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In vitro Propagation of Red Lotus (*Nelumbo nucifera* Gaertn) - An Aquatic Edible Plant in Vietnam

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ABSTRACT

Background: Lotus is an important aquatic plant with great economic value, not only as an ornamental flower but also as a source of herbal medicine. In general, lotus is usually propagated through the rhizome or tuber but the normal propagation rate is very low and it often depends on the quality of water environment and the weather conditions where it is grown. Lotus propagation by tissue culture has many predominant advantages compared with the traditional propagation methods. The current study aims to study the *in vitro* propagation of Hue's Red Lotus, a famous local lotus in Hue city, Vietnam.

Methods: Fresh mature seeds of Red lotus (23 to 25 days old) were used as initial materials for *in vitro* propagation. Experiments are carried out to assess sterilization ability by $HgCl_2$, 0.1% and determine the appropriate formula for shoot regeneration, multiple shoot clumps induction, root induction of multiple shoot clumps and acclimatization.

Result: The optimum formula for seeds of Vinh Thanh Red Lotus is to sterilize surface of seeds by 70% ethanol for 30 seconds and then $HgCl_2$ 0.1% for 16 minutes. The samples were cultured on MS (Murashige Skoog) medium supplemented without plant growth regulators for *in vitro* shoot regeneration. MS medium added with 0.5 mg/L BAP gave the highest multiplication rate (5.60 shoots per explant) and good quality shoots. Adding 0.5 mg/L NAA in rooting medium provided the highest rooting effect and good root quality (18.17 roots/shoot clumps). The survival rate of transplanting plants was 40% on mud medium which were soaked for one week in 2g/L $KMnO_4$ after two weeks in the greenhouse.

Key words: *In vitro* propagation, MS medium, *Nelumbo nucifera* Gaertn, Red lotus, Shoot regeneration.

Abbreviations: BA = Benzyladenine, BAP = 6-Benzylaminopurine, IBA = Indole-3-butyric acid, MS = Murashige and Skoog (1962) medium, NAA = 1-Naphthaleneacetic acid.

INTRODUCTION

Lotus *(Nelumbo nucifera)* is a potential perennial aquatic plant, commonly grown in China, Japan, Northeastern Australia and many other countries (Guo, 2009; Nguyen, 2001). Lotus is an important economic aquatic plant, not only as a food (Shad *et al*., 2013, Zhang *et al*., 2015) and ornamental flower but also as a source of herbal medicine (Hwang *et al*., 2015, Nguyen *et al*., 2004). Almost every parts of Lotus can be used, with the rhizome (sometimes called root) and seeds being the main consumption parts (Wu *et al*., 2011). Besides, its embryo, leaf and flower are used as herbal medicines which have strong bioactive ingredients (Dhanarasu *et al*., 2013; Zhu *et al*., 2015), such as alkaloids, flavonoids (Nagarajan, 1965), antioxidants (Hwang *et al*., 2015; Sridhar *et al*., 2007), antisteroids (Pal and Dey, 2015), anticancerous (Zhao *et al*., 2017), anti-HIV (Kashiwada, 2005), antiviral, anti-inflammatory (Mukherjee *et al*., 1997), antipyretic (Mukherjee *et al*., 1996; Paudel and Panth, 2015), anti-diabetic (Maqbool *et al*., 2019) and anti-obesity properties (Mukherjee, 2009; Ono, 2006). In addition, this flower is very beautiful which is used as an ornamental plant (Guo, 2010b). According to the history, lotus has been honoured by China, India and Egypt. It appears in a wide range of art products of these countries as a symbol of cultures, perfection, purity and beauty (Ming *et al*., 2013; Paudel and Panth, 2015).

With high values associated to this flower, lotus has more than 300 cultivars (Guo, 2009). In general, lotus is usually propagated through the rhizome or tuber, but the normal propagation rate is very low and it often depends on the quality of water environment and weather where it is grown (Shou *et al*., 2008). It can be propagated by seed. The seed needs to be carried out by scarifying the hard seed coat and then incubating at 25°-30°C. Seeds should be submerged in a water saturated media. If the hard coat remains intact, the seed will remain viable for centuries and it may take a few years for the seed to sprout if placed in water (Mahmad *et al*., 2014). However, propagation by seed will not be true-to-type as lotus has a high degree of genetic variability (Nguyen, 2001). Therefore, it is important to design an efficient method to improve the propagation effectively in lotus.

