The Journal of Animal & Plant Sciences, 29(5): 2019, Page: 1336-1343
ISSN: 1018-7081

POLYPLOIDY INDUCTION BY COLCHICINE TREATMENT IN GOLDEN BERRY (*PHYSALIS PERUVIANA*), AND EFFECTS OF POLYPLOIDY ON SOME TRAITS

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ABSTRACT

Goldenberry (*Physalis peruviana L*) an important small fruit that is growing rapidly around the globe. Different colchicine treatments (0.0, 0.3, 0.6, and 0.9% w/v) were used to induce tetraploidy in goldenberry plants. Three colchicine treatment methods were evaluated; i) immersion of seeds in colchicines solution, ii) immersion of germinated seeds (with roots about 2 mm long) in colchicine and iii) incubation of seeds on semi solid Murashige and Skoog (MS, 1962) medium containing colchicines. Experiments were set up with three factors factorial experimental design with three replicates, where the colchicine application method was the main factor of variation, the concentrations was the secondary factor and the exposure time was the third factor. Twenty seeds for each treatment, with three replications were used. Diploid plants grown from untreated seeds were used as controls. The incubation of seeds on colchicine containing MS medium was found to be the only effective method and tetraploid plants were produced in this way. The highest tetraploidy was achieved by adding 0.06 and 0.09% colchicine to MS medium. An exposure time 21-30 d was sufficient to produce tetraploid plants. Ploidy determination was made by flow cytometry. The polyploidization rate ranged between 4.1-58.8%. Tetraploid plant could not be obtained from other treatments. Statistical analysis was not performed since data could not be obtained from the lowest groups. The induced tetraploid plants were with larger plants, fruits, leaves, and stomata, higher chlorophyll content and lower stomata density compared to diploid control plants. The results have shown that colchicine should be added to the *in vitro* culture medium at least 0.6% and 0.9% doses and the seeds should be cultured for at least 30 days. An analysis of variance (two sample T-test) was performed at the 5% significance level to comparison of the morphologic characteristics of diploid and tetraploid plants.

Keywords: chromosome doubling; flow cytometry; colchicine; goldenberry; stomata.

INTRODUCTION

Goldenberry (*Physalis peruviana L*) belonging to the Solanaceae family, is a species of South American origin. It is a small fruit growing rapidly all around the world. Fruits, roots, and green parts of the plant are used for multipurpose such as food, medicine and, industry due to high contents of nutrients.

Polyploidy is one of the important tools to produce genetically superior plants. Polyploidy forms as a result of multiple sets of chromosomes in excess of the diploid number in plants. In breeding and genetic studies, reduction or increase all sets of chromosomes is a powerful mechanism for generating genetic variation and creation and changing genotypes (Wendel, 2000; Dhooghe *et al*. 2010; Omidbaigi *et al*. 2010; Dar *et al*. 2017).

Polyploidy has been encouraged by plant breeders to increase some useful fruit quality characteristics such as fruit size, ripening, and color. Polyploidy generally increase flower number, seed and fruit size, photosynthesis and respiration rate, resistance to high temperatures, drought and floods (Hunter and Hunter, 2004; Hannweg *et al*. 2016). Polyploid plants take attention with their superior agronomic characteristics compared with diploids. Polyploid plants generally produced bigger and better quality fruits, higher biomass production, stronger plants (Joshi and Verma, 2004; Rubuluza *et al*. 2007; Głowacka *et al*. 2010; Xing *et al* 2011), larger leaves & flowers, thicker stem and roots (Kermani *et al*. 2003; Shao *et al*. 2003; Chen *et al*. 2011; Hennweg *et al*. 2016; Noori *et al*. 2017). In addition, polyploid plants were found to have deep dark green color due to larger cells and more chlorophyll content (Ilarslan, 1990; Kehr 1996; Kermani *et al*. 2003; Rubuluza *et al*. 2007, Allum *et al*. 2007; Xu *et al*. 2010). Photosynthesis potential was also higher compared with diploid plants (Vyas *et al*. 2007; Ojiewo, 2007). An increase in the number of chromosomes could sometimes improve basic secondary metabolites and concentrations of preservatives (Gao *et al*. 1996; Głowacka *et al*. 2010; Hannweg *et al*. 2016; Nori *et al*. 2017). It has been reported that increased enzyme activity and gene diversity, low transpiration rate but higher photosynthesis, late flowering but longer flowering time, low growth rate but higher tolerance to mineral and nutritional stress, higher tolerance to disease, extreme temperatures or drought stress in polyploid

