Development of *ex vitro Rhynchostylis* plantlets in cutting ball containing different concentrations of naphthaleneacetic acid

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Abstract

The study was aimed to evaluate the effects of various concentrations of naphthaleneacetic acid (NAA) in encapsulated alginate as a cutting ball on *ex vitro* development of *Rhynchostylis gigantea* plantlets. NAA concentrations in a cutting ball caused particular effects on development of roots and leaves of *R. gigantea* plantlets. There was the changing of root diameter coincidentally followed by the altering of the rate of root initiation and average number of originated roots among treatments. The optimum time to carry *ex vitro R. gigantea* plantlets was 4 weeks. The NAA concentrations ranging from 1 to 2 ppm promoted better root development of *ex vitro R. gigantea* plantlets than other treatments. NAA concentration at 1 ppm gave the highest rate of leaf initiation, but it showed the lowest content of chlorophyll.

Keywords*: Rhynchostylis*, cutting ball, alginate encapsulation, NAA

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Introduction

Ex vitro acclimatization has been considered as the most difficult phase for the culture of orchids (Colombo, Faria, Assis, & Fonseca, 2005). The most important requirement of *ex vitro* plantlet is *in vitro* complete development, and almost all previous studies have focused on the improvement of *in vitro* conditions to accelerate plantlets acclimate after transplantation from *in vitro*, including survival rate and developmental ability. In orchids, roots are invariably colonized by mycorrhizal endophytes (Rasmussen, 2002) similar to *Rhizoctonia* – like endophytes that are active as the symbiosis to support growth and development of plants. Due to that symbiosis proceeding, in *Rhynchostylis retusa*, dense and brownish hyphae in the inner cortex as compared to living hyphae in the outer cortex could be attributed to digestion of fungal mass by lytic enzymes produced by host for release of nutrients (Trappe, 2005).

Even parts of plants are fully developed while the *in vitro* plantlets are acclimatizing and transplanting, however, the abiotic stresses from out-of-bottles environment affect less vigorous plantlets and can make them weak and die. The idea to create a parallel nursery *in vitro* and *ex vitro* to adapt the improvement of survival rate and healthy growth, development of plantlets causes the initiation of cutting ball technique. In general, there are two ways to form a ball, the agar-media ball and cutting balls developed following alginate encapsulation. The use of alginate encapsulation of *in vitro* cultured shoot tips as an alternative to somatic embryos to develop synthetic seeds has increased (Sarkar, & Naik, 1998). This technology is particularly useful for the propagation of rare hybrids, elite genotypes, and genetically engineered plants whose seeds are either too expensive or not readily available (Mandal, Pattnailk, & Chand, 2000). In begonia, the germination percentages when the encapsulated seeds were grown in MS media *in vitro* were significantly better than in peat pellet *ex vitro* (Sakhanokho, Pounders, & Blythe, 2003).

Orchids are considered as the important export agricultural products of Thailand. Only in 2020, the total global net export of Thai orchids was recorded around 21,872 tons, which is worth 1,370 million baht (Thitiphonkachit, 2021). *Rhynchostylis* is one of important commercial orchid in Thailand, and is known popularity under the name “Chang” or “Elephant Orchid”. Now, *Rhynchostylis* has become a high-income business and important part of Thai agriculture (Kanchit, 2016).

Recently, the most popular methods have been applied in acclimatization and transplanting of *ex vitro Rhynchostylis* plantlets quite directly, both for research and commercial purposes.

Following that, regenerated plantlets those appear 4-6 leaves, with 5-6 cm in stem height and 4-5 cm in root length from culture bottles have been removed to outside, washed, dried the remaining water and exposed to natural light. Until the roots become white, plantlets have been transplanted into crushed pine bark or charcoal (Xi, Zeng, & Huang, 2021). This method has been confirmed, applied widely and considered as suitable way for native or popular hybrid varieties for commercial use, due to direct contact between plantlets and environment of the method. However, for rare hybrid genotypes, the adaptation of varieties to the environment has not been approved. Moreover, the development of roots outside the culture bottles can shorten *in vitro* culture period and reduce the stress of plants during acclimatization and transplanting. Therefore, in this research, the cutting ball method has been chosen as an expected alternative way to improve survival rate, vitality, growth and development of plantlets during acclimatization and transplanting.

