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Research article

Effect of Chitosan, Coconut Water and Potato Extract on Protocorm Growth and Plantlet Regeneration of *Cymbidium aloifolium* (L.) Sw.

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Abstract

Effective and rapid *in vitro* plantlet regeneration from protocorms of *Cymbidium aloifolium* using chitosan, coconut water and potato extract were investigated. Two experiments were performed using twelve week old protocorms of size 2-3 mm. In the first experiment, the protocorms were propagated on solid New Dogashima (ND) medium supplemented with various concentrations of chitosan (0, 0.05, 0.10 and 1.00 mg/l). For the second experiment, the protocorms were cultured on the same medium supplemented with coconut water (0, 100, 150 and 200 ml/l) and potato extract (0, 5, 10 and 15 g/l). Survival and response percentages, shoot number and length, root number and length, leaf number and fresh and dry weight were collected after 10 weeks of culture. The results showed that 0.05 mg/l chitosan treatment was the most effective for protocorm growth, producing the highest shoot number and length, root number and length, leaf number, and fresh and dry weight. The combination of coconut water and potato extract treatments revealed that 150 ml/l coconut water alone was the best for plantlet regeneration and growth, shoot length, root length, and fresh and dry weight. This treatment gave significantly different results from the other treatments. Findings will provide useful information for other *in vitro* commercial or conservation orchid propagation programs.

1. Introduction

Orchids, one of the most fascinating and beautiful flower producing plant species, belong to the family Orchidaceae [1]. They are globally popular as ornamental plants and grow naturally in some countries [2]. Thailand is renowned for exporting orchids, both as cut flowers and potted plants [3].

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Several species of orchids are cultivated because of their benefits in the medical field and for cultural purposes [4]. The annual export value of orchid flowers and seedlings is over one billion Thai Baht. Recently, the orchid business has drastically increased in terms of quantity and value [5]. There are many genera of economically important orchids including *Cymbidium*, *Dendrobium*, *Epidendrum*, *Spathoglotis* and *Vanda*. *Cymbidium aloifolium* is an ornamental orchid that is very popular in Thailand because of its economic value [6]. *Cymbidium* is also used in traditional medicine as it has a rich phytochemical content [7]. The natural occurrence of this ornamental plant in Thailand is threatened because of anthropogenic activities, with habitat destruction reducing population abundance [6]. Numbers of orchids are decreasing while demand is increasing. Plant tissue culture can generate an increase in plant numbers by supplying the necessary nutrients to promote plant growth and development [4].

Improvements in orchid quantity can be attained by tissue culture techniques [8]. In addition, *in vitro* tissue culture is an effective way to save orchid species from extinction [9]. This propagation technique can be used for quick mass multiplication using appropriate growing media and hormones [10]. Hormones such as auxins and cytokinins, and natural substances, are important factors that can be used to support and improve orchid development from protocorm to plantlet [11]. Chitosan is a chemical substance that is often applied for *in vitro* breeding due to its various benefits on plant development and environmental friendliness [2]. Chitosan has the ability to stimulate the differentiation of plant tissue [12]. As a plant growth promotor, chitosan has a significant effect on roots, leaves and flower growth and numbers [13]. Moreover, chitosan stimulates a signal that has a role in synthesizing hormone such as gibberellins [14], auxins and cytokinins [15]. Besides, chitosan can optimize plant growth due to its role in reducing stress damage in plant cells by regulating ABA activity [16]. The use of chitosan not only promotes plant growth but also stimulates defense mechanisms by increasing jasmonic acid concentrations, resulting in greater disease resistance [15]. Chitosan is appropriate for promoting plant growth because it is cheap, easy to find, replenishable and safe for the environment and humans [15]. Chitosan is generally considered as a biocompatible and non-toxic chemical substance [17]. In agriculture, chitosan has been used for seed, leaf, fruit and vegetable coating, as a fertilizer, and in controlled agrochemical release to increase plant production, stimulate the immunity of plants, protect plants against microorganisms and stimulate plant growth [12]. Abiotic stress such as salt stress can also be reduced by chitosan application [18].

