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Elicitors (Chitosan and Hyaluronic Acid) Affect Protocorm-Like Body Formation in Hybrid *Cymbidium*

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ABSTRACT

Protocorm-like body (PLB) production from hybrid *Cymbidium* Twilight Moon 'Day Light' was examined on a new medium, Teixeira *Cymbidium* (TC) medium in response to chitosan and other elicitors. TC_{PLB}, the control medium specific for induction of PLBs, contained a unique set of micro- and macro-nutrients, 0.1 mg/l α-naphthaleneacetic acid and 0.1 mg/l kinetin, 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar. Crude deacetylated chitin (i.e., chitosan), chitosan and hyaluronic acid (HA) had a pronounced effect on PLB formation, depending on the concentration, when added to TC medium without plant growth regulators (PGRs). In all cases, the performance was never better than TC, although 0.1-1.0 mg/l chitosan or HA produced more PLBs/PLB and a greater fresh weight than TC basal medium without PGRs. This indicates that both these elicitors could induce PLBs and thus acted as phytohormones. Peroxidase activity, a signal of stress induction, increased in PLB extracts of PLBs grown on medium containing chitosan and HA, peaking when these elicitors were supplied at 50 mg/l.

Keywords: basal medium, chitosan, hyaluronic acid or hyaluronan, PLB, Teixeira *Cymbidium* (TC) medium
Abbreviations: HA, hyaluronic acid; **NAA**, α-naphthaleneacetic acid; **PLB**, protocorm-like body; **PGR**, plant growth regulator; **TDZ**, thidiazuron (*N*-phenyl-N-1,2,3-thidiazuron-5'-ylurea); **VW**, Vacin and Went

INTRODUCTION

Chitosan, obtained primarily from the exoskeletons of crustaceans, but also found in cuticles of insects as well as in the cell walls of fungi and some algae, is one of the most common natural polymers that can be found. It is a polysaccharide derived from a low acetyl form of chitin, mainly composed of glucosamine and *N*-acetyl-glucosamine, and has a structure and composition similar to both cellulose and chitin (Hadwiger and McBride 2006). Its unique physiological and biological properties have allowed it to be used in a variety of industries: a) bioremediation and waste removal; b) cosmetics, including moisturizers, bath lotions, and face, hand and body creams; c) pharmacy, especially controlled release of drugs, control of blood cholesterol; d) medicine, such as tissue engineering (Kim *et al*. 2008); e) agriculture as a coating material for fruits, seeds and vegetables (e.g., Photchanachai *et al*. 2006), for controlled agrochemical release of fertilizers, to stimulate plant immune systems, plant growth and plant production and also to protect plants against attack by microorganisms (e.g., Nge *et al*. 2006; Yin *et al*. 2010), or to elicit secondary metabolite production (e.g., Karwasara *et al*. 2010). Chitosan is an exogenous elicitor of response mechanisms and has been demonstrated to induce plant defences in many crops (reviewed in Uthairatanakij *et al*. 2007). Based on that review and on Bautista-Baños *et al*. (2006), when chitosan is used in many circumstances, a chain of events allows it to be considered as an elicitor: 1) an increase in phenolic substances following chitosan and chitin oligomers application; 2) a subsequent increase in the activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL), the key enzymes of the phenylpropanoid pathway; 3) modification of the products of PAL and TAL via the phenylpropanoid pathway to produce precursors of secondary metabolites, including lignin, flavonoid pigments, and phytoalexins, which play an important role in plant-pathogen interactions; 4) phytoalexin production, suggesting that chitosan may be involved in the signalling pathway for the biosynthesis of phenolics; 5) chitosan can induce chitinase and chitosanase, which are members of a group of plant pathogenesis-related (PR) proteins. Even though chitosan has had a strongly positive effect on many growth responses in a number of plants, including orchids (Uthairatanakij *et al*. 2007) the mechanism of action remains unknown. Of relevance to this study are the few studies that have been conducted with *in vitro* regeneration of orchids. The formation of *Dendrobium* Sonia Jo 'Eiskul' protocorm-like bodies (PLBs) was enhanced when 10-20 mg/l of 70-90% deacetylated chitosan was used while all other forms, molecular weights and concentrations bleached and killed off PLB formation (Pornpienpakdee *et al*. 2010). Nge *et al*. (2006) studied the effects of chitosan of various molecular weights on the growth of *Dendrobium phalaenopsis* PLBs *in vitro*. The application of shrimp chitosan with a molecular weight of 1 kDa accelerated the growth and development of the meristematic tissue of orchids from PLB explants in liquid media, more than 10 and 100 kDa chitosan. Fungal chitosan (10 kDa) at 15 mg/l was more effective than shrimp oligomer chitosan at 1 and 10 kDa for increasing growth of orchid PLBs after six weeks of cultivation. Moreover, application of fungal chitosan generated the highest number of orchid plantlets (Nge *et al*. 2006). Chitosan also stimulated PLB development in *Cymbidium finlaysonianum* (Shimasaki *et al*. 2003), although one of the weaknesses of the use of chitosan in *in vitro* tissue culture is its insolubility in media with high pH.