plants (Cohen and Yao, 1996; Song *et al*. 1997; Meyer *et al*. 2009; Nilanthi *et al*. 2009; Zhang *et al*. 2010). Increase of chromosomes affected anatomical and structural changes such as leaf size and stomatal density (Yen *et al*. 2010; Omidbaigi *et al*. 2010; Chen 2011). Polyploid plants were widely used in plant breeding programs to develop fertility of inter species, and genus hybrids to create new varieties (Comai, 2005). Polyploid plants can evaluated for commercial properties, selected directly as new variety or can be use as parents in hybridization with diploid to generation triploid plants (Sun *et al.* 2009).

In vitro chromosome doubling in plants could be encouraged by many of antimitotic agents. Colchicine, oryzaline and trifluralin are commonly used chemicals (Chauvin *et al*. 2003; Sun *et al*. 2009; Dhooghe *et al*. 2010; Chen *et al*. 2011). Colchicine is the most commonly and effectively used antimitotic agent for encouraging the polyploidy in many plants. Different treatments methods such as seed treatment, flower bud or apical meristem application and root application were used for promoting polyploidy (Gao *et al*. 1996; Adaniya and Shirai, 2001; Joshi and Verma, 2004; Klima *et al*. 2008; Zhang *et al*. 2010; Omidbaigi *et al*. 2010). Traditionally polyploidy has been induced by immersing of seeds, roots or whole plants to colchicine solution or culturing shoots on plant growing media containing colchicine in greenhouse. Different treatment concentrations and time period of the antimitotic agent were important parameters and had a remarkable interaction, while low doses failing and extremely high doses were detrimental (Dhooghe *et al*. 2010). However, the frequency of polyploidy plants with colchicine application was low and chimeras occurred at high rates. Colchicine sensitivity was specific to species for inducing of polyploidy. In other words, colchicine concentration varied according to plant species for promoting polyploidy (Omidbaigi *et al.* 2010).

This experiment aimed to establish an efficient procedure for induction polyploidy in goldenberry plants by treating diploid seeds with colchicine. It was also aimed that determine optimum concentration and exposure time period identify certain morphological and cytological traits of diploid or tetraploid plants.

MATERIALS AND METHODS

This study was conducted in the university laboratories and greenhouses in 2016-2018. Goldenberry *(Physalis peruviana* L.) was used in this study as plant material. Three experiments were conducted to induce polyploidy. All seeds were disinfected by dipping in 70% ethanol for 1 min, followed by 10 min of calcium hypochlorite (10%) solution and then rinsed three times with sterile distilled water. Twenty seeds for each treatment, with three replications were used. Untreated diploid plants grown from untreated seeds were used as control.

Doubling procedure

a) **Seed treatment:** Disinfected seeds were soaked in three different colchicine concentrations (0.3, 0.6, and 0.9 % w/v) for three exposure times (12, 24 and 36 hours; h). Then the treated seeds were rinsed thoroughly with distilled water.

b) **Germinated seed treatment:** Disinfected seeds were germinated in Petri dishes on water-moistened filter paper in growth chamber at 24 ± 2 ºC. Seeds with 2-3 mm radicles were evaluated as germinated. The germinated seeds were soaked in three different concentrations of colchicine solutions (0.3, 0.6, 0.9 % w/v) for three exposure time (6, 12, and 24 h). Then the treated seeds were rinsed thoroughly with distilled water and planted carefully in plastic seedling tray filled with peat.

c) ***In vitro*** **culture (**incubation of seeds on colchicine containing agar medium)

Disinfected seeds were cultured on semisolid MS medium (Murashige and Skoog, 1962) supplemented with 20 g l^{-1} sucrose, 7 g l^{-1} agar and three different concentrations of colchicine (0.3, 0.6, and 0.9 % w/v) for 14, 21 and 30 days (d) in growth chamber at 24 ± 2ºC.