Natural substances like coconut water and potato extract are widely used because they can enhance *in vitro* plantlet shoot growth [19]. Coconut water contains amino acids, organic acids, nucleic acids, several vitamins, sugars and sugar alcohols, plant hormones (auxins, cytokinins), minerals and other unidentified substances that have growth-promoting qualities [20]. Potato extract is an effective natural supplement for seed germination and seedling growth *in vitro*. Addition of potato extract can selectively enhance seed germination and protocorm production [21]. The effects of hormones, natural substances and carbohydrate sources on shoot development depend on their types and concentrations [22]. Treating protocorms with hormones and natural supplements has proven to be advantageous in promoting and improving plant development [11]. Disparate species show diverse reactions under different concentrations, types and hormone combinations. Appropriate concentrations of medium supplements either alone or in combination can greatly impact orchid quantity. The objective of this research was to study how chitosan, coconut water and potato extract affected protocorm growth in *Cymbidium aloifolium in vitro*.

2. Materials and Methods

2.1 Plant materials

For protocorm induction, dehisced capsules of *C. aloifolium* were sterilized using 5% (v/v) sodium hypochlorite (Clorox) with two drops of Tween 20 for 15 min before washing in sterilized distilled water 3 times and dissecting into two pieces. The mature seeds were transferred and cultured on solid New Dogashima (ND) medium [23] that had been supplemented with 20 g/l sucrose and had its pH adjusted to 5.4. The cultures were incubated at 25±2ºC with a 16/8 h (light/dark) cycle providing 40 µmol·m^{-2}·s^{1} for 12 weeks.

2.2 Chitosan, coconut water and potato extract treatments

Twelve week old protocorms of size 2-3 mm were used as explants. Two experiments were conducted; chitosan treatment and coconut water (CW) with potato extract (PE) treatment. For chitosan stock solution preparation, 500 mg chitosan powder was dissolved using 100 ml of 5% acetic acid and 100 ml of distilled water. After chitosan had completely dissolved, distilled water was added until the final volume was 500 ml. Potato extract stock solution was prepared by chopping potato (50 g) into small pieces before boiling in distilled water three time. Distilled water was further added until the final volume was 1000 ml. The protocorms were cultured on solid ND medium supplemented with various concentrations of chitosan (0, 0.05, 0.1, 1 mg/l), coconut water and potato extract in various concentrations (CW: 0, 100, 150, 200 ml/l and PE: 0, 5, 10, 15 g/l) alone or in combination. There were five different treatments for the chitosan experiment and 16 treatments for coconut water with potato extract, each with 10 replicates and 10 protocorms per replicate. All cultures were incubated at 25±2ºC with a 16/8 h (light/dark) cycle providing 40 µmol·m^{-2}·s^{-1} and then subcultured after 5 weeks of culture. Survival percentage, response percentage and growth performance for both experiments were recorded after 10 weeks of culture. Fresh and dry weights (g) were obtained by weighing the whole plant in each replication before incubation and after drying at 70°C for 72 h. The response sample was defined as an explant that was still alive and growing in response to the culture medium, whereas the survival sample was characterized as an explant that was still alive with growth or no growth. Therefore, the survival and response percentages were calculated as follows.

Survival percentage = (Final number of survival plants/Initial number of explants) x 100

Response percentage = (Final number of response samples/Initial number of explants) x 100

2.3 Statistical analysis

Both experiments were conducted using completely randomized design (CRD) with five replications. Data were analyzed by one-way analysis of variance (ANOVA) with difference between means separated by Duncan's multiple range test ($p \leq 0.05$). All data were presented as mean value ± standard error (SE).

3. Results and Discussion

3.1 Chitosan treatment

Chitosan promoted the growth of orchids when it was used at appropriate concentrations. Higher chitosan concentrations resulted in lower survival rate and reduced shoot growth. The highest

growth performance of protocorms was recorded at 0.05 mg/l chitosan treatment, with all growth performances higher than the other treatments and the control group (Figure 1). After 10 weeks of culture, all protocorms of every treatment were regenerated into plantlets with shoots and roots (Figure 2).

