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In Vitro Self and Cross Pollinated Seed Culture of *Nymphaea rubra* 'Maeploi', A Night – Blooming Tropical Waterlily Hybrid (Nymphaeaceae) from Thailand

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ABSTRACT

This research was aimed to develop culture medium enhancing the germination of self and cross-pollinated seeds in *Nymphaea rubra* 'Maeploi' and *Nymphaea pubescens* Willd. The self-pollinated seeds of *N. rubra* 'Maeploi' were investigated for surface sterilization method, seed culture medium and light conditions. The self-pollinated seeds were surface sterilization with 20% (v/v) sodium hypochlorite (NaOCl) at various times (5, 10, 15 and 20 min), followed by the second surface sterilization with NaOCl at different concentrations (5, 10, 15 and 20% (v/v)) for 10 min. MS medium at different strengths (MS, 1/2MS, 1/4MS, 1/8MS) was also studied to rescue the cross-pollinated seeds, compared to control. The results showed that the fruit set percentages of *N. pubescens* x *N. rubra* 'Maeploi' and *N. rubra* 'Maeploi' x *N. pubescens* were low at 5% and 3.33%, respectively. The *N. rubra* 'Maeploi' self-pollinated seeds were soaked with 20% (v/v) NaOCl for 5 min, followed by the sterilization with 5% (v/v) NaOCl for 10 min showed without the microbial contamination. Moreover, the suitable medium for *N. rubra* 'Maeploi' self-pollinated seeds was 1/8MS semi solid medium under dark condition. The germinated seedlings were developed shoots (60%), immature leaves (51.67%), and roots (48.33%) after 4 weeks of culture. Germination

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percentages of *N. rubra* 'Maeploi' and *N. pubescens* self-pollinated seeds cultured on 1/8MS were 51.67 higher than those of cross-pollinated seeds of *N. pubescens* x *N. rubra* 'Maeploi' and *N. rubra* 'Maeploi' x *N. pubescens* (35 and 31.67%, respectively).

INTRODUCTION

Nymphaea is the largest genus of waterlilies that classified into the family Nymphaeaceae, which consists of two groups: Apocarpiae and Syncarpiae, (1-4). The Apocarpiae group contains three subgenera, especially *Anecphya* (an Australian tropical waterlily), *Confluentes* (an Australian tropical waterlily) and *Brachyceras* (a day-blooming tropical waterlily). In the Syncarpiae group also consists of three subgenera, such as *Hydrocallis* (a night-blooming tropical waterlily), *Lotos* (a night-blooming tropical waterlily) and *Nymphaea* (a hardy waterlily) (3). The members of this genus are approximately 50 species which are widely distributed in both tropical and temperate areas (1; 5) for example, *Nymphaea rubra* Roxb. ex Andrews and *N. pubescens* Willd., which are classified into the subgenus *Lotos*. They are a night-blooming tropical waterlily and their flowers are opened during 07.00 p.m. to 10.00 a.m.) in the Thai climate (3). *N. rubra* 'Maeploi' is a Thai waterlily hybrid cultivar in the nature that registered to the international waterlily and water gardening society (IWGS) by Dr. Slearmlarp Wasuwat in 2002 (Figure 1A). This waterlily cultivar has the light brown on adaxial side and dark green on abaxial side of leaf. Its dark pink flowers like a ruby which are smaller size than its related waterlily species, especially *N. rubra* Roxb. ex Andrews. For *N. pubescens* Willd., a beautiful night-blooming tropical waterlily with the white flowers and yellow stamen, which is beyond to be its unique characteristics. It has been reported that the ethanolic extract of its flowers could induce apoptosis in human cervical and breast carcinoma cells *in vitro*, and therefore, this plant species was used as a medicinal plant for cancer therapy with anti-proliferation activity (6). These two waterlily species have been chosen because they are tropical potted aquatic plants and used as ornamental waterlilies for building decoration and water gardening (7). In order to conserve this natural waterlily hybrid for genetic resource and to produce a mass numbers of germinated seedlings of the interspecific waterlily hybrids, the both of self and cross pollination were investigated. However, the report on seed culture under aseptic technique of these two waterlily species is still limited.