All germinated seeds planted to peat containing plastic seedling tray then seedlings (at 4-5 true leaf stage) were transplanted to peat containing pots (15 liter). All plants were irrigated with drip irrigation system and fertilized with 13 g of 15: 8: 25: 3.5 nitrogen: phosphorus: potassium: magnesium oxide per plant.

Ploidy analysis: The ploidy level of samples, stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride), were measured by flow cytometry (using a CyFlow® Ploidy Analyser, Partec, Germany) according to the procedures given by Partec.

The comparison between diploid and tetraploid plants was done on the basis of changes in agricultural characteristics including leaf area (cm^2, LI-3000C portable area meter, USA), stem diameter (cm), plant height (cm), days to flowering, chlorophyll index (Field Scout CM 1000 Chlorophyll Meter, USA), fruit yield per plant (g), fruit weight (g), soluble solid content (Greinorm Brix refractometer, Germany), seed number per fruit, weight of 1000 seeds (g), ascorbic acid content (mg 100 g^{-1}, by Iodine titration), fruit set rate (%), stomata number per 1 mm^2 of leaf, stomata length (µm) and width (µm), pollen number per flower and pollen viability (%). All measurements were done with three replicates and 5 samples were used per replicate to comparison of the morphologic characteristics of diploid and tetraploid plants.

The fourth leaves from the shoot tip of the selected plants were collected and leaf area measured with a leaf area meter 44 days after planting. Stem diameter and plant height were measured after 12 weeks of seed sowing.

Five diploid (control) and five tetraploid plants (two leaves from each plant) were selected for determination of the stomata density (number of stomata per mm^2 of leaf), stomata length and stomata width. Width and length of the stomata were measured by ocular micrometer. A small leaf area on the abaxial leaf surfaces was measured with nail polish. After the nail polish solution dried, the nail polish impression was removed and placed on a microscope slide and observed under light microscope (under magnification of 10x40). Two slides were prepared for each leaves and 5 stomata were measured on each slide (one hundred stomata for each diploid and tetraploid plants).

Ten flower buds from each control and tetraploid plants were collected one day before anthesis for counting pollen grain per flower. Flower buds separated into two groups. Each five flower buds were incubated in glass bottle at room temperature for 24 hours until the open. Counting was made two times per group of pollen. Pollen number was counted by hemacytometer slide with a light microscope (Eti, 1990).

Viability rates were determined by tetrazolium test in seeds obtained from diploid and tetraploid plants. An analysis of variance (two sample T-test) was performed at the 5% significance level to comparison of the morphologic characteristics of diploid and tetraploid plants.

RESULTS AND DISCUSSION

The first measureable effectiveness of colchicine treatment was a significant decrease in germination rate of the seeds. Colchicine treatment methods and concentrations did not significantly affect seed germination. Germination percentages of colchicine treated seeds were low as compared to the control. The seed germination rates were 90% in untreated (control) seeds, 84.5% for seed treatment method and 81.4% for *in vitro* treatment method. For germinated seed treatment colchicine application was made to seeds have already germinated. Exposure time was found to have a significant effect on the germination in the experiment. Longer exposure time reduced the germination rates. The average germination rate was 92.78% when the shortest application periods were considered in each application method, but this rate decreased to 86.65% at the longest time.

In this study, an efficient procedure was established for successful induction of polyploidy goldenberry by treated diploid seeds with colchicine on agar MS medium. Doubling chromosome was successfully developed and tetraploid plants were obtained.

