British Biotechnology Journal
4(5): 531-540, 2014

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The Effect of Colchicine on Adventitious Shoot Regeneration from Cultured Leaf Explants of *Petunia hybrida*

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Authors' contributions

This work was carried out in collaboration between both authors. Author HAQ designed the study, performed the statistical analysis, and wrote the first draft of the manuscript. Author MJYS managed the analyses of the study, managed the literature searches and wrote the protocol. Both authors read and approved the final manuscript.

Original Research Article

Received 14th December 2013
Accepted 23rd March 2014
Published 28th April 2014

ABSTRACT

Aims: The effect of various concentrations of colchicine on adventitious shoot regeneration of two *Petunia hybrid* cultivars was studied.
Study Design: All collected data were analyzed using the SAS software. One-way analysis of variance of the treatments was conducted for each experiment followed by means separation using the least significant difference (LSD) at 5% probability level.
Place and Duration of the Study: The study was conducted during January 2012 to December 2012 at the Faculty of Agriculture, An-Najah National University, Palestine.
Methodology: *In vitro* leaf explants of both 'Daddy Blue' and 'Dreams White' cultivars were used. Before incubation on the shoot regeneration medium, the leaves were cut transversely into two pieces and were soaked for three hours in different concentrations of colchicine solution (0.025, 0.05 and 0.1% w/v), colchicines application was done at zero incubation time and after 1, 2 or 3 weeks of culture incubation. Wet control was used by soaking explants in sterile distilled water.
Results: The ability to form shoots was highly reduced with colchicine application. Significant reduction in the regeneration frequency was observed with the two cultivars. Shoot regeneration percentage was reduced from 78.3% and 90.0 % without colchicine to 13.3% and 5.2% when colchicine was added after two weeks of incubation at 0.025% in

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'Daddy Blue' and 'Dreams White', respectively. The colchicine treatments at different intervals gave significantly lower regeneration percentages than the control in both cultivars. No regeneration was observed when colchicine was added after three weeks of incubation, and at 0.1% colchicine level at all incubation treatments. A similar trend of average number of shoots produced was observed.
Conclusion: This study showed a high reduction in regeneration percentage due to colchicine application with slight difference in response between the two tested cultivars; therefore, other evaluation methods are needed in the future.

Keywords: Colchicine; in vitro; Petunia hybrid; regeneration; thidiazuron.

1. INTRODUCTION

Chromosome doubling has been intensively studied over the years and has found its way to several applications in plant breeding; it was mainly used to facilitate interspecies crosses, including cotton [1], wheat [2] tomato [3], and chili pepper [4]. Chromosome doubling can be induced by several antimitotic agents. Colchicine is the most widely used chemical to induce polyploidy [5].

As plant tissue culture evolved, *in vitro* polyploidization became more popular. From the 1990s onwards, *in vitro* chromosome doubling was an established method in plant tissue culture [6]. *In vitro* polyploidy has been induced in many herbaceous ornamental species: Dieffenbachia [7], Dracaena [8], Hypericum [9], *Lagerstroemia indica* [10], Rhododendron [11], *Rhododendron simsii* [12], Rosa spp [13,14,15], Dianthus [16], Gladiolus spp. [17], *Helleborus* [18], *Lilium longiflorum* [19], *Ranunculus asiaticus* [20], *Spathiphyllum wallisii* [21] and *Tulipa gesneriana* [22].

A high mitotic index (MI) value was obtained when petunia cell suspension culture was treated with a sequential application of 30 µg/ml aphidicolin and 0.1% w/v colchicine; the MI values obtained were 62.8% and 65.7% respectively for aphidicolin and colchicines [23]. The effect of polyploidy was also found to interfere with flavonol differentiation in Petunia 'Mitchell' [24].

