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ARTICLE

Colchicine effect on the DNA content and stomata size of *Glycyrrhiza glabra* var.glandulifera and *Carthamus tinctorius* L. cultured *in vitro*

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Abstract *In vitro* induction of polyploids using colchicine causes an increase in DNA content in plants. This is of high importance especially for plants that have medicinal and commercial values. Seeds of two medicinal plants, licorice *Glycyrrhiza glabra* L. var.glandulifera and safflower *Carthamus tinctorius* were treated with different concentrations of colchicine, 0%, 0.03%, 0.05%, 0.08%, 0.1% (W/V) *in vitro* for 24 and 48 h. Treated seeds then were cultured on solid Murashige and Skoog (MS) media under controlled conditions. After a month, the length of the stomata was measured to study the effect of colchicine on stomata size. Cellular DNA content of the regenerated plants was measured by spectrophotometry. Flow cytometry was used for confirming the results obtained from stomata size measurement and spectrophotometry. Results suggested that treated plants have a fair amount of larger stomata, significantly in licorice plantlets that were treated with 0.1% colchicine for 24 h and safflower plantlets that were treated with 0.03%, 0.05% and 0.1% colchicine. Safflower DNA content in all treatments enhanced significantly, but in licorice only DNA content of plantlets that were treated with 0.05% colchicine for 24 h and 0.1%, 0.03% colchicine for 48 h found to be increased significantly. The morphological features of treated plantlets such as shoot and leaf thickness were found to be increased. Flow cytometry confirmed the previously mentioned results and suggested tetraploids in all treated safflower plantlets and licorice plantlets obtained from treatment with 0.08% of colchicine and mixoploids in licorice plantlets obtained from treatment with 0.1% of colchicine.

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1. Introduction

Licorice (*Glycyrrhiza glabra* L.) and safflower (*Carthamus tinctorius*) are medicinal plants containing various compounds and have long been used for medicinal and industrial purposes [5,33]. The roots and stolons of *G. glabra* are used as natural sweetener [14] and anti-inflammatory drug [3]. Safflower

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pigments have been shown to have pharmaceutical properties and are also safely used in the food industry [33,17,32].

Developing a suitable method for increasing the secondary metabolites found in these plants and also enhancing their resistance against environmental stress can help the conservation of these precious species. The induction of artificial polyploidy has long been considered as a valuable tool that can improve genetic of many plants and as a result changes the morphological, anatomical, and physiological characteristics of plants [18,20,22]. It also increases the production of important medicinal compounds and makes the plants more resistant to stresses [1,24,8]. Colchicine as an anti-mitotic agent is used for chromosome doubling under *in vitro* conditions. The effect of colchicine for *in vitro* chromosome doubling is different regarding to its concentration, method and duration of treatment, and also genetic factors of the treated plants [1,10].

An important step in polyploidization is to determine ploidy levels in a quick and simple way. The measurement methods are classified into direct and indirect. The direct method is the classic method of counting chromosome in mitotic cells of root tips. This method is accurate, but time consuming and needs a lot of experience [1,10,2]. Therefore, indirect methods have been developed for ploidy determination [27]. Cytologic characteristics such as size of stomata cells, stomata density, pollen grain diameter, and number of chloroplasts in guard cells can be used as indirect ways for determining polyploids [10,27,23].

DNA content can also be a good indicator of ploidy level as it increases by chromosome doubling that happens in polyploidy. Therefore, optical density of extracted DNA, measured by spectrophotometry, will rise as well. This method also speeds the determination process of ploidy of *in vitro* regenerated plants and gives a rough estimation of ploidy changes after ploidy induction [28].

Another method for measuring DNA content is Flow cytometry (FCM). Unlike spectrophotometry this method needs small amounts of tissue and can analyze a large number of cells especially when mixoploids or aneuploids exist [10,11]. It was proved that flow cytometric analysis is faster and more convenient compared with the other methods [1,10,27].

