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Colchicine and duration time on survival rate and micropropagation of *Dionaea muscipula* Ellis

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Young leaf bases and leaf blades about 0.5 cm in height of a carnivorous plant *Dionaea muscipula* were used as explants for determining the callus multiplication. Explants were cultured on 1/2MS medium supplemented with various concentrations of benzyladenine (BA; 0, 0.1, 0.2, 0.5, 1.0 mg/l). 1/2MS medium supplemented with 1.0 mg/l BA gave the highest average diameter of callus about 0.55 cm after culturing for nine weeks. Callus was subcultured into the same medium every three weeks four times. 1/2MS supplemented with 0.5 mg/l BA gave the highest average plant height, number of leaves, number of roots, and root length. When young shoots about 0.5 cm long treated with a combination of different concentrations (0, 5, 10, 15 and 20 mg/l) of colchicine within different incubation times (24, 48 and 72 h), the survival rate was dependent on the concentration of colchicine and incubation time. Their survival rate was the lowest, when young shoots were soaked in 20 mg/l colchicine for 72 h (70%).

Key word: *Dionaea muscipula*, colchicine, incubation time, micropropagation.

INTRODUCTION

Dionaea muscipula Ellis (Venus fly trap) is a carnivorous plant which is endangered and on the brink of extinction, and belongs to the *Droseraceae* family. This plant is native to the eastern coast of the U.S.A. (Slack, 1981). This ornamental plant is attractive and can be used as a medicinal plant. This plant has been used for years as a source for an anticancer drug and various secondary metabolites which have been used as immunomodulator, antileprosy, antifertility, abortifacient and chitin synthetase inhibitor (Finnie and Staden, 1993; Pakulski and Budzianowski, 1996). Due to the insect-trapping characteristics, many people want to grow them as ornamental plants and their demand is increasing. The plant tissue culture technique plays an important role in preservation and micropropagation of this plant. There is a number of reports on the *in vitro* propagation of other carnivorous plants as an effort toward their preservation. Jala (2012) reported micropropagation of the pitcher plant *Nepenthes mirabilis* by using shoot tip. Crouch et al. (1988, 1990) reported *in vitro* propagation of a sundew, *D. rotundifolia* L., by a leaf culture. Jang and Park (1999) reported a method for mass propagation of *D.*

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Table 1. Effect of various concentrations of BA on size and red color of *Dionaea muscipula* Ellis callus after culturing for 9 weeks (±SE).

Concentration of BA (mg/l)	Size of callus (diameter: cm)*	Level of red callus (red)*
0.0	0.0 ± 0.00^{c}	0 ± 0.00^{c}
0.1	0.29 ± 0.28^{ab}	1.2 ± 0.79^{ab}
0.2	0.40 ± 42^{ab}	0.80 ± 0.79^{a}
0.5	0.16 ± 0.18^{a}	0.90 ± 0.99^{a}
1.0	0.55 ± 0.25^{b}	2.20 ± 0.79^{b}

[abc]Values in the same columns not significantly different using the Tukey test at 95% ($p\leq0.05$). Level of the intensity of red color of the callus: 1, light red; 2, red; 3, dark red.

L. through a shoot culture. A few reports on micropropagation of Venus fly traps are available. Teng (1999) used flower stalks as explants. Intact plants can be readily cultured by micropropagation (CZany et al., 1992).

This study examined an effect of colchicine and time for soaking which can change their morphological, mutation characters in suitable formula of medium and plant growth regulator (benzyladenine, BA) for rapid *in vitro* micropropagation of *D. muscipula* Ellis.