Colchicine is very toxic to humans [25]. Colchicine binds only poorly to plant tubulins, thus it must be used in relatively high concentrations. Chromosome doubling protocols vary in explant type, exposure time, concentration, application method and confirmation technique. In ornamentals, different explant types have been successfully used in the past: plantlets or shoots, buds or shoot tips, callus, somatic or zygotic embryos, seeds, seedlings, nodal segments and tuber segments using colchicine at a relatively high concentration ranging from 0.25 mM in Cyclamen to 15 mM in Alstroemeria plant [6]. However leaf explant was not widely used. In addition, using antimitotic agents is associated with negative side effects after polyploidization. It can include infertility, brittle wood and watery fruit [26]. High-level polyploids (e.g., octaploids) can be stunted and malformed, probably resulting from the extreme genetic redundancy and somatic instability that leads to chimerical tissue. Also, the occurrence of albinism after polyploidization has been described [27]. Moreover, chromosome doubling often has a later effect on the proliferation, regeneration rate and shoots elongation, retarded after treatment with antimitoticagents. In *Dioscorea zingiberensis*, 70% lethality was observed when explants were treated with 0.3% colchicines [28], in another study, sprouting and growth delay were observed in *Morus alba* explants treated with 0.2% colchicines [29].

Regeneration rate in Marigold explants was reduced significantly from 87.83% on 0.001% colchicine to 27.26% on 0.05% colchicines [30]. Higher colchicine concentrations exhibited higher mortality rates in banana somatic embryos ranging from 8–20%, 48–62% and 80–90% mortality on concentrations 0.3, 0.5, and 1.0% colchicine respectively [31]. In *Vitis vinifera*, the number of surviving embryos and regenerated plantlets following colchicine treatment decreased with increasing colchicine concentration and treatment time [32], while in citrus embryogenic callus, shoot development was partly suppressed in the presence of colchicines [33]. But there is not enough data on the effect of these mutagenic agents on *in vitro* shoot regeneration of ornamental plants using leaf explant. Therefore, this work was conducted to study the effect of lower concentrations with lower exposure time of colchicine on adventitious shoot regeneration of petunia leaf explants. Colchicine was added before and during the incubation time for shoot regeneration.

2. MATERIALS AND METHODS

In vitro leaf explants from two *Petunia hybrida* cultivars ('Daddy Blue' and 'Dreams White') established in the laboratory were used. The explants were cultured on regenerated media consisting of MS [34] basal medium containing 3% (w/v) sucrose, 100 mgL^{-1} myoinositol and solidified with 0.8% (w/v) agar supplied with 2.7 µM NAA and 4.0 µM TDZ [35]. The pH of all media was adjusted to 5.6-5.7. Full-expanded leaves were plated onto Petri dishes containing 30 ml of regeneration medium. Leaves were cut transversely into two pieces The leaf explants were soaked in 0.025, 0.05 and 0.1% (w/v) colchicine solution for 3 hours [18,20], then transferred back to adventitious shoot-regeneration medium. To synchronize the mitotic divisions and thus ameliorate the effect of the antimitotic agent [22], soaking was done at plating time (zero incubation time), and after 1, 2 or 3 weeks of culture incubation. Wet control was used by soaking explants in sterile distilled water. The colchicine solutions were filter sterilized using 0.45 µm sterile filter (Schleicher & Schuell Comp.). Explants were cultured with the abaxial side in contact with the media. Five explants (half leaf) per Petri dish were used as an experimental unit; each treatment was replicated four times. The treatments consisting of the different levels of colchicine combined with the different application times. Thus factorial treatments for each cultivar was arranged in a completely randomized design. Two experiments were conducted, one for each cultivar. Each experiment was repeated two times and the averages are presented in this article. All cultures were incubated under dark conditions for 3 weeks at 22±1ºC. Regenerated shoots were excised and cultured on MSO medium [Murashigue and Skoog basal medium without hormones] [34]. The cultured ones were maintained in a growth chamber at 22±1ºC for four weeks with 16 h of photoperiod illumination of 40 µ mol $m^{-2}s^{-1}$ supplied from cool white fluorescent. The rooted shoots were acclimatized as described by Abu-Qaoud [35]. All collected data were analyzed using the SAS software [36]. One-way analysis of variance of the treatments was conducted for each experiment followed by mean separation using the least significant difference (LSD) at 5% probability level.

3. RESULTS AND DISCUSSION

The effect of colchicine on shoot regeneration of the two petunia cultivars is shown in Table 1 and Table 2. Significant reduction in the regeneration percentage was observed with the two cultivars.

