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Induction of polyploidy in citrus rootstocks

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Abstract
The present investigation was undertaken to Induction of polyploidy in citrus rootstock through Colchicine Treatment. Seed were immersed in different concentrations of colchicine treatments (0, 0.2%, 0.6%, 1.2%) for different rootstock (Citrange, Citromelo and Sour orange). The seedling ploidy was determined via flow cytometry. Higher colchicine concentrations decreased the survival. Mixoploeid Citrange produced by 0.6% Colchicine treatment. Morphological data confirmed the results of flow cytometry. The mixoploid from Citrange can be used for breeding programs

Keywords: Citrus Rootstocks, Colchicine, flow cytometry.

Introduction

The genus Citrus is economically very important and is known for its juice and pulp throughout the world. The genus belongs to the family Rutaceae that includes 162 species (Hynniewta, 2011) and is grown in tropical and subtropical areas of the world. Citrus is the third most important fruit crop of Iran with an estimated production of 2.6 million tons from an area of 0.176 m ha (FAO, 2013). The phenomenon of polyploidy has played a vital role in the evolution of many crops. Some of the economically important plants whose triploids are in commercial use include several varieties of apple, bananas, mulberry, sugar beets, tea and watermelon. In citrus and its relatives, there are a few known tetraploid and triploid types. The great majority of the species of Citrus, Fortunella and Poncirus are diploid, having 18 chromosomes. Doubling of an entire chromosome complement may result in an increase of cell volume and consequently in an increase of plant parts. This can be a useful tool in breeding and selecting for larger fruit size(Elyazid and Shereif, 2014). Polyploidy is a major component of eukaryote evolution and particularly in angiosperms (Grant, 1981; Soltis and Soltis, 1993; Wendel and Doyle, 2005). Many plant species result from autopolyploidization or allopolyploidization events and polyploidization should be considered as the most common sympatric speciation mechanism (Otto and Whitton, 2000). According to Thompson and Lumaret (1992), the dynamics of polyploid plants is based on three processes: the origin (polyploidization events), the establishment and the persistence of new polyploids. The mechanisms leading to polyploidy were variously discussed during the 1970s, and for a long time chromosome doubling was considered the major cause. Most authors (e.g. Stebbins, 1971) considered that meiotic restitution played only a minor role in the evolution of polyploid complexes. Diploidy is the general rule in Citrus and related genera of Aurantioideae, with a basic chromosome number x = 9 (Krug, 1943). However, some higher euploid genotypes have been found in the citrus germplasm. The most common euploid variations are triploid and tetraploids (Lee, 1988). Longley (1925) was the first to formally identify a tetraploid wild form: the ‘Hong Kong’ kumquat (Fortunella hindsii Swing.). Triploid ‘Tahiti’ lime (Citrus latifolia Tan.), tetraploid strains of Poncirus trifoliata (L.) Raf., allotetraploid Clausena excavata Burm. F., tetraploid Clausena harmandiana Pierre (Guill) and hexaploid Glycosmis pentaphylla Retz. (Corrêa) are other examples of the few natural polyploids found in the germplasm of Aurantioideae (Ollitrault et al., 2008). Aleza et al (2011) said that tetraploidization by chromosome doubling of nucellar cells are frequent events in apomictic citrus, and are affected by both genotypic and environmental factors. Colder conditions in marginal climatic areas appear to favour the expression of tetraploidization. Tetraploid genotypes arising from chromosome doubling of apomictic citrus are extensively being used as parents in breeding programmes to develop seedless triploid cultivars and have potential direct use as new rootstocks. Kainth and Grosser (2010) showed that The seeds that received higher concentrations and longer durations turned brown completely or had a dead meristematic bud. A similar trend was observed for the number of mutated shoots (tetraploids and mixoploids). Colchicine treatment decreased the growth rate of the affected seedlings. Reversion of the tetraploids and mixoploids into diploids was also observed. The stable pink/red-fleshed tetraploid plants generated should be useful as breeding parents in grapefruit/pummelo improvement programs. Elyazid and Shereif (2014) observed The highest DNA content was recorded at 0.2% for 24 hr. Stomata No. per unit area was decreased by colchicine treatments; moreover stomata length and width were studied. The results indicated that colchicine treatment at 0.1% for 48 hr had the highest tetraploid induction efficiency percentage. However, this hypothesis was not formally demonstrated for a large range of genetic diversity. Today, there is renewed interest in citrus tetraploid lines as parents for seedless triploid breeding programmes (for a bibliographic review see Ollitrault et al., 2008) and for rootstock breeding (Saleh et al., 2008). the present study was designed to Induction of polyploidy in citrus rootstock through Colchicine Treatment.