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Lotus propagation by tissue culture is a promising breeding method in the future associated with industrial exploitation of good lotus varieties specialized in seeds or tubers (Resende *et al*., 2015; Yu *et al*., 2015). At the same time, propagation by tissue culture techniques will solve difficulties such as high propagation coefficient, uniform seedlings, high growth and disease freeness (Hoang, 2013). Hence, there are predominant advantages of *in vitro* culture compared with the traditional propagation methods (Borah *et al*., 2019). Although there are studies on *in vitro* propagation of lotus (Shou *et al*., 2008); (Mahmad *et al*., 2014). There is no study describing such studies on lotus in Vietnam especially Red Lotus variety or Vinh Thanh Red Lotus.

This study presents the results of the *in vitro* propagation of Hue's Red Lotus, a famous Hue's local plant, to develop a large number of *in vitro* plants rapidly and use these plants as a material to serve the conservation and development of local lotus varieties in Thua Thien Hue province.

MATERIALS AND METHODS

Plant materials

Red Lotus's mature seeds (23 to 25 days old) collected from Hue city were used as initial materials for the *in vitro* propagation (Fig 1).

Methods

Seed sterilization and culture initiation

After removing the seed coat, seeds were initially washed thoroughly under running tap water three times and then soaked in detergent for three times and rinsed with distilled water three times (5 min. each time). The surface-sterilized seeds were sterilized by 70% ethanol for 30 seconds followed by $HgCl_2$ 0.1% for 6-16 minutes. The samples were again washed for four to five time using sterile distilled water. Dormant embryos were collected from sterilized seeds and cultured on germination medium consisting of MS basal medium supplemented with 30 g/L sucrose and 8 g/L agar to assess sterilization ability after four weeks of culture.

Shoot regeneration

The samples were cultured on MS medium supplemented with 30 g/L sucrose, 8 g/L agar and different concentrations of BAP from 0.0-2.0 mg/L for shoot regeneration. The results were assessed after 5 weeks of culture.

Multiple shoot clumps induction

Shoot tips and nodal stem segments from *in vitro* shoot were cultured on MS medium supplemented with different concentrations of BAP (0.0-2.0 mg/L) or KIN (0.0-2.0 mg/L) or combination supplemented with different concentrations of BAP (0.0-2.0) with NAA (0.1; 0.5 mg/L) and fresh coconut water (5%-20%) to determine the multiple shoots capacity. The results were assessed after 5 weeks of culture. After five weeks, multiple shoot clumps were divided and sub-cultured on the same fresh regeneration medium, incubated at the same conditions described above.

Root induction of multiple shoot clumps

The shoot clumps were selected from shoot multiplication and were transferred to MS basal medium supplemented with different combinations of IBA (0.1; 0.5 mg/L) and NAA (0.5 mg/L) for root induction.

Culture conditions

All experiments used the MS basal medium containing 30 g/L sucrose and 8 g/L agar; the pH of all culture media was adjusted to 5.8 and autoclaved at 121°C for 15 minutes. Explants were maintained at 25 ± 2°C with 16h photoperiod of light at an intensity of 2000-3000 lux.

Acclimatization

Plantlets with well-developed shoots (5-8 cm in length) with roots (3-4 cm in length) were removed from the flasks and washed thoroughly in running tap water. They were then transferred to the plastic pots containing five mediums: mud,

Fig 1: Flower and seed morphology of red lotus variety cultivated in Vietnam.

clay loam soil, coir fiber: rice husk (1:1), coir fiber: rice husk: clay loam soil (1:1:1), mud was oaked for one week in 2g/L KMnO4. The treatments were placed in a greenhouse for acclimatization for four weeks. The survival rate of *in vitro* plant in five treatments was observed after two weeks.

Statistical analysis

All the experiments were repeated three times and there were 30 explants for each treatment. Least square means and analysis of variance (ANOVA, general linear model procedure) were calculated using SPSS (Statistical Product and Service Solutions) software.

RESULTS AND DISCUSSION

The effect of sterilization time

Table 1 and Fig 2 show that there was a greaty effect of the sterilization time by 0.1% solution of $HgCl_2$ in the number of samples survived. The seed treated with 0.1% $HgCl_2$ within 6 to 15 minutes showed that the survival rate increased from 34.29% to 75%. The rate of infected samples decreases gradually from 65.71% to 25%, especially all samples do not die. Sterilization time increases up to 16 minutes and 17 minutes; the number of infected samples decreases; the rate of survival samples is high from 76.67% to 88.00%, but the death sample appears with the rate of 2%-16.66%.