Hence, factors affecting on fertilization, fruit setting and germination of cross-pollinated seeds between *N. rubra* 'Maeploi' and *N. pubescens* were observed for the interspecific waterlily hybrid production. The main goal of this study was aimed to develop the culture medium for increasing the germination and survival percentage of self-pollinated seeds of *N. rubra* 'Maeploi' and *N. pubescens,* and cross-pollinated seeds between *N. rubra* 'Maeploi' and *N. pubescens.* According to the results from

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this study will be used as an effective procedure for further studies and research involved in *in vitro* and *ex vitro* propagation, varietal improvement and *ex situ* conservation of a night-blooming tropical waterlilies and several aquatic plants.

MATERIALS AND METHODS

Experiment I: Self and cross pollination

Self-pollination of *N. rubra* 'Maeploi', *N. pubescens*, and cross pollination between *N. rubra* 'Maeploi' and *N. pubescens* (*N. pubescens* x *N. rubra* 'Maeploi' and *N. rubra* 'Maeploi' x *N. pubescens*) were performed by hand pollination that is described as conventional breeding. The vigorous parental plants of two selected waterlily species were collected at lotus museum, Division of Building and Site Administration, Office of the President, Rajamangala University of Technology Thanyaburi (RMUTT), Pathum Thani, Thailand, which were used as plant materials in this study (Figure 1A-B). Because of the receptivity of stigma former their stamen within the same flower on the first day (1st day) blooming, therefore, the 1st day blooming flowers were only used as the female parent plant (seed plant) while the second day (2nd day) or the third day (3rd day) blooming flowers were used as male parent plants (pollen plant). To cross pollinate these two selected waterlily species, the pollen grains were collected from one flower and then taken to deposit them into the nectar or stigmatic fluid on the center of stigma of a first day bloom, following by reciprocal cross pollination. Each pollination of self and interspecific cross in *N. rubra* 'Maeploi' and *N. pubescens* was performed by sixty flower blooms. Fruit set percentages of self and cross pollination were monitored and recorded after hand pollination for one week.

Experiment II: Surface sterilization on self-pollinated seeds of *N. rubra* 'Maeploi'

Self-pollinated seeds of *N. rubra* 'Maeploi' (Figure 1E) were pre-sterilized by mild detergent and then dipped into 70% ethanol for 60 seconds before sterilization with sterilizing reagent. The first surface sterilization was examined by 20% (v/v) sodium hypochlorite (NaOCl) solution in various soaking times (5, 10, 15, and 20 min). Regarding to the second surface sterilization was studied at different concentrations (5%, 10%, 15%, and 20% (v/v)) of NaOCl solution for 10 min. Seeds were cultured on semi solid Murashige and Skoog (MS) (8) medium without plant growth regulators (PGRs) and then incubated at 25 ± 2 ℃, under the cool white fluorescent light at intensity of 37 μmol/m^2/s for 16 hours per day. Each treatment contained six replications and each replication had ten seeds. Contamination and seed germination percentages were recorded after four weeks of culture.

Experiment III: Culture medium development for seed culture of *N. rubra* 'Maeploi'

In case of culture medium development, self-pollinated seeds of *N. rubra* cv 'Maeploi', 0.2 – 0.3 cm in diameter were cultured on different strengths of MS semi-solid medium: MS, 1/2MS, 1/4MS and 1/8MS, pH at 5.6 – 5.8 and then maintained under the same light condition in experiment II, when compared to dark condition. Six replications were performed with ten seeds

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per replication. Seed germination percentage, seedling survival percentage, shoot formation, leaf formation and root formation were recorded after four weeks of culture.

Experiment IV: In vitro culture of cross-pollinated *N. rubra* 'Maeploi' seeds

Self and cross-pollinated seeds of two selected waterlily species were obtained from the experiment I. The sample seeds were surface sterilized using the suitable procedures from the experiment II, cultured on the suitable strength of MS semi-solid medium from experiment III, compared to the control. The experiment was also taken to place under the standard culture conditions that consisted of incubation temperature at 25 ± 2 ℃, under the cool white fluorescent lamps originating the light intensity of approximately 37 μmol/m^2/s for 16 hours photoperiod. Each treatment was performed with six replications and ten seeds per replication. Seed germination percentage, seedling survival percentage, shoot formation, leaf formation and root formation were also monitored and recorded after four weeks of culture.

Data collection and statistical analysis

All experiments were arranged by a completely randomized design (CRD). The data were statistically subjected to a one-way analysis of variance (ANOVA, F-test) using SPSS version 22.0. The means ± standard error (SE) were compared by using Duncan's multiple range test (DMRT) with the level of significance at 5%.

