Micropropagation of Tropical Night Blooming Waterlily (*Nymphaea pubescens*)

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Abstract

Micropropagation of tropical night blooming waterlily (*Nymphaea pubescens*) was studied. Rhizomes of *Nymphaea pubescens* were surface sterilized in different methods of disinfection. The best method of explant disinfection was achieved by agitating in 30% ethanol for 1 minute and agitated in 9% hydrogenperoxide for 5 minutes each, and then shaken in 0.1 % mercuric chloride solution for 15 minutes each, and shaken in 20% Clorox for 15 minutes and washed in sterile distilled water 3 times. This method gave the lowest percentage of contamination which was 0% and also gave 100% of shoot induction and the explants gave normal shoots. The cleaned explants were cultured in liquid Murashige and Skoog medium supplemented with a combination of 0, 2, 4 and 6 mgl^{-1} BA and the same concentration of NAA. After 60 days of incubation, the average maximum number of shoots (4.0 shoots per explant), average shoot length (0.50 cm) and average number of leaves (8.46 leaves per explant) were achieved on medium with 2 mgl^{-1} BA and 6 mgl^{-1} NAA. The average longest shoot length (0.79 cm) were achieved on medium with free growth regulator. The average maximum number of leaves (19.10 leaves per explant) were achieved on medium with 4 mgl^{-1} NAA.

Keywords: micropropagation, waterlily.

1. Introduction

Waterlilies are commercially important aquatic plants, which can be divided into two groups: tropical waterlily and hardly waterlily. For tropical waterlily can be divided into two subgroups : tropical night blooming waterlily and tropical day blooming waterlily. The conventional propagation of waterlily is through underground rhizomes with a rather low propagation rate. In vitro culture methods have been used for multiplication in many plant species, but its application in waterlily is rarely reported possibly. Until now, a protocol for waterlily regeneration has been reported (Lakshmanan, 1994 and Arunyanart, *et al*., 2008). *In vitro* multiplication of economically valuable waterlily through shoot proliferation from rhizomes was studied. There are many factors affecting the success of in vitro generation, such as the cultivar differences, the physiological status of explants, developmental stages of explants and plant growth regulators added to media. Micropropagation might be useful to increase the production of disease – free clonal material and rapid multiplication. The purpose of this study was to develop an in vitro propagation system for tropical night blooming waterlily (*Nymphaea pubescens*) for high propagation rate and disease-free clonal material to commercial production and breeding programs.

2. Materials and Methods

2.1 Methods of disinfection

Rhizomes were excised from the donor plants, immersed for 5 minutes in soapy water, washed throughly under running tap water for 120 minutes. The explants were then disinfested in different methods of disinfection and washed in sterile distilled water 3 times (Table 1). The cleaned explants were cultured on solidified Murashige and Skoog medium supplemented with 0.5 mgl^{-1} activated charcoal. All media were adjusted to pH 5.8 with 1 N NaOH, 0.8% agar was added, 20 ml were

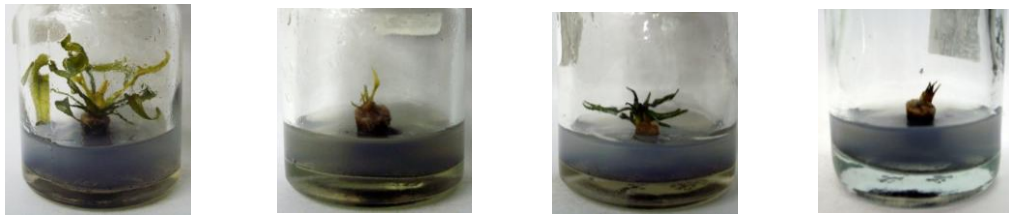
dispensed into each 4 x 6.5 cm glass bottle and then autoclaved at 15 psi. for 15 minutes at 121 °C. All cultured were incubated at 25±1 °C with a 16- h photoperiod (white fluorescent lamps, 40 µmol $m^{-2}s^{-1}$).For each treatment, one explant per glass bottle was cultured and the treatments were replicated at 5 times using a completely randomized design. The number of shoot initiating days, percentage of contaminated explants, frequency of shoot initiation, the number of leaves per explant and general characteristics were evaluated 30 days after culture.