In this study we used colchicine treatments in order to obtain polyploids of licorice and safflower. We used indirect methods such as spectrophotometer to compare optical density of DNA content in treated and control plants for estimating the DNA increase as the result of chromosome doubling. Also stomata size as an indirect method was used for recognition of putative polyploids in these species and then flow cytometry for confirming ploidy levels and comparing it with other methods of ploidy screening used in this experiment.

2. Materials and methods

2.1. Seed treatment

Seeds of Licorice (*G. glabra* var. glandulifera L.) were soaked in sulfuric acid 98% for 30 min in order to remove the hard seed coat that prevents the germination. Then seeds were sterilized in 70% for 1 min and 1% sodium hypochlorite for 20 min and finally rinsed with distilled water for three times. Safflower's seeds also were sterilized using 0.1% $HgCl_2$ for 8–10 min followed by three rinses with distilled water. Seeds then were cultured on sterile liquid MS medium (Murashige and Skoog, 1962) [26]. supplemented with colchicine (0%, 0.03%, 0.05%, 0.08%, 0.1%, pH = 6) for 24 or 48 h on an orbital shaker (100 rpm). Then they were transferred on solid MS media and allowed for germinating in the culture room under normal condition (16 h light period, temperature 25–27 °C and humidity 65% and 2000 Lux light).

2.2. Ploidy analysis

Putative polyploid plants were selected based on morphology, as they seemed to be thicker, darker and showed delayed growth. Size of the stomata in selected plants was measured and also cellular DNA content of them was analyzed by spectrophotometry, and finally flow cytometry as an accurate method was carried out to confirm the efficiency of above mentioned methods.

2.3. Size of stomata

Epidermal cells of two leaves from 5 diploid and 5 putative tetraploid plants from each treatment were randomly examined. Imprint of epidermal cells of plantlets treated with different concentrations of colchicine for 24 h were obtained by applying thin layer of clear nail polish on the lower leaf surface and removing it after drying the polish (abaxial side) [1,19,31,25]. Imprints were stuck on a microscope slide and observed through the light microscope at 1000× magnification (for guard cells measurement). (BX50; Olympus Optical Co. Ltd.). Stomata length was measured as an indicator of stomata size which by itself is an indicator of ploidy using the Image tool software.

2.4. Spectrophotometry

The DNA of 5 control and 5 treated plants from each treatment were randomly extracted by the standard CTAB procedure. Optical density of total DNA content was measured at 260 nm wavelength using spectrophotometry [28].

2.5. Flow cytometry (FCM)

Flow cytometric analysis was carried out using a PA-1 (Partec, Germany) flow cytometer. Leaf tissue (0.5 mg) from selected plants was chopped with a sharp blade in 0.5 ml CyStain DNA 1 step nucleus extraction buffer (HR, high resolution, A solution, Partec, CA) after being filtered through a 30-μm nylon mesh and then incubated in the same buffer for 5 min. The different flow cytometer parameters were adjusted with untreated material to secure well defined and reproducible readings. The nuclear DNA of 150 colchicine treated plants was used in these determinations.

2.6. Statistical analysis

All data in this study were statistically analyzed using the LSD test and analysis of variance (ANOVA) by the SPSS 18 software. Diagrams were prepared using the Microsoft Excel 2007.

