mg/l BA + 1 mg/l HA was found to be effective for maximum number of PLBs, highest formation of PLB and shoot formation was effective for single addition of BA and HA treatment. Our present study reports establish a new protocol on organogenesis of *Cymbidium* spp. through the combination treatment of cytokinin (BA) and elicitor (HA) *in vitro*.

Key words: Cytokinin • Elicitor • Organogenesis, Phytohormone, Plant growth regulator

INTRODUCTION

Orchids are highly valued ornamentals, due to their beautiful flowers and *Cymbidium* is historically and commercially an important orchid since it was the first ever orchid to be propagated *in vitro* [1, 2]. To meet the demand of this orchid in the future, new technologies *in vitro* condition is very important. Plant growth regulators (PGRs) are one of the most effective means of controlling plant organogenesis *in vitro* [2]. Plant growth regulators (PGRs) are molecular structures with a specific configuration in order to join specific receptors, thus transmitted to the cell patterns of development and differentiation that must be followed. The modification of the culture medium in concentration and type permitted a wide variety of species a significant increase in both biomass production and on the accumulation of

secondary metabolites [3, 4]. PGRs such as 6-benzylaminopurine (BA) is a first-generation synthetic cytokinin that elicits plant growth and development responses, setting blossoms and stimulating fruit richness by stimulating cell division [5]. BA improves plant regeneration of several orchids, such as *Cymbidium* [6, 7], *Dendrobium* [8], *Cattleya* [9], *Vanda* [10] orchids; because of its ability to induce organogenesis. Recently in several studies, hyaluronic acid (HA) has been reported to act as a plant growth regulator and considered to elicit the induction of plant defense mechanisms in many plants [11]. One kind of elicitor HA positively affects the number of PLBs in *Cymbidium* and *Dendrobium* *in vitro* [2, 7, 8, 12, 13]. Hyaluronic acid (HA), is a naturally occurring biopolymer, which serves important biological functions in bacteria and higher animals including humans. The functions of HA include

cell adhesion and migration, dynamic process that are mediated through interaction with extracellular matrix components, regulation of protein secretion, gene expression and cell proliferation and differentiation [14, 15]. Bacterial fermentation methods for large-scale economic production of HA have been well standardized as HA is used in medical and cosmetic industry extensively [16, 17]. Although there are several reports found on single addition of HA and BA can support the induction and development of PLBs of different orchids *in vitro*, but the combination of cytokinin (BA) and elicitor (HA) on PLBs culture of *Cymbidium* spp would not be done. These study reports focused on combination of HA and BA, to assess its effect on PLB and shoot formation of *Cymbidium insigne* and *Cymbidium finlaysonianum* *in vitro*. However, the combinations, concentrations and the ratio of plant growth regulators (PGRs) are critically important for *in vitro* culture techniques.

MATERIALS AND METHODS

Plant Materials: Protocorm-like bodies (PLB) of *C. insigne* and *C. finlaysonianum* were proliferated in the modified Murashige and Skoog [6] medium by transferred new medium every two months. After excision of PLB (ca. 3 mm in diameter) into pieces, they were used for explants.

Plant Growth Regulators: 6-benzylaminopurine (BA; Sigma, USA) and hyaluronic acid (HA 9; Shiseido, Japan) were used as plant growth regulators.

Culture Conditions: MS medium with 412.5 mg/l ammonium nitrate, 950 mg/l potassium nitrate, 20 g/l sucrose and 2 g/l Phytigel (Sigma) were used. After preparing the media pH was adjusted 5.5-5.8 using 0.1mM 2-(N-morpholino) ethanesulfonic acid sodium salt (MES-Na) before autoclaved at 121° C for 15 minutes. BA and HA9 at concentrations of 0, 0.1, 1 and 10 mg/l, were added to culture media before sterilization. Jars of 250 ml (UM culture bottle, as one, Japan) with plastic caps were used, each bottle receiving 30 ml of medium. Five *in vitro* PLBs were placed in each culture vessel and three culture vessels were used for each treatment. All cultures were maintained at 25±1° C under 16 hour photoperiod illuminated with white fluorescent tube (National FL20SS; 54 µmole m⁻² • s⁻¹) during 6 weeks.