2.2 Induction of shoots

Rhizomes were excised from the donor plants, immersed for 5 minutes in soapy water, washed throughly under running tap water for 120 minutes, agitated in 30% ethanol for 1 minute and agitated in 9% hydrogenperoxide for 5 minutes each, and then shaken in 0.1% mercuric chloride solution for 15 minutes and then shaken in 20% Clorox for 15 minutes and washed in sterile distilled water for 3 times. The cleaned explants were cultured in liquid Murashige and Skoog medium with growth regulators free for 14 days, then transferred to culture in liquid Murashige and Skoog medium supplemented with a combination of 0, 2, 4 and 6 mgl^{-1} BA and the same concentration of NAA. All media were adjusted to pH 5.8 with 1N NaOH, 0.8% agar was added, 25 ml were dispensed into each 5 x 8.5 cm glass bottle and then autoclaved at 15 psi. for 15 minutes at 121 °C. All cultured were incubated at 25± 1 °C with a 16-h photoperiod (white fluorescent lamps, 40 µmol $m^{-2}s^{-1}$). For each treatment, one explant per glass bottle was cultured and treatments were replicated at 6 times using a completely randomized design. The number of shoots per explant, shoot length, the number of leaves per explant and general characteristics were evaluated 60 days after culture.

3. Results and Discussion

The percentage of contamination, mean number of shoot initiation days, frequency of initiation, mean number of leaves and general characteristics in different methods of disinfection are indicated in Table 1. Of all methods tested, method by agitating in 30% ethanol for 1 minute and agitated in 9% hydrogen-peroxide for 5 minutes each and then shaken in 0.1% mercuric chloride solution for 15 minutes each, and shaken in 20% Clorox for 15 minutes and washed in sterile distilled water 3 times was found to be optimum for rhizomes explant disinfection. This method gave the lowest percentage of contamination which no contamination and also gave 100% in frequency of shoot initiation and the explants gave normal shoots and leaves. (Figure 1)



A B C D

Figure 1 Effects of different methods in disinfection on percentage of contamination and shoot formation from the rhizomes *Nymphaea pubescens* after 30 days in culture on MS medium.

(A) 30% ethanol 1 min + 9% H_2O_2 5 min + 0.1% $HgCl_2$ 15 min + 20% Clorox 15 min

(B) 30% ethanol 1.5 min + 9% H_2O_2 10 min + 0.1% $HgCl_2$ 20 min + 20% Clorox 20 min

(C) 50% ethanol 1 min + 18% H_2O_2 5 min + 0.2% $HgCl_2$ 15 min + 25% Clorox 15 min

(D) 50% ethanol 1.5 min + 18% H_2O_2 10 min + 0.2% $HgCl_2$ 20 min + 25% Clorox 20 min

We found that the concentration and duration of surface sterilized is effected for the selective in optimum method of disinfection for rhizomes of *Nymphaea pubescens*. And there results unlike in other researches, Arunyanart et al.(2008) reported that the best method of explant sterilization was achieved by agitating in 70% ethanol for 1 minute and agitated in 0.1% mercuric chloride solution twice for 5 minutes each, and then shaken in 5% calcium hypochlorite solution twice for 15 minutes each, and shaken in 1% calcium hypochlorite solution twice for 5 minutes each, and washed in sterile distilled water 3 times for 5 minutes each. This differential requirement of surface sterilized for rhizomes of *Nymphaea pubescens* may be attributed to genotypic differences and donor plants.

Table 1 Effects of different methods in disinfection on percentage of contamination and shoot formation from the rhizomes of *Nymphaea pubescens* after 30 days in culture on MS medium.