Therefore, the sterilization time is the best for seed's Red Lotus with 88% survival rate at 0.1% $HgCl_2$ for 16 minutes treatment.

Table 1: The effect of sterilization time by 0.1% solution $HgCl_2$.

Sterilization time with $HgCl_2$ (minute)	infected sample rate (%)	Death sample rate (%)	Survival sample rate (%)
6	65.71	0	34.29
7	48.57	0	51.43
9	39.58	0	60.42
11	34.04	0	65.96
13	30.00	0	70.00
15	25.00	0	75.00
16	**10.00**	**2.00**	**88.00**
17	6.67	16.66	76.67

Table 2: The effects of BAP on shoot regeneration on Red Lotus.

BAP (mg/l)	Shoot number/ explant	Leave number/ shoot clumps	Shoot height (cm)	Shoot formation (%)
0.00	2.53^{a}	5.87^{a}	1.65^{a}	100
0.50	1.43^{b}	4.20^{b}	1.00^{b}	100
1.00	1.23^{b}	3.43^{c}	$0.90^{b,c}$	100
1.50	1.30^{b}	3.37^{c}	$0.75^{c,d}$	100
2.00	1.23^{b}	3.2^{c}	0.67^{d}	100

*Values marked with same letter are not significantly different at $p<0.05$.

The effects of BAP on shoot regeneration

The samples were cultured on MS medium with the addition of 0.5-2.0 mg/L BAP for shoot regeneration. The effect of BAP on the rate of shoot formation was monitored for 5 weeks of culture (Table 2 and Fig 3).The results show that the maximum number of shoot was formed from explant on MS medium containing 30 g sucrose, 8g/L agar without BAP with 2.53 shoot per explant. The average height of shoots was 1.65 cm and the average number of leaves per sample was 5.87 leaves.

The sample cultured on MS media supplemented with 0.5-2.0 mg/L BAP gave the lowest number of shoots per explant (1.23-1.43 shoots). There was no significant difference in the number shoot formed by a various concentration of BAP. The increase of BAP had a negative effect on the induction of shoot. Thus, the addition of BAP growth regulator is not necessary for shoot regeneration stage from Red Lotus seed.

The effects of BAP and KIN on multiple shoot clumps induction

Phytohormones play an important role in multiple shoot induction (Yu *et al.*, 2015). The results indicated effect of various concentrations of BAP (0.0-2.0mg/L) or KIN (0.0-2.0 mg/L) on the induction of multiple shoot clumps in Red Lotus. The addition of BAP and KIN led to a significant increase in shoot formation compared to the media without plant growth regulators (Table 3).

The culture medium supplemented with 0.5 mg/L BAP gave the highest number of shoot with 5.10 shoots per explant after five weeks of culture. The best shoots' growth

Fig 2: The sample after being sterilized was cultured on MS basal medium to monitor infection rate, death rate and survival rate.

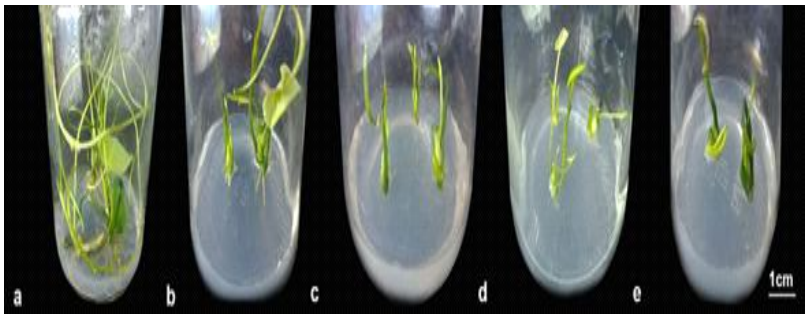

Fig 3: Shoot regeneration after 5 weeks cultured on MS medium supplemented with 0.5-2.0 mg/L BAP. (a). BAP 0 mg/L; (b). BAP 0.5 mg/L; (c). BAP 1.0 mg/L; (d). BAP 1.5 mg/L; (e). BAP 2.0 mg/L.

ability with 1.56cm in height/shoot and 4.50 leaves/shoot clumps and quality of shoots were also good (Fig 4).

