Chondroitin Sulfate Can Be a New Plant Growth Regulator for *Cymbidium* Micropropagation©

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Chondroitin sulfate is unbranched polysaccharides of variable length containing two alternating monosaccharides: D-glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc). It is a major component of extracellular matrix, and is important in maintaining the structural integrity of the tissue. It is widely used as a material for food ingredient, cosmetics, and medicine. The present study was conducted to investigate the effect of chondroitin sulfate on in vitro regulation of protocorm-like bodies (PLB) of *Cymbidium insigne* under different quality of light (white fluorescent tube, green LED, and red LED). Recently, LEDs have drawn considerable interest as an alternative light source for in vitro propagation. New PLB and shoots were successfully regenerated on modified Murashige and Skoog medium supplemented with chondroitin sulfate under different lights. The highest PLB formation and shoot formation rate (80%) were found amongst explants cultured on medium supplemented with 0.1 mg·L^{-1} chondroitin sulfate under white fluorescent light. The chondroitin sulfate at every concentration enhanced the growth of PLB formation rate (100%) and the shoot formation rate 90% found at 1 and 10 mg·L^{-1} chondroitin sulfate under green LED. The highest percentages of PLB formation was 100% and shoot formation was 90% obtained from the medium supplemented with 0.1 mg·L^{-1} chondroitin sulfate under red LED, but the color of new PLB and shoots was not green. These results indicated that chondroitin sulfate would work as a plant growth regulator which enhances both PLB and shoot formation and this newly developed light source could be used as an energy efficient light source for the propagation of *Cymbidium* PLB in vitro and comparatively green light showed best formation of PLB and shoot.

Cymbidium are among the most important and popular orchids in horticulture. *Cymbidiums* are easy to grow, undoubtedly one of the main reasons for their popularity in horticulture (Du Puy and Cribb, 2007). Thus, many attempts have been made to develop methodologies, culture media, and culture conditions for the micropropagation of *Cymbidium* (Morel, 1960; Shimasaki and Uemoto, 1990; Hamada et al., 2010; Hossain et al., 2010). Considering its high potential in the ornamental market, a reliable propagation method for large-scale production is therefore important for its commercialization. The use of polysaccharides as a plant growth regulator for orchid plants has attracted considerable interest in recent years (Kaewjampa et al., 2010; Nahar et al., 2011, 2012) because it is widely available and generally viewed as a safe material for humans and the environment.

Chondroitin sulfate is an unbranched polysaccharides of variable length containing two alternating monosaccharides: D-glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc). It is usually found attached to proteins as part of a proteoglycan. Chondroitin sulfate is an important structural component of cartilage and provides much of its resistance to compression (Baeurls et al., 2009). Along with glucosamine, chondroitin sulfate has become a widely used dietary supplement for treatment of osteoarthritis. The

study also found chondroitin sulfate to have a significant effect in reducing joint swelling, effusion, or both. It is widely used as a material for food ingredients, cosmetics, and medicine (Toida, 2007).

On the other hand, light-emitting diodes (LEDs) have been considered as a novel radiation source for growth and development of plant species because of several unique characteristics (Tanaka et al., 1998). In recent years, the effects of the light spectra on the growth and development of orchids have attracted considerable interest. The LEDs have features which are far better than the commonly used radiation sources – fluorescent, metal halide, high pressure sodium, and incandescent. The most attractive features of LEDs are small mass, volume, solid state construction, and long life (Brown et al., 1995). Because of these unique characteristics, LEDs may be suitable for the culture of plants in a tightly controlled environment such as space-based plant culture systems (Barta et al., 1992). In an earlier paper, we reported the enhanced growth of *C. insigne* plantlets cultured in vitro with modified Murashige and Skoog (1962) (MS) medium and low concentration of chitosan and hyaluronic acid under different light sources (Nahar et al., 2012). The objective of this study was to investigate the effects of chondroitin sulfate with modified MS medium under different LED light sources on *C. insigne* in vitro. A secondary objective was to identify the most effective light for the rapid propagation of *Cymbidium* plantlets in vitro.