Material and method

Seeds were extracted in 2014 from the fruits of Citrange, Citromelo and Sour orange located at the Fajr Citrus Research in Sari. The extracted seeds were washed under running water 3–4 times. The seeds were dried, peeled, and sterilized using 10% sodium hypochlorite for 8 min followed by 2–3 rinses with deionized water. Seeds were then placed onto seed germination medium under sterile conditions. The seeds were put on this medium for 12–14 d until they germinated and the hypocotyl had emerged about 5–8 mm out of the cotyledons. At this point,the seeds were undergoing high meristematic activity and were ready to be treated.

Colchicine treatments: The experimental design was a two-way factorial consisting of four colchicine concentrations (0, 0.2, 0.6, 1.2) and three rootstock (Citrange, Citromelo and Sour orange). There were three replications of each treatment. Colchicine stock solution was prepared by dissolving colchicine in a few drops of dimethylsulfoxide (DMSO) followed by the addition of sterile water to bring the final concentration to 1 g/mL. This solution was filter sterilized. Per treatment, seedlings were incubation with Colchicine treatments in pot with final colchicine concentrations of 0, 0.2, 0.6 and 1.2 g/L. Emergent seedlings were analyzed for their ploidy via flow cytometry at a stage when the seedling had at least three fully expanded leaves. The seedlings confirmed to be tetraploid were micrografted onto vigorous rootstocks. The micrografted tetraploids were put under shade for 10–14 d prior to moving them to the greenhouse with set points of 21 to 17 °C day/night temperatures.

Ploidy Analysis: Ploidy was analyzed using a tabletop flow cytometer (Partec GmbH, Münster, Germany). This technique makes it possible to analyze 150–200 genotypes per day. Flow cytometry works by estimating the volume and florescence of isolated nuclei. The ploidy is presented in form of a histogram of integral fluorescence with the peaks depicting the ploidy level of the respective sample. The protocol is a series of steps starting with excision of a 0.2- to 0.3-cm2 piece of fully expanded leaf tissue and placed in a 50-mm plastic petri dish. The sample was prepared for analysis using a High Resolution Staining Kit (Partec GmbH). The tissue is chopped with a sharp razor blade after adding few drops of Nuclei Extraction Buffer. After chopping, 6–7 more drops of Nuclei Extraction Buffer were added and the sample was filtered through a yellow 50-µm filter into a 3.5-mL (55 mm × 12 mm) Sarstedt tube. The staining buffer (DAPI) was added drop by drop through the filter to infiltrate the remaining cells, until half of the tube was filled. Each sample was incubated for 10–15 s at room temperature before running it on the flow cytometer. The sample moves as a very narrow, laminar flowing sample stream through the flow cuvette. When the cells or the particles labeled with fluorescent coloring due to the staining buffer pass through the measuring area one after the other, the individual cells or particles get illuminated by the excitation light and the fluorescent light intensity which is proportional to DNA content is measured and analyzed to depict the respective number of chromosomes and hence the ploidy level of the sample.

Results and discussion

The colchicine treatment induced mixoploiedy in citrange selections. However, the frequency of mixoploied varied among treatments. Some treatments also produced cytogenic chimeric plants having tetraploid and diploid nuclei in varying proportions of cells. Chimeric plants have been recovered in similar in vitro studies conducted by Wu and Mooney (2002).

The most important factors that determine the tetraploid induction efficiency are colchicine concentration and the exposure period for which seeds were exposed to colchicine. Higher colchicine concentrations and longer duration period hampers seedling growth, causes hyperploidy, browning, necrosis in the meristematic tissue and death of the seedling (Sanford, 1983). In this study, all colchicine treatments greatly decreased the growth rate of the treated seedlings in Citrange. Table 1 showed that chlorophyll a, b and total in mixoploied (9.45, 3.27 and 12.72) was higher than diploeid citrange (6.73, 2.33 and 9.07). highest Carotenoid (2.20) was observed in mixoploid to produce with 0.6% colchicine treatments. Average highest of

length, width and Leaf Area Index (3.64, 2.72, 3.59) observed in diploid, however decreased in mixoploid (3.33, 2.07, 2.46) (Table 1).