RESULTS AND DISCUSSION

The results showed that fruit set percentage of self-pollinated flowers of *N. rubra* 'Maeploi' and *N. pubescens* were 1.67 and 11.17%, respectively. Whereas, the cross-pollinated flowers of *N. pubescens* x *N. rubra* 'Maeploi' and *N. rubra* 'Maeploi' x *N. pubescens* resulted in 5% and 3.33% of fruit set (Table 1). This data indicated that fertilization processes rarely occurred in both self and cross pollinations of these two *Nymphaea* species. Thus, the self and cross barriers in the same species or interspecific hybridizations, including the pre- and post-fertilization incompatibility of self and cross pollination are required for further investigation.

Previous study, Sun et al. (21) reported the pollen viability, pollen germinability and time after pollination (hour, h) influenced on self and cross-pollination of four tropical waterlilies, especially *N. odorata* 'Peter Slocum', *N. colorata*, *N. micrantha* and *N. gigantea*. The results showed that the highest number of germinated pollen grains on stigmas in the self-pollinated *N. odorata* 'Peter Slocum' was peaked at 12 hours after pollination (HAP). At the same time, the self-pollination of *N. odorata* 'Peter Slocum' gave a high percentage (81.2%) of normal embryos. Whereas, the normal embryos and seeds were not produced from the *N. odorata* 'Peter Slocum' x *N. micrantha*, *N. odorata* 'Peter Slocum' x *N. colorata* and *N. odorata* 'Peter Slocum' x *N. gigantea* crosses. From the results suggested that pre- and post-fertilization barriers existed together in these crosses, which may be the main causes resulting in the failure and embryo abortion of interspecific hybridizations in waterlily

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(21). Like the low seed set in some crosses between *Nymphaea* 'Fen Zhuang', *Nymphaea* 'Bai Lu' and *Nymphaea* 'Hong Ying' was from the low pollen viability, low pistil receptivity and embryo abortion (22). In another plant species, hand pollination in *Oxalis corymbosa* for evaluating the flower characteristics in sample population. The resulted showed that some sample populations were not produced seeds after fertilization that caused from limitations of pollen transfer. Almost cross-pollinated seeds were less germination and also failed to grow in early development of embryos and seedlings, which indicated that the embryo rescue or embryonic culture are necessary (10).

According to surface sterilization of self-pollinated *N. rubra* 'Maeploi' seeds, the sample seeds were soaked in 20% (v/v) NaOCl for 5 min, followed by the sterilization with 5% (v/v) NaOCl for 10 min showed without the microbial contamination and the highest seed germination percentage of approximately 5% after four weeks of culture (Table 2). This sterilizing treatment could inhibit the microbial contamination and maintain the clean culture in the long period of cultivation time. In preparation of plant tissues and explants before culturing, the selected explants must have to be rendered aseptic techniques, especially surface sterilization. The various dilutions (10-30% v/v) of Clorox® or commercial bleach containing sodium hypochlorite, (5.25% w/v NaOCl) often used as the active ingredient and disinfectant for removal microorganisms (23). Likewise, the ability of sodium hypochlorite (NaOCl) to inhibit the growth of microbial contaminants in *Ziziphus spina* [christti] seeds has been investigated *in vitro*. The experiment revealed that the surface sterilization with 4% NaOCl for 20 min, followed by dipping seeds in 2% benomyl for 5 min and then rinsed with distilled water was the best sterilization treatment for culturing *Z. spina* [christti] seeds, when compared to other sterilization treatments. Seeds tested in each treatment were aseptically cultured on agar-solidified MS medium in culture tubes. However, this sterilization treatment also gave the highest number of the germination of sterilized seeds in *Z. spina* [christti] (24). Moreover, sodium hypochlorite was used as media sterilant in the production of sugarcane plantlets at commercial scale, which was the total active chlorine concentration at 0.002% in the culture medium (25). On the other hand, *in vitro* surface sterilization of *Nymphaea gigantea* 'Atrans' hybrid seeds have been previous studied. This waterlily hybrid cultivar was from the cross pollination between *N. gigantea* var. violacea and *N. gigantea* 'Atrans'. Regarding to break the seed dormancy and *in vitro* culture of *Nymphaea gigantea* 'Atrans' hybrid seeds, some selected surface sterilization procedures were also investigated. The sample seeds were soaked in 70% (v/v) ethanol (EtOH) for 30 seconds, followed by a two-step procedure: 7.5% and 4% (v/v) hydrogen peroxide (H_2O_2) for 5 min in each step and then rinsed thrice in sterile distilled water for 5 min, respectively. From the results showed that without the microbial contamination and gave the highest percentage of surface sterilization

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(100%) of 60 days-stored seeds after two weeks of culture, compared to the others (26).