Hyaluronic acid (HA), composed of glucuronic acid and

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N-acetyl glucosamine joined alternately by β-1-3 and β-1-4 glycosidic bonds, has biological functions in lower and higher organisms, including in cell adhesion, migration, proliferation and differentiation, regulation of protein secretion, and gene expression (reviewed by Fraser *et al*. 1997; Kogan *et al*. 2007). HA suppresses disease in cucumber, tomato and pepper (Park *et al*. 2008) and has shown to have antioxidant activity (Ke *et al*. 2011). Polysaccharides (chitosan and HA) in culture media have also been shown to improve orchid (*Cymbidium* Waltz 'Idol' and *Cymbidium dayanum*) organogenesis *in vitro* (Nahar *et al*. 2011; Kaewjampa *et al*. 2012). Most work is in biomedical science and the applications to plant biotechnology remain rare.

This study focused on comparing HA and chitosan (purchased and prepared fresh), to assess its effect on PLB formation.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were of the highest analytical grade available and were purchased from either Sigma-Aldrich (St. Louis, USA), Wako (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), unless specified otherwise.

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan) were originally developed spontaneously from shoot-tip culture on Vacin and Went (VW, 1949) agar medium without PGRs. Then they were induced and subcultured every two months on Teixeira *Cymbidium* (TC) medium (Teixeira da Silva 2012) supplemented with 0.1 mg/l α-naphthaleneacetic acid (NAA), 0.1 mg/l kinetin (Kin), 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA), according to procedures and advice outlined by Teixeira da Silva *et al*. (2005) and Teixeira da Silva and Tanaka (2006). All media were adjusted to pH 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 ml medium in 100 ml Erlenmeyer flasks, double-capped with aluminium foil, at 25°C, under a 16-h photoperiod with a light intensity of 45 $\mu mol/m^2/s^1$ provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally bisected PLB (3-4 mm in diameter) segments, 10 per flask, were used as explants for PLB induction and proliferation and for all treatments as follows: TC, TC without PGRs, TC + chitosan (commercial), TC + chitosan (non-commercial) or TC + HA. Culture conditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva *et al*. 2005), biotic (Teixeira da Silva *et al*. 2006b) and abiotic factors (Teixeira da Silva *et al*. 2006a) for PLB and callus induction, formation and proliferation.