Methodology

Plant materials

The plantlets of 6 months old of white-flower hybrid *R. gigantea* obtained from *in vitro* culture were used as plant materials. They were removed the old roots and attached to cutting balls (Figure 1). Samples were grown in coco-peat under 25°C and 16 hours lighting.

Cutting ball preparation

The cutting balls were prepared following the alginate encapsulation formation. MS medium was supplemented with different concentrations of NAA (0, 0.5, 1, 1.5 and 2 ppm). The sodium alginate ($C_6H_9NaO_7$) solution in liquid MS media (30 g/L) was mixed with calcium dichloride ($CaCl_2$) solution (11.2 g/L) by the pipette, which the 1.6 mL of sodium alginate solution per drip was added to calcium dichloride. The encapsulations initiated immediately after the drip of $C_6H_9NaO_7$ contacted $CaCl_2$ solution. This took 12 hours to solidify the encapsulated cutting balls. After solidification, encapsulated cutting balls were collected, shaped and sterilized by autoclaving at 121°C for 15 minutes (15 psi).

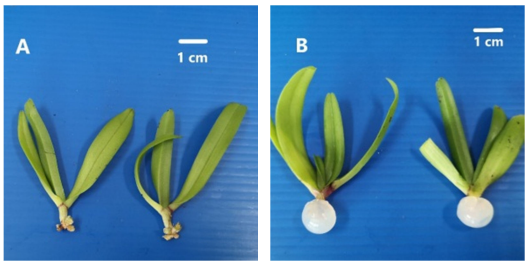

Figure 1 *R. gigantea* plantlets were removed the old roots (A) and attached to cutting balls (B).

Data collection and analysis

The CRD experiment with 15 replications (1 Rep: 1 plantlet) per treatment was applied

in this research. Number of new roots, leaves and root, leaf indexes were observed and measured after 4 and 8 weeks attaching plantlets to cutting balls.

Chlorophyll content determination was measured following Arnon (1949), leaves of each sample were extracted with acetone at 80%. Chlorophyll a and b were measured at wavelengths of 663 and 645 nm, respectively in a digital spectrometer.

Data were analyzed with mean separation using the LSD test at $P \leq 0.05$ by SAS software – University Edition.

Results

Effects of different contents of NAA in cutting ball on root and leaf initiation

Root and leaf initiations of all samples were observed and measured at 2 points of time, after 4 and 8 weeks. The number of observed initial roots and leaves after 8 weeks were not included in these observed indexes after 4 weeks and shown in (Figure 2). The difference of results between treatments that were supplemented with and without NAA was clearer after 4 weeks. Root and leaf initiation rates of any treatment in the cutting balls supplemented with NAA were higher than no NAA.

Figure 2 Observed *R. gigantea* plantlets carried in cutting balls after 4 and 8 weeks (A and B, respectively).