After the four weeks of *in vitro* culture, self-pollinated *N. rubra* 'Maeploi' seeds cultured on 1/8MS semi solid medium and incubation under dark condition gave the highest germination percentage (75%) and survival percentage (75%). Most of the germinated seedlings produced shoots (60%) , immature leaves (51.67%) and roots (48.33%), respectively (Table 3). However, the immature leaves of germinated seedlings formed under dark condition were pale yellow when compared to those of germinated seedlings grown under light condition (Figure 1 F-G). Culture medium is an important factor affecting on growth and development of cultured explants (i.e. seeds, shoot tips, dormant buds, plant meristematic tissues and callus). Application of culture medium depends on many factors, especially plant species, plant organs and tissues. In various plant tissue culture media, MS (Murashige and Skoog) basal medium is widely used in plant tissue culture, which is attainably enrich in both macro and micronutrients (17). Many culture media have been examined in both seed and tissue culture but not all of them were effective to use, depending on plant species and other factors. The highest germination percentages of *N. rubra* 'Maeploi' and *N. pubescens* self-pollinated seeds were at 51.67% on 1/8MS culture medium whilst the germination percentages of *N. pubescens* x *N. rubra* 'Maeploi' and *N. rubra* 'Maeploi' x *N. pubescens* cross-pollinated seeds were at 35% and 31.67% on 1/8MS culture medium, respectively (Table 4). The results showed that the interspecific hybrid seeds of *Nymphaea* species germinated well on 1/8MS culture medium and self-pollinated seeds could germinate and growth better than cross-pollinated one. This may be because *Nymphaea* seeds required low concentraions of MS medium. Buitendijk et al. (11) denoted that the interspecific hybridization in *Alstroemeria* spp. was also found the problems about early growth and development of hybrids, which known as post-fertilization barriers. The developmental rate of hybrid seedlings was gradually decreased after 18 days of fertilization. On the other hand, Liu et al. (12) studied on culture medium used for embryo rescue of *Leucadendron* hybrids after the interspecific hybridization. MS medium containing 2% (w/v) sucrose and 3.0 g/L Phytogel was suitable culture medium for accelerating growth and development of the interspecific hybrid embryos and seedlings in this plant genus. Manzur et al. (13) reported that *Capsicum* spp. seeds culture on half strength MS (1/2MS) agar medium containing 4% (w/v) sucrose gave the highest germination rate that used to rescue the hybrid embryos. Bodhipadma et al. (18) reported that the suitable culture medium and condition used for *in vitro* leaf induction in *Nymphaea nouchali* var. *versicolor* 'Bua Phuean' tuberous rhizomes, it was found in explant cultured on MS semi-solid medium supplemented with 2.5 mg/L BAP and 0.1% activated charcoal under light condition for four weeks of culture. However, more related studies and database of *in vitro* propagation and improvement of lotuses and waterlilies are needed. In addition, this study was to develop the suitable culture medium used for enhancing the

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seed germination and produce vigorous seedlings of self and cross-pollinated seeds of *N. rubra* 'Maeploi'. Moreover, these practical treatments provide basic information for future research and studies on the *ex situ* conservation, propagation and improvement of tropical night-blooming waterlilies and their hybrids. For further studies, the biotechnological approaches and molecular techniques are also important and necessary for developing the plant variety production, hybridization and other breeding programs that applied to precise any quantitative and qualitative trait of interests, for example; the application of plant genomic selection and deoxyribonucleic acid (DNA) marker selection (14).