Method of disinfection	Number of shoot initiation days	Percentage of contamination	Frequency of shoot initiation	Number of leaves	General characteristics
Method 1 30%ethanol 1 min 9%H_2O_2 5 min 0.1%$HgCl_2$ 15 min 20%Clorox 15 min	6.6	0	100	8[a]	normal shoot, green leaves and long petiole
Method 2 30%ethanol 1.5 min 9%H_2O_2 10 min 0.1%$HgCl_2$ 20 min 20%Clorox 20 min	21.0	60	0	0[b]	no develop shoots and leaves
Method 3 50%ethanol 1 min 18%H_2O_2 5 min 0.2%$HgCl_2$ 15 min 25%Clorox 15 min	9.3	0	60	2.6[b]	normal shoot, green leaves and short petiole
Method 4 50%ethanol 1.5 min. 18%H_2O_2 10 min 0.2%$HgCl_2$ 20 min 25%Clorox 20 min	10.0	20	0	0[b]	no develop shoots and leaves
C.V.(%)	-	-	-	70.37	
F-test	-	-	-	**	

** highly significant

The mean of number of shoots, shoot length, number of leaves and general characteristics in different sets of media are indicated in Table 2. The concentration of growth regulators in the medium highly significantly influenced shoot formation in vitro. Of all the media tested, medium containing a combination of 2 mgl^{-1} BA and different concentrations (2, 4, and 6 mgl^{-1}) of NAA was found to be optimum for induction of shoots. By after 60 days of incubation, the average maximum number of shoots (4.00, 3.40 and 3.20 shoots per explant respectively), average shoot length (0.50, 0.55 and 0.62 cm respectively), average number of leaves (8.46, 8.45 and 11.82 leaves per explant respectively) and normal shoots, leaves and no root were achieved on medium with a combination of 2 mgl^{-1} BA and different concentrations (6,4 and 2 mgl^{-1}) of NAA. The average longest shoot length (0.79, 0.63 and 0.62 cm respectively) and normal shoots were achieved on medium with growth regulator-free, 2 mgl^{-1} NAA and a combination of 2 mgl^{-1} BA and NAA. And the average maximum of number of leaves (19.10, 18.00 and 16.80 leaves per explant respectively) and normal shoots, leaves and roots were achieved on medium with 4 mgl^{-1} NAA, 2 mgl^{-1} NAA and 6 mgl^{-1} NAA. (Figure 2)