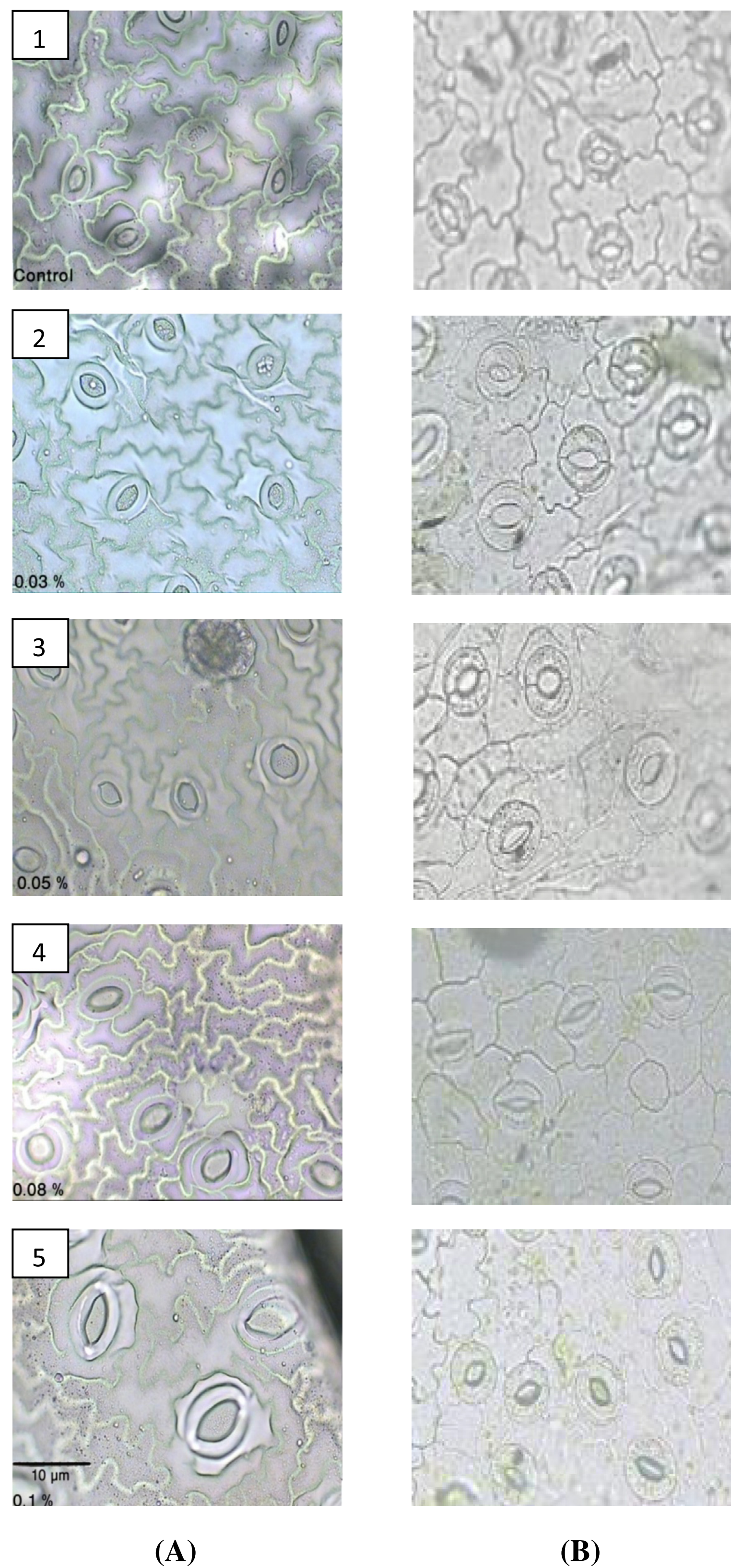

Figure 1 Stomata of leaves obtained from treatments with (1) 0%, (2) 0.03%, (3) 0.05%, (4) 0.08%, (5) 0.1% of colchicine for 24 h in (A) licorice (B) safflower. Each stoma was obtained from the same part of leaves in all treatments (1000×).

3. Results and discussion

3.1. *In vitro* plant polyploidization

The growth of seedlings under the effect of colchicine was assessed a month after treatment. All of the control seedlings and the seedlings resulted from treatment with different concentrations of colchicine for 24 h, survived. Not all of the seedlings treated for 48 h grew and just some in lower concentrations. It indicates that the 24 h colchicine treatments used were enough to induce cell polyploidization and did not have serious toxic effects, while 48 h treatments had some toxic effects and reduced the growth rate [20,1,13].

