

## INVESTIGATION OF MORPHOLOGICAL AND ANATOMICAL CHANGES IN *CATHARANTHUS ROSEUS* (L.) G. DON DUE TO COLCHICINE INDUCED POLYPLOIDY

A.Y. Shala\* and Z. Deng\*\*

\* Medicinal and Aromatic Plants Research Department, Horticulture Research Institute,  
Agricultural Research Center, Giza, Egypt.

\*\* Department of Environmental Horticulture, Gulf Coast Research and Education Center,  
University of Florida, USA.



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**ABSTRACT:** *Catharanthus roseus* (L.) G. Don is valuable medicinal plant grown as an ornamental plant. The study was carried out at Gulf Coast Research and Education Center, University of Florida, USA. to evaluate the morphological and anatomical changes from induced polyploidy in periwinkle for this purpose colchicine was applied at four different concentrations 0.0, 0.05, 0.1 and 0.2% for seedling treatment. The results showed that raising colchicine doses caused an increasing of survival percentage and tetraploid plants number. Polyploids showed an increment in leaf thickness, stomatal size, pollen diameter and pollen viability compared to diploid plants. Flow cytometry verified the existence of tetraploids and mixoploids from various colchicine-treated plants especially 0.2 which was found efficient on inducing polyploidy in the plants followed by 0.1% as compared to other concentration under study.

**Key words:** Vinca, chromosome doubling, colchicine, nuclear DNA content, tetraploid.

### INTRODUCTION

*Catharanthus roseus* (L.) G. Don is a perennial plant from Apocynaceae Family and is generally identified as the Madagascar periwinkle. It was originally classified as *Vinca rosea* by Linnaeus, and later renamed as *Catharanthus roseus* built on their morphological features (Van Der Heijden *et al.*, 2004). Physiologically it produces important antineoplastic alkaloids such as vincristine and vinblastine, which are chiefly existing in the leaves, while antihypertensive alkaloids (ajmalicine, serpentine and reserpine) are located in roots (Mishra *et al.*, 2001). Leaves of *C. roseus* are utilized for the therapy of menorrhagia, rheumatism, dyspepsia, indigestion, dysmenorrhea, diabetes, hypertension, cancer, menstrual disorders, skin diseases, bleeding, diarrhea and antiviral properties (Farnsworth *et al.*, 1968 and Holdsworth, 1990). It is grown as a

decorative plant in gardens and parks for its different colored flowers and blooming nature. The wild type of *C. roseus* is a diploid ( $2n = 16$ ; 1500 Mbp =  $12 \times$  Arabidopsis thaliana genome) plant (Levêque *et al.*, 1996).

Chromosome doubling is a beneficial implement in horticulture for gaining beneficial features, such as strong growth, bigger and extra heavily textured flowers, longer or delayed flowering period, and higher levels of resistance to disease (Hilu, 1993; Rose *et al.*, 2000; Väinölä, 2000; Shao *et al.*, 2003 and Zhang *et al.*, 2008). Also, polyploid plants can be more vigorous, have thicker leaves, bigger fruit, a better degree of drought and disease tolerance, enhanced adaptability, and endurance to environmental stress (Chakraborti *et al.*, 1998 and Eeckhaut *et al.*, 2004). Furthermore, chromosome doubling might offer an opportunity for

novel phenotypic differences causing from gene duplications (Udall and Wendel, 2006).

Chemicals utilization to produce variations in chromosome number has been well established (Thao *et al.*, 2003 and Jaskani *et al.*, 2005). Moreover, many inhibitors can be applied to block cell cycle progression in cycling plant cells, such as the anti-mitotic drug, one of these is colchicine that used to generate impeding at metaphase of mitosis. Colchicine is an alkaloid isolated from *Colchicum autumnale* L. which acts by binding to the tubulin dimmers inhibiting microtubules establishment and consequently, spindle fibers during cell division (Petersen *et al.*, 2003). In addition, it is a conventional polyploidy producing compound, which effectively used to produce polyploidy by stopping mitosis at the anaphase period (Thao *et al.*, 2003).

Stomata of polyploids are generally larger (Griesbach and Bhat, 1990 and Kadota and Niimi, 2002), and are one of an easy diagnostic trait that is often used to identify potential polyploids. Furthermore, this technique is rapid, inexpensive, and nondestructive, does not require advanced equipment, and has a fairly high accuracy rate (up to 90%) in some cases (Cohen and Yao, 1996) conversely, it is an indirect way for ploidy evaluation. If mixoploid plants are, formed in that case stomatal size can be an inaccurate way and should be joined with another method (Chen *et al.*, 2006). Chromosome counts are possibly the best precise, and aneuploidy might be discovered, while this technique can be laborious if many plants are being tested.