When BAP increased from 1.0 mg/L to 2.0 mg/L, the shoot regeneration was decreased from 3.90-3.23 shoots per explant.

It is known that cytokinin is very important for shoot organogenesis. So, we studied the effects of 0.5-2.0 mg/L KIN on shoot clumps induction. But, the result in Table 3 indicated that the addition of KIN on the cultural media had a negative effect on the induction of multiple shoot clumps. The shoot clumps formed with 1.87-2.57 shoots on MS medium supplemented range 1.0-2.0 mg/L KIN, lower than compare to MS media.

Overall, the shoot clumps multiple occurred with the addition of cytokinin at lower concentrations, while a higher concentration of cytokine was found to induce formation of shoots. This finding is consistent with result from the other reports. Yu *et al.* (2015) had reported MS medium supplemented with 2.22 µM 6-BA successfully produced 21.33 shoots after four weeks of culture. Shou *et al.* (2008) had reported that the maximum number of shoots was induced from lotus bud explants on MS medium containing 8 g/L agar, 30 g/L sucrose and 4.44 µM BA added with 0.54 µM NAA for 4 weeks, with low rates of lotus multiplication (3.50 ± 0.05 number of shoots per bud). In our study, MS medium culture (pH 5.8) containing 30g sucrose, 8g/L agar and 0.5 BAP is the best result in shoot formation from Red lotus's mature fresh seeds.

The effects of various combination of BAP and NAA on multiple shoot clumps induction

Table 4 and Fig 5 show the effects of various combination of BAP (0.5-2.0 mg/L) and NAA (0.1, 0.5 mg/L) on multiple shoot clumps induction of explant from dormant embryos.

Table 3: The effects of BAP and KIN on multiple shoot clumps induction.

Concentration of plant growth regulator (mg/l)	BAP				KIN			
	Shoot number	Leave number /shoot clumps	Shoot height (cm)	Shoot formation (%)	Shoot Number/ shoot clumps	Leave Number/ shoot clumps	Shoot height (cm)	Shoot formation (%)
0.0	2.73^{c}	3.37^{b}	0.96^{b}	100	2.50^{a,b}	3.37^{a}	0.96^{a}	86.67
0.5	5.10^{a}	4.50^{a}	1.56^{a}	100	3.07^{a}	1.83^{b}	0.90^{a,b}	93.33
1.0	3.90^{b}	2.43^{c}	0.89^{b}	100	1.87^{b}	1.47^{b}	0.73^{b,c}	76.67
1.5	3.57^{b,c}	2.37^{c}	1.17^{b}	100	2.13^{b}	1.67^{b}	1.03^{a}	83.33
2.0	3.23^{b,c}	2.63^{b,c}	1.03^{b}	96.67	2.57^{a,b}	2.00^{b}	0.69^{c}	83.33

*Values marked with same letter are not significantly different at p<0.05.

Table 4: The effects of various combination of BAP and NAA on multiple shoot clumps induction on Red Lotus.

BAP (mg/l)	NAA (mg/l)	Shoot number/ explant	Leave/ shoot clumps	Shoot height (cm)	Shoot formation (%)
0.5	0.1	3.60^{a}	3.03^{a}	1.22^{a}	96.67
0.5	0.5	2.30^{b}	2.87^{a,b}	1.03^{a,b}	93.33
1.0	0.1	1.00^{d}	2.40^{b,c}	0.94^{b}	76.67
1.0	0.5	1.53^{c}	2.17^{c}	0.98^{b}	83.33
1.5	0.1`	2.00^{b,c}	2.00^{c}	0.67^{c,d}	86.67
1.5	0.5	1.53^{c}	1.03^{d}	0.60^{d}	76.67
2.0	0.1	1.77^{c}	1.40^{d}	0.55^{d}	76.67
2.0	0.5	1.83^{c}	0.97^{d}	0.83^{b,c}	80

Values marked with same letters are not significantly different at p<0.05.

Table 5: The effects of various combination of BAP and fresh coconut water on multiple shoot clumps induction.