MATERIALS AND METHODS

Plant Material, Culture Media, and Culture Conditions

Protocorm-like bodies (PLB) approximately 5 mm in length derived from meristem cultures of *C. insigne* which proliferated on modified MS medium (Shimasaki and Uemoto, 1990) served as explants. The MS medium with 412.5 $mg \cdot L^{-1}$ ammonium nitrate, 950 $mg \cdot L^{-1}$ potassium nitrate, 20 $g \cdot L^{-1}$ sucrose, and 2 $g \cdot L^{-1}$ Phytagel™ powder (Sigma) was adjusted to pH 5.5- 5.8 before autoclaving. Chondroitin sulfate (Coach Industries Inc., Japan) at concentrations of 0, 0.1, 1, and 10 $mg \cdot L^{-1}$, 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 explants were placed in each culture vessel and two culture vessels were used for each treatment. All cultures were maintained at 25°C under different intensity of light such as white fluorescent tube, green LED (55 $\mu mole \cdot m^{-2} \cdot s^{-1}$), and red LED (55 $\mu mole \cdot m^{-2} \cdot s^{-1}$) during 16-h photoperiod for 8 weeks.

Data Analysis

Experimental data were collected after 8 weeks of culture by counting the number of PLB and shoots; percentage of PLB and shoots, and measuring the fresh weight of PLB. The data was statistically analyzed by calculating standard errors of the means (means ±SE, n=10). Average number of PLB: number of PLB/one PLB explants.

$$\text{Percentage of formation} = \frac{\text{Number of culture explants with new PLB/shoots}}{\text{Total number of culture explants}} \times 100$$

RESULTS

Effect of Chondroitin Sulfate on PLB and Shoot Formation

Chondroitin sulfate at every concentration in *C. insigne* growing media enhanced the growth of PLB and shoot formation when compared to control treatment as shown in Table 1. Chondroitin sulfate clearly stimulated PLB growth and induced shoot formation (Fig. 1). After 8 weeks of culture, the order of increase in the average number of PLBs under white fluorescent tube and green LED was as follows: chondroitin sulfate 1 $mg \cdot L^{-1}$ >0.1 $mg \cdot L^{-1}$ >10 $mg \cdot L^{-1}$ >control. The highest average number of shoot was 1.8 shoots/explants whereas control treatment showed 0.3 shoots/explants; the greatest

number of shoots was found on the medium supplemented with 0.1 mg·L^{-1} chondroitin sulfate under white fluorescent tube. Under red LED, the color of new PLB and shoots was not green and the order of increase in the average number of PLB and shoots was as follows: chondroitin sulfate 0.1 mg·L^{-1} >1 mg·L^{-1} > 10 mg·L^{-1} > control, the higher fresh weight (272 mg) of PLB also found under red LED (Table 1).