Data Analysis: Experimental data were collected after 6 weeks of culture (when root formation would be occurred) by counting the number of PLB and shoot; percentage of PLB and shoot and the fresh weight of PLB were measured. The experiment was randomized design and was performed by taking 15 replicates for each treatment. Data were statistically analyzed by calculating standard error of the means (means±SE).

RESULTS

Effect of 6-benzylaminopurine (BA) and hyaluronic acid (HA9) on Organogenesis Responses from PLB of *Cymbidium insigne*: Combination treatment of 6-benzylaminopurine (BA) and hyaluronic acid (HA9) with modified MS medium on organogenesis in PLB of *C. insigne* after 6 weeks of culture is shown in Table 1 and Fig 1. The results revealed that the combination of BA and HA had effect on different parameters studied in *C. insigne*. The highest percentage of PLB formation rate 93% and shoot formation rate 73% were observed when medium containing 0.1 mg/l BA+ 0.1 mg/l HA whereas lowest formation rate of PLB was found at high concentration of BA and HA (10 mg/l BA+10 mg/l HA) treatment. The maximum average number of PLBs (7.1 PLBs/explant), shoots (1.7 shoots/explant) and maximum fresh weight of PLBs (74.1 mg) were obtained at 0.1 mg/l BA+ 0.1 mg/l HA treatment.

Effect of 6-benzylaminopurine (BA) and hyaluronic acid (HA9) on organogenesis responses from PLB of *Cymbidium finlaysonianum*: In *C. finlaysonianum* result revealed that combination of 6-benzylaminopurine (BA) and hyaluronic acid (HA9) with modified MS medium had effect on different parameters studied (Table 2, Fig 2). The highest percentage of PLB formation rate 93% and the maximum average number of PLBs (30.8 PLBs/explant) were observed on medium containing 0.1 mg/l BA + 1 mg/l HA, whereas the lowest formation rate (20%) was found at 10 mg/l BA + 10 mg/l HA treatment. The highest shoot formation rate 53% observed at single addition of BA (1 mg/l BA+ 0 HA) and single addition of HA (0 BA + 1 mg/l HA) treatment. The maximum average number of shoots (1.3 shoots/explant) and the maximum fresh weight of PLBs 342.5 mg was obtained at 1 mg/l BA with modified MS medium.

Combination Treatment of 6-Benzylaminopurine (BA) and Hyaluronic Acid (HA) on PLB Culture of *Cymbidium* spp. *in vitro*



Fig. 1: Effect of 6-benzylaminopurine (BA) and hyaluronic acid (HA9) on organogenesis in PLB culture of *Cymbidium insigne* after 6 week of culture. A: Control; B & C: 0.1 mg/1 BA+ 0.1 mg/1 HA; Bar: 10 mm

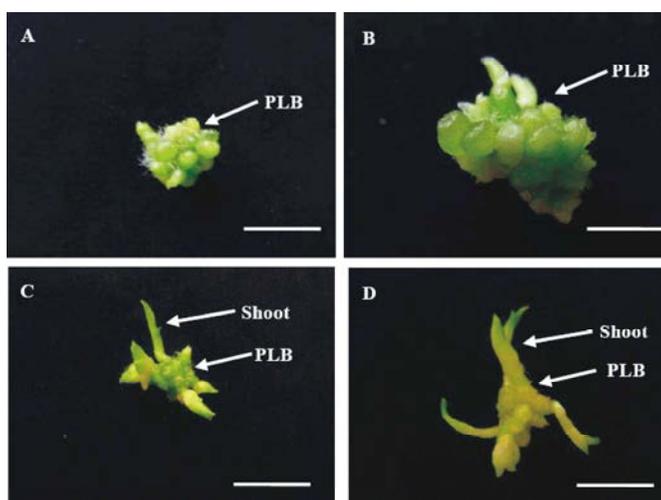


Fig. 2: Effect of 6-benzylaminopurine (BA) and hyaluronic acid (HA9) on organogenesis in PLB culture of *Cymbidium finlaysonianum* after 6 week of culture. A: Control; B : 0.1 mg/1 BA+ 1 mg/1 HA; C: 1 mg/1 HA; D: 1 mg/1 BA; Bar: 10 mm