The morphological features of treated plantlets such as root shoot and leaf thickness were found to be increased, while their length was decreased. Also, treated plants' growth was slower than the control's especially in plantlets that resulted from 48 h of treatment in which only cotyledons and a few leaves emerged. So for the rest of the experiments for which we needed a large amount of tissues we used only plantlets that were treated for 24 h.

These morphological changes were used for selecting putative polyploids. There was an inverse relationship between survival of plantlets and colchicine concentration and it is in agreement with similar reported result in ex vitro [12,29] and *in vitro* [1,19,6,7] polyploidization using other plants.

These morphological changes were reported in similar studies [7]. The slower rate in growth may be the result of physiological disturbance induced by colchicine, which causes a reduction in cell division rate [30]. The growth of both control and treated plantlets was equally well in subculture, and it shows that colchicine only causes an initial reduction of growth [1,6].

3.2. Stomata size

Length of the stomata in putative morphologically polyploid plants was measured. We selected robust and darker green plants that were all from the group which was treated with different concentrations of colchicine for 24 h. The results suggested that treated plants both in licorice and safflower have a fair amount of larger stomata (Fig. 1). In licorice length of the stomata in treated plants was larger significantly in plantlets treated with 0.05% and 0.1% of colchicine for 24 h with average length of 128.01 and 181.86 nm respectively compared with 84.7 nm in control plantlets. In Carthamus average length of the stomata in plantlets treated with 0.03%, 0.05%, 0.1% of colchicine was 127.17, 132.67 and 123.83 nm respectively in which all were significantly larger in comparison with the control group with an average length of 99.44 nm (Fig 2.).

Stomata length as a measure of stomata size can be an indicator of ploidy levels and it has been used in different plant types for determining ploidy levels [22,19,25,6,21,15].

3.3. DNA content

In this study, average concentrations of colchicine had the best effect on increasing the total DNA content of treated licorice plantlets. Optical density of total DNA content of licorice plantlets that were treated with 0.05% and 0.1% of colchicine for 24 h and 0.1% and 0.03% of colchicine for 48 h found to be almost doubled that confirms tetraploids. In safflower total DNA content of all treated plantlets for 24 h was almost twice more than that of control groups representing tetraploids and in 48 h treatments sometimes (in 0.03%, 0.08% and 0.1% of colchicine) three times more than that of control groups representing hexaploids (Fig 3).

Total DNA content of plants increases as the result of chromosome doubling that happens in polyploidization. This can be used as an indicator for ploidy determination, so we

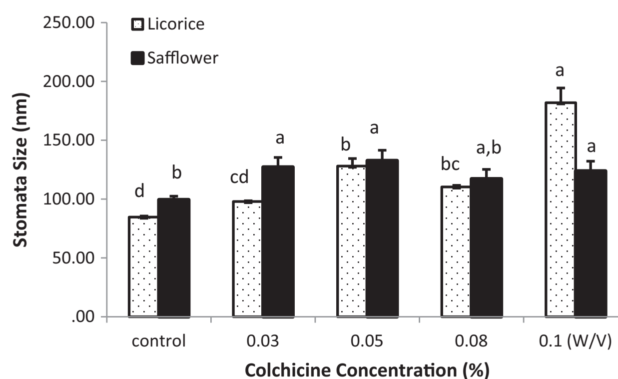

Figure 2 Effect of colchicine concentration on length of the stomata in licorice and safflower plants.