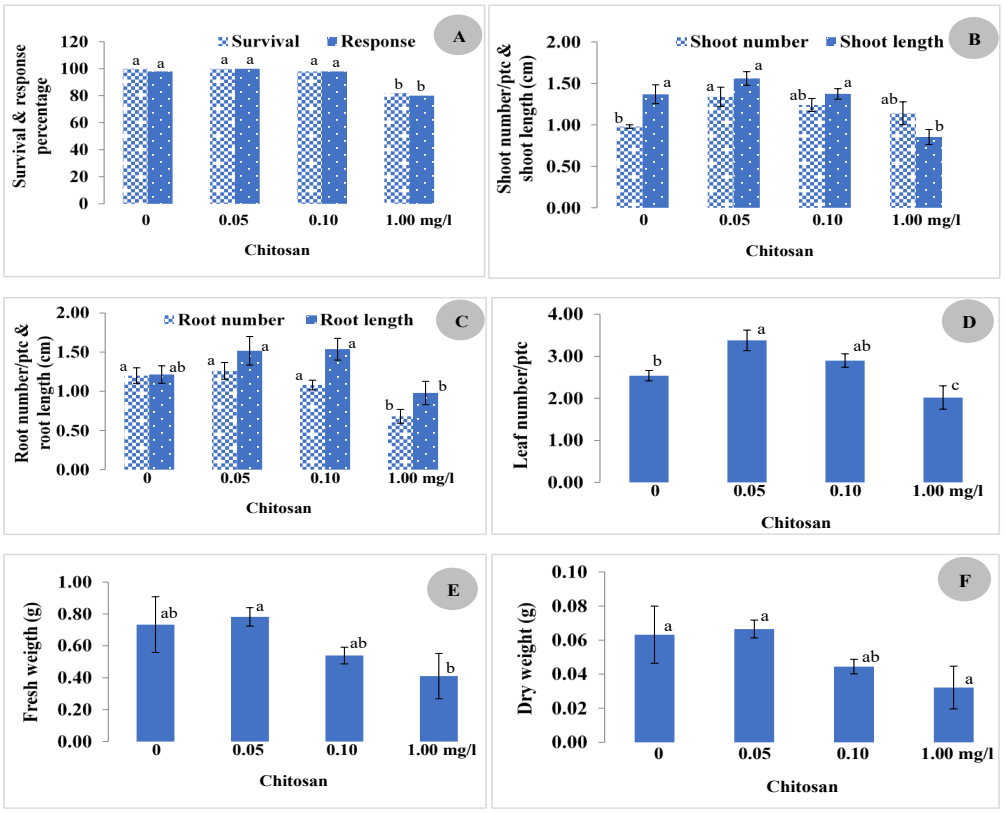

Figure 1. Effects of chitosan at different concentrations on *C. aloifolium* protocorm growth after 10 weeks of culture: (A) survival and response, (B) shoot number and length, (C) root number and length, (D) leaf number, (E) fresh weight, and (F) dry weight.
Data are shown as mean ± SE, ptc = protocorm.

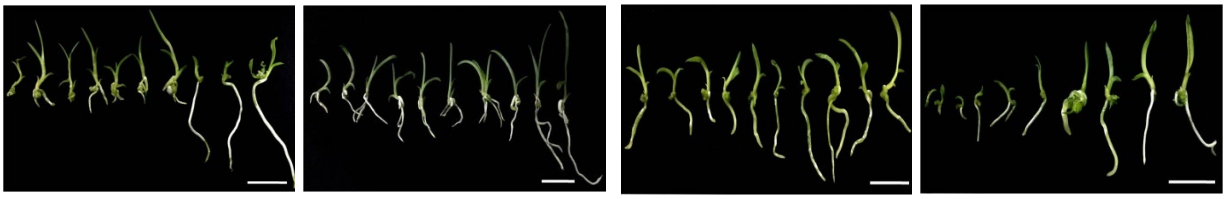

Figure 2. Effects of chitosan addition at different concentrations in solid ND medium on *C. aloifolium* protocorm growth after 10 weeks of culture (scale = 2 cm)