Table 1. average of morphological characteristics in diploid and mixoploid

morphological characteristics	diploid	mixoploid
chlorophyll a	6.73	9.45
chlorophyll b	2.33	3.27
chlorophyll total	9.07	12.72
Carotenoid	1.43	2.20
Length of leaf	3.64	3.33
Width of leaf	2.72	2.07
Leaf Area Index	3.59	2.46

Figure 1 shows an example of histograms obtained from the ploidy analyzer for a diploid and mixoploid samples.

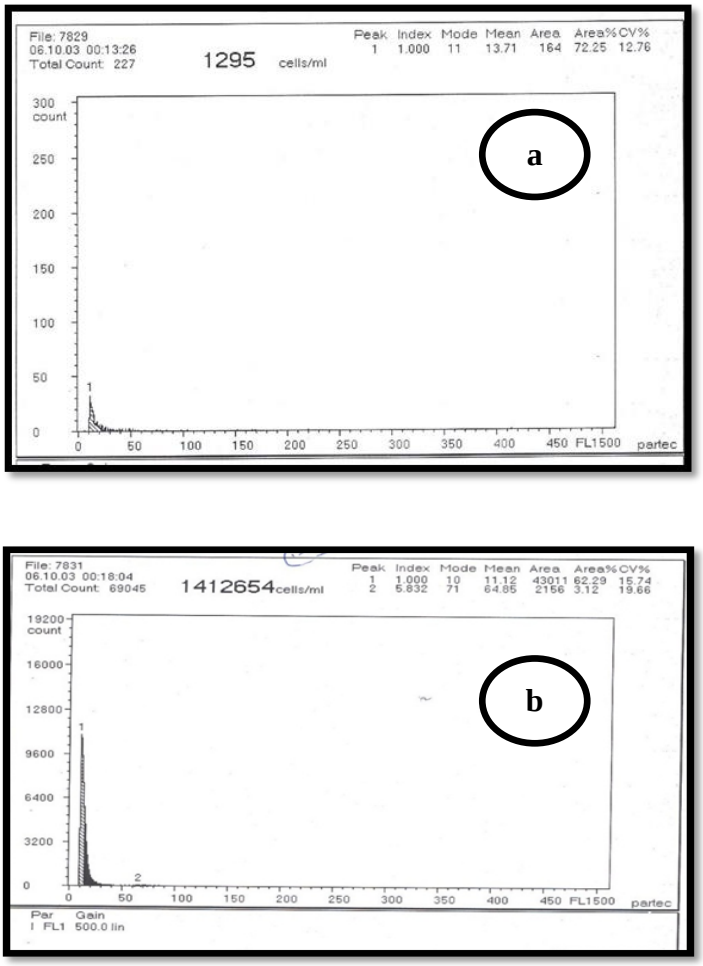

Fig 1. Flow cytometry histogram representing Citrange seedling:
a) diploid profile, b)mixoploid

The most effective concentration at which the mixoploid showed that in 0.6%. This in agreement with Oiyama and Okudai (1968), who previously reported that 0.1% of colchicine was the best concentration for tetraploid induction in shoot tips in citrus.