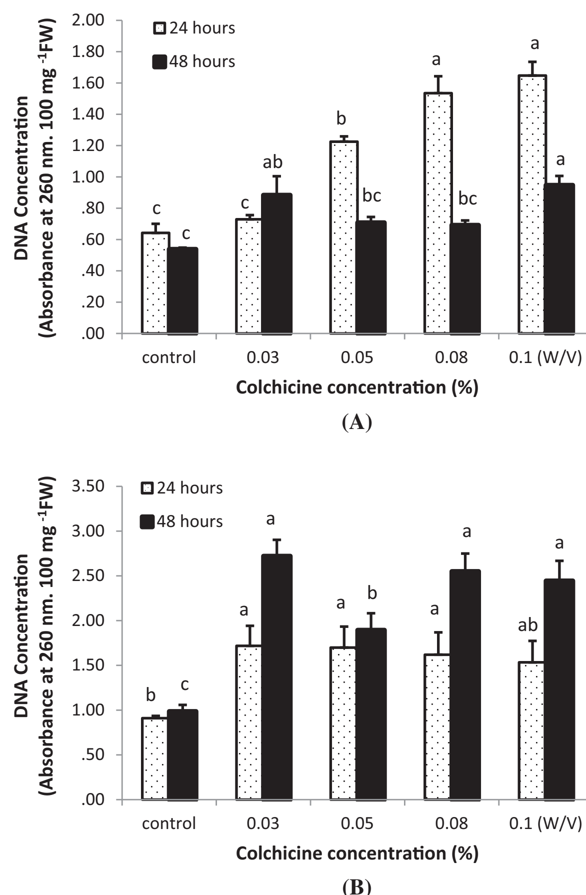

Figure 3 Absorbance of extracted DNA content of regenerated seedlings of Licorice and safflower treated for (A) 24 and (B) 48 h with 0%, 0.03%, 0.05%, 0.08% and 0.1% of colchicine.

extracted DNA and then measured its optical density in treated plants and compared it with the control group using a spectrophotometer. This method is easy and fast and reduces the time to determine ploidy of *in vitro* regenerated plants. Raza et al. reported the efficiency of this method in determining of ploidy levels in watermelon [28]. Our results are also in agreement with the similar studies carried out by Colijn-Hooymans et al. (1994) and Gao et al. (1997) [9,16].