Colchicine application methods, duration (exposure time) and doses were important for chromosome duplication in the experiment. The most important factor for chromosome duplication noted to be colchicine application methods. While the colchicine concentration was the second and exposure period the third important source of variation in our experiments. It has been determined that a shorter exposure time than 3 weeks is not sufficient for chromosome doubling and that longer exposure is required to produce tetraploids. Tetraploid seedlings obtained only from seeds cultured on 0.6% and 0.9 % cholchicine supplemented with MS medium for 21 and 30 days. The best induction was obtained with *in vitro* treatment with %0.9 colchicine. 4.1% and 10.6% of tetraploid plants were induced when the seeds were kept for 21 days in 6% and 9% colchicine containing MS medium, respectively. At the same doses, 23% and 58.8% tetraploid plants were obtained from 30 days of application, respectively. No mixoploid plant was induced in treated plants. Since the concentration and exposure duration of colchicine were too low for induction tetraploidy in this study, it is considered that they were insufficient to induce mixoploid plants to. Tetraploid plants obtained only from in vitro culture method. Tetraploid plant could not be obtained from other treatments. Statistical analysis was not performed since data could not be obtained from the other groups.

In vitro polyploidisation method is easy to apply and could be effective one for chromosome doubling.

Ploidy levels of the plants were determined by flow cytometry. The cytometric DNA histograms of the diploids and the induced tetraploids are shown in Fig. 1.

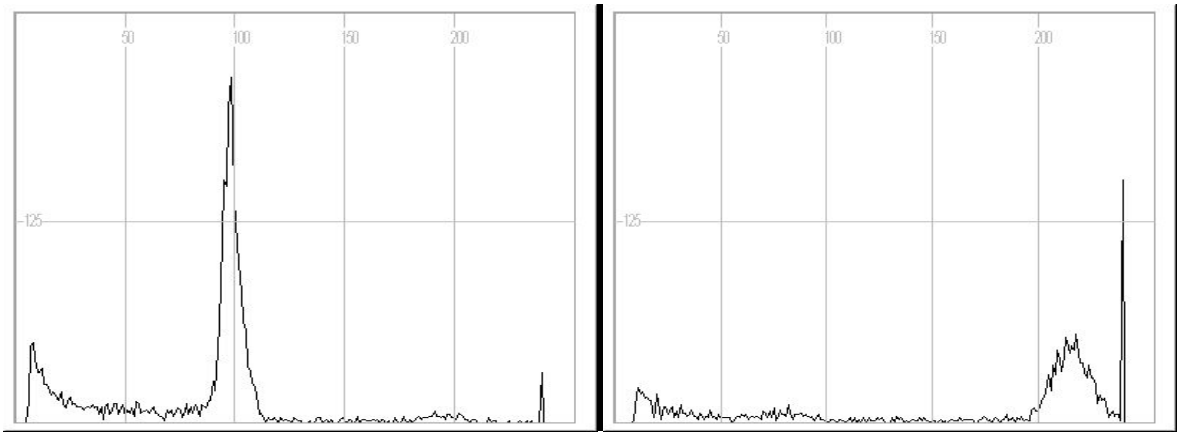

Fig 1. Flow cytometry histograms of nuclei isolated from leaves of colchicine untreated (diploid) plant (left) and colchicine treated (tetraploid) plant (right)

Chromosome doubling was accompanied by changes in morphological and physiological traits of plants. Certain morphologic and physiologic characteristics were compared between diploid and tetraploid plants and the results are presented in Table 1.

Significant differences were observed between diploids and tetraploids in the morphological structure of the leaves and floral characters. The induced tetraploid plants had longer and larger plants, bigger leaves (Fig2), fewer but larger flowers (Fig 3) and bigger fruits (Fig 4), compared with the diploid as a common effect of chromosome doubling. The tetraploid seedlings were significantly taller than the diploid. The diploid and tetraploid plants had 46.17 cm and 58.75 cm height, respectively. In relation to chromosome doubling, increases in flower size (5.05 cm^2) were observed compared to control flowers (3.95 cm^2). Chromosome doubling caused to significant increases in thickness and size of the leaves, chlorophyll index and conversely decreasing in fruit number per plant, fruit set rate and seed number per fruit. These morphological changes were reported in similar studies (Mathura *et al*. 2006; Tulay and Unal, 2010; Chen *et al*. 2011; Xing, 2011; Hannweg *et al*. 2013; Moghbel *et al*. 2015; Xie, 2015; Diallo *et al*. 2016; Widoretno, 2016; Wang *et al*. 2017).