MATERIALS AND METHODS

Young leaf bases and leaf blades of *D. muscipula* were used as explants. Explants were surface sterilized by soaking with 70% alcohol for 1 min and soaked with 10% Clorox (NaOCl) for 10 min, followed by 5% clorox and washed with sterilized distilled water 3 times to remove the Clorox. They were cultured on 1/2 MS (Murashige and Skoog, 1962) supplemented with 0, 0.1, 0.2, 0.5, or 1.0 mg/l BA and 3% sucrose, 0.25% gelrite at pH 5.7, which had been autoclaved at 121° C for 20 min. The cultures were maintained at 25 ± 2°C under 16-h photoperiod with illumination provided by cool fluorescent lamps at an intensity of 60 µmol m^{-2} s^{-1} (TLD 36 w/853350 lm Phillips Thailand). These cultures were maintained in a proliferating state by subculturing every three weeks into the same medium 4 times. Callus formation was found at the edge of explants in ½ MS after culturing for four weeks.

A bunch of young leaf bases and leaf blades, about 0.5 cm in height (4-5 leaf bases and leaf blades) were soaked in various concentrations (0, 5, 10, 15 and 20 mg/l) of colchicine for 24, 48 and 72 h. Young leaf bases and leaf blades soaked in combinations of different concentrations of colchicine and incubated for different times were then cultured in 1/2MS medium and subcultured every 3 weeks 4 times. Percentage of leaf bases and leaf blades that survived after culturing in 1/2MS were counted as shown in Table 3. When these leaf bases and leaf blades were cultured longer, it was found that shoots were proliferated and formed; their growth are displayed in Table 4. Mutant characters from plantlets were recorded when these plantlets were cultured for 12 weeks as shown in Figure 2.

Statistical analysis

Experiments were set up in Completely Randomized Design (CRD) with six treatments; each treatment consisted of 20 replicates for the first experiment and 25 replicates for the second experiment. The test of statistical significance was done by applying Tukey's test at 5% confidence level using SAS statistical software, Release 6.03 (SAS Institute Inc., Cary, NC).

RESULTS

Explants of *D. muscipula* approximately 0.5 - 1 cm in size were cultured on half-strength MS (1/2MS) medium supplemented with various concentrations of BA. During the second week, the explants turned brown. During the fourth week, the explants which were cultured in ½ MS medium turned from dark brown to red (Figure 2). It was found that some red callus occurred at the edge of the explants. There was a significant difference ($p<0.05$) in size and red color of callus. The size of callus increased after subculturing every 3 weeks and 3 times and culturing on ½ MS medium supplemented with 0.1, 0.2, 0.5 or 1.0 mg/l of BA. Callus from 1/2MS supplemented with 1.0 mg/l BA gave the highest average diameter of callus, about 0.55 cm, and the level of red color of callus was 2.2 (Table 1).

When the red callus was subcultured into the same medium every 3 weeks 4 times, it was found that red callus was induced to form new young shoots and formed roots at the base. Plant height, number of leaves, number of roots and root length in each concentration of BA are shown in Table 2. No parameter from various concentration of BA between 0.1-1.0 mg/l was significantly different (Table 2). The average plant height with 0.1 and 0.5 mg/l BA was about 0.96 and 0.93 cm, respectively. The values of all parameters at 1.0 mg/l BA were always the lowest (but not significant).

Survival rate

Explants, about 0.5 cm in height, were soaked with combination of different concentrations (5, 10, 15 or 20 mg/l) of colchicine and incubated for various (24, 48, 72 h) time. After being treated, all explants were cultured in 1/2MS supplemented with 0.2 mg/l BA and subcultured every three weeks at two times. After 6 weeks, survival rate was recorded as shown in Table 3.

The results show that the survival rate of *D. muscipula*

Table 2. Effect of various concentrations of BA on growth of *Dionaea muscipula* in terms of plant height, number of leaves, number of roots and root length after being cultured for 12 weeks. (±SE)

Concentration of BA (mg/l)	Plant height (cm)	Number of leaves	Number of roots	Root length (cm)
0.0	0.24±0.23[a]	0.05±0.56[a]	0.0±0.0[a]	0.0±0.0[a]
0.1	0.96±0.36[b]	2.20±0.42[b]	3.60±2.01[b]	0.98±0.57[b]
0.2	0.85±0.42[b]	2.30±1.06[b]	4.40±3.24[b]	0.69±0.55[b]
0.5	0.93±0.36[b]	2.30±0.67[b]	5.90±2.64[b]	0.96±0.50[b]
1.0	0.58±0.47[b]	1.70±1.34[b]	3.20±3.29[b]	0.69±0.53[b]

[abc]Different letters within the same columns indicate statistically significant difference using the Tukey test at 95% ($p \leq 0.05$).

