Research article

In vitro Cultures and Colchicine-Induced Tetraploidy of Sundew (*Drosera spatulata* Labill.)

PIYAVADEE CHAROENWATTANA*
Rajamangala University of Technology Thanyaburi, Pathumthani, Thailand
Email: piyavadeec@yahoo.com

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Abstract Sundew (*Drosera spatulata* Labill.) has become a favorite ornamental plant because of their carnivorous nature, the beauty of their glistening traps and their value as medicinal herb. It is also because of their low propagation rate in their natural environment that *in vitro* propagation of carnivorous plants is pursued. The objective of this research was to cultivate sundew *in vitro* to increase its propagation rate. The tetraploid of *D. spatulata* was induced by the colchicine treatment, providing valuable plant material for further breeding programs. Plantlets of *D. spatulata* were cultured on MS agar medium (pH 5.4-5.8), and growth and survival rates were observed. The results showed the highest survival of 100 percent in an MS (pH 5.6) medium. Results observed after nine weeks showed that a 1/3MS (pH 5.6) medium promotes the highest growth with an average plant diameter of 25.95 mm. For tetraploids induction, plantlets were immersed in aqueous solutions of colchicine (6 and 12 mg l^{-1}) for the duration of one week. The effects of different concentrations on survival and growth were recorded. Putative polyploids were identified by stomata density and stomata size. The ploidy level was determined by chromosome counting. The survival rate of treated plantlets decreased in all treatments. The most efficient colchicine concentrations were in the range from 6 to 12 mg l^{-1}. The chromosome number of the tetraploid plants was $2n = 4x = 40$, while that of the control diploid plants was $2n = 2x = 20$. The diameter of the tetraploid plants increased, and the length of the leaves and number of inflorescences were more than those of the diploid. The tetraploid plants derived from exposure to colchicine exhibited variations, such as larger stomata and lower density of stomata.

Keywords *Drosera spatulata*, colchicine, tetraploid, micropropagation, MS media

INTRODUCTION

The genus *Drosera* (Droseraceae), commonly called sundew, is the largest group of carnivorous plants and consists of approximately170 species. *Drosera spatulata* Labill. is one of the most widespread sundews in nature. *D. spatulata* is a carnivorous species native to south-east Asia and Australia (Junipers et al., 1989). It is a rosette-forming spoon-leaved sundew. This species is widely variable, but generally plants are about 4 cm in diameter. Each leaf is attached to the central rosette by a narrow 8 mm long petiole that is only glandular on the upper half. Individual leaf laminae are typically 5 mm long and 4 mm wide. In early summer, plants will produce 8 cm tall erect shapes with around six small white or pink flowers on each one-sided racemose inflorescence. Each flower can be up to 6 mm across (International Carnivorous Plant Society, 2013).

Carnivorous plants are a phenomenon in the plant kingdom and are valued as ornamental plants. They capture their prey by means of specialized trapping devices, which is an additional way to obtain protein - a source of nitrogen that is essential for the proper development of all organisms (Williams and Bennet, 1982). Apart from their ornamental value, the *Drosera* has medicinal significance. Extracts of these plants, containing 1,4-naphthoquinones, are used as antispasmodic agents in the treatment of respiratory tract ailments (Junipers et al., 1989). Due to the pharmacological significance of *Drosera* spp., there is great demand for plant material that has been met mainly through the exploitation of natural resources. As a solution for saving endangered species as well as alternative sources of plant material for pharmacological purposes, *in vitro*

culture of plants from the Droseraceae family has been employed (Jayram and Prasad, 2005).

For *D. spatulata* the chromosome numbers of 2n= 20, 40, 50 and 60 have been reported (Rivadavia and Preto, 2005).Changes in chromosome numbers may be induced via exposure of plant tissue to various chemical preparations and forms of electromagnetic radiation (van Harten, 1998). Colchicine is an alkaloid derived from the bulbous plant *Colchicum autumnale* L. (autumn crocus) (Ranney, 2003; Eigsti and Dustin, 1955). The chemical induces tetraploidy by disrupting the metaphase stage of cellular mitosis through inhibition of the spinder fibers that segregate replicated chromosomes into daughter cells (Eigsti and Dustin, 1955; van Harten, 1998; Recupero, et al., 2005). Colchicine has been found to have a significant effect on polyploidy induction and thus has been widely used for inducing polyploidy in plants. Inducing polyploidy is an effective means for the generation of innovative germplasm resources suitable for selective breeding.