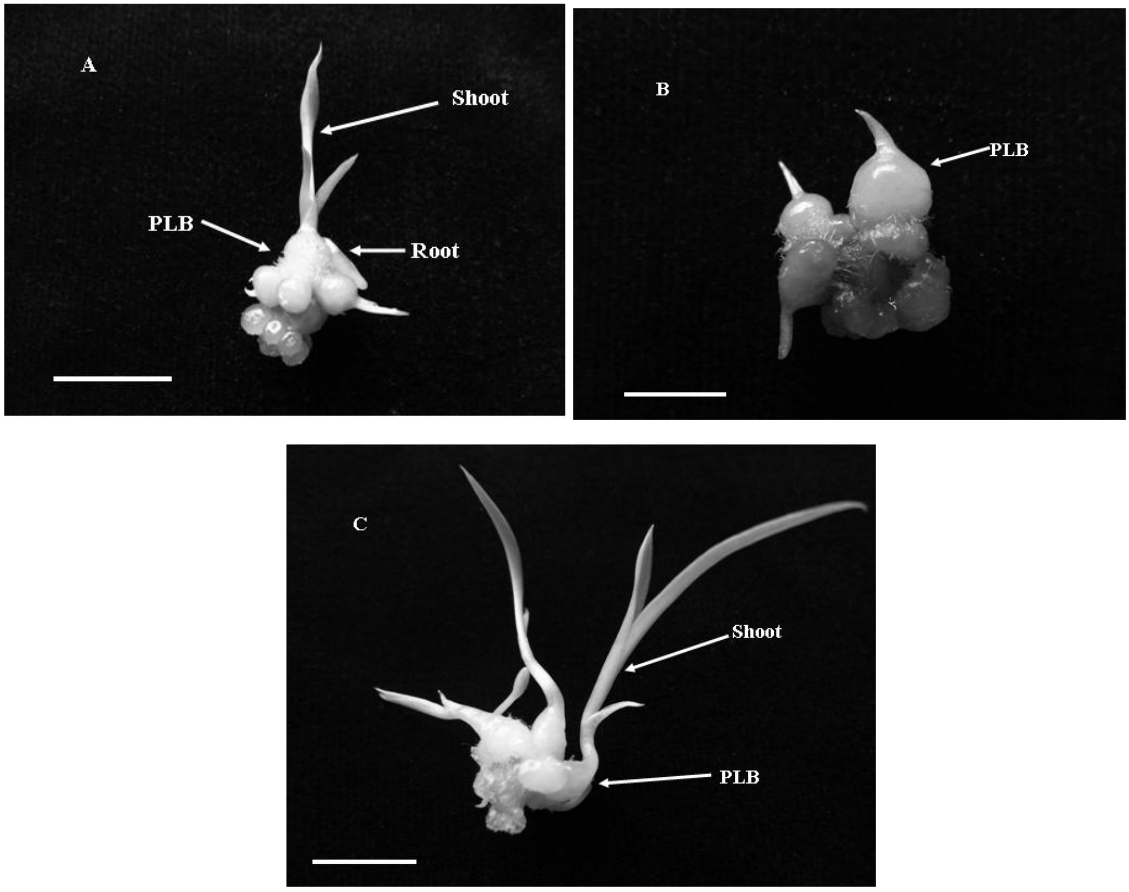

Fig. 1. Effects of chondroitin sulfate in modified MS media on organogenesis in PLB of *Cymbidium insigne* after 8 weeks of culture. A: 0.1 mg·L^{-1} chondroitin sulfate under White fluorescent tube; B: 1 mg·L^{-1} chondroitin sulfate under green LED; C: 0.1 mg·L^{-1} chondroitin sulfate under red LED (Bars= 10 mm).

Effect of Light Quality on PLB, Shoot and Root Formation

The growth and development of PLB of *C. insigne* were markedly affected by different light treatments in vitro. Under white fluorescent light, the highest percentage of PLB and shoot formation rate of 80% was observed on the medium containing 0.1 mg·L^{-1} chondroitin sulfate whereas control treatment showed 40 and 30% formation rate respectively within 8 weeks of culture (Fig. 2). Under green LED, almost every concentrations of chondroitin sulfate with modified MS medium produced the highest percentage of PLB formation rate of 100% and the maximum shoot formation rate of 90% was observed with media containing 1 and 10 mg·L^{-1} chondroitin sulfate with the lowest formation rate found in the control treatment (Fig. 3). On the other hand, the highest percentage of PLB formation was 100% and shoot formation was 90% obtained from the medium supplemented with 0.1 mg·L^{-1} chondroitin sulfate under red LED, but the color of new PLBs and shoots were not green (Fig. 4).

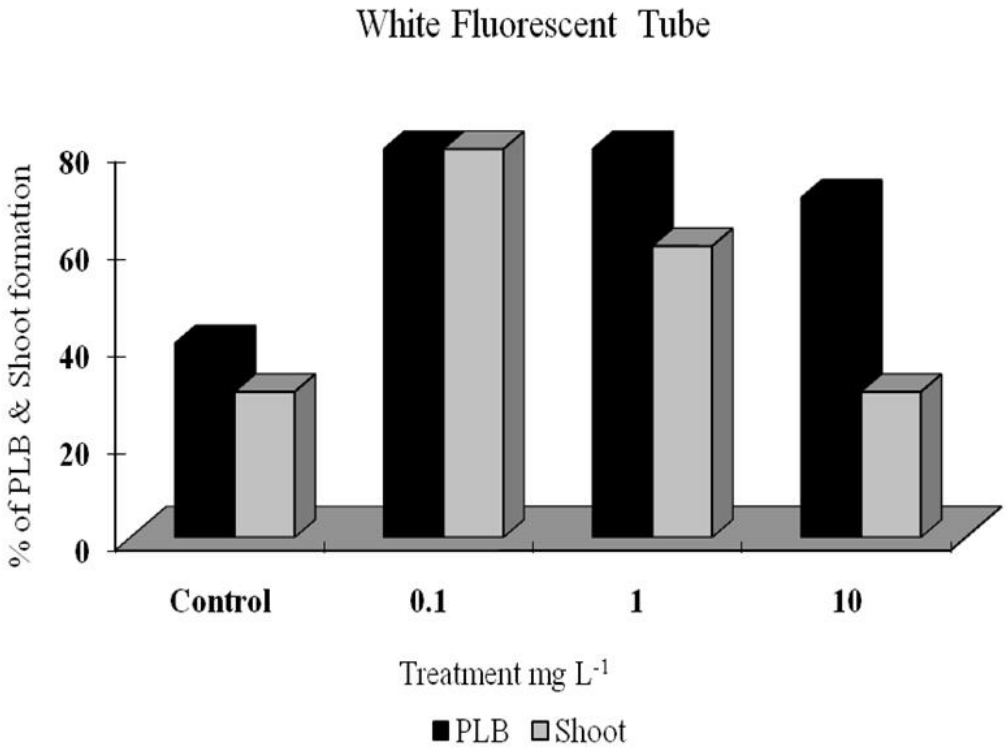

Fig. 2. Effects of chondroitin sulfate on the growing percentage of new PLB and shoot formation under white fluorescent tube.