The average fruit weight and the total number of fruits per plant showed a reverse relationship with the ploidy level. As fruit number per plant decreased, mean fruit weight of tetraploid had a tendency to increase. Despite the significant reduction in the number of fruit per plant in tetraploid, there was no significant difference in fruit yield per plant due to the important increases of tetraploid fruit weight. Diploid and tetraploid fruit weights were 3.35 g and 4.01 g respectively. There was an increase in ascorbic acid content of fruits of tetraploid plants.

It has been determined that colchicine application caused considerable morphological changes that not associated with chromosome doubling, such as increases in plant height, stem diameter, leaf area, flower size and chlorophyll content (data not shown). This result agreed with Siddiqi *et al*. (1983) reported that colchicine acted as stimulant effects to plant. Colchicine treated plant exhibited some morphological changes due to colchicine influence certain metabolic processes effecting the rate of enzymatic reactions.

Tetraploid goldenberry plants show a delay in flowering time. Diploid plants flowered in 87 days after seed sowing whereas tetraploid plants in 93.33 days. Similar results were reported in previous work with different crop species (Gu, 2005; Tulay and Unal, 2010; Yao *et al*. 2011; del Pozo and Ramirez-Parra, 2014). The rate of fruit set in control plants was calculated as 96.65%. Chromosome doubling caused to decrease this ratio to 82.87%.

In contrast, the fruit diameter, fruit length, soluble solid content, total dry matter, fruit yield per plant, seed yield per fruit, 1000 seeds weight, and viability of seed were similar in diploid and tetraploid plants. There were no significant differences between tetraploid and diploid plants in terms of the above mentioned characteristics. Certain stomatal characteristics and pollen number per flower, pollen viability were compared between diploid and tetraploid plants.

In the present study, there was no significant difference in pollen viability while the number of pollen per flower showed a significant decrease with increasing chromosome. The tetraploid plants produced viable and fertile pollen. The pollen viability as tested by 0.1% TTC was calculated as 63.30% for diploid and 65.90% for polyploid plants. The induced tetraploid plant showed significantly lower pollen grains per flower ($40.17x10^4$) than their diploid ($45.83 x10^4$), but the viability of pollen increased significantly in tetraploids. As similar observations by Kermani *et al*.(2003) we observed higher pollen number per flower and viability in tetraploid than diploid plant in contrast with some previous work with

different crop species (Dhoran *et al*. 2011; Tulay and Unal, 2010). Allum *et al*. (2007) shown that doubling the chromosome did not affect the rate of viable to non-viable pollen grains. Wang *et al*. (2017) reported that the pollen diameters of tetraploid plants were significantly larger than those of diploid plants.

Significant variation was found in stomatal frequency between the diploid and tetraploid plants. The stomatal density was lower than in diploid plant. Diploid plants have almost twice as many stomata (175.53) per mm^2 in comparison to the tetraploid (77.73). In the case of *P. peruviana*, stomatal density can be considered the most reliable discriminating criterion in ploidy levels. The diploid and tetraploid plants also exhibited clear differences for stomatal sizes. There was an increase in the size of stomata by chromosome doubling. Mean stomata length-width were 34.75-19.02 for diploid and 47.75-30.19 for tetraploid respectively. No other characteristic was changed as important as stomata density in tetraploid plant. It has been reported that the increase in chromosomes provided by colchicine has a significant effect on the density and size of stomata which is commonly used properties in determining some physiological characteristics in plant breeding studies. Stomatal traits have been used successfully in distinguishing plants with different ploidy levels in many studies with different plant types. It has been reported that diploid plants tend to have smaller and lower density of stomata than their induced tetraploid (Omidbaigi *et al*. 2010; Głowacka *et al*. 2010; Chen *et al.* 2011; Xing, 2011; Beck, 2003; Hannweg *et al*. 2013; Xie, 2015; Widoretno, 2016). The number of stomata per unit leaf area has proved to be a good indicator of ploidy in *P. peruviana*.