From a single plant cultivated *in vitro*, many genetically identical clonal lines can be obtained through vegetative propagation. This technique allows for an increase in the propagation rate of valuable plant material. *In vitro* micro-propagation offers a means of multiplication of ornamental plants, rare and endangered species, as well as plants that are a source of secondary metabolites. Mitotic polyploidization induced through chemical treatment was also used to increase the genetic variability. Polyploids exhibiting valuable new phenotypic traits can occupy new niches and become agricultural and horticultural importance.

OBJECTIVE

The aim of this research was to develop the *in vitro* micro-propagation of *D. spatulata* through optimization of the culture medium and its pH to increase the propagation rate. The application of colchicine to induce tetraploidy in *D. spatulata* was also determined.

METHODOLOGY

Plant materials and growth conditions: The seeds of *D. spatulata* were surface sterilized and cultured on Murashige and Skoog (1962) for 18 months. Once the cultures were established, these plantlets were used as initial materials in all experiments. The plants were maintained at 25±2 °C under 8 h photoperiods with a light intensity of 3,200 lux. The experiments were carried out during 2012-2013 at the Faculty of Agricultural Technology, Rajamangala University of Technology Thanyaburi, Pathumthani, Thailand.

Effect of pH levels: The plantlets were cultured on Murashige and Skoog (MS) basal medium, containing 3% (w/v) sucrose and 0.7% (w/v) agar at pH 5.4, 5.6 and 5.8. For each treatment four replications of 16 plantlets were tested. The experiment was repeated two times and data were recorded on survival rate, plant diameter, and the length and width of leaf for 7 weeks.

Effect of different strengths of MS medium: The experiment was carried out in a completely randomized design (CRD) with four replications of 16 plantlets. The plantlets were cultured on the different strengths of MS medium (1/3, 1/2 and full strength) at the optimum pH based on the previous experiment. Their survival rate, plant diameter, and the length and width of leaf over the course of 9 weeks were observed and recorded.

Tetraploid induction: In order to compare the effect of colchicine on *D. spatulata*, the plantlets were transferred to an MS medium for five weeks prior to the tetraploid induction. The experiment was carried out in a completely randomized design (CRD) with three replications of 12 plantlets. For inducing tetraploidy, the plantlets were immersed in aqueous solutions of colchicine (0, 6 and 12 mg l^{-1}) on a rotary shaker at 25±2 °C in the dark for one week. The plantlets were then cultured on an MS medium at pH 5.6. The effects of colchicine on the survival and growth of plantlets were recorded over the course of five weeks. Putative polyploids were identified by measuring the density and size of stomata. The ploidy level was determined by chromosome counting.

Morphological observation of diploid and tetraploid plants: A set of morphological characters were measured. Plant diameter and the length and width of leaf were measured. The width and length of stomata in the lower epidermis of mature leaves were measured using an eyepiece

micrometer. The stomatal density was also observed.

Chromosome preparation: Root tips of ~1.5 cm in length were excised and immersed in 0.002 M 8-hydroxy quinolone at 4 °C for 2-3 hours and washed with distilled water before fixing in 3:1 ethanol: glacial acetic acid at 4 °C for 24 hours. These were then digested in 10% HCl at 60 °C for 10 minutes and washed 3 times with distilled water. Root tips were stained with basic fuchsine at 4 °C for 30-45 minutes in the dark. Afterwards, the root tips were stained with 2% (w/v) aceto-carmine and squashed. The number of chromosomes was observed under a light microscope (Olympus CX21, Japan).

Statistical analysis: Comparisons of quantitative data between two variable groups were made using student's *t*-test and morphological variation rates were subjected to an analysis of variance (ANOVA) and multiple comparisons; these factors were analyzed using Duncan's New Multiple Range Test (DMRT) or Least Significant Difference (LSD).

RESULTS AND DISCUSSION

Effect of pH Levels

The pH levels had an effect on the plantlets at the second week after cultured in an MS medium. Percentage of surviving plantlets was very low at pH 5.4 (18.75%) and was higher at pH 5.8 (87.5%), while all plantlets survived at pH 5.6. Survival was severely inhibited in more acidic media (pH 5.4).The results showed a highly significant difference on plants diameter and length of leaf, but no significant difference on leaf width after 7 weeks. Plant diameter and length of leaf of *D. spatulata* at pH 5.6 (23.22 and 14.09 mm) were observed to be higher than at pH 5.8 (17.19 and 9.16 mm) (Table 1; Fig. 1). These results were similar to those of Jayaram and Prasad (2007), which found that the highest percentage of shoot proliferation observed in *D. indica* were at pH 5.7. However, the effect of pH levels on nutrient uptake and shoot proliferation has also been reported (Parliman et al., 1982).