3.1 Cultivar Daddy Blue

The shoot regeneration percentage was reduced from 78.3% without colchicine to 13.3% at week two treatment with 0.025% colchicine. No regeneration was observed when colchicine was added after three weeks of incubation. A similar trend was observed when colchicine was used at a higher 0.1% level. The colchicine treatments at different intervals gave significantly lower regeneration percentages than the control. The percentages range from 12.5% at week two, to 35.5% at zero incubation time, but without differences among the colchicine treatments. No regeneration was observed from the three week treatments. When colchicine was used at higher concentration (0.1%), no regeneration was obtained except at the zero incubation time application which gave a non significant regeneration percentage of 6.3.

Regarding the average number of shoots produced per explants, regenerated treated explants produced a low number of shoots compared to the non-treated ones. Only the week two treatments (0.025%) gave a significantly similar number of shoots (6.5) to the control; however all other treatments resulted in lower average number of shoots per explant, which ranges from 3.5 to 6.5 shoots.

Table 1. The effect of different concentrations of colchicine on adventitious shoot regeneration and average shoot number from Petunia leaf explants of cv. Daddy Blue

Application time	Colchicine conc (%)	Shoot regeneration%	Average number of shoots
Control	0.00	78.3 a	8.5 a
Zero time	0.025	28.3 b	4.0 bc
1 week	0.025	10.5 bc	4.5 b
2 weeks	0.025	13.3 bc	6.5 ab
3 weeks	0.025	0.0 c	0.0 c
Zero time	0.050	35.5 b	5.5 b
1 week	0.050	16.3 bc	5.0 b
2 weeks	0.050	12.5 bc	4.5 b
3 weeks	0.050	0.0 c	0.0 c
Zero time	0.100	6.3 c	3.5 bc
1 week	0.100	0.0 c	0.0 c
2 weeks	0.100	0.0 c	0.0 c
3 weeks	0.100	0.0 c	0.0 c

Means in the same column followed by the same letter are not significantly different ($p < 0.05$; Least Significant Difference test LSD)

3.2 Cultivar Dream White

For Dreams White cultivar, the regeneration percentage was significantly reduced with colchicine treatment. A 90% regeneration was observed without colchicine treatment; this percentage was reduced to 5.2 and 14.2 when colchicine was added after two weeks of incubation at 0.025 and 0.05%, respectively. No regeneration was observed when colchicine was applied after three weeks of incubation at all levels and in all intervals with the high level of colchicine (0.1%). The same trend was exhibited with the average number of shoots. However, no significant differences in the average number of regenerated shoots were observed between non-treated explants and explants treated with colchicine at zero time of incubation. 8.5 shoots were produced with 0.025%, and 7.2 shoots with 0.05% colchicine treatments.

Table 2. The effect of different concentrations of colchicine on adventitious shoot regeneration and average shoot number from Petunia leaf explants of cv. Dreams White

Application time	Colchicine conc (%)	Shoot regeneration%	Average number of shoots
Control	0.00	90.0 a	10.3 a
Zero time	0.025	35.5 b	8.5 ab
1 week	0.025	20.3 b	4.0 cd
2 weeks	0.025	5.2 c	2.0 de
3 weeks	0.025	0.0 c	0.0 e
Zero time	0.050	28.8 b	6.5 bc
1 week	0.050	25.5 b	7.2 abc
2 weeks	0.050	14.2 bc	2.0 de
3 weeks	0.050	0.0 c	0.0 e
Zero time	0.100	15.5 bc	4.5 cd
1 week	0.100	0.0 c	0.0 e
2 weeks	0.100	0.0 c	0.0 e
3 weeks	0.100	0.0 c	0.0 e

Means in the same column followed by the same letter are not significantly different ($p< 0.05$; Least Significant Difference test LSD)

The results indicate that there was an inverse relationship between colchicine concentration and regeneration rate. These results agree with the work of Sajjad et al. [30] who found that a negative effect of high colchicine concentration on shoot regeneration of African marigold explants. The retarded growth was due to reduced rate of cell division caused by colchicine. Similar observations in which shoot length decreased due to initial retardation of growth have also been reported [28,37].