Analysis of DNA content by flow cytometry is often desired, many thousands of cells can be investigated quickly, mixoploids can be promptly distinguished. In some cases, for instance, in genome doubling from wide hybridization, genomic rearrangement can lead to changed genome size and complicate interpretation of ploidy based on DNA content (Levin, 2002). In addition, flow cytometry needs buying of costly apparatus, at the same time

chromosome performance cannot be detected with this method.

The aim of this study was to find a suitable level of colchicine that would effectively induce maximum polyploids in *Catharanthus roseus* (L.) G. Don from diploid plant, characterize the morphological and anatomical changes and determine the nuclear DNA content of newly induced tetraploid Vinca.

## MATERIALS AND METHODS

Vinca seeds cv. Vinca Little Bright Eye were brought from Neseed Company 3580 main street, Hartford CT 06120 (its website is <http://www.neseed.com/aboutus.asp>) then were sown on Apr. 1<sup>st</sup> 2015 and grown in a greenhouse at the University of Florida, Gulf Coast Research and Education Center, Wimauma, Florida, USA. into 128-celled trays containing Fafard 3B Mix, the components of the mix were Canadian sphagnum peat (50%), processed pine, bark, perlite and vermiculite.

### Colchicine treatment and polyploidy production:

Following 10 days of germination in the greenhouse, four concentrations of colchicine (Austratec Pty. Ltd) (0.0, 0.05, 0.1 and 0.2 percent) were used. An amount of 15 $\mu$ l of colchicine solution was applied to each seedling on three consecutive days. The colchicine solution was containing 1 ml semi-solid agar and 0.2% dimethyl sulphoxide (DMSO) to increase cell permeability and absorption of colchicine. Measurements of survival percentage were determined two times, four and eight weeks after colchicine treatment by counting the number of survival seedlings per treatment.

### Polyploidy analysis:

Selection of tetraploid plants was done on the basis of morphology, followed by a selection on the size of stomata, pollen viability and diameter and finally flow cytometry.

### **Leaf area determination:**

Leaf area was measured by scanning leaves using leaf area measurements software while, leaf thickness was measured with an electronic caliper.

### **Stomata length:**

Diploid and tetraploid plants leaves were removed and a small piece of the lower epidermis was peeled off (the stomata were separated by removing a thin layer from the lower side of leaves and studied under a microscope). Each leaf peel was placed in a drop of water on a glass slide, coated with a cover slip, then used for measuring stomata length under a light microscope (Olympus BX41, Canada Q- Color-5, Cooled, RTV) by using 40X objective magnification, (Stomatal size was measured in micrometers by evaluating the length and width of stomata). The stomata were photographed with an M Eye Digital camera and measured using Vision Explore software (Q Capture Pro).

### **Pollen viability and diameter:**

For evaluation of pollen viability, pollens were collected from opened flowers for diploid and tetraploid plants. Pollens were stained with 40 mL of fluorescein diacetate (FDA, Sigma-Aldrich, St. Louis, MO). FDA solution containing  $10^{-6}$  M FDA and 0.25 M sucrose and incubated at room temperature for a least 60 min in the dark. Stained pollen was located on a glass slide and coated with a coverslip. Pollen grains were scanned utilizing a fluorescence microscope (Olympus BX 41). Consistently round, non-wrinkled, brilliantly fluorescing pollen grains were deemed viable while, non-fluorescing or delicately fluorescing and wrinkled or twisted pollen grains were deemed non-viable. Pollen diameter was measured in micrometers by placing the scale end to end along the diameter of pollen grains. Pollens were photographed with an M Eye Digital camera and measured using Vision Explore software (Q Capture Pro). Diploid pollen viability was observed at 10× lens while tetraploid pollen was observed at

4× lens under a fluorescence microscope (Olympus BX 41).