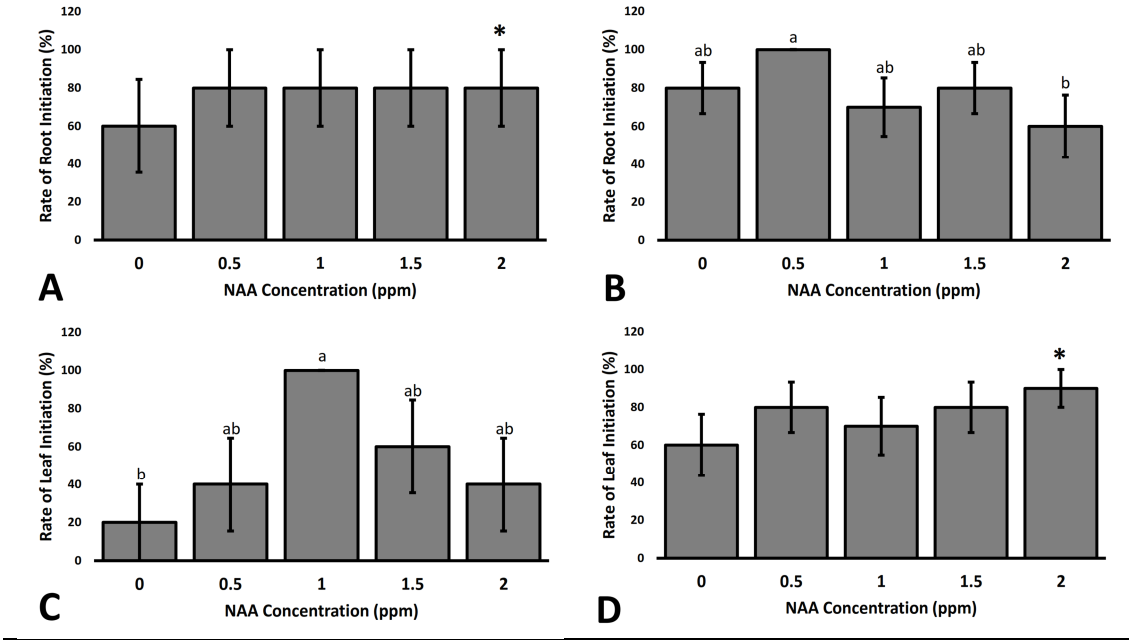

Figure 3 Rate of root initiation (A, after 4 weeks and B, after 8 weeks); and leaf initiation (C, after 4 weeks and D, 8 weeks) of *R. gigantea* plantlets carried in cutting balls containing different concentrations of NAA.

After 4 weeks, there was no significant difference in the rate of formation of roots between all treatments. The proportion of root initiation was similar in treatments with NAA concentrations, and it was the lowest in treatment without NAA in cutting balls (Figure 3A). After 8 weeks, the difference of root initiation became more significant, whereby treatment with 0.5 ppm NAA had the highest rate of root initiation (Figure 3B).

In the process of leaf initiation, the number of new leaves initiated after 4 and 8 weeks was not significantly different. Almost all of the samples appeared only 1 new leaf. However, there was a significant difference in the rate of leaf initiation. After 4 weeks, the highest rate belonged to treatment with 1 ppm NAA in cutting balls, followed by treatments with NAA of 1.5, 2 and 0.5 ppm, respectively, while treatment without NAA in cutting balls got the lowest rate of leaf initiation (Figure 3C). After 8 weeks, this difference became less significant, with the highest rate in treatment with 2 ppm NAA, and the lowest rate in treatment without NAA (Figure 3D).

Effect of different contents of NAA in cutting ball on root development

Multiple comparison procedures were used to perform the different responses of NAA concentrations to root indexes, including root diameter and length. In (Table 1), there was no significant difference among root indexes of all treatments measured after 4 weeks. There was only the significant difference in root diameter measured after 8 weeks. Following that, the highest of root diameter belonged to treatment with 1.5 ppm NAA in cutting balls, and the lowest in treatment without NAA.

Table 1 Root indexes of *R. gigantea* plantlets carried in cutting balls containing different concentration of NAA.

NAA concentration (ppm)	root indexes of samples after 4 weeks		root indexes of samples after 8 weeks	
	root diameter (mm)	root length (mm)	root diameter (mm)	root length (mm)
0	2.87±0.09	8.74±0.65	2.45±0.32$^{b\ 1/}$	5.78±1.37
0.5	2.72±0.31	7.60±2.03	2.48±0.21^{b}	6.95±2.00
1	3.01±0.28	11.37±1.95	2.99±0.26^{ab}	7.84±1.43
1.5	3.06±0.16	10.86±1.39	3.22±0.24^{a}	9.48±1.88
2	4.03±0.20	10.89±1.95	3.12±0.24^{ab}	10.32±2.47