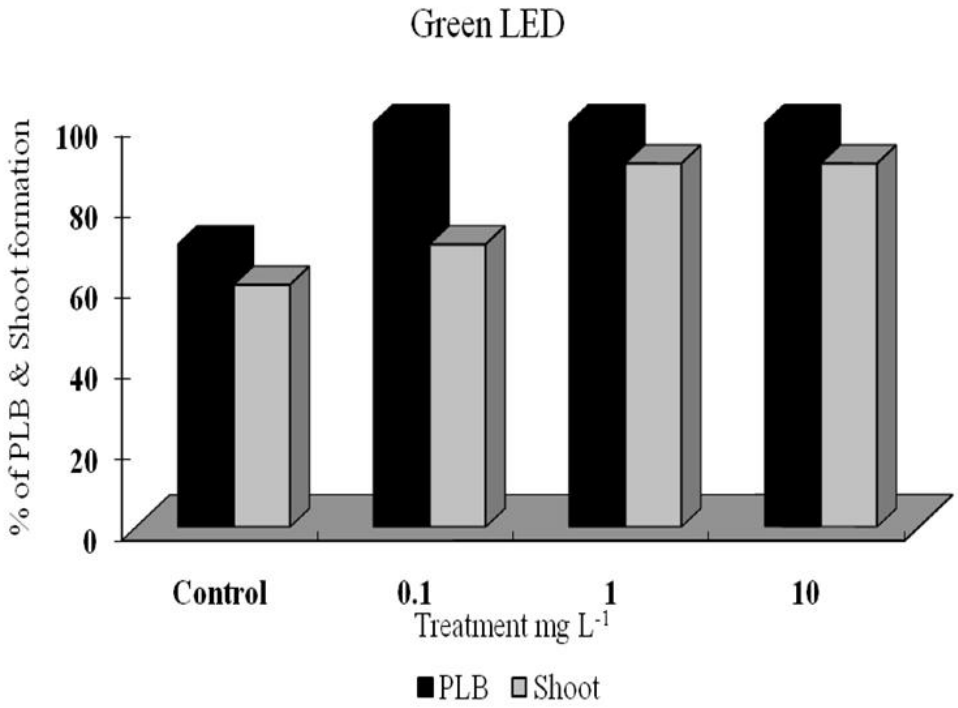

Fig. 3. Effects of chondroitin sulfate on the growing percentage of new PLB and shoot formation under green LED.

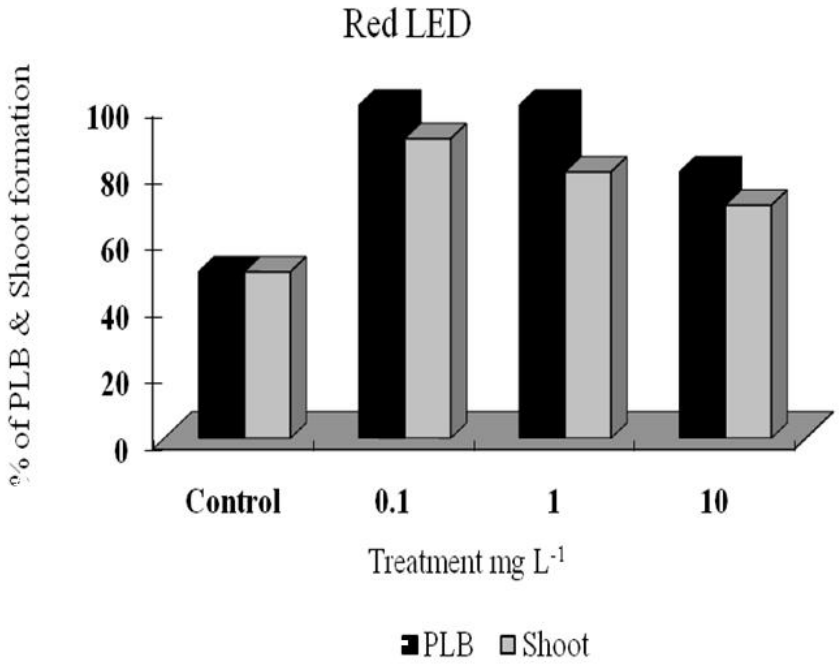

Fig. 4. Effects of chondroitin sulfate on the growing percentage of new PLB and shoot formation under red LED.