Colchicine ($C_{22}H_{25}O_6N$) is a product extracted from the seeds and bulbs of *Colchicum autumnale* L., in the late 1930s. Colchicine inhibited the formation of spindle fibers, which resulted in polyploid cells. Colchicine, as an antimitotic agent, binds to plant cell tubulin dimmers causing depolymerization of microtubules, thus disrupting the cell cycle [6]. Changes in microtubular organization indicated a transition to organogenesis in petunia leaf protoplast [38]. Subsequently such interference will affect regeneration. In petunia, when Olomoucine (a specific inhibitor of kinases) was tested it arrested the differentiation of mesophyll protoplasts induced to divide at G1 phase [39]. In another study, it was found that the DNA methylase inhibitors (5-azacytidine and 5-aza-2'-deoxycytidine) inhibited adventitious shoot induction in petunia leaf cultures. These results demonstrate that cytosine methylation at CCGG and CGCG sites within a MADS-box gene and a CDC48 homologue, among others, shows strong positive correlation with adventitious shoot bud induction in petunia leaf explants. The p34cdc2 kinase (the product of the cdc2 gene) is required during the G1 cell cycle phase at the initiation of DNA replication and also in G2-M phases for entry into mitosis. A cdc2 was isolated and reported in *Petunia hybrid* PCR fragment named as (cdc2Pet). It was differentially expressed in petunia leaves and protoplast during different cell cycles [40].

This study showed that the regenerated shoots from colchicine treated explants, have thick abnormal dark colour leaves (Fig. 1), and they were short and grew slowly. This finding was similar to the work of other researchers in different plants [28,29,30,31]. In addition, regenerated plants were difficult to be acclimatized compared to non treated shoots, so it was not possible to maintain them for further evaluations. Therefore, in this study we only rely on morphological evaluation. Flow cytometry is the pre-eminent method for evaluation of the induced polyploidization. However, alternative confirmation methods, such as chromosome counts and morphological observations, are also used [6]. Our results on plant height were in

agreement with Wright [41] who reported that induced tetraploid plants seemed to grow more slowly and abnormally. Growth inhibition, after colchicine treatment, was also confirmed by Stebbins [42] who showed that the decreased growth rate of polyploids was caused by the reduced rate of cell division.