Table 1 Effect of pH levels on plant diameter, length and width of leaf in *D. spatulata*

pH	Plant diameter (mm)	Leaf length (mm)	Leaf width (mm)
MS pH 5.6	23.22±0.27[1/]	14.09±0.65	4.79±0.08
MS pH 5.8	17.19±0.97	9.16±0.35	4.84±0.28
sig.(2-tailed)	0.006	0.001	0.798
t-test	**	**	ns

**, ** indicate significant difference by student's t-test at 5% and 1% levels of probability, respectively.*
ns = not significant [1/]Data represent means± S.D. of four replications each.

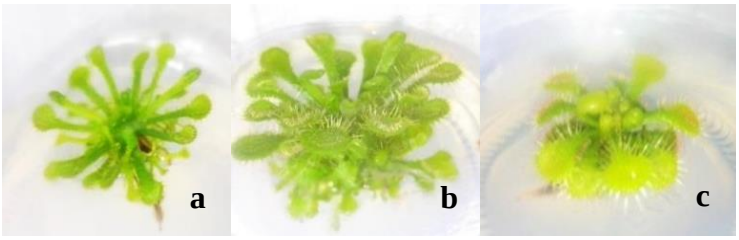

Fig. 1 *D. spatulata* after 7 weeks of culture in an MS medium at pH 5.4(a), 5.6(b) and 5.8(c)

Effect of Different Strengths of MS Medium

The plantlets survived in all strengths of MS medium. Plant diameter and length of leaf exhibited highly significant differences in the fourth week. The results showed no significant difference after nine weeks. Plant diameter and length of leaf were 25.95, 24.61, 25.81 and 13.31, 12.84, 13.57 mm after 9 weeks in 1/3, 1/2 and full strength MS, respectively. The width of leaf showed a highly significant difference after 9 weeks. The width of leaf were 5.65, 5.39, 4.50 mm after 9 weeks in

1/3, 1/2 and full strength MS, respectively (Table 2; Fig. 2). The strength of MS medium was not related to the survival rate of *D. spatulata*, but had an effect on growth and development of the plantlets during the first four weeks. According to Kim and Jang (2004), the shoot proliferation and tuber formation of *D. peltata* were most effective on 1/2 MS medium at pH 5.7. Carnivorous plants usually grow in nutrient-poor environments and in poor soils; carnivore, therefore, has evolved as an additional pathway to supplement nutrients such as nitrogen and phosphorus (Adamec, 1997).

Table 2 Effect of different strengths of MS medium on plant diameter, length and width of leaf of *D. spatulata* after 9 weeks of culture at pH 5.6

MS strength	Plant diameter (mm)	Leaf length(mm)	Leaf width(mm)
1/3MS	25.95	13.31	5.65 [a]
1/2MS	24.61	12.84	5.39 [a]
MS	25.81	13.57	4.50 [b]
F-test	ns	ns	**
CV (%)	7.61	6.18	7.25

Means in columns followed by different letters are significant difference at 5% level according to DMRT
**, ** indicate significance at 5% and 1% levels of probability, respectively. ns = not significant*

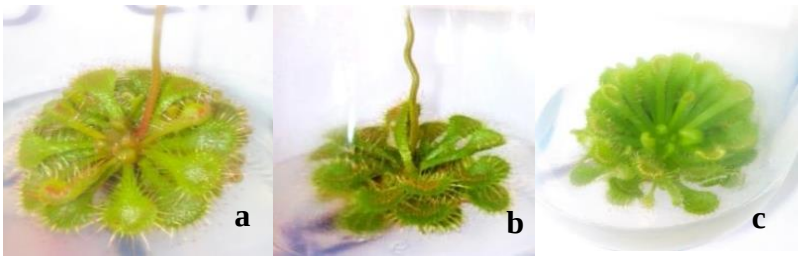

Fig. 2 *D. spatulata* on 1/3MS (a), 1/2MS (b), and MS (c) after 9 weeks of culture at pH 5.6

Induction and Identification of Tetraploid *D. spatulata*

Twenty-four out of 36 (66.7%) colchicine-treated plants survived after five weeks in all treatments. The plant diameter and length of leaf of colchicine-treated plants (35.48, 35.28 and 20.76, 20.42 mm) were significantly larger than those in not colchicine-treated (30.02 and 17.33 mm) (Table 3). The numbers of stomata of colchicine-treated plants (4.40 and 4.40 cells) were significantly less than those not colchicine-treated (5.22 cells). The width and length of stomata of colchicine-treated plants (38.75, 37.93 and 50.43, 46.37 µm) were significantly larger than those of not colchicine-treated (33.87 and 37.43 µm) (Table 3; Fig. 3). Furthermore, the earlier group matured with 2 or more inflorescences in some colchicine-treated plants. The chromosome number from root tips of *D. spatulata* in non-treated colchicine were 20 chromosomes, whereas 40 chromosomes resulted from treated colchicine at 6 and 12 mg l^{-1}.