### **Flow cytometry:**

Ploidy levels were screened by using flow cytometry (Cube 6 Flow Cytometry, Germany). Leaf squares of approximately 0.5 g were cut from edges of the newly matured leaves, placed in a 55 mm diameter plastic petri dish along with 1.0 ml nuclei extraction buffer (LB01 lysis buffer ), and chopped using a razor sharp edge into pieces. The contents of the petri dish were then filtered (pore size = 50 mm) into a 3.5 ml test tube to which 0.5 ml of staining buffer (propidium iodide, 1 mg/1) and 0.5 ml of Rnase was added and kept in darkness for 10 min. The flow cytometer's gain value was set at 490.0 and speed was set at 0.40 ml s<sup>-1</sup>. The standard peak of a known diploid *Vinca* was calibrated to appear at approximately 100 of fluorescence intensity (channel number) and histograms of DNA content were generated by using the Partec software package. To determine genome size *Glycine max* cv. Polanka, with a known DNA content of 2C= 2.50 pg, was utilized as an internal standard to verify relative genome size that was done by flow cytometry.

## **RESULTS**

### **Survival percentage:**

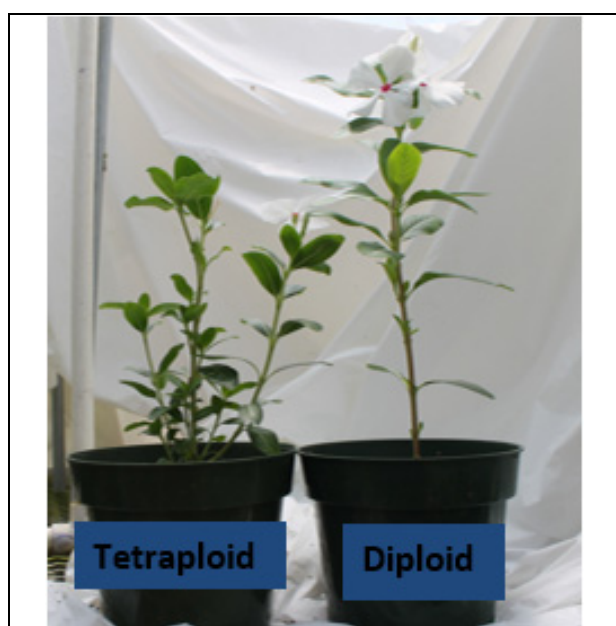
Higher colchicine concentrations tended to have greater survival percentage (Table, 1). The highest colchicine concentration (0.2%) recorded the best survival percentage (55.4%) while, the survival percentage sharply declined as colchicine rate decreased.

### **Morphological changes:**

In all morphological observations, colchicine concentration had a marked influence on the treated plants growth accordingly, control plants were taller than colchicine treated plants also, tetraploids had more branches than diploids (Fig., 1). Moreover, tetraploid had smaller and thicker

**Table 1. Effect of colchicine concentrations on survival percentage of *Catharanthus roseus* (L.) G. Don plants.**

| Treatments         | Number of plants before the treat. | Survival (%) after one month | Survival (%) after two months |
|--------------------|------------------------------------|------------------------------|-------------------------------|
| T0                 | 89                                 | 59 (66.0%)                   | 59 (66.0%)                    |
| Colchicine (0.05%) | 84                                 | 22 (26.0%)                   | 22 (26.0%)                    |
| Colchicine (0.1%)  | 82                                 | 40 (48.7%)                   | 40 (48.7%)                    |
| Colchicine (0.2%)  | 92                                 | 53 (57.6%)                   | 51 (55.4%)                    |

**Fig. 1. Diploid and tetraploid *Catharanthus roseus* (L.) G. Don plants.**

leaves therefore, leaf area of tetraploid was smaller than diploid plants (Table, 2).

#### Stomata observation:

Stomata size showed significant differences among diploid and tetraploid plants consequently, mean of control stoma length was  $18.82 \pm 1.77 \mu\text{m}$  and  $16.64 \pm 2.26 \mu\text{m}$  for width (Table, 2 and Fig., 2) while, mean stoma for tetraploid plants was

$28.59 \pm 3.74$  and  $21.87 \pm 2.80 \mu\text{m}$  for length and width respectively.

#### Pollen diameter and pollen viability:

There was a clear difference between pollen sizes in colchicine-treated plants and non-treated ones (Table, 3 and Fig., 3) accordingly, the greater part of tetraploid pollen grains were larger than diploids pollen grains. Tetraploid and diploid pollen diameter were  $64.57 \pm 3.22$  and  $59.41 \pm 1.67 \mu\text{m}$ , respectively. Otherwise, the results showed pollen viability differences among diploid and tetraploid plants (Table, 3 and Fig., 4) hence, tetraploid plants had the highest pollen viability percentage (64.0%) comparing with diploids, which had the moderate average pollen viability percentage (55.0%).