Table 3. Survival rates of *Dionaea muscipula* soaked with a combination of different concentrations of colchicine, incubated for various time and transferred to the culturing medium 1/2 MS supplemented with 0.2 mg/l BA for next 6 weeks.

Colchicine Concentration (mg/l /h)	Number of explants (shoot)	Number of survivors (shoot)	Survival rate (%)
Control	40	40	100
5/24	40	34	85
10/24	40	32	80
15/24	40	34	85
20/24	40	32	80
5/48	40	33	82.5
10/48	40	33	82.5
15/48	40	31	77.5
20/48	40	30	75
5/72	40	34	85
10/72	40	30	75
15/72	40	30	75
20/72	40	28	70

depended on the concentration of colchicine and duration time. Tend of Explants which were soaked at higher concentrations of colchicine and for longer incubation times gave lower survival rate. Concentrations of 5 and 15 mg/l colchicine and 24 h incubation gave the highest average survival rate (85%) and the same as 5 mg/l colchicine for 72 h (85%) as in Table 3. For explants which were soaked with combination of 20 mg/l colchicine for 72 h, the survival rate, had the lowest (70%).

Regeneration

All explants of *D. muscipula* were soaked with a combination of different concentrations of colchicine and incubation times, and subcultured every 2 weeks for 6 times. After 12 weeks, variations occurred in plant traits of colchicines and incubation times were induced. The variations, such as size of callus, level of red callus, plant height, number of leaves, number of roots and root length were obtained by treatment with different concentrations of colchicine and varying incubation times as shown in Table 4. All parameters were significantly different ($p \leq 0.05$). It was shown that low concentrations of colchicine (5 and 10 mg/l) and a short incubation time (24 h) gave the highest result in size of callus, level of red callus, number of leaves, number of roots and root length.

Explants which were soaked with 5 mg/l colchicine for 72 h induced callus and gave the highest average diameter of callus (2.4 cm) as shown in Figure 1a. Explants soaked with 15 mg/l colchicine for 24 h gave the most intensive red callus (2.15cm) as shown in Figure 1b. The highest average plant height was achieved with explants soaked in 5 mg/l colchicine for 24 h, about 2.66 cm (Figure 1c). Explants soaked in 10 mg/l colchicine for 24 h gave the highest average number of leaves approximately 2.85 leaves (Figure 1d). Number of leaves was the highest for explants soaked in 15 mg/l colchicine for 48 h, approximately 13.45 roots (Figure 1e), and the longest average root length occurred with explants soaked with 15 mg/l colchicine for 72 h, approximately 2.15 cm. (Figure 1f). From the experiment, it was shown that explants soaked with higher concentrations of colchicine and for longer incubation times had decreased

Table 4. Effect of colchicine concentrations and incubation time on plant height, number of leaves, number of roots and root length in *Dionaea muscipula* after culturing for 12 weeks (±SE).