Production of chitosan scaffold

Non-commercial fresh chitosan was prepared according to Entsar *et al*. (2008) with some modification according to El-Mazney *et al*. (2010), and following advice from Maksimov *et al*. (2003), Kim and Rajapakse (2006) and Setthakaset *et al*. (2008) to obtain more pure/purified chitosan from crustaceans. Frozen shrimp (size 26, origin Vietnam, Gyomu) peels were collected, washed, cut into small pieces and boiled in a solution of 1% sodium bicarbonate (Sigma-Aldrich) to which 5% hydrochloric acid (HCl) was added. This was left for 36 h according to Islam *et al*. (2011) to demineralize the shells. The mixture was then filtered through Whatman No. 1 filter paper after the formation of foam. The resulting solid was boiled a second time in a 1% sodium bicarbonate solution (Sigma-Aldrich), for 4 h before being treated with 50% sodium hydroxide (NaOH) (Sigma-Aldrich) for 24 (Islam *et al*. 2011), to transform the chitin to chitosan, i.e., deproteinization of shells. Finally the mixture was poured into excess ethanol for 6 h to eliminate any proteins and traces of colour. One g of chitosan was washed with 100 ml of distilled water, stirred, heated and filtered. The resulting mixture was washed again several times to bring it up to neutrality before being treated with a solution of 1% acetic acid. The treated mixture was then heated, stirred and filtered 5 times through Whatman No. 1 filter paper for higher purity. After cooling, it was placed in an incubator to dry at 60°C for 2 days. The resulting dried chitosan scaffold was broken into smaller pieces and ground into a fine powder using a chilled mortar and pestle, at room temperature, in as a dry a condition as possible. Powdered chitosan (i.e., deacetylated chitin) was stored indefinitely at 4°C in the dark.

Elicitors tested

Chitosan (2-amino-2-deoxy-(1→4)β-D-glucopyranan; water-soluble for plant tissue culture, Wako), self-produced deacetylated chitin (described above), hyaluronic acid (poly(β-glucuronic acid-[1→3]-β-N-acetylglucosamine[1→4]); Sigma-Aldrich) were added to TC basal medium without PGRs at 0.1, 1 and 10, and 50 mg/l.

Morphogenic analysis

The number of PLBs formed per PLB segment was measured. All measurements were made after 45 days in culture (by exceeding 45 days, and if left on the same medium, PLBs tend to induce shoots spontaneously).

Crude enzyme preparation and POX activity

A protocol based on and modified from a cotton protocol was used (Khashimova *et al*. 2013). One PLB explant per treatment, 7 days after explants were plated, was ground in liquid nitrogen, and 1 g of material was extracted with 30 ml of 0.1 M sodium phosphate buffer (pH 6.6). The homogenate was then filtered through Whatman No. 1 filter paper (M-3) and then through a polyamide filter pore size 0.45 mm (Whatman). POX (EC 1.11.1.7) activity was determined according to Khashimova *et al*. (2013). 0.1 ml crude enzyme was diluted with 1.9 ml of 0.01 M sodium phosphate buffer (pH 5.8) and 0.05 ml of a 0.02 M guaiacol in 0.01 M sodium phosphate buffer (pH 6.1 based on Gonçalves *et al*. 1998). Then, 0.05 ml of 0.03% H_2O_2 was added. Assays were initiated by adding H_2O_2 and the change in optical density at 470 nm was measured for 1 min specific POX activity was calculated by the formula (Khashimova *et al*. 2013).

$$A = 2\Delta d / ab \text{ Unit/mg}$$

where A = specific POX activity; Δd = difference in UV absorption from time zero to one minute; a = protein concentration in mg/ml; b = volume of protein sample (0.1 ml).

The total protein concentration was determined by the method of Lowry *et al*. (1951) using bovine serum albumin (BSA, Sigma-Aldrich) as a standard.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment (i.e., each medium). All experiments were repeated in triplicate (n = 90, total sample size per treatment). Data was subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's New Multiple Range test (DNMRT) using SAS® version 6.12 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Chitin, a crystalline polymer, is an important component of fungal pathogenicity since fungal pathogens with defects in chitin synthesis are significantly less virulent on the original susceptible hosts (Soulie *et al*. 2006) and it has enormous tensile strength, thus contributing to the overall integrity of the cell wall (Bago *et al*. 1996). The cell wall becomes disordered and the fungal cell becomes malformed and osmotically unstable when chitin synthesis is disrupted (Specht *et al*. 1996). Chitin is not present in vertebrates or plants, but is abundant in the environment, being a major constituent of arthropod exoskeleton, fungal cell wall, and

Table 1 Effect of chitosan, crude chitosan and HA on PLB formation from half-PLB culture of hybrid *Cymbidium* Twilight Moon 'Day Light'.