#### Flow cytometry analysis:

Screening of 106 plants by flow cytometry ascertained that 62 treated plants were diploid while, 31 treated plants were tetraploid and 13 were mixoploid (Table, 4). Histograms with excitation peak of fluorescent intensity at channel 100 matched by diploids (Fig., 5A) and channel 200 corresponded to tetraploids (Fig., 5B).

**Table 2. Morphological characteristics of diploid and tetraploid *Catharanthus roseus* (L.) G. Don plants (mean  $\pm$  SD).**

| Ploidy | Leaf thickness (mm) | Leaf area (cm <sup>2</sup> ) | Stoma size ( $\mu\text{m}$ ) |                  |
|--------|---------------------|------------------------------|------------------------------|------------------|
|        |                     |                              | Length                       | Width            |
| 2x     | $0.25 \pm 0.02$     | $14.33 \pm 1.95$             | $18.82 \pm 1.77$             | $16.64 \pm 2.26$ |
| 4x     | $0.32 \pm 0.02$     | $10.58 \pm 1.45$             | $28.59 \pm 3.74$             | $21.87 \pm 2.80$ |

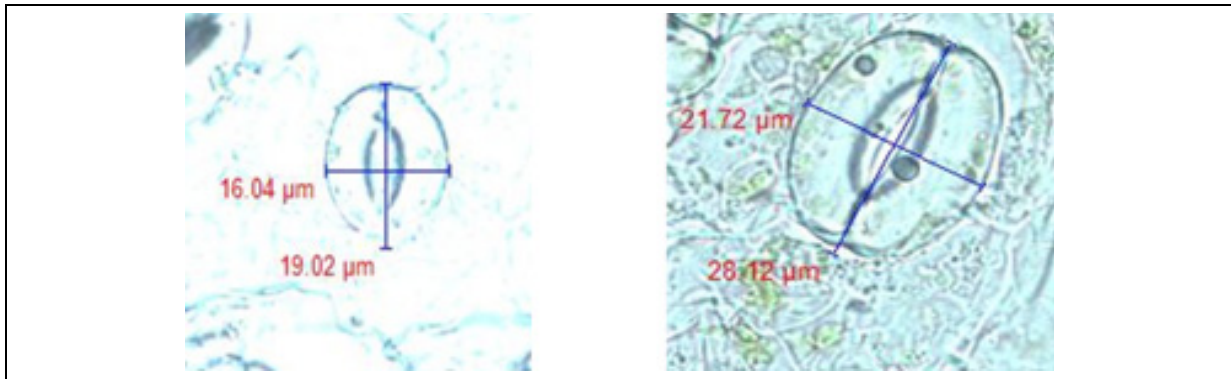


Fig 2. Stoma size in *Catharanthus roseus* (L.) G. Don diploid plant left, tetraploid plant right.

Table 3. Pollen diameter and pollen viability percentage of diploids and induced tetraploids of *Catharanthus roseus* (L.) G. Don plants (mean  $\pm$  SD).

| Ploidy | Pollen diameter ( $\mu\text{m}$ ) | Pollen viability % |            |
|--------|-----------------------------------|--------------------|------------|
|        |                                   | Viable             | Non-viable |
| 2x     | 59.41 $\pm$ 1.67                  | 55.0%              | 45.0%      |
| 4x     | 64.57 $\pm$ 3.22                  | 64.0%              | 35.8%      |