Coconut water (%)	Shoot number/ explant	Leave/ shoot clumps	Shoot height (cm)	Shoot formation (%)
0.00	5.10[a]	4.50[a]	1.56[a]	100
5.00	4.07[b]	3.43[b]	1.13[b]	90
10.00	1.93[c]	1.07[c]	0.68[c]	60
15.00	2.37[c]	1.70[c]	0.77[c]	66.67
20.00	1.90[c]	1.67[c]	0.66[c]	56.67

*Values marked with same letter are not significantly different at p<0.05.

Table 6: The effects of various combination of NAA and IAA on root induction of multiple shoot clumps.

IAA (mg/l)	NAA (mg/l)	Root No/shoot clumps	Root length (cm)	Root formation (%)
0.50	0.00	12.30[b]	1.36[a,b,c]	100
1.00	0.00	15.83[a,b]	1.17[c]	96.67
0.00	0.50	18.17[a]	1.64[a]	100
0.50	0.50	11.67[b]	1.08[c]	100
1.00	0.50	14.63[a,b]	1.41[a,b]	96.67

*Values marked with same letter are not significantly different at p<0.05.

Fig 4: Multiplication shoots formation after 5 weeks cultured on MS medium supplemented with 0.5 mg/L BAP.

After five weeks, the growth of shoot was different. Explants were cultured on MS basal media supplemented with combination of 0.5 mg/L BAP and 0.1 mg/L NAA was observed with the highest results, that is 3.60 shoots per explant at the mean; the length of shoot is 1.22 cm and the number of leaves is 3.03 leaves/shoot clumps. However, when combination with 0.5 mg/L NAA inhibited, the formation of shoots is 2.30 shoots per explant. Continuing the combination range of 1.0-2.0 mg/L BAP with 0.1 mg/L and 0.5 mg/L NAA, the shoot formation was decreased from 2.00-1.00 shoots per explant. Although cytokinins are usually supplied in culture media aiming at the stimulation of the shoots formation (Resende *et al*., 2015; Golada *et al*., 2018), in this study, the combination of NAA had a negative effect on the induction of multiple shoot clumps. It is probably be the NAA weakened the positive effect of BAP in multiple shoot clumps induction in some species and this finding was also indicated in Yu's report on the lotus (2015). Thus, BAP alone can increase the formation of shoots in lotus; and the optimal medium for multiple shoot induction was MS medium supplemented with 0.5 mg/L BAP, which obtained 5.10 shoots per explant. These multiple shoots were healthy and dark green colour.

The effects of various combination of BAP and fresh coconut on multiple shoot clumps induction

Many studies show that the composition of coconut water contains amino acids, organic acids and sugar as plant growth regulator. The previous studies often use coconut water to supplement the culture media. In this study, we studied the effects of combination range of 5-20% coconut water with 0.5 mg/L BAP on the shoot formation of Red lotus. The results are summarised in Table 5 and Fig 6.

MS media supplemented 0.5 mg/L BAP resulted in the best number of shoots (5.10 shoots/explant), the best shoot length (1.56 cm) and the largest number of leaves (4.50 leaves/ shoot clumps). The 0.5 mg/L BAP media supplemented coconut with the various concentration from 5% to 20%, the shoot formation was decreased from 3.43-1.07 shoots per explant, the length of shoot and the number of leaves per shoot clumps also were lower than the control

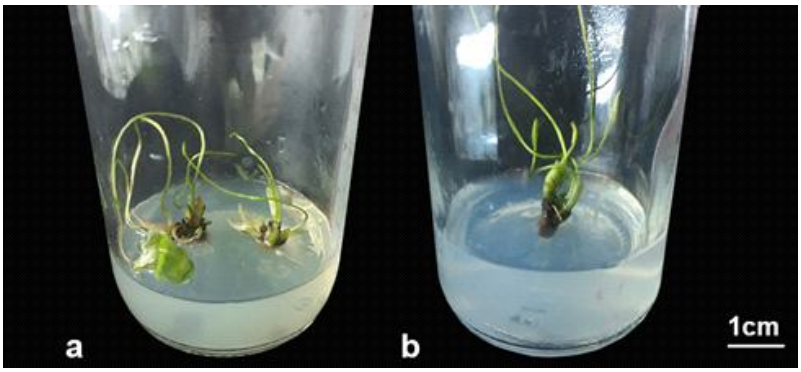

Fig 5: Multiple shoot clumps on MS basal medium supplemented with combination of BAP and NAAa. BAP (1.0 mg/L) and NAA (0.1 mg/L) b. BAP (1.5 mg/L) andNAA (0.1 mg/L).