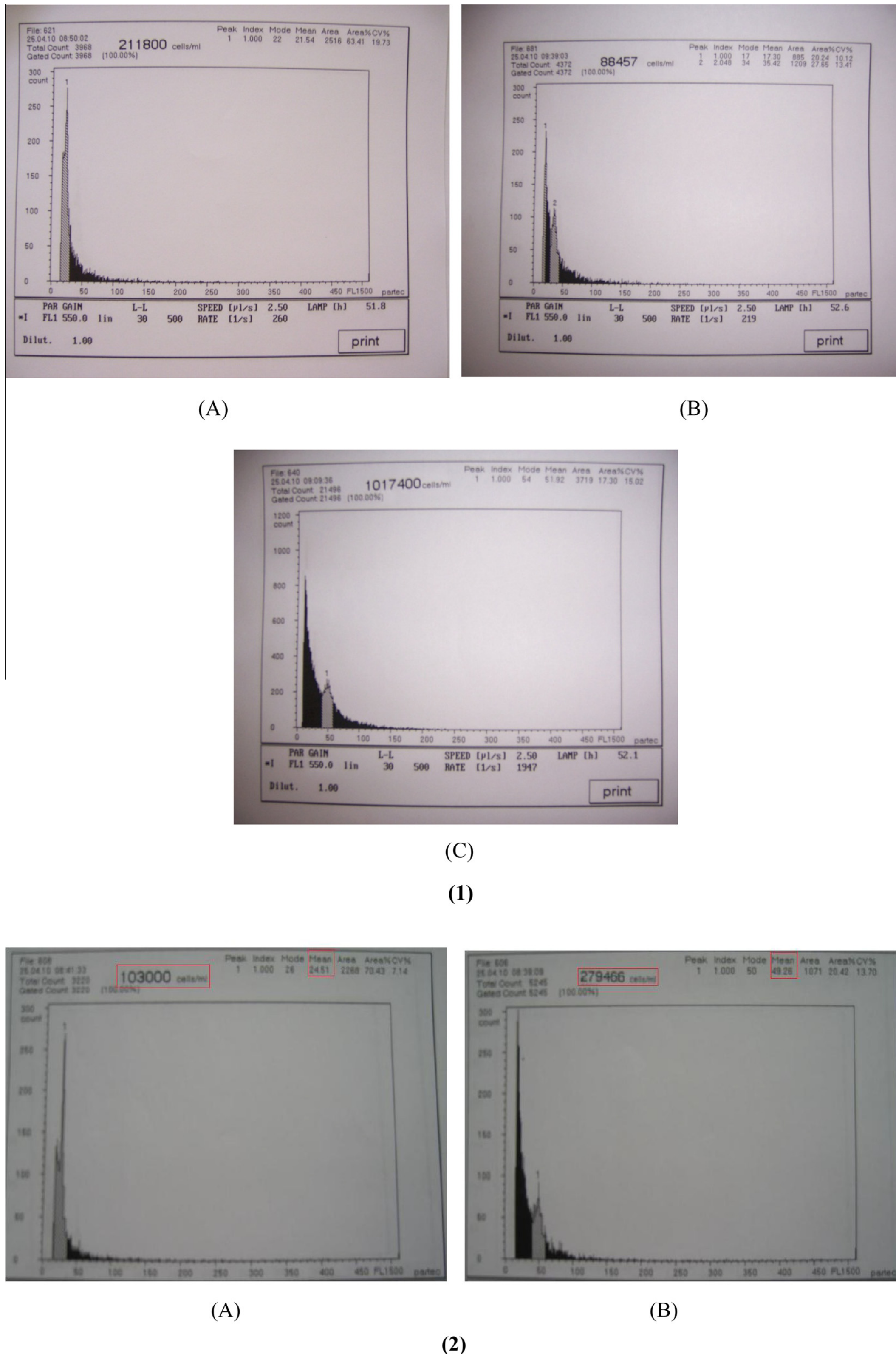

Figure 4 DNA-histograms of nuclei isolated from leaves of (1) Licorice (A) a diploid, (B) mixoploid and (C) tetraploid. (2) Safflower (A) a diploid, (B) tetraploid.

3.4. Flow cytometric analysis

The putative polyploid plants were selected on the basis of morphology, stomata length and total DNA content for analyzing by flow cytometry.

Results of flow cytometry in putative polyploid plants in licorice confirmed the previously mentioned results and suggested tetraploids in plantlets obtained from treatment with 0.08% of Colchicine and mixoploids in plantlets obtained from treatment with 0.1% of Colchicine. Fig. 4 shows the histogram obtained by flow cytometry of a control plantlet treated with 0% colchicine and 2 treated plantlets treated with 0.08% and 0.1% of colchicine. In plantlets treated with 0.1% of colchicine mixture of diploid and tetraploid tissues was observed. Peak "1", with a mean relative DNA content of 17.3, corresponds to the control cells, whereas peak "2", with mean relative DNA content of 35.4 corresponds to the tetraploid cells.

In plantlets treated with 0.08% of colchicine a peak with mean relative DNA of 51.9 was observed which corresponds to tetraploids cells.

In safflower the peak with mean relative DNA of 24.51 observed in control plantlets analysis corresponds to diploid cells. Fig 4 shows the histogram obtained by flow cytometry analysis of treated plantlets in which a peak with mean relative DNA of 49.26 was observed that corresponds to tetraploids cells (Fig 4).

Flow cytometry is very accurate and powerful for estimating plant nuclear DNA content as it measures florescence intensity of stained nuclei sensitively within seconds [4].

4. Conclusions

In this study, efficient concentrations of colchicine for tetraploid licorice and safflower generation in *in vitro* were introduced. Measuring optical density of total DNA content makes it possible to easily recognize putative tetraploids. Also Tetraploids were morphologically thicker but shorter than controls and their growth was slower and they have larger stomata than the diploid plants. Nuclear DNA measurement via flow cytometry confirmed the above mentioned results. Using different methods we conclude that all methods can be efficient in determining ploidy levels, although flow cytometry is easier, faster and more accurate.

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