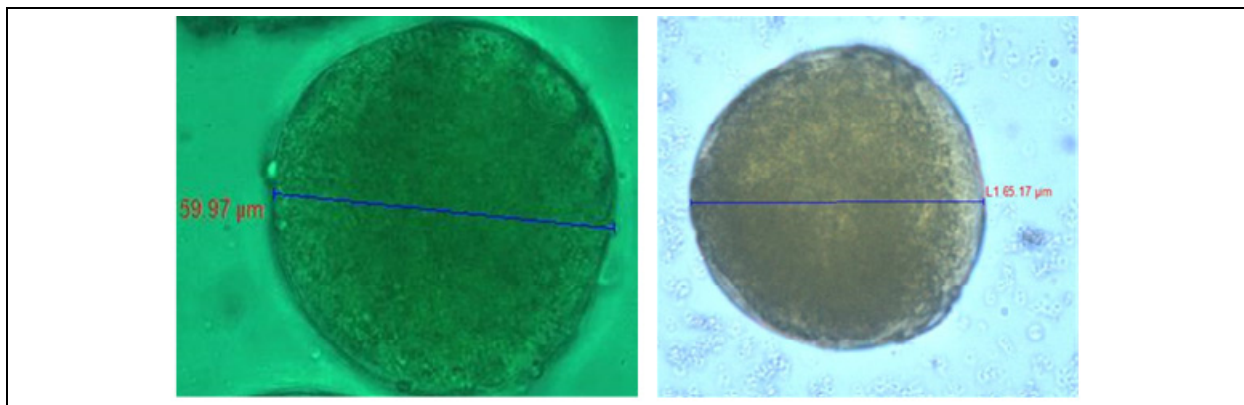


Fig. 3. Pollen size in *Catharanthus roseus* (L.) G. Don diploid plant right, left tetraploid plant.

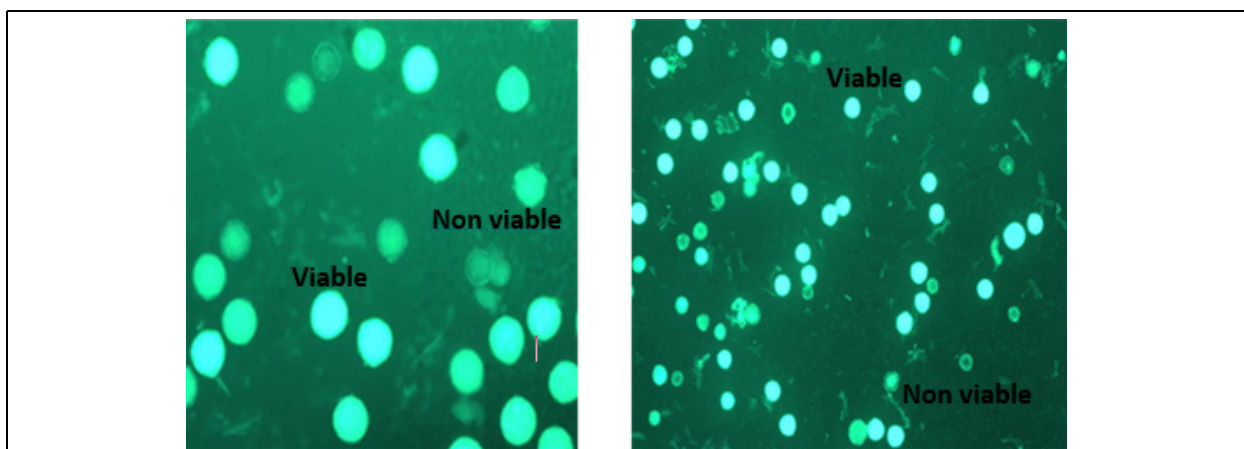
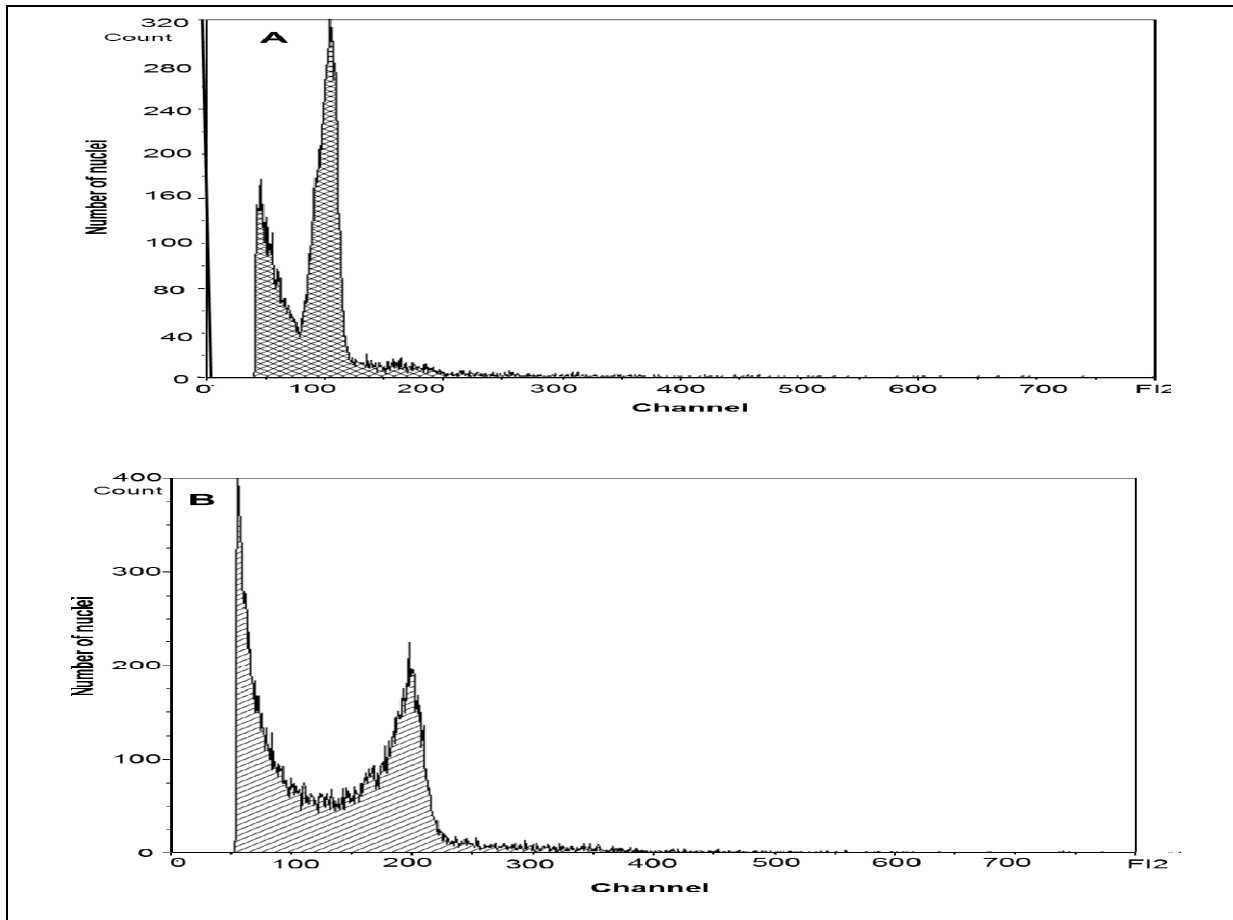