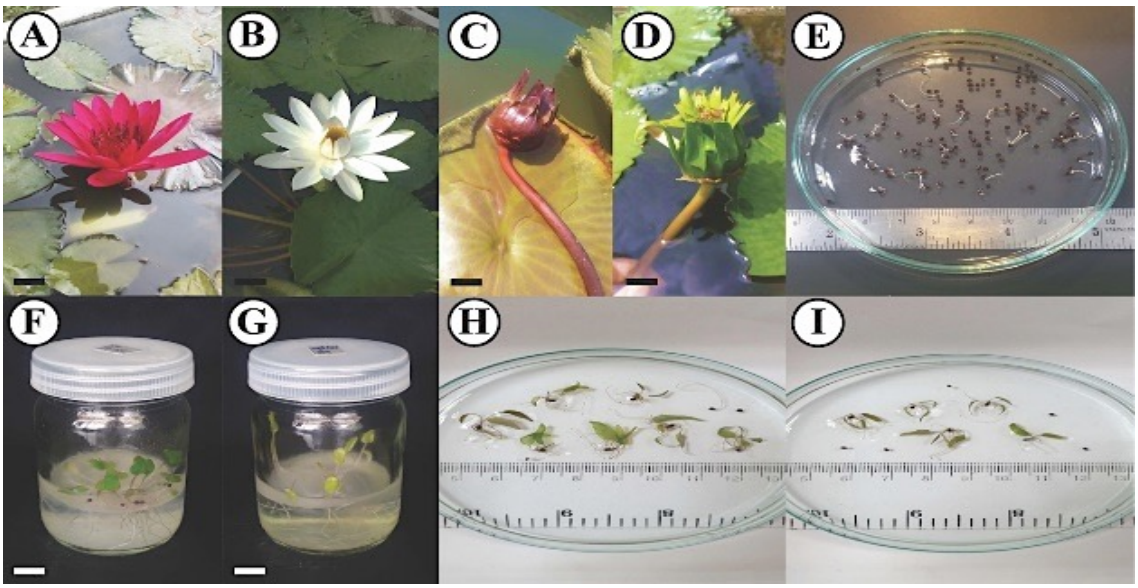

Figure 1 Interspecific hybridization of two selected *Nymphaea* species, **(A)** *Nymphaea rubra* 'Maeploi' (black scale bar = 2 cm), **(B)** *Nymphaea pubescens* Willd. (black scale bar = 2 cm), **(C)** *N. rubra* 'Maeploi' seed pod (black scale bar = 2 cm), **(D)** *N. pubescens* Willd. seed pod (black scale bar = 2 cm), **(E)** self-pollinated *N. rubra* 'Maeploi' seeds, **(F)** self-pollinated *N. rubra* 'Maeploi' seedlings cultured on one-eighth strength MS (1/8MS) semi-solid medium under light condition (white scale bar = 1 cm), **(G)** self-pollinated *N. rubra* 'Maeploi' seedlings cultured on 1/8MS semi-solid medium under dark condition (white scale bar = 1 cm), **(H)** cross-pollinated seedlings between *N. pubescens* and *N. rubra* 'Maeploi' cultured on 1/8MS semi-solid medium under light condition, **(I)** cross-pollinated seedlings between *N. rubra* 'Maeploi' and *N. pubescens* cultured on 1/8MS semi-solid medium under light condition.

Table 1 Fruit set percentages of self and cross-pollination of two selected *Nymphaea* species.

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Treatment	Fruit set (%)
Self-pollination of *N. rubra* 'Maeploi'	1.67 ± 1.67[b]
N. pubescens x *N. rubra* 'Maeploi'	5.00 ± 2.84[ab]
N. rubra 'Maeploi' x *N. pubescens*	3.33 ± 2.34[ab]
Self-pollination of *N. pubescens*	11.17 ± 4.18[a]

Value presented as mean ± SE; different letters within a column that indicate significant differences at p = 0.05 according to analysis of variance (ANOVA, F-test).

Table 2 Utilization of NaOCl for surface sterilization of self-pollinated *N. rubra* 'Maeploi' seeds.

1st surface sterilization		2nd surface sterilization		Contamination (%)	Seed germination (%)
NaOCl (% V/V)	Time	NaOCl (% V/V)	Time		
20	5	-	-	0.00 ± 0.00[b]	5.00 ± 2.84[a]
20	10	-	-	3.33 ± 2.34[b]	3.33 ± 2.34[a]
20	15	-	-	0.00 ± 0.00[b]	1.67 ± 1.67[a]
20	20	-	-	0.00 ± 0.00[b]	3.33 ± 2.34[a]
20	5	5	10	0.00 ± 0.00[b]	5.00 ± 2.84[a]
20	10	10	10	0.00 ± 0.00[b]	1.67 ± 1.67[a]
20	15	15	10	0.00 ± 0.00[b]	3.33 ± 2.34[a]
20	20	20	10	16.67 ± 4.85[a]	6.67 ± 3.25[a]

Value presented as mean ± SE; different letters within a column that indicate significant differences at p = 0.05 according to analysis of variance (ANOVA, F-test).