The use of chitosan as a plant growth stimulator is of interest as it is widely available, cheap, replenishable and generally viewed as a safe material for humans and the environment [2]. Chitosan affects the growth of orchids, especially in tissue culture, as shown by the increased fresh weight and number of protocorm-like bodies (PLBs) of *Dendrobium* sp. Chitosan can stimulate orchid tissue because it enables the cells to further differentiate, resulting in increased number of plantlets [14]. Pornpienpakdee *et al*. [2] identified chitosan as an appropriate growth stimulator in orchid micropropagation, especially in *Dendrobium* sp. In this study, chitosan at 0.05 mg/l resulted in the highest number and length of protocorm roots and shoots, leaf number and also fresh and dry weights. High PLB growth using chitosan was also recorded by Restanto *et al*. [14] but at different concentrations. Their results showed that chitosan at 15 ppm produced the highest number and fresh weight of PLBs and highest number of plantlets. Pornpienpakdee *et al*. [2] found that 10 or 20 mg/l of chitosan oligomers was the most appropriate to induce the formation of PLBs and shoots, while Nahar *et al*. [24] determined that chitosan impacted shoot formation, with the highest rate obtained at 1 mg/l. For *Dendrobium formosum*, chitosan significantly enhanced seed germination in *in vitro* propagation [25], and also significantly affected yield and quality in the flower stage. Kumari *et al*. [26] found that chitosan treated plants yielded higher numbers of spikes per square meter, with greater spike length, increased internodal length, enhanced flower diameter and more florets per spike. Similar results were recorded by Anusuya and Sathiyabama [27], who revealed that growth parameters (shoot height, leaf number/plant, plant fresh weight) increased with application of chitosan. However, in contrast, Sukma *et al*. [28] suggested that chitosan had a negative effect on *Phalaenopsis* protocorm growth by decreasing protocorm multiplication and plantlet regeneration. Optimal orchid growth at various chitosan concentrations depends on several factors, including plant species, explants, and developmental stage. Increase in fresh weight and number of PLBs and plantlets caused by chitosan may induce signals to synthesize plant hormones such as gibberellins [14], which do not only stimulate plant growth but may also induce differentiation of plant cells and tissues, and especially in *Dendrobium* orchid [2].

3.2 Coconut water and potato extract treatment

The addition of natural supplements to orchid growth media induced shoot and root development. The survival rate of protocorms was 100% in all treatments, but not all responded. Shoot numbers were not significantly different in each experiment, but shoot length demonstrated significant differences. The highest shoot numbers were obtained with 10 g/l PE + 100 mg/l CW and 5 g/l PE + 150 ml/l CW treatments (1.28 ± 0.06), while the highest shoot length was obtained with 150 ml/l CW without PE treatment (2.09 ± 0.23). The root number of the control group was the highest (0.98 ± 0.04) followed by 5 g/l PE+ 150 ml/l CW treatment (0.96 ± 0.14). However, the highest root length, which was produced by 150 ml/l CW without PE (2.77 ± 0.26), was significantly different from the other treatments. The highest leaf number, fresh weight and dry weight were also obtained from 150 ml/l CW without PE treatment. By contrast, the lowest growth performance was found in the 5 g/l PE + 200 ml/l CW treatment (Figures 3 and 4).