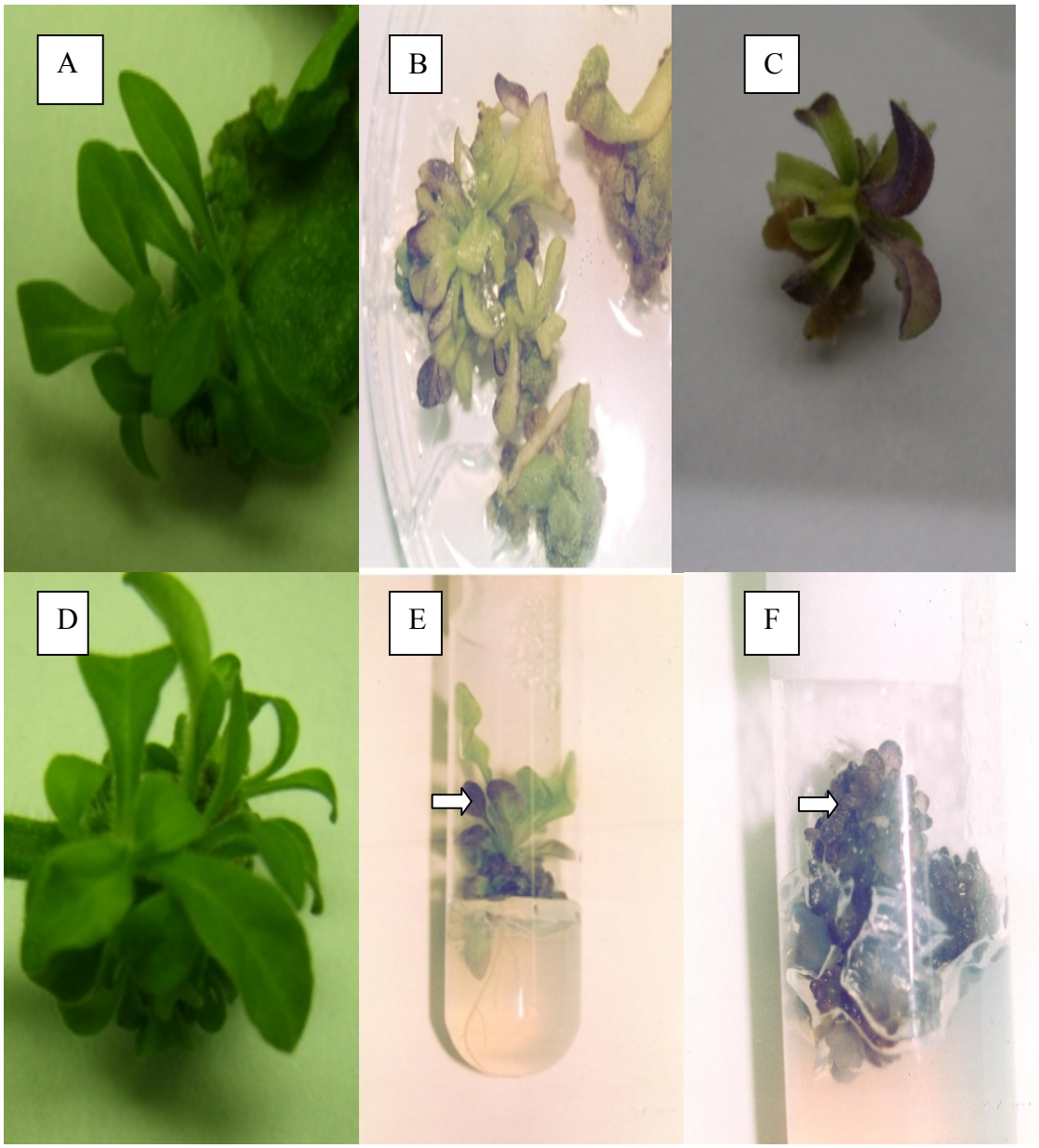

Fig. 1. A. Normal adventitious regenerated shoots form non colchicine treated leaf explants of Petunia Daddy Blue cultivar, B+C: Regenerated shoots from Petunia leaf explants treated with 0.025 % colchicines after two weeks of incubation of cv 'Dreams White'. D: Growing shoots from control treatment of Daddy Blue Petunia cultivar. E: Slow growing regenerated shoots form colchicine treated (0.05%) explant with dark reddish leaf color.F: Thick leaves of adventitious shoots from colchicine treated explants of cultivar Dreams White

The increase in dimensions of the leaves was probably due to the fact that cells with a larger complement of chromosomes grow larger to maintain a constant ratio of cytoplasmic to nuclear volume, and express more proteins [5]. Therefore, these cells were larger than diploid counterparts and greater cell volume frequently developed into thicker tissues, thus resulting in large size plant organs. The morphological and growth characteristics obtained in our study were also consistent with the finding of Griesbach et al. [24] who reported an increase in the width of the leaves and the length, width and the volume of palisade cells in petunia 'Mitchell' as a result of increase in ploidy. They reported that octaploid plants produced both deformed leaves and flower buds which aborted before opening. The results are also consistent with Ye et al. [43] who obtained larger and thicker leaves with dark green coloration in seedlings of three crape myrtle cultivars treated with 0.5% colchicine. This study showed a slight difference in response between the two tested cultivars; however, other studies indicated genotype dependent efficiency of colchicine application [15,22].

4. CONCLUSION

This study, demonstrated a simple application method of antimitotic agent (colchicine) compared to both *in vivo* and *in vitro* published methods [14].This protocol could be easily implemented to other crops. In this study, *in vitro* leaf explant was used; in addition, lower concentrations of colchicine with lower exposure time (3 hours) were used. The result revealed a significant negative effect of colchicine on shoot regeneration; this might add more clarification on the mechanism of shoot regeneration. Morphological and anatomical observations which demonstrate chromosome doubling are simple but often inaccurate [44]. Therefore, other evaluation methods are needed in the future.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Mrs. Eman Al-Qadi for technical help and An-Najah National University for financial support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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