Table 3 Effect of colchicine treatment on growth, number and size of stomata of *D. spatulata* on MS medium at pH 5.6 after 5 weeks

Colchicine Concentration (mg l^{-1})	Plant diameter (mm)	Leaf length (mm)	Leaf width (mm)	Survival rate (%)	Number of stomata (cell)	Size of stomata (µm)	
						width	length
0	30.02 [b]	17.33 [b]	4.34	66.66	5.22 [a]	33.87 [b]	37.43 [b]
6	35.28 [a]	20.42 [a]	4.68	66.66	4.40 [b]	37.93 [a]	46.37 [a]
12	35.48 [a]	20.76 [a]	4.81	66.66	4.40 [b]	38.75 [a]	50.43 [a]
F-test	*	*	ns	-	**	*	*
CV (%)	4.87	6.21	7.91	-	7.16	6.83	10.92

Means in columns followed by different letters are significant difference at 5% level according to LSD
**, ** indicate significance at 5% and 1% levels of probability, respectively. ns = not significant*

Hence, the tetraploid induction using colchicine treatment at 6 and 12 mg l^{-1} can double chromosomes from 2n=2x=20 to 2n=4x=40 (Fig. 3). Increases in ploidy can affect very specific traits, including plant diameter, length of leaf, number and size of stomata in *D. spatulata*. Key morphological variations observed in tetraploid plants included larger and thicker leaves, darker green coloration, larger stomata, lower stomata across the lower leaf epidermis, increased numbers of chloroplasts in the stomata guard cells and increased pollen diameter (Glowacka et al., 2010).

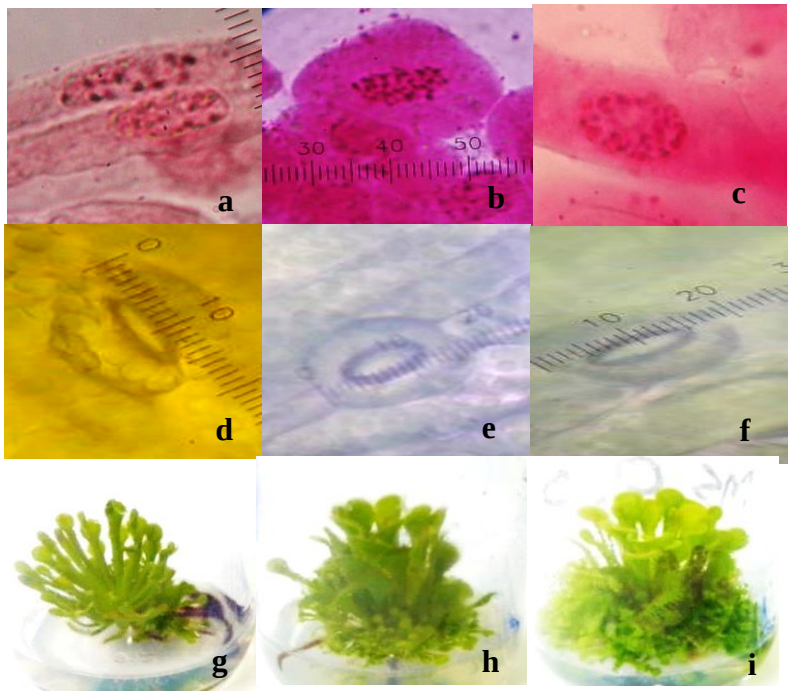

Fig. 3 Effect of the colchicine treatment on chromosome number, size of stomata and growth of *D. spatulata* at 0 (a, d, g), 6 (b, e, h) and 12 (c, f, i) mg l^{-1}after 5 weeks

CONCLUSION

A pH level of 5.4 had a highly significant effect on the rate of survival and growth of the plants. More than 80% died after cultured in an MS medium for 2 weeks. The growth of *D. spatulata* was limited due to sensitivity of low pH levels. The 1/3MS medium at pH 5.6 was the most effective medium for the micro-propagation of *D. spatulata*. Moreover, the plants exhibited leaves with red color, which are valuable and popular phenotypic traits and have market demand. Tetraploid induction affected plant diameter and length of leaf. A concentration of colchicine at 12 mg l^{-1} gave the maximum plant diameter and length of leaf of 35.48 and 20.76 mm, respectively. The numbers of stomata decreased in all colchicine-treated plants in contrast to the sizes of stomata, which increased. The chromosome number of colchicine-treated plants doubled (2n=40).Successful use of colchicine to induce tetraploids in *D. spatulata* demonstrate an effective means to explore new characteristics by increasing genetic variability for plant breeding in agriculture.

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