Table 1: Combination effect of 6-benzylaminopurine (BA) and hyaluronic acid (HA9) at different concentrations on *in vitro* PLBs and shoots formation of *Cymbidium insigne* after 6 weeks

Treatment (mg/l)		PLB		Shoot		Fresh weight (mg)
BA	HA	No./explant	Rate (%)	No./explant	Rate (%)	
0.0	0.0	1.6±0.8	53	0	0	18.9±3.6
0.0	0.1	2.4±0.5	80	0.3±0.3	27	29.5±0.5
0.0	1.0	2.1±1.2	60	0.4±0.5	20	29.9±10.1
0.0	10.0	2.3±1.0	67	0.3±0.4	20	33.7±8.5
0.1	0.0	2.2±0.7	73	0.6±0.6	27	30.7±4.7
0.1	0.1	7.1±1.9	93	1.7±0.3	73	74.1±12.6
0.1	1.0	2.1±0.5	80	0.4±0.4	27	36.4±5.0
0.1	10.0	1.7±0.6	60	0.7±0.4	40	38.2±6.9
1.0	0.0	0.8±0.5	40	0	0	28.1±4.2
1.0	0.1	1.0±0.5	47	0.4±0.5	20	39.7±8.2
1.0	1.0	1.3±0.5	60	0	0	24.9±4.9
1.0	10.0	1.3±0.7	47	0	0	30.2±7.2
10.0	0.0	0.9±0.4	47	0.4±0.3	33	23.4±4.2
10.0	0.1	1.4±0.6	53	0	0	28.3±5.5
10.0	1.0	1.9±0.6	73	0.2±0.2	20	37.4±5.2
10.0	10.0	0.9±0.9	33	0.3±0.3	20	16.3±6.0

Data were statistically analyzed by calculating standard error of the means (means±SE)

Table 2: Combination effect of 6-benzylaminopurine (BA) and hyaluronic acid (HA9) at different concentrations on *in vitro* PLBs and shoots formation of *Cymbidium finlaysonianum* after 6 weeks

Treatment (mg/l)		PLB		Shoot		
BA	HA	No./explant	Rate (%)	No./explant	Rate (%)	Fresh weight (mg)
0.0	0.0	5.0±2.8	47	0	0	60.8±17.3
0.0	0.1	10.1±4.2	73	0	0	93.4±23.2
0.0	1.0	4.7±1.2	73	0.8±0.3	53	49.8±9.1
0.0	10.0	5.2±2.1	73	0	0	42.9±12.7
0.1	0.0	18.5±5.4	80	0	0	153.8±41.6
0.1	0.1	15.3±4.1	80	0	0	111.1±30.4
0.1	1.0	30.8±7.2	93	0.7±0.5	33	251.4±58.0
0.1	10.0	15.9±5.3	73	0	0	124.2±37.2
1.0	0.0	22.3±6.5	73	1.3±0.7	53	342.5±91.8
1.0	0.1	4.5±4.2	27	0	0	52.1±13.2
1.0	1.0	17.6±5.5	67	0.7±0.5	33	312.3±93.7
1.0	10.0	21.3±3.3	87	0	0	280.7±46.5
10.0	0.0	12.2±4.7	67	0.5±0.4	27	153.0±46.6
10.0	0.1	13.1±6.9	67	0	0	159.9±72.2
10.0	1.0	14.3±8.1	47	0	0	133.6±48.4
10.0	10.0	4.3±5.8	20	0	0	48.9±28.2