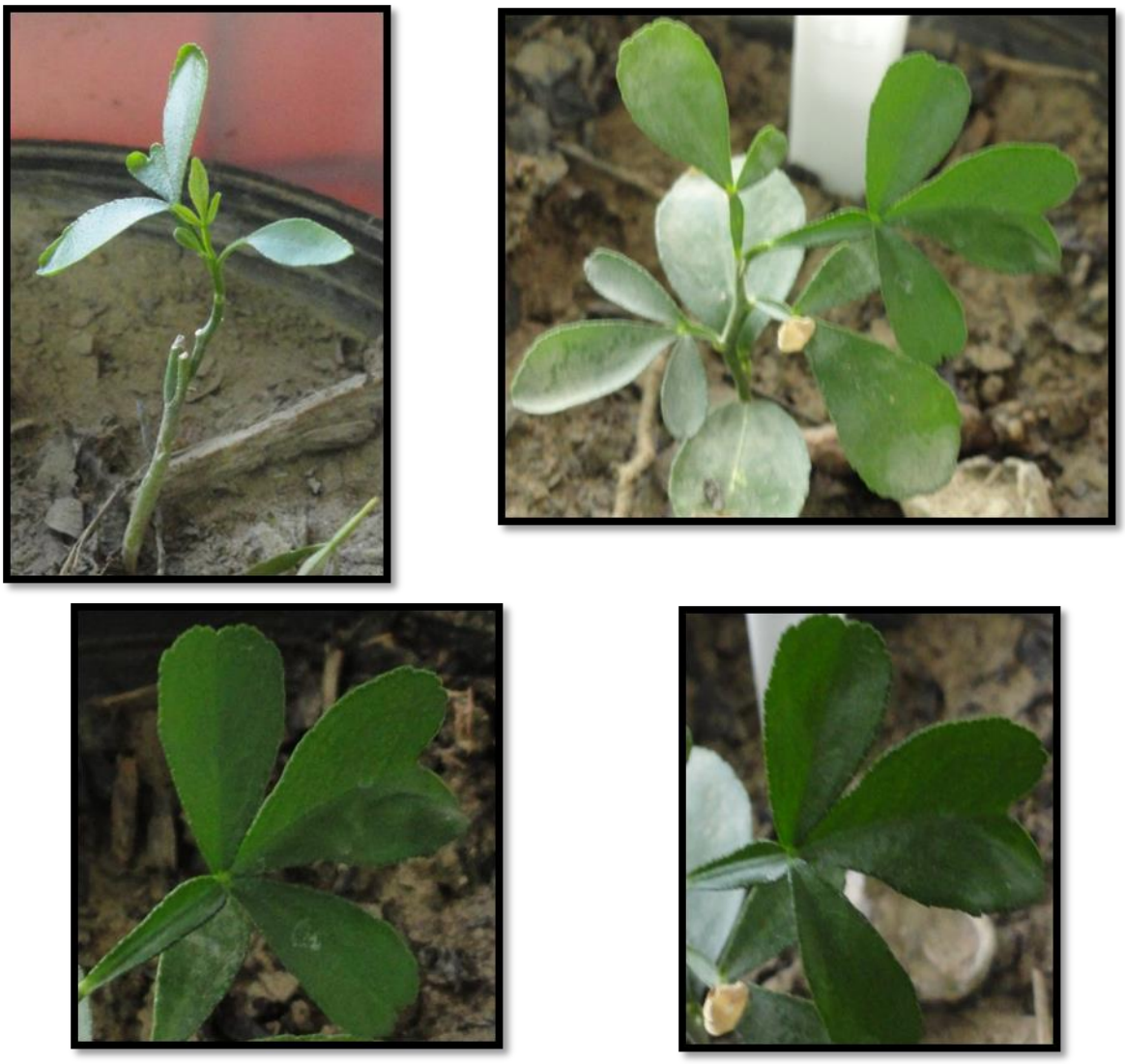

Figure 2. Mixoploeid Citrange produced by Colchicine treatment

Colchicine interferes with cell division and the affected cells divide at a slower rate than unaffected cells. The stable mixoploid plants confirmed by flow cytometry were micrografted onto vigorous rootstocks for further growth and were moved to the greenhouse (Figure 2).
Recent work on citrus mixoploid rootstocks suggests that they could be more tolerant to salt and water stress than their parental diploids (Saleh et al., 2008), probably due to modified abscisic acid constitutive synthesis (Allario et al., 2009). Furthermore, tetraploid rootstocks generally reduce canopy size, which is a desirable trait in modern orchards (Barrett and Hutchison, 1978; Lee, 1988). The selection of doubled-diploid lines from seedlings of traditional rootstocks could be an interesting way to improve tolerance to abiotic stress without modifying allelic constitution, with high probabilities of transmitting traits related to

disease resistance. Agronomical trials are currently underway to evaluate the agronomic performance of some of these mixoploid rootstocks under abiotic stress conditions.

Conclusion

The mixoploid from monoembryonic Citrange selections selected for breeding programs. In this study, a method to induce mixoploidy in Citrange seedlings by treating pre-germinated seeds with colchicine at various concentrations and exposure periods is described. Stable mixoploid were successfully and were confirmed by flow cytometry. This method facilitates treatment of large number of seeds at the same time perhaps reducing safety risks when working with colchicine since with shoot tip grafting far more handling is necessary as individual shoot tips that have to be treated separately.

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