Fig. 4. Viable and nonviable pollen in *Catharanthus roseus* (L.) G. Don diploid plant left, tetraploid plant right.

**Table 4. Effects of colchicine concentrations on ploidy level of *Catharanthus roseus* (L.) G. Don plants.**

| Treatments         | Plants number before screening | Diploid plants No. | Tetraploid plants No. | Mixoploid plants No. |
|--------------------|--------------------------------|--------------------|-----------------------|----------------------|
| T0                 | 59                             | 59                 | -                     | -                    |
| Colchicine (0.05%) | 19                             | 15                 | 3                     | 1                    |
| Colchicine (0.1%)  | 40                             | 23                 | 12                    | 5                    |
| Colchicine (0.2%)  | 47                             | 24                 | 16                    | 7                    |



**Fig. 5. Flow cytometry histogram of (A) an untreated (control) *Catharanthus roseus* (L.) G. Don. The diploid peak is fixed at channel 100, (B) tetraploid peak is observed at channel 200.**

The number of transplants that were tetraploids and mixoploid containing both diploid and tetraploid cells increased as colchicine rate increased (Table, 4), accordingly, all colchicine concentration produced tetraploid plants, however colchicine at 0.2% produced more tetraploids and mixoploids number than all other treatments. Therefore, these data recommend that colchicine concentrations at 0.2% followed by 0.1% were the optimum

concentration under the conditions of this experiment for induction of tetraploidy in *Vinca* transplants.

#### **Ploidy confirmation:**

Tetraploids of *Catharanthus roseus* (L.) G. Don had a mean genome size of  $2C = 3.09 \pm 0.04(\text{SD})$ , which is approximately twice that of the genome size of diploid *Catharanthus roseus* (L.) G. Don,  $2C = 1.57 \pm 0.02 (\text{SD})$ .

## DISCUSSION

This study displayed that *Vinca* tetraploids could be induced by treating plant tips with colchicine at concentrations of 0.05, 0.1 and 0.2%, respectively. Usage of colchicine at 0.2% on the plant tips increased survival percentage and produced more tetraploids than all other colchicine concentrations while other concentrations significantly reduced survival compared with untreated plants. Our findings were in harmony with Marzougui *et al.* (2011) who applied an aqueous solution of 0.5% colchicine on *Trigonella foenum-graecum* L. populations between the two cotyledons for three days per each population as resulted in a significant increment in survival percentage. Conversely, our outcomes were not consistent with the results of Henny *et al.* (2009) on *Dieffenbachia* who reported that shoot clumps survival considerably declined as colchicine concentrations increased also, Oates *et al.* (2012) found that survival of *Rudbeckia* diploid hybrid genotypes ranged from 0% to 82% without any improvement in the allotetraploid hybrids.