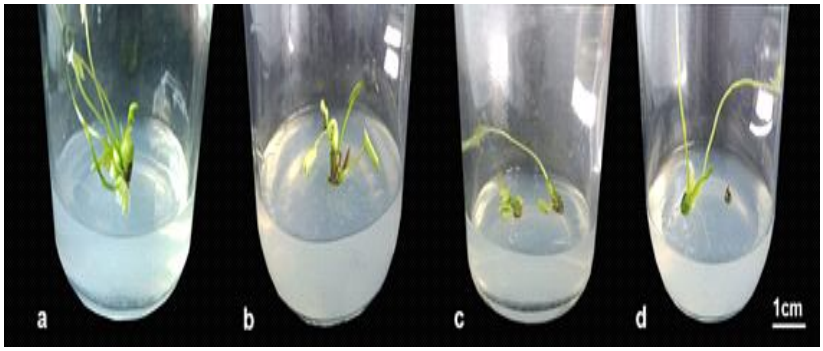

Fig 6: Multiplication shoots formation after 5 weeks cultured on MS medium supplemented with 0.5 mg/L BAP and (5-20%) fresh coconut water. a.MS medium supplemented with 0.5 mg/L BAP b .MS medium supplemented with 0.5 mg/L BAP and 5% fresh coconut water c. MS medium supplemented with 0.5 mg/L BAP and 15% fresh coconut waterd.MS medium supplemented with 0.5 mg/L BAP and 20% fresh coconut water.

Fig 7: Root formation of the *in vitro* plants on MS medium supplemented with 0.5 mg/l NAA.

Fig 8: Regenerated plants of Red Lotus growing in plastic pots after two weeks in the greenhouse on mud medium soaked for one week in 2g/L $KMnO_4$.

media. Thus, lower or higher concentration of coconut water inhibited the formation of shoot clumps.

The effects of various combination of NAA, IAA on root induction of multiple shoot clumps

After five subcultures, multiple shoot clumps were transferred to MS basal medium supplemented with various concentrations of IAA, NAA for root induction. After five weeks, the highest rate of rooting was 100% and the maximum mean number of roots per cluster of shoots was 18.23 on MS basal medium supplemented with 0.5 mg/L NAA, with 1.64 cm in length (Table 6 and Fig 7). The number of roots was low and the roots were thin and short on the other various combination of NAA and IAA concentrations.

Transplantation of *in vitro* culture plantlets derived from shoot apical meristems of lotus

After four weeks, the plantlets with approximately 8-15 cm in height and well-developed roots were obtained on MS basal medium supplemented with 0.5 NAA and 8 g/L agar, 30 g/L sucrose. After one week of acclimation, the successfully acclimated plantlets were planted in pots on five mediums and were placed in the greenhouse. After two weeks, plants grew well with much bigger leaves in the greenhouse. The survival rate of transplanting plants was 40% on mud medium soaked for one week in 2g/L $KMnO_4$ (Fig 8).

CONCLUSION

In this study, the plant regeneration system for lotus was established by using dormant embryo from 23 to 25 days old fresh seed. The regeneration technique based on the embryos of lotus solves the problem in lotus cross breeding.

This method is possible to conserve the precious hybrid seeds and expand them in a short time for the further research. Our result indicated:

1. The surface-sterilized seeds were sterilized by 70% ethanol for 30 seconds and then they were sterilized by $HgCl_2$ 0.1% for 16 minutes is optimum of seed's Vinh Thanh Red Lotus.
2. The MS basal medium with 30g sucrose and 8g/L agar provides the highest shoot regeneration rates with 2.53 shoot/explant;
3. The MS basal medium supplemented 30g sucrose, 8g/L agar and 0.5 BAP provides the highest multiplication rates for five weeks, which gave the highest number of shoot per explant (5.10 shoots) and the tallest shoot (1.56 cm), the largest leaves shoot clumps (4.50 leaves);
4. The NAA at 0.5 mg/L provides a rooting 100%, with the number of root is higher than in the other medium (18.17 roots/shoot clumps);
5. The survival rate of transplanting plants was 40% on mud medium were soaked for one week in 2g/L $KMnO_4$ after two weeks in the greenhouse.

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Competing Interests

Authors have declared that no competing interests exist.

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