Data were statistically analyzed by calculating standard error of the means (means±SE)

DISCUSSION

Tissue culture offers an excellent technique for rapid multiplication of plants. The main aim of this study was to find the effect of combination concentration of cytokinin and elicitor in *Cymbidium* tissue culture. In this study a report explained that combination of BA and HA increases new PLB and shoot in *Cymbidium* tissue culture. According to this study, in *C. insigne*, low concentration of BA and HA induced maximum formation (93%) of PLB and shoot. The highest number of PLB and shoot also increased at low concentration of BA and HA (0.1 mg/l+ 0.1 mg/l) added with modified MS medium within 6 weeks of culture. Another study Nahar *et al.* [18] on *C. insigne* reported that 73% PLB formation and 67% shoot formation were found on 1 mg/l BA treatment. But high concentration of BA (10 mg/l) induced less formation of PLB (13%) and shoot (13%). Similar results was found at this study that in *Cymbidium insigne* high concentration of BA and HA (10 mg/l+10 mg/l) had lowest formation of PLB (33%) also in *C. finlaysonianum* lowest formation of PLB (20%) observed at high concentration of BA and HA and there was no shoot formation observed at 10 mg/l BA + 10 mg/l HA treatment. Kaewjampa *et al.* [7] reported that in *Cymbidium* Walt 'Idol' shoot formation significantly promoted at BA 1.0 mg/l, similar results observed at this study that 1 mg/l BA (single addition) induced maximum shoot formation. Shimasaki and Fukomoto [19] suggested that benzylaminopurine is

effective for adventurous shoot regeneration from hypocotyls segments of snapdragon. In *C. finlaysonianum*, single addition of HA (1 mg/l) also induced highest formation of shoot (53%). Among all the concentrations and combinations, 0.1 mg/l BA+0.1 mg/l HA were found to be the most effective combination concentration for PLB, shoot formation and maximum fresh weight of PLBs of *Cymbidium insigne*. In *C. finlaysonianum* 0.1 mg/l BA + 1 mg/l HA was found to be effective for maximum number of PLBs, highest formation of PLB and shoot formation was effective for single addition of BA and HA treatment.

Culture media is commonly supplemented with plant growth regulators (e.g. auxins and cytokinins) to control the proliferation of PLBs and formation of shoots in Orchidaceae plants *in vitro* [20, 7, 21]. Previous reports found that both auxin and cytokinin (including the ratios of auxin/cytokinin) play a role in organogenesis during *Cymbidium* micropropagation. However, achieving acclimatization in *Cymbidium* spp. is difficult since a high concentration of BA (6-Benzylaminopurine) in culture medium is known to increase mutation rates in regenerated plants [22, 23]. The optimum shoot multiplication and regeneration of healthy, vigorous shoots is dependent on plant growth regulators and media supplements used. In general, the balance between auxins and cytokinins is critical for shoot proliferation. Cytokinins at higher concentrations have deleterious effect on shoot growth, while the presence of auxins can

nullify the suppressive effect of cytokinins on shoot growth [24]. In the present study, during 6 weeks of culture, there was no malformation observed at regenerated shoots. HA has been one of agents for enhancing secondary metabolite production in plant tissue culture which elicit systemic resistance in several plants [11]. Elicitors generally refer to the agents that stimulate the defense responses of plants and 6-benzylaminopurine (BA) is well known plant growth regulator and widely used for micropropagation of orchids. Micropropagation of orchids in large scale have to be developed further because of commercial value and conserve them. The efficiency of type and concentration of plant growth regulators varied with kind of species or varieties [25]. The proper ratio of plant growth regulator is required for organogenesis. Our present study reports establish a new protocol on organogenesis of *Cymbidium* spp. through the combination treatment of 6-benzylaminopurine (BA) and hyaluronic acid (HA) *in vitro*.

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