The growth of colchicine-treated plants was slower than control. The delayed growth was due to a reduced rate of cell division caused by colchicine for that reason *Vinca* shoot length decreased (Fig., 1). Similar observations in which the average of shoot length decreased due to initial retardation of growth by colchicine application on *Impatiens patula* Craib (Tharawoot *et al.*, 2012), *Echinacea purpurea* L., (Abdoli *et al.*, 2013) and *Brassica campestris* L. (Kumar and Dwivedi, 2014) that plant height was lower in tetraploid plants related to diploid untreated plants. (Shao *et al.*, 2003) illustrated that during polyploidy induction process, plants are seriously injured, prohibiting normal growth or even killing the plants, depending on the concentrations of colchicine and the period of the treatment.

In the present study colchicine induction in *Vinca* produced tetraploid significantly different in leaf area and leaf thickness (Table, 2). These results were in harmony

with those of Tharawoot *et al.* (2012) on *Impatiens patula* Craib who found that tetraploid plants had the highest average of leaf thickness than diploid plants, Kumar and Dwivedi, (2014) found a reduction in leaf length and leaf breadth of *Brassica campestris* L. tetraploid and Iannicelli *et al.* (2016) reported that tetraploid displayed considerable changes from the field mother plant in size of *Lippia integrifolia* leaves. Similarly, the increment in the mean length and width of the stomata of tetraploid plants than in diploid plants (Table, 2 and Fig., 2) hence, stomata size is another source of data which can be used as a tool for primarily verify the status of tetraploid. This is in agreement with the results on mulberry (Chakraborti *et al.*, 1998), *Dieffenbachia* × 'Star bright M-1, (Henny *et al.*, 2009), *Catharanthus roseus* (L.) G. Don (Xing *et al.*, 2011), *Tagetes erecta* (Sajjad *et al.*, 2013), *Echinacea purpurea* L. (Abdoli *et al.*, 2013) and *Brassica campestris* L. (Kumar and Dwivedi, 2014) that, stomata in polyploids are larger than diploids consequently, stomata size can be utilized to reveal the ploidy of *Vinca* plants. Accordingly, changes in leaves anatomical properties that generally associate with ploidy in plants due to the increment in cell size (Dwivedi *et al.*, 1986) are commonly operated to evaluate polyploidy induction in plants.

The broad literature on phenotypic influences on polyploidy has been studied by Ramsey and Schemske (1998). Transitions to polyploidy are joined by physical changes in the size and geometry of cells, which in turn may involve biochemistry, development, anatomy and ultimately whole-plant morphology, growth and physiology. Therefore, many of the gigas traits of polyploids, such as enlarged cell size, expanded floral structures, stronger foliage and vigorous stems, may be a direct result of increased DNA content (Randolph, 1941). Moreover, having more chromosomes may also slow mitotic division and consequently result in the lower overall growth of the whole plant (Ramsey and Schemske, 2002).

The increase in the size of *Vinca* pollen diameter (Table, 3 and Fig., 3) was documented in the present study which agrees with previous observations of Abdoli *et al.* (2013) on *Echinacea purpurea* (L.) who found that the diameter of tetraploid pollen grains was considerably larger than those in the corresponding diploid plants, Kumar and Dwivedi (2014) applied aqueous solution of colchicine of 0.3, 0.4, 0.5, 0.6% to *Brassica campestris* L. seedlings and found that pollen diameter in autotetraploid and diploid plants was  $67.95 \pm 0.02$  and  $26.05 \pm 0.28$   $\mu\text{m}$ , respectively and Iannicelli *et al.* (2016) exposed *Lippia integrifolia* explants to 0.01% of colchicine and obtained differences in pollen grains size between the new tetraploids and the diploids. In addition, our results displayed the increment in tetraploid pollen viability percentage (Table, 3 and Fig., 4) over diploid, which consistent with Oates *et al.* (2012) on *in vitro* treated shoot apices with 30 mM oryzalin for 5 days as increased pollen staining of allotetraploids of *Rudbeckia subtomentosa* compared to their diploid counterparts. Likewise, Adaniya and Shirai (2001) stated that all induced ginger tetraploid strains showed higher pollen fertility than original diploid, which may be due to the differences in the types and frequencies of chromosome aberrations (Adaniya and Shoda, 1998).