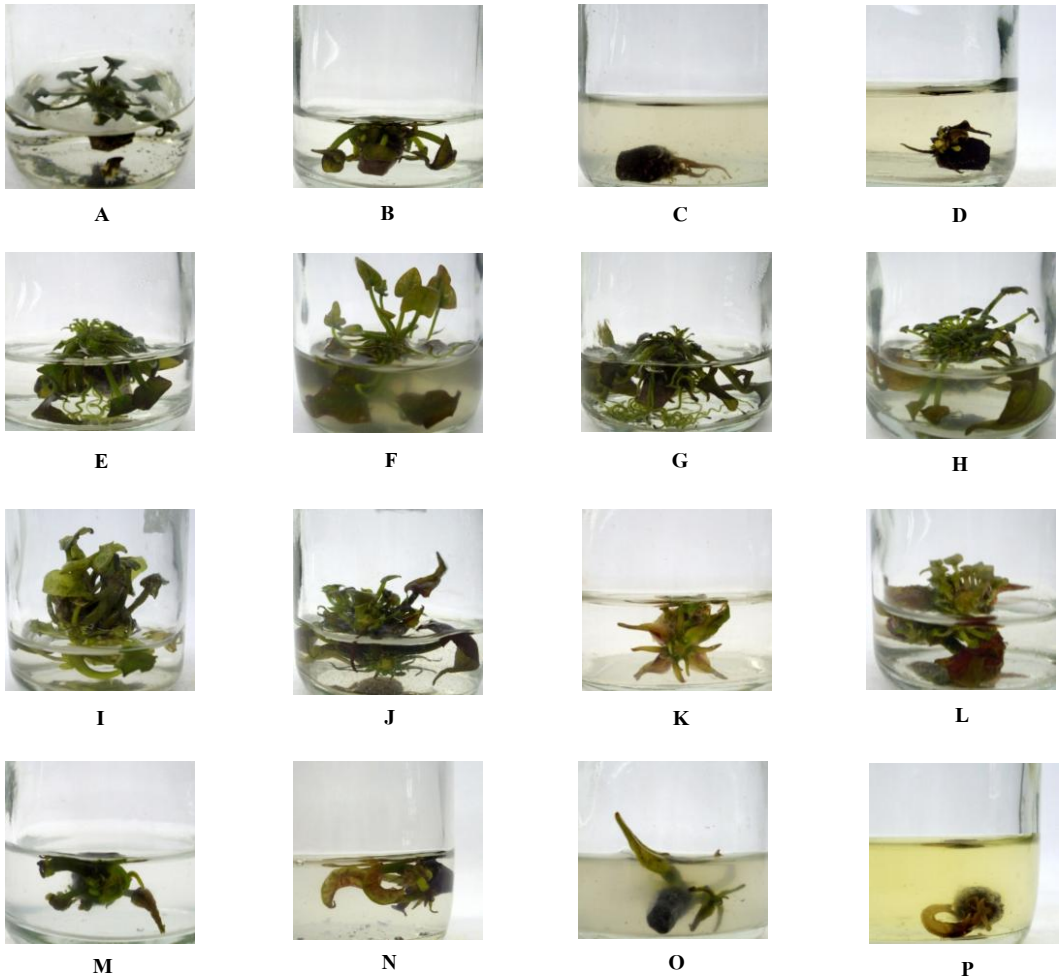


Figure 2 Effects of BA and NAA on shoot formation from the rhizomes of *Nymphaea pubescens* after 60 days in culture on MS medium

(A) MS (B) MS + BA 2 mgl^{-1} (C) MS + BA 4 mgl^{-1} (D) MS + BA 6 mgl^{-1}

(E) MS + NAA 2 mgl^{-1} (F) MS + NAA 4 mgl^{-1} (G) MS + NAA 6 mgl^{-1} (H) MS + BA 2 mgl^{-1} + NAA 2 mgl^{-1}

(P) MS + BA 6 mgl^{-1} + NAA 6 mgl^{-1} (I) MS + BA 2 mgl^{-1} + NAA 4 mgl^{-1}

(J) MS + BA 2 mgl^{-1} + NAA 6 mgl^{-1} (K) MS + BA 4 mgl^{-1} + NAA 2 mgl^{-1}

(L) MS + BA 4 mgl^{-1} + NAA 4 mgl^{-1} (M) MS + BA 4 mgl^{-1} + NAA 6 mgl^{-1}

(N) MS + BA 6 mgl^{-1} + NAA 2 mgl^{-1} (O) MS + BA 6 mgl^{-1} + NAA 4 mgl^{-1}

The cleaned explant were cultured in liquid MS medium supplemented with a combination 0, 2, 4 and 6 mgl^{-1} BA and the same concentration of NAA. The results showed that the medium with NAA gave root induction and medium with a combination of 2 mgl^{-1} BA and different concentrations (2, 4 and 6 mgl^{-1}) of NAA gave the average maximum number of shoots (3-4 shoots), normal shoots and leaves and no root which the same results as Aranyanart *et al.* (2007) reported that a combination of 6 µM NAA and 7.5 µM BA strongly favored induction of shoots in *Nymphaea* spp.cv. 'Joey Tomocik'. These results of researches showed that the different level of concentration but the same disinfectant in disinfection which according the theory reported that the different genotype responsed to different growth regulators (Skoog and Miller, 1957) but the results unlike in *Nymphaea* hybrid 'James Brydon' where shoot induction and shoot proliferation media contained a combination of 2ip, BA and NAA (Lakshmanan, 1994) and in *Nymphaea* spp.cv 'Joey Tomocik' where the average maximum number of shoots (3.8 shoots per explant) media contained with 6 µM NAA, 7.5 µM BA and 40 µM 2ip (Aranyanart *et al*., 2008).