Colchicine concentration (mg/l /h)	Size of callus (cm)	Level of red color	Plant height (cm)	Number of leaves	Number of roots	Root length (cm)
Control	1.35±0.67^{a}	1.6±0.75^{ab}	3.58±0.53^{d}	3.40±0.60^{c}	15.3±4.66^{c}	2.80±0.45^{d}
5/24	1.35±0.88^{a}	1.25±0.85^{a}	2.66±0.60^{c}	2.65±0.67^{abc}	9.30±3.39^{ab}	1.66±0.62^{abc}
10/24	1.55±0.76^{ab}	1.85±0.75^{ab}	2.41±0.56^{bc}	2.85±0.59^{bc}	10.00±4.12^{abc}	1.80±0.44^{abc}
15/24	1.90±0.72^{abc}	2.15±0.59^{b}	2.15±0.52^{abc}	2.50±0.69^{ab}	9.40±4.12^{ab}	1.63±0.56^{abc}
20/24	1.50±0.95^{ab}	1.65±0.99^{ab}	1.85±0.84^{ab}	2.40±0.82^{ab}	10.55±5.66^{abc}	1.64±0.79^{abc}
5/48	1.85±0.67^{abc}	2.00±0.79^{ab}	2.26±0.78^{abc}	2.40±0.82^{ab}	13.20±6.21^{bc}	1.78±0.73^{abc}
10/48	1.80±0.77^{abc}	1.80±0.89^{ab}	1.60±0.78^{a}	2.00±0.79^{a}	7.80±4.72^{a}	1.21±0.77^{a}
15/48	2.15±0.75^{bc}	1.50±0.69^{ab}	2.41±0.58^{bc}	2.85±0.67^{bc}	13.45±4.68^{bc}	2.01±0.55^{bc}
20/48	1.95±0.83^{abc}	1.65±0.81^{ab}	2.10±0.70^{abc}	2.40±0.75^{ab}	10.11±6.76^{abc}	1.66±0.86^{abc}
5/72	2.40±0.68^{c}	1.65±0.67^{ab}	1.91±0.69^{ab}	2.35±1.04^{ab}	10.40±6.25^{abc}	1.40±0.71^{ab}
10/72	1.85±0.59^{abc}	1.90±0.55^{ab}	2.31±0.76^{abc}	2.65±0.75^{abc}	13.00±4.15^{abc}	1.76±0.53^{aabc}
15/72	1.55±0.76^{ab}	1.80±0.83^{ab}	2.31±0.73^{abc}	2.65±0.81^{abc}	13.00±4.63^{abc}	2.15±0.76^{cd}
20/72	1.85±0.75^{abc}	1.70±0.73^{ab}	2.19±0.75^{abc}	2.60±0.82^{abc}	13.15±4.75^{bc}	1.80±0.68^{abc}

^{abc}Different letters within the same columns indicate statistically significant difference using the Tukey test at 95% ($p \leq 0.05$).

growth rate.

Explants of *D. muscipula* treated with a combination of different colchicine concentrations and various incubation times were cultured for 90 days. It was found that there are different characteristics which were a change from the control and were counted as mutations. Their mutation rate was recorded in each treatment as shown in Table 5. It was shown that high concentrations of colchicine (10, 15, 20 mg/l) and soaking for long incubation times (48 and 72 h) affected leaf base and leaf blade and gave the highest mutation rate, especially at 20 mg/l colchicine soaked for 72 h which gave 95% mutation (Table 5). But the same concentration of colchicine (20 mg/l) and shorter incubation time (24 h) gave only 55% mutation. The color of the leaf blade and leaf base was still green as only callus phase turned red.

Mutation characteristics of *D. muscipula* that occurred after being soaked with a combination of different concentrations of colchicine and various incubation times and cultured for 12 weeks are shown in Figure 2. The edge of the leaf base changed from smooth to curved edge (Figure 2a), leaf blade doubled (Figure 2b) and size of the leaf blade was bigger with more teeth (Figure 2c) when compared to the control. Their growth rate was decreased also.

DISCUSSION

In this experiment, young leaf base and leaf blade used as explants and cultured on 1/2MS medium supplemented with 0.5 mg/l BA gave the highest average diameter of red callus and red callus regenerated to new shoots within six weeks. This result was the same as that of Crouch et al. (1990) that reported that MS medium was suitable for mass propagation of a sundew, *D. rotundifolia* L. from the young leaf. They cultured the young leaf on MS medium supplemented with 1.0 mg/l BA and got the highest red callus that regenerated to new shoots later. But Jang and Park (1999) used the shoot of *D. rotundifolia* L. cultured on 1/3MS medium supplemented with 0.5 mg/l kinetin for mass propagation. Slack (1981) used a young leaf for micropropagation of *Pinguicula moranensis* (Butterwort) on MS medium. For the Venus fly trap, only a few reports are available. Beebe (1980) and Parliman et al. (1982b) used the leaf for producing adventitious buds on MS medium supplemented with NAA and BA. Parliman et al. (1982a) cultured the rhizome on 1/2MS medium supplemented with 1.9 mg/l NAA and 0.2 mg/l BA and got the best new shoots with