Medium composition	Concentration (mg/l)	Percentage of explants forming *neo*-PLBs (%)	Number of PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Peroxidase activity (mmol min^{-1} mg^{-1} protein)
TC (control)		100 a	8.3 a	526 a	23.2 e
TC minus PGRs		100 a	1.2 de	321 bc	28.6 d
Chitosan	0.1	100 a	2.3 d	299 c	27.8 de
(commercial)	1.0	100 a	2.6 d	382 b	31.2 d
	10.0	67 c	4.2 c	301 c	41.4 c
	50.0	18 e	0.8 e	183 d	65.5 a
Chitosan crude	0.1	100 a	1.4 de	285 c	29.1 d
	1.0	81 b	6.8 b	315 bc	46.6 bc
	10.0	46 d	0.9 e	191 d	48.4 bc
	50.0	0 f	0 e	56 f	56.4 b
HA	0.1	100 a	2.1 d	335 bc	30.1 d
	1.0	88 ab	5.4 bc	386 b	31.1 d
	10.0	83 b	4.8 c	481 ab	48.2 bc
	50.0	9 ef	0 e	109 e	47.8 bc

Mean values followed by the same letter in the same column are not significantly different based on DMRT ($P = 0.05$). See text for media constituents. n = 90 (9 Petri dishes × 10 for each treatment).
PGR = plant growth regulator; HA = hyaluronic acid; TC = Teixeira *Cymbidium* medium (Teixeira da Silva 2012), includes 0.1 mg/l α-naphthaleneacetic acid and 0.1 mg/l kinetin, 2 g/l tryptone and 20 g/l sucrose (see reference for modified micro- and macro-nutrients)

helminth eggshell, pharynx and/or the cuticle (Whitrman *et al*. 2011).