$^{1/}$ means with different letters in the column indicate differences between standard errors according to LSD test, P<0.05; Data represent mean±SE; (n=15)

Results performed by using the LSD test showed that there was a more variability of root length among treatments than root diameter. The variability of indexes among treatments measured after 8 weeks was also higher than measured in the 4th week. While there was no significant difference between treatments measured in 4th and 8th week in root diameter, that difference became more significant in root length. Root diameter also decreased in 2 ppm NAA treatment, that trend in root length completely decreased for all treatments. Linking to data from (Figure 3), root diameter index increased or decreased from 4th week to 8th week coincidentally followed by the increasing of the rate of root initiation and average number of root initiation, respectively.

Figure 4 Different colors in roots of *R. gigantea* plantlets carried in cutting balls: creamy color roots (A) and green roots (B).

The transformation of root colors coincidentally followed by the level of root completion. Level of completion followed by the changing of root, from white to yellow and to green, respectively. From the experiment, all of roots initiated after 4 weeks were white. (Figure 4) showed that the greeny roots contained more root hairs than the creamy (light yellow) color of the roots. The completion of the root does not relate to the maturation of the root. There were some roots that got green color. While some roots were still white or yellow even the root length approach the particular length.

Effect of different contents of NAA in cutting ball on leaf development

The chlorophyll content of samples measured after 4 weeks were more variability among different concentrations of NAA than after 8 weeks.

After 4 weeks, the chlorophyll content had the trend to significantly linearly increase as the increase of levels of NAA concentrations (except the indexes in 0.5 ppm NAA which were higher than in 1 ppm NAA). Followed by the stability of chlorophyll indexes from 4 weeks to 8 weeks, those indexes in treatments with 0 and 0.5 ppm NAA concentrations trended to increase but in treatments with 1, 1.5 and 2 ppm NAA trended to decrease (Table 2).

Table 2 Chlorophyll content of R. *gigantea* plantlets carried in cutting balls containing different concentration of NAA.

NAA concentration (ppm)	chlorophyll content of samples after 4 weeks (g/m^2)		
	chlorophyll a	chlorophyll b	total chlorophyll
0	0.05±0.00$^{b\ 1/}$	0.05±0.00^{b}	0.02±0.00^{b}
0.5	0.09±0.03^{ab}	0.10±0.04^{ab}	0.06±0.02^{ab}
1	0.07±0.00^{ab}	0.07±0.00^{ab}	0.04±0.00^{b}
1.5	0.10±0.04^{ab}	0.11±0.05^{ab}	0.07±0.03^{ab}
2	0.14±0.04^{a}	0.21±0.08^{a}	0.14±0.06^{a}
NAA concentration (ppm)	chlorophyll content of samples after 8 weeks (g/m^2)		
	chlorophyll a	chlorophyll b	total chlorophyll
0	0.07±0.01	0.10±0.01	0.07±0.01^{ab}
0.5	0.08±0.01	0.13±0.02	0.09±0.01^{a}
1	0.07±0.01	0.10±0.01	0.07±0.01^{ab}
1.5	0.07±0.00	0.10±0.01	0.06±0.01^{ab}
2	0.08±0.00	0.09±0.00	0.05±0.00^{b}

$^{1/}$ means with different letter in the column indicate differences between standard errors according to LSD test, P≤0.05; Data represent mean±SE; (n=15)

Linking with data of leaf initiation from (Figure 3), the total chlorophyll content of samples after 4 weeks was contrast to the rate of leaf initiation. Following that, among treatments with NAA, the treatment with 1 ppm which got the highest of rate of leaf initiation was coincidently showed the lowest chlorophyll content. While the treatment with 2 ppm NAA which got the lowest rate of leaf initiation showed the highest chlorophyll indexes. All of treatments with NAA showed the higher chlorophyll content than without NAA.