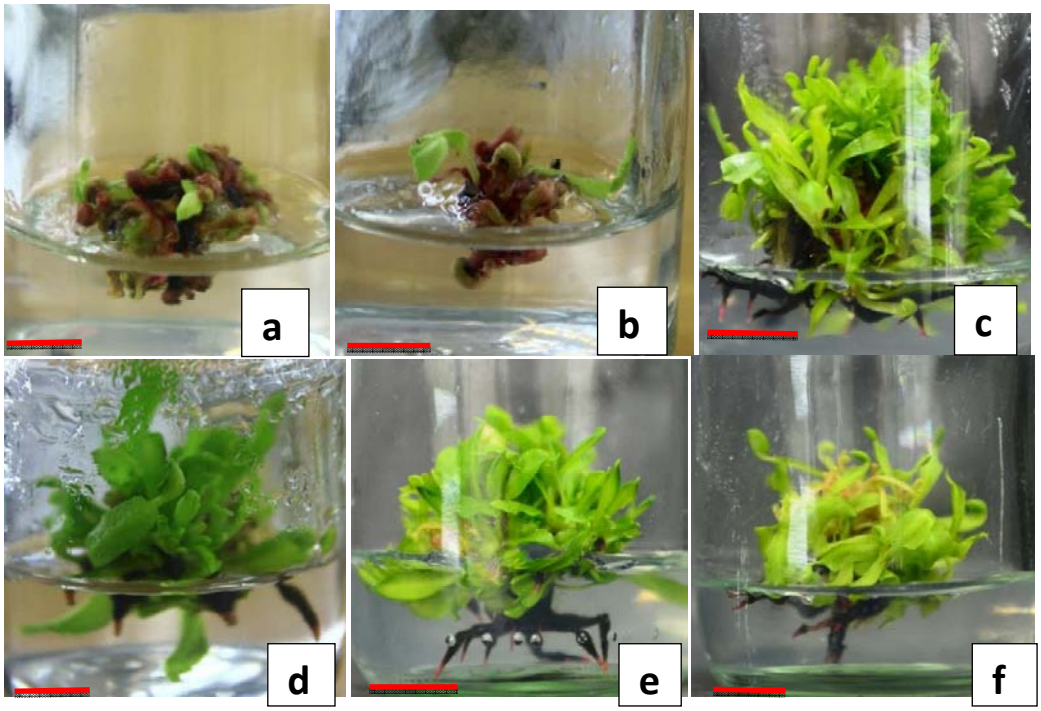

Figure 1. Growth and development of *Dionaea muscipula* after being soaked with a combination of different concentration of colchicine and incubation times, and then cultured on 1/2 MS medium for 30 days: (▬ bar = 1 cm). a) soaked in 5 mg/l colchicine for 72 h; b) soaked in 15 mg/l colchicine for 24 h; c) soaked in 5 mg/l colchicine for 24 h; d) soaked in 10 mg/l colchicine for 24 h; e) soaked in 15 mg/l colchicine for 72 h, f. soaked in 15 mg/l colchicine for 72 h.

Table 5. Mutation rate in explants treated with combination of different concentration colchicine and various incubation times and cultured for 12 weeks.

Colchicine concentration (mg/l /h)	Number of explants (shoot)	Number of mutation (shoot)	Mutation rate (%)
Control	20	0	0
5/24	20	5	25
10/24	20	8	40
15/24	20	8	40
20/24	20	11	55
5/48	20	10	50
10/48	20	11	55
15/48	20	16	80
20/48	20	15	75
5/72	20	12	60
10/72	20	16	80
15/72	20	17	85
20/72	20	19	95

roots. Minocha (1985) used the mature leaf segments of *D. muscipula* for *in vitro* propagation. (Hutchinson and Zimmerman,1984) used the shoot tip to culture and produced plantlets by culturing them on LS medium (Linsmaier and Skoog, 1965) supplemented with 10 µM kinetin and 0.5 µM NAA. Teng (1999) used the flower stalk as explants for *in vitro* culture. Grevenstuk et al. (2010) reported a method for mass propagation of *D. muscipula* by culturing protocorm on 1/4MS supplemented with kinetin.