Table 2 Effects of BA and NAA on shoot formation from the rhizomes of *Nymphaea pubescens* after 60 days in culture on MS medium

Medium formula	Number of shoots	Shoot length (cm)	Number of leaves	General characteristics
MS	1.17^{c}	0.79^{a}	6.92^{bcde}	normal shoots and leaves, a little roots
MS+BA 2 mgl^{-1}	2.00^{bc}	0.54^{bc}	10.67^{bc}	short shoots and short petiole, no root
MS+BA 4 mgl^{-1}	1.00^{c}	0.50^{bc}	3.00^{de}	abnormal shoots, brown leaves and no root
MS+BA 6 mgl^{-1}	1.00^{c}	0.50^{bc}	4.00^{de}	abnormal shoots and short petiole, no root
MS+NAA 2 mgl^{-1}	1.00^{c}	0.63^{ab}	18.00^{a}	normal shoots and leaves, a little roots
MS+NAA 4 mgl^{-1}	1.20^{c}	0.50^{bc}	19.10^{a}	normal shoots and long petiole, a little roots
MS+NAA 6 mgl^{-1}	1.20^{c}	0.55^{bc}	16.80^{a}	normal shoots and long petiole, many roots
MS+BA 2 mgl^{-1}+NAA 2 mgl^{-1}	3.20^{ab}	0.62^{ab}	11.82^{b}	normal shoots and leaves, no root
MS+BA 2 mgl^{-1}+NAA 4 mgl^{-1}	3.40^{ab}	0.55^{bc}	8.45^{bcd}	normal shoots and leaves, no root
MS+BA 2 mgl^{-1}+NAA 6 mgl^{-1}	4.00^{a}	0.50^{bc}	8.46^{bcd}	normal shoots and short petiole, no root
MS+BA 4 mgl^{-1} +NAA 2 mgl^{-1}	2.17^{bc}	0.54^{bc}	5.60^{cde}	short shoots and short petiole, no root
MS+BA 4 mgl^{-1}+NAA 4 mgl^{-1}	2.33^{bc}	0.50^{bc}	8.20^{bcd}	short shoots and short petiole, no root
MS+BA 4 mgl^{-1} +NAA 6 mgl^{-1}	1.33^{c}	0.45^{bc}	6.14^{cde}	short shoots and short petiole, no root
MS+BA 6 mgl^{-1} +NAA 2 mgl^{-1}	1.33^{c}	0.54^{bc}	4.47^{de}	small shoots and short petiole, no root
MS+BA 6 mgl^{-1} +NAA 4 mgl^{-1}	1.40^{c}	0.50^{bc}	3.60^{de}	short shoots and vitrification leaves
MS+BA 6 mgl^{-1} +NAA 6 mgl^{-1}	0.67^{c}	0.33^{c}	2.00^{c}	abnormal shoots and short petiole, no growth and no root
C.V.%	64.78	28.27	43.73	
F-test	**	**	**	

** highly significant

4. Conclusion

The best method of explant disinfection was achieved by agitating in 30% ethanol for 1 minute and agitated in 9 % hydrogenperoxide for 5 minutes each, and then shaken in 0.1% mercuric chloride solution for 15 minutes each, and shaken in 20% Clorox for 15 minutes and washed in sterile distilled water 3 times. This method gave lowest percentage of contamination and also gave 100% of shoot induction. Of all the media tested, medium containing a combination of 2 mgl^{-1} BA and different concentrations (2, 4 and 6 mgl^{-1}) of NAA was found to be optimum for induction of shoots because its gave the average maximum number of shoots and normal shoots, leaves but no root.

5. References

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