DISCUSSION

Chondroitin sulfate is a major component of extracellular matrix, and is important in maintaining the structural integrity of the tissue. The extracellular matrix can serve many functions, such as providing support, segregating tissues from one another, and regulating intercellular communication, due to its diverse nature and composition. Chondroitin sulfate contributes to the tensile strength of cartilage, tendons, ligaments, and walls of the aorta (Hensch, 2005). Recently, the use of chondroitin sulfate for the symptomatic treatment of osteoarthritis has become very popular. On the other hand, there is abundant in vitro and in vivo evidence from animal and human clinical studies demonstrating the efficacy and safety of chondroitin sulfate (Toida, 2007). This is the first report demonstrating chondroitin sulfate as a new plant growth regulator for growing new PLB and shoot formation in *Cymbidium* tissue culture. The mechanisms by which chondroitin sulfate elicits plant growth are not yet understood fully. These experiments indicated that if chondroitin sulfate is added to culture media it acts as a plant growth stimulator to induce PLB and shoot formation of *C. insigne*. During 8 weeks of culture, there was no malformation observed in regenerated shoots. We have hypothesis that the effect of chondroitin sulfate on orchid tissue culture is partly depend on the action of an elicitor. The present study demonstrated the potential of another polysaccharide, chondroitin sulfate, to induce PLB and shoot formation in *Cymbidium* orchid plants. When compared to the control, very low concentrations of chondroitin sulfate with modified MS medium produced maximum percentage of PLB and shoot formation within 8 weeks of culture under different light sources (Table 1).

Light emitting diodes (LEDs) represent a fundamentally different technology from the gaseous discharge-type lamps currently used in horticulture and has a number of advantages when compared to traditional forms of lighting. These optoelectronic devices feature high radiant efficiency, long lifetime, cool emitting temperature, relatively narrow emission spectra, short switching time, and contain no mercury as some conventional light sources do (Massa et al., 2008; Morrow, 2008). In this study, we showed that green light with low concentrations of chondroitin sulfate could promote PLB and shoot formation in *C. insigne*. Comparing the three light treatments, green LEDs appear to

provide the best light conditions for promoting new PLB and shoots with *C. insigne*. In other studies with *Cymbidium* orchid cultures, red LEDs were the most effective light source for inducing callus tissue from PLB (Huan and Tanaka, 2004), but this study showed the results that green LEDs had good effects on new PLB and shoot formation. In this study, the highest percentage of PLB and shoot formation occurred under green light treatment (Fig. 2) and also the highest number of PLB found under green light with chondroitin sulfate (1 $mg \cdot L^{-1}$) of *C. insigne* but the highest number of shoot observed under white light with chondroitin sulfate treatment (0.1 $mg \cdot L^{-1}$ with modified MS media). Similar results were obtained on our earlier paper that green LEDs promote the proliferation of protocorm-like bodies of *C. insigne* in vitro by using chitosan and hyaluronic acid under different lights (Nahar et al., 2012) and also in strawberry plants, the growth and enlargement of fruit of strawberry plants were promoted by green light irradiation (Kudo et al., 2009).

Table 1. Effects of chondroitin sulfate and different light quality on organogenesis responses from PLB of *Cymbidium insigne* after 8 weeks of culture.

Chondroitin ($mg\ L^{-1}$)	Light quality	PLB No./explants	FW (mg)	Shoot No./explants
Control	White fluorescent	1.5±1.0	40.0±8.0	0.3±0.3
	Green LED	2.2±0.7	89.0±16.2	0.8±0.3
	Red LED	1.3±0.6	45.0±11.3	0.5±0.2
0.1	White fluorescent	2.0±0.6	52.0±8.9	1.8±0.4
	Green LED	5.8±1.3	184.0±39.2	0.9±0.3
	Red LED	7.2±2.0	120.0±12.6	1.6±0.2
1	White fluorescent	5.1±1.5	143.0±45.8	0.9±0.4
	Green LED	7.2±0.6	185.0±22.0	1.5±0.3
	Red LED	5.7±1.2	272.0±67.2	1.3±0.3
10	White fluorescent	1.9±0.5	55.0±8.3	0.6±0.7
	Green LED	3.8±0.7	109.0±16.2	1.1±0.2
	Red LED	3.2±0.8	106.0±19.5	0.7±0.2

Each value represents means±SE with 10 PLB samples. Average number of PLBs: number of PLBs/one PLB explants.

CONCLUSIONS

Based on our present study, we concluded that chondroitin sulfate worked as a plant growth regulator which inducing PLB and shoots with very low concentration and green LEDs promote on the proliferation of protocorm-like bodies of *C. insigne* in vitro. Further work is warranted to evaluate the use of chondroitin sulfate in vivo.

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