Chitin, chitosan and plant (orchid) growth and development

Chitin and chitosan have been shown to stimulate plant growth and development (reviewed by Uthairatanakij *et al*. 2007) and can also serve as a powerful elicitor for the induction of secondary metabolites *in vitro* or in bioreactors (e.g., Sivanandhan *et al*. 2012) but this topic is beyond the scope of our discussion. The discussion in this paper, however, will be limited to orchids. Chitosan at 50 mg/l enhanced *Dendrobium* 'AW 179' plantlet growth *in vitro* (Tantasawat *et al*. 2010) while 60 mg/l enhanced shoot and root parameters of *Rhynchostylis gigantea* (Obsuwan *et al*. 2010a). *In vitro*-derived *Mokara* seedlings treated with 20 mg/l chitosan and *Phalaenopsis* seedlings treated with 10-20 mg/l chitosan performed better than controls (Obsuwan *et al*. 2010b). Kaewjampa *et al*. (2012) found the highest number of *Cymbidium* Waltz 'Idol' PLBs on medium containing 0.1 mg/l HA9 (12.4 PLBs/explant) (1 mg/l HA9 for *Cymbidium dayanum*; Nahar *et al*. 2011) while 6-benzyladenine (BA) and chitosan H at 10 and 0.1 mg/l formed 8.1 and 5.8 PLBs/explant, respectively. In that study, PLBs formed in 100% of all treatments except for the control (PGR-free medium) (82.7%), HA9 at 0.1 mg/l (93.3%) and chitosan H at 10 mg/l (93.3%). HA9 is the product name of sodium hyalunomate produced by Shiseido (Japanese cosmetic company) and depending on the molecular weight (MW), there are three types of HA: 9, 12, and 20. HA9 has a smaller MW than HA12 or HA20. Recently, the names have been changed to HA9N, HA12N and HA20N, respectively (http://ha.shiseido.co.jp/cosmetic/powder.htm). In contrast, our results indicate fewer PLBs in the presence of HA and chitosan (**Table 1**). Chitosan H, which is 84.8% deacetylated, had a much more growth-promotive effect on vegetables that other forms of chitosan that had different levels of acetylation (Fukumoto *et al*. 2003). HA, like chitosan, is an abiotic elicitor that enhances secondary metabolite production in plant tissue culture (Zhou and Wu 2006). Kaewjampa *et al*. (2012) reported that PLBs allowed to develop further in media supplemented with 1 mg/l chitosan H had the highest shoot formation percentage (100%), followed by HA9 and BA (10 mg/l = 85% and 1 mg/l = 80%, respectively). In that study, maximum fresh weight of PLBs was possibly on medium containing BA (451.1-467.3 mg), while the lowest PLB fresh weight was found in media supplemented with 10 mg/l chitosan H (173.1 mg). *n*-Acetyl-glucosamine, also a polysaccharide and elicitor, at 0.01 mg/l improved *Cymbidium insigne* PLB formation 5-fold more than in the control (Shimasaki, unpublished results). Kananont *et al*. (2010) found that almost all types of chitosan at 10 mg/l, except O-90, were able to significantly improve the growth of *Dendrobium bigibbum* var. *compactum* protocorms; 10 or 20 mg/l of P-70 chitosan enhanced the growth of *D. formosum* protocorms; none of the chitosans tested affected seed germination. Chitosan O-80 at 1, 10, 50, and 100 mg/l induced early flowering and increased *Dendrobium* 'Eiskul' floral production (Limpanavech *et al*. 2008).

Chitin, chitinases, hyaluronic acid and induction of a stress-induced enzyme system, POX

When chitin oligomers infiltrate into plant tissue, they causes massive and sometimes analogous innate immune responses, including upregulation of chitinases (Zhang *et al*. 2002; Reese *et al*. 2007) that are detrimental to parasite development (Lawrence end Novak 2006), and likely cause allergies in humans. Thus, elicitors such as chitin that exist in plant parasites can be recognized by and cause parallel immune responses in plant hosts. Plant cells are equipped with chitin-degrading enzymes (chitinases) to digest fungal cell walls and are capable to perceive chitin fragments (chitin oligosaccharides) released from fungal cell walls during fungal infection (Wan *et al*. 2008a). Plants pretreated with chitin produce chitinases and protease inhibitors (Zhang *et al*. 2002) that are ecologically important plant defenses against insects (Lawrence and Novak 2006). Chitin recognition by the plant results in the activation of defense signaling pathways. Chitosan is a deacetylated form of chitin by chitin deacetylase (EC3.5.1.41) and has eliciting activities leading to a variety of defence responses in host plants to microbial infections, including the accumulation of phytoalexins, pathogen-related (PR) proteins, and proteinase inhibitors, lignin synthesis, and callose formation (Adams 2004; Baker *et al*. 2011).

So what is the link between elicitors (chitin, chitosan, HA) and POX? Small, diffusible chitin oligosaccharides can initiate a wide range of biological responses in plants, inducing POX activity. This was clearly evident in the increase in POX activity in media containing chitin relative to control media (TC and TC without PGRs) (**Table 1**), suggesting that there may be a chitin perception and signal transduction pathway in *Cymbidium*, although this theory could be challenged by the fact that TC without PGRs also showed higher POX activity than the control. No such other conclusion has yet been drawn for any other orchid, although chitosan induced POXs in pea (*Pisum sativum*) roots with pI 5.3 and 5.7, which may be specifically related to pathogen defense (Kukavica *et al*. 2012). We suspect that a challenge by a fungus, or another stress, such as salt, or another biological agent, would further increase POX acti-