As a result; tetraploid plants were obtained in goldenberry by colchicine treatment of seed. The incubation of seeds on colchicine containing agar medium was found more effective than the other studied methods in polyploidization. It has been determined that application method is important factor in induction of polyploidy. At the same time, concentration and time duration also affected the success. Low doses and short duration have failed to induce polyploidy. Results of our study shown that, the colchicine should be added to the *in vitro* culture medium at least 0.6% and 0.9% doses and the seeds should be cultured for at least 30 days. It is necessary to test longer exposure periods for increasing the polyploid plant frequency. The obtained polyploidy have been found to be superior to some of their diploids in terms of agronomic properties. Results showed that the induced tetraploid plants are characterized by larger, darker green and thicker leaves, larger stomata, lower density of stomata, larger flowers, lower fruit set rate in comparison with diploid plants.

The stomata size and density could be used as a useful indicator of successful conversion from diploidy to tetraploid if a flow cytometer is unavailable. It allows a fast evaluation when dealing with a high number of cells.

As future perspectives; in order to increase polyploidization rate, different explants such as root tip, leaf and meristem should be tried in *in vitro* methods. Beside, the seeds of the obtained tetraploid plants should be grown to determine whether the offspring are stable and their resistance to various stress conditions.

Table 1. Certain characteristics of diploid and tetraploid *P. peruviana* plants.

Characteristics	Diploid	Tetraploid	P
Plant height, cm	46.17±0.65 b	58.75 ±1.20 a	0.001
Stem diameter, cm	9.13± 0.20 b	11.35±0.66 a	0.033
Leaf area, cm^2	49.67±2.80 b	73.00±6.10 a	0.026
Flower size (area), cm^2	3.90 ±0.03 b	5.05± 0.18 a	0.003
Fruit weight, g	3.35 ±0.11 b	4.01±0.08 a	0.008
Fruit diameter, mm	29.52±0.77	30.42±0.57	0.400
Fruit length, mm	31.63±0.67	33.35±0.62	0.131
Soluble solid content, brix	14.87±0.44	15.40±0.42	0.427
Dry matter, %	17.18±0.25	17.12±0.29	0.875
Ascorbic acid, mg100 g^{-1}	56.22 ±1.10 b	61.30± 0.95 a	0.025
Days to flowering	87.00 ±0.58 b	93.33±0.33 a	0.001
Fruits yield per plant, g	646.70±48.00	647.00±14.00	0.995
Fruits number per plant	181.33± 6.40 a	152.33±5.80 b	0.029
Fruit set rate, %	95.90±0.73 a	80.07± 5.40 b	0.047
Seed number per fruit	192.37± 3.50 a	161.37± 2.50 b	0.002
Seed yield per fruit, g	0.20±0.00	0.19±0.00	0.457
1000 seed weight, g	1.09±0.02	1.09±0.04	1.000
Chlorophyll index	316.67 ±7.00 b	536.00±42.00 a	0.007
Seed viability rate, %	87.57±0.40	86.45±1.30	0.459
Pollen number per flower ($x10^4$)	45.83± 1.10 a	40.17 ±0.83 b	0.015

Pollen viability rate, %	63.30± 0.81 a	65.96±0.25 b	0.035
Stomata width, µm	19.02± 2.20 b	30.19±0.27 a	0.007
Stomata length, µm	34.75±2.80 b	47.75 ±0.63 a	0.010
Stomatal densities, no mm^{2-1}	175.53±1.30 a	77.73 ±3.80 b	0.000

Analysis of variance (two sample T-test) was performed at the 5% significance level.

Fig 2. Leaf of diploid (left) and tetraploid (right) goldenberry plants

Fig 3. Flowers of diploid (left) and tetraploid (right) goldenberry plants

Fig 4. Fruits of diploid (left) and tetraploid (right) goldenberry plants

Acknowledgments: This article is a part of research project No. 2013-191 supported by the Scientific Research Projects Commission of Eskisehir Osmangazi University.

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