When explants of *D. muscipula* were treated with a combination of different concentrations of colchicines and

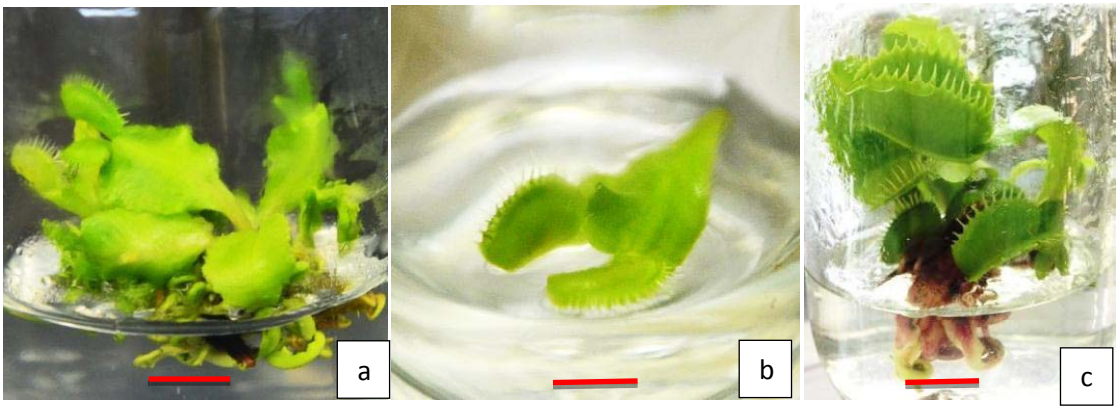

Figure 2. Mutation in *Dionaea muscipula* after being treated with combinations of colchicine And various incubation times and cultured for 90 days in 1/2MS : a) leaf base with curved edge, b) twin leaf blade, c) bigger leaf blade and more teeth(bar = 1 cm).

various incubation times and, then cultured on 1/2MS medium supplemented with 2 mg/l BA for 90 days, it was found that the survival rate depended on the concentration and duration of treatment. The growth rate of explants treated with high concentrations of colchicine (10, 15 and 20 mg/l) and soaked for longer incubation times (48 and 72 h) was very slow. This result corresponded to those of Chaicharone et al. (1995) and Thao et al. (2003). Zhang et al. (2008) reported that induced polypoid in *Phlox subulata* L. by soaking the shoot tip in high concentrations of colchicine (0.005%) for 10 days got the highest growth rate, about 40%. When soaked with 0.4% colchicine for 30 days, their survival and growth rate decreased to zero. (Sun and Hong, 2009) showed that the survival rate depends on the concentration and duration of treatment. Colchicine is a toxic chemical that is often used to induce polyploidy in plants. Sometimes the polyploid plants have larger leaves and flowers (Barry, 2000). *D. muscipula* explants treated with 20 mg/l colchicine for 72 h gave the highest variation which was the same as Gibson (1999) which reported that the *Drosera binata* plants looked larger than the control due to the higher colchicine treatment also and started to generate clearly stunted plantlets. Swanson (1957) explained that reduced growth rate was due to reducing rate of cell division that results from the physiological disturbances caused by colchicine.

Conclusion

Young leaf base and leaf blade were suitable for callus induction on *D. muscipula* when cultured on 1/2MS medium supplemented with 0.1-1.0 mg/l BA. Red callus differentiated to new shoots for micropropagation when cultured callus on 1/2MS supplemented with 0.5 mg/l BA.

The effect of colchicine concentration and duration time for incubation on *D. muscipula* could be observed in morphological change and their growth rate, survival rate and variation which occurred in high concentrations of colchicine and long incubation times as compared to the control.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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