Discussions

Results from the experiment represent the similarities in responsibility of root indexes to different levels of NAA concentrations. Those results can be concluded that there were the effects of NAA concentration in encapsulation cutting balls on development of the *ex vitro* roots of *R. gigantea* plantlets. However, the non-significant differences in LSD test of means of root indexes indicated that there was not optimum concentration of NAA for root development of *ex vitro R. gigantea* plantlet among treatments applied in the experiment. Different studies about micropropagation of Rhynchostylis have indicated that different levels of NAA concentration affect *in vitro* rooting, which also depend on various factors such as the stages of micropropagation, type of media or the combination with other types of plant growth regulators (PGRs). Almost all of the results depend on the purposes of studies. The highest number of protocorm like bodies (PLBs) from each callus highest can be achieved when the tissue of Rhynchostylis are cultured in Vacin and Went (VW) medium supplement with 15% coconut water, 1 ppm of bezyladenine (BA) and 1 ppm of NAA (Parab, & Krishnan, 2012). After culture for 50 days of the *R. retusa* shoots, the rooting rate and average root length are highest at 4 μM NAA is used (Thomas, & Michael, 2007). In this study, even the results could not show the optimum recommendation of NAA concentration used in encapsulations, however, the testing proved that there were responses of root indexes to the levels of NAA concentration. However, the changing of root diameter coincidentally followed by the changing of the rate of root initiation and average number of root initiation among treatments might refer to the relatives of NAA concentrations in cutting balls on initiation and indexes of *Rhynchostylis* plantlet roots.

NAA is not considered as the key factor of leaf development, especially in orchids. However, in the study of hybrid *Origanum x intercedens* Rech. (Lamiaceae), results indicate that leaves treated with NAA undergo a reduction in blade surface area, while simultaneously the amount of the mesophyll increases significantly. Chlorophyll

content was less than that of the without treated NAA leaves. NAA further increases the density of the glandular trichomes (peltate) on both leaf surfaces as well as the size of the secretory head of the trichomes. The epidermal cells radially surrounding the foot cell of the trichomes increase in size and number (Bosabalidis, & Exarchou, 1995). In our study, *R. gigantea* plantlets treated with NAA in encapsulation cutting balls did not show the efficiency in chlorophyll content increasing in leaves, but it indicated that there was more stability of chlorophyll content in all of the treatments as the increasing level of NAA. Further experiments need to be conducted to clear the relative of NAA and chlorophyll in *R. gigantea* leaf.

Cutting ball method is the solution for shortening the period of plant tissue cultural performance. On the orchid objects, the traditional way of propagation from plant tissues includes 3 stages: *in vitro* mass multiplication, separation, acclimatization and transplanting. If the cutting ball technique is applied, after shoots are separated inside the glasses, the plantlets attached in the cutting ball can initiate root out-of-glass and can be moved directly to the nursery, the acclimatization and transplanting can be rejected. However, the alginate cutting ball technique still keeps the disadvantage that limits the popularity and wide application. The progress to make alginate cutting balls is just performed in a laboratory with the fulfillment of a facility that is not easy for farmers or mass production in the orchid industry. In this way, agar ball seems to be a better alternative method with the productivity of the ball being released. However, there are some studies that need to be informed to clear the effects of agar ball to orchid, exclusive *Rhynchostylis* plantlet.

Conclusions

There were the differences between NAA treatments and control treatment (no NAA in cutting balls) proving that NAA caused particular effects on *ex vitro* development *R. gigantea* plantlets that were carried in cutting balls. Those differences were significant just after 4 weeks, the longer time could not cause explainable changes. Follow that, the optimum time to carry *R. gigantea* plantlets was 4 weeks.

The optimum concentrations of NAA in alginate cutting ball for root development were from 1 to 2 ppm, however, there will be more expand experiment to be carried to determine the particular content of NAA.

The NAA content also affected leaf initiation and development of *ex vitro R. gigantea* plantlets. The NAA concentration of 1 ppm showed the highest rate of leaf initiation but it also caused the lowest in chlorophyll content of samples. By the way, treatments in that higher rate of leaf

initiation showed lower content of chlorophyll, respectively. Further experiments will be carried out in order to improve the quality of leaf, indicating by increasing in chlorophyll content.

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