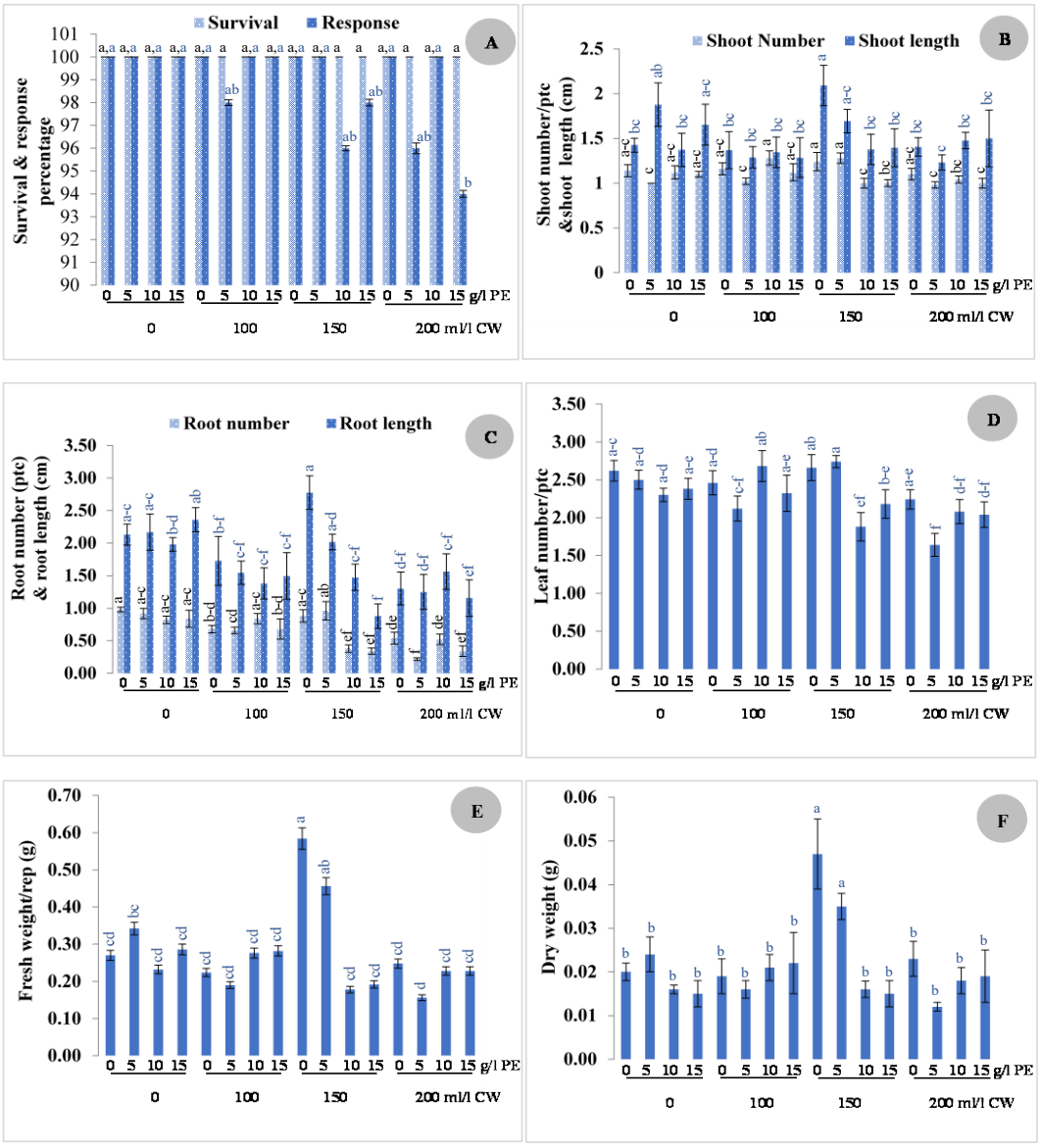

Figure 3. Effects of coconut water (CW) and potato extract (PE)on *C. aloifolium* protocorm growth after 10 weeks of culture: (A) survival and response, (B) shoot number and length, (C) root number and length, (D) leaf number, (E) fresh weight, and (F) dry weight. Data are shown as mean ± SE, ptc = protocorm.

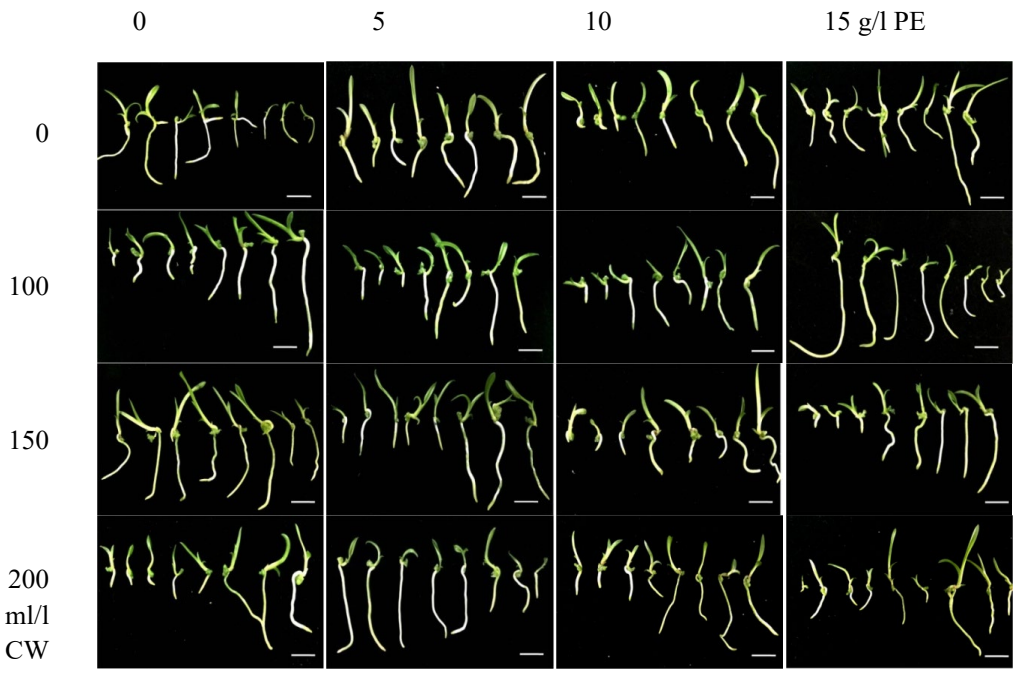

Figure 4. Effects of coconut water and potato extract addition in solid ND medium on *C. aloifolium* protocorm growth through *in vitro* propagation after 10 weeks of culture (scale= 1 cm)