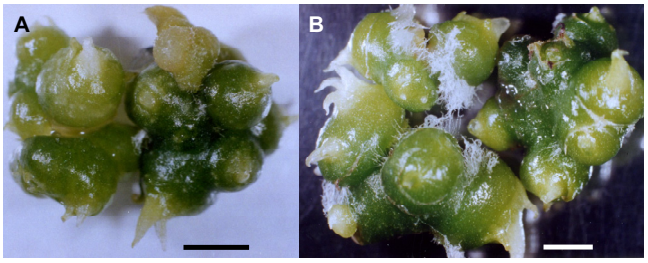

Fig. 1 (**A**) PLB induction on Teixeira *Cymbidium* medium (TC_{PLB}) with PGRs (0.1 mg/l α-naphthaleneacetic acid + 0.1 mg/l kinetin). (**B**) PLB formation on TC_{PLB} without plant gorowth regulators but supplemented with 1.0 mg/l chitosan (commercial, Wako). Bars = 5 mm (**A**), 2.5 mm (**B**).

vity, but this remains to be tested, although initial evidence shows that the addition of PGRs (e.g. TC, which includes PGRs, or the inclusion of HA, a new PGR-like substance, **Table 1**) can induce POX activity, i.e., the addition of PGRs to plant *in vitro* tissue culture medium constitutes in itself a stress. This theory also remains to be tested in greater detail. Caution should be taken when extrapolating the POX data since it represents only a single data point 7 days after PLB explants were plated, and may not reflect the true impact of these elicitors on PLB enzyme stress activity over time. A much more detailed experiment that examines the evolution of POX over the entire experimental period, 45 days, ideally on a daily basis, is required to draw a much stronger conclusion. Plant POXs function in diverse physiological processes including disease resistance and wound response and some POXs produced oxygen species as signal mediators and antimicrobial agents (Schweikert *et al*. 2002). The function of individual POXs may differ from each other, as suggested by their organ-specific or characteristic stress responsive expression profiles. In addition to some POXs, there are other proteins that interact with pectins (Penel and Greppin 1996). Similarly, modified chitin oligosaccharides play a central role in the establishment of a host-specific symbiosis between legumes and their rhizobial symbionts (Cohn *et al*. 1998; Stacey and Shibuya 1997). Khashimova *et al*. (2013) found that POX activity increased in presence of cotton-derived chitin, leading them to conclude that cotton POX, which adsorbed to chitin, contained sites that specifically interact with chitin acetyl residues. Chitooligosaccharides are a potent regulator of plant gene expression (e.g., Wan *et al*. 2008b). Chitosan induced hydrogen peroxide resulting in the increase of peroxidase activity (Yin *et al*. 2012). The role of POX in formation of diphenyl bridges, cross-linking of hydroxyproline-rich proteins (extension) in the cell wall matrix and during stress related physiological processes is well founded (Low and Merida 1996; Asthir *et al*. 2010). IBA and water stress dipping treatments increased POX activity in chestnut roots *in vitro* (Gonçalves *et al*. 1998) while colchicine caused a POX burst in chrysanthemum (Liu *et al*. 2011). HAs, which demonstrate antioxidant activity *in vitro* (Ke *et al*. 2011), but so far only demonstrated for non-plant systems, may be responsible for the lower POX activity at 1 mg/l whereas the POX activity was significantly higher when the same concentration of chitosan or deacetylated chitin were used. Peroxidase has also been shown to stimulate the growth of hybrid aspen (*Populus sieboldii* X *Populus grandidentata*) (Kawaoka *et al*. 2003).

CONCLUSIONS

Chitosan and hyaluronic acid have the ability of inducing new PLBs in hybrid *Cymbidium* in a medium lacking plant growth regulators (**Fig. 1B**), suggesting that they themselves also act as a form of phytohormone, although the levels of production are lower than the optimized medium, TC (**Fig. 1A**).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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