Table 3 Effects of different MS medium strengths on germination and early development of self-pollinated *N. rubra* 'Maeploi' seedlings after 4 weeks of culture.

MS medium	Condition	Germination (%)	Survival (%)	Shoot formation (%)	Leaf formation (%)	Root formation (%)
1	Light	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[d]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
1	Dark	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[d]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
1/2	Light	35.00 ± 6.21[b]	35.00 ±6.21[b]	23.33 ± 5.51[c]	21.67 ± 0.05[b]	21.67 ± 5.36[b]
1/2	Dark	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[d]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
1/4	Light	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[d]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
1/4	Dark	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[d]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
1/8	Light	43.33 ± 6.45[b]	43.33 ±6.45[b]	43.33 ± 6.45[b]	43.33 ± 0.07[a]	43.33 ± 6.45[a]
1/8	Dark	75.00 ± 5.64[a]	75.00 ± 5.64[a]	60.00 ± 6.38[a]	51.67 ± 0.07[a]	48.33 ± 6.51[a]

Value presented as mean ± SE; different letters within a column that indicate significant differences at p = 0.05 according to analysis of variance (ANOVA, F-test).

Table 4 *In vitro* culture of self and cross-pollinated seeds of two selected *Nymphaea* species on

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1/8 MS medium, compared to control after 4 weeks of culture.

Treatment			Germination percentage (%)	Survival Percentage (%)	Shoot formation (%)	Leaf formation (%)	Root formation (%)
Hand pollination		MS medium					
(Self)	*N. rubra* 'Maeploi'	Full MS	5.00 ± 2.84^{c}	5.00 ± 2.84^{c}	1.67 ± 1.67^{c}	1.67 ± 1.67^{c}	0.00 ± 0.00^{c}
(Self)	*N. rubra* 'Maeploi'	1/8 MS	51.67 ± 6.51^{a}	51.67 ± 6.51^{a}	50.00 ± 6.51^{a}	50.00 ± 6.51^{a}	43.33 ± 6.45^{a}
(Cross)	*N. pubescens* x *N. rubra* 'Maeploi'	Full MS	13.33 ± 4.43^{c}	13.33 ± 4.43^{c}	1.67 ± 1.67^{c}	1.67 ± 1.67^{c}	1.67 ± 1.67^{c}
(Cross)	*N. pubescens* x *N. rubra* 'Maeploi'	1/8 MS	35.00 ± 6.21^{b}	35.00 ± 6.21^{b}	28.33 ± 5.87^{b}	28.33 ± 5.87^{b}	25.00 ± 5.64^{b}
(Cross)	*N. rubra* 'Maeploi' x *N. pubescens*	Full MS	6.67 ± 3.25^{c}	3.33 ± 2.34^{c}	1.67 ± 1.67^{c}	1.67 ± 1.67^{c}	1.67 ± 1.67^{c}
(Cross)	*N. rubra* 'Maeploi' x *N. pubescens*	1/8 MS	31.67 ± 6.06^{b}	31.67 ± 6.06^{b}	25.00 ± 5.64^{b}	25.00 ± 5.64^{b}	25.00 ± 5.64^{b}
(Self)	*N. pubescens*	Full MS	11.67 ± 4.18^{c}	11.67 ± 4.18^{c}	1.67 ± 1.67^{c}	1.67 ± 1.67^{c}	0.00 ± 0.00^{c}
(Self)	*N. pubescens*	1/8 MS	51.67 ± 6.51^{a}	51.67 ± 6.51^{a}	45.00 ± 6.48^{a}	45.00 ± 6.48^{a}	43.33 ± 6.45^{a}

Value presented as mean ± SE; different letters within a column that indicate significant differences at p = 0.05 according to analysis of variance (ANOVA, F-test).

CONCLUSIONS

Low fruit set percentages were obtained from self and cross pollination of *N. rubra* 'Maeploi' and *N. pubescens*. Using 20% (v/v) NaOCl for 5 min followed by 5% (v/v) NaOCl for 10 min was the optimum for seed surface sterilization. One-eighth strength MS (1/8MS) semi-solid medium was suitable for *in vitro* culture of self-pollinated *N. rubra* 'Maeploi' seeds and cross-pollinated seeds between *N. rubra* 'Maeploi' and *N. pubescens*.

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