A number of complex organic additives including peptone, beef extract and natural additives such as tomato juice, potato extract (PE) coconut water (CW) and banana extract (BE) are commonly added to orchid plant tissue culture media [11]. Coconut water has been applied to stimulate PLB development [2]. Based on our experiments, highest protocorm growth was achieved from the coconut water medium, with optimal concentration at 150 ml/l. This result concurred with Asghar *et al*. [10], who revealed that the highest protocorm number, longest shoots and highest fresh weight were obtained in *Dendrobium nobile* with coconut water medium at 100 ml/l. A similar result was achieved by Obsuwan and Thepsithar [29], who showed that the addition of coconut water yielded maximum plant height. Enhanced shoot development using CW has been observed in a number of *Dendrobium* sp. and in *Cymbidium pendulum* [10, 30, 31]. Coconut water contains a complex combination of compounds including amino acids, organic acids, nucleic acids, several vitamins, sugars, plant hormones (auxins, cytokinins), minerals, and other unidentified substances that promote plant growth. Natural supplements can efficiently support shoot induction, multiplication and multiple root formation [11]. In this study, higher concentrations of coconut water resulted in lower plant growth. Similarly, Asghar *et al*. [10] revealed that higher coconut water concentration had an inhibiting effect on shoot development. By contrast, potato extract addition, either alone or in combination with coconut water, generated lower growth in this study. This result was incompatible with Obsuwan and Thepsithar [29], who found that maximum fresh weight, and the number of roots and shoots on *Vanda* and *Mokara* orchids were obtained when PE was added to the medium. Vitamin B6 obtained from PE induced PLB proliferation due to the production of essential amino acids [29]. However, natural supplements affected protocorm growth differently depending on several factors such as orchid species or explant developmental stage [32, 33].

In our study, the most suitable concentration for protocorm growth in chitosan experiment was 0.05 mg/l chitosan, while the most appropriate treatment quantities of CW and PE were 150 ml/l CW without PE. Shoot, root and leaf number per protocorm and fresh weight of the 0.05 mg/l chitosan treatment were higher than the 150 ml/l CW treatment. As a result, (0.05 mg/l chitosan treatment value/ 150 ml/l CW treatment value) x 100 was used to calculate the change percentage of this result. When comparing the growth performance of these two conditions, their response, survival rate, shoot number, fresh weight and dry weight were not significantly different, while shoot length, root number, root length and leaf number of the chitosan treatment were significantly higher than the coconut treatment (Table 1). This result proved that chitosan treatment was more suitable for *Cymbidium aloifolium* protocorm growth than coconut and potato extract treatment.

Table 1. Comparison of response, survival percentage and growth performance between chitosan and coconut water treatments (mean ± SE)

Observation	Chitosan treatment (0.05 mg/l)	Coconut water treatment (150 ml/l)	Change (%)
Response percentage	100	100	100
Survival percentage	100	100	100
Shoot number/ptc	1.34 ± 0.12	1.24 ± 0.10	92.53
Shoot length (cm)	1.56 ± 0.08	2.09 ± 0.23	133.97*
Root number/ptc	1.26 ± 0.11	0.88 ± 0.10	69.84*
Root length (cm)	1.52 ± 0.18	2.77 ± 0.26	182.23*
Leaf number/ptc	3.38 ± 0.25	2.66 ± 0.17	78.69*
Fresh weight/replicate	0.78 ± 0.06	0.58 ± 0.09	74.35
Dry weight/replicate	0.07 ± 0.01	0.05 ± 0.01	71.42

*Significant difference at $p < 0.05$, ptc = protocorm

4. Conclusions

Medium addition of chitosan and natural substances impacted *C. aloifolium in vitro* propagation and protocorm growth. Survival and response percentages, shoot and root number, length and fresh and dry weight of treated protocorms improved compared to untreated protocorms. The highest protocorm improvement was achieved in ND medium containing 0.05 mg/l chitosan. At this concentration, the protocorms showed higher growth performance than the control. This information can be applied to assist the orchid propagation industry, especially for *in vitro* culture.

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