The results displayed that high colchicine concentration increased proportions of mixoploids in *Vinca* among the population of diploids and tetraploids (Table, 4) which were in agreement with the previous reports by Henny *et al.* (2009) who stated that higher levels of colchicine tended to produce mixoploids in *Dieffenbachia*, Tharawoot *et al.* (2012) applied colchicine at 0, 0.03, 0.05 and 0.09% on shoots explants of *Impatiens patula* with the average length of 3 cm and found that mixoploid plants produced only from the treatment with 0.03% colchicine. Similarly, Bagheri and Mansouri, (2015) demonstrated that the maximum mixoploids proportion in *Cannabis sativa* L. was obtained from shoot tips treated with colchicine for 24-h in 0.2

and 0.1% w/v. Increasing production of mixoploids could be legitimized by the fact that the antimitotic agent cannot influence all meristematic cells at the time of treatment.

Flow cytometry is a beneficial instrument in confirming ploidy level of *Vinca* transplants (Table, 4). The advantage of this procedure is simple, rapid, efficient and convenient more than 20 leaf samples can be carried out in 1 h to confirm *Vinca* ploidy levels. Due to the expense of running an enormous number of samples, reducing sample size based on morphology can greatly lessen flow cytometry screening costs and increase tetraploids identification chances so, for accurate ploidy level identification of *Vinca* plants flow cytometry is highly recommended. Furthermore, the morphological markers can also be utilized to accurately confirm the ploidy.

## CONCLUSION

Tetraploid plants of *C. roseus* (L.) G. Don were gained from colchicine-treated plants. The tetraploid plants have different morphological and anatomical features than those original diploid plants. Tetraploid plants have shorter stems, smaller and thicker leaves as well as exhibited much larger stomata size, pollen diameter and increment of pollen viability than diploid plants and the previous characters were very effective for assessing the ploidy in *Vinca* plants. It might be worthy to recommend that colchicine could be utilized to raise possible fertile tetraploid *Vinca* plants, flow cytometry to be used for accurate identification of ploidy level in *C. roseus* (L.) G. Don. Due to the beneficial medicinal importance of *C. roseus* (L.) G. Don and the useful outcomes of polyploidy on secondary metabolite yield, additional research is required to determine in detail the effect of polyploidy on alkaloid accumulations of *C. roseus* plants.

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## تقييم التغيرات المورفولوجية والتشريحية في الونكا الراجعة الى التضاعف الكروموسومى المستحدث بالكولشسين

عوض يوسف شعله\* ، زناو دنج\*\*

\* قسم بحوث النباتات الطبية والعطرية، معهد بحوث البساتين، مركز البحوث الزراعية، الجيزة، مصر.  
\*\* قسم البستنة البيئية، مركز جولف كوست للبحوث والتعليم، جامعة فلوريدا، الولايات المتحدة الأمريكية.

الونكا من النباتات الطبية الهامة كما أنها تنمو كنبات زينة. تم إجراء التجربة بجامعة فلوريدا بالولايات المتحدة الأمريكية بهدف قياس التغيرات المورفولوجية والتشريحية الناتجة عن التضاعف الكروموسومى في الونكا ولهذا الغرض تم استخدام الكولشسين لاستحداث التضاعف الكروموسومى بأربعة تركيزات هي ٠،٠٥، ٠،١، ٠،٢ و ٠،٢ % وذلك لمعاملة البادرات. وقد تبين من النتائج أن زيادة تركيزات الكولشسين تؤدي لزيادة نسبة البقاء وعدد النباتات الرباعية العدد الكروموسومى. أظهرت النباتات المتضاعفة زيادة في سمك الورقة وحجم الثغور وقطر حبوب اللقاح وحيوية حبوب اللقاح بالمقارنة بالنباتات ثنائية العدد الكروموسومى. وقد أظهر جهاز الفلوسايتومتر وجود النباتات المتضاعفة والخليطة العدد الكروموسومى من النباتات المعاملة بالتركيزات المختلفة من الكولشسين وبخاصة تركيز ٠،٢ % والذي كان فعالاً في تسجيل أعلى عدد من النباتات المتضاعفة يليه ٠،١ % بالمقارنة بباقي التركيزات تحت الدراسة.