

Full Length Research Paper

Induction and flow cytometry identification of mixoploidy through colchicine treatment of *Trigonella foenum-graecum* L.

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Polyploidy plays an important role in plant evolution and constitutes an important mechanism of diversification and genetic variations creation. The objective of this study was to produce a polyploid cultivar of *Trigonella foenum-graecum* L. ($2n=16$) and to evaluate its cytological potentialities. Polyploidy induction was carried out by using a 0.05% colchicine solution. Ploidy level was determined by an analysis of flow cytometry, which is suitable, quick and easy for the identification of ploidy level. Quantification of DNA by spectrophotometric methods showed that treated plants presented higher contents of cellular DNA than diploid plants, and this quantification was checked by fluorescence method and comparison of the DNA profiles showed a more significant thickness of the DNA band in the extract from the treated plants than the diploid plants. In addition, the tested plants were classed as diploid and mixoploid by flow cytometry. The cytological characteristics such as the stomata size and pollen grain diameter of the mixoploid were significantly larger than those of the diploid. Polyploidy induction is an effective method to increase plant performance.

Key words: Fenugreek, colchicine, flow cytometry, polyploidy, mixoploidy.

INTRODUCTION

Polyploidy is a prominent process and has been significant in the evolutionary history of plants (Adams and Wendel, 2005). It has been estimated that up to 70% of land plants and 95% of ferns have some polyploidy in their evolutionary history (Otto and Whitton, 2000). Thus, the polyploidy is an important evolutionary factor for genesis of new plant species as well as creation of cultivated plant species. Due to this fact, it is

possible that polyploidy would allow developing such plant forms, which would contain higher amounts of biologically active compounds, compared to their initial diploid forms (Audrius et al., 2010).

The polyploidisation can result in larger and darker leaves, delays in flowering, larger inflorescences, prolongations of the flowering period, apomixes, larger fruits, greater secondary metabolite production and yield (Predieri, 2001; Urwin et al., 2007) and overcoming barriers to hybridization, no seed or fewer seed, enhancing pest resistance and stress tolerance (Predieri, 2001). Additionally, interspecific hybrid fertility can be

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restored by polyploidisation (Nimura et al., 2006).

Accordingly, the induction of polyploids is a useful tool in plant breeding. There are different methods for induction of polyploidy in plants such as seed treatment (Quan et al., 2004), flower bud (Wu et al., 2007), apical meristem (Saharkhiz, 2007) and root (Taira et al., 1991). Other methods of inducing polyploids include treatments with mitotic inhibiting chemicals such as colchicine (Derman, 1940), oryzalin (Alberts et al., 1994), trifluralin (Eeckhaut et al., 2004), amiprofos- methyl (Hansen et al., 1998) and N_2O (Kitamura et al., 2009).

Furthermore, colchicine, a compound that effectively arrests mitosis at the anaphase stage, is widely used to induce polyploidy in plants. Traditionally, tetraploid parents have been obtained by treating newly emerged diploid seedlings with colchicine (Suying et al., 1995). However, this treatment produces a limited number of tetraploids and mostly chimeric seedlings that possess vines of mixed ploidy (Jaskani et al., 2004). The production of polyploid regenerants from tissue culture colchipooids has been reported for many plant species (Veilleux and Johnson, 1998).

In vivo treatment of shoots, smaller axillary or sub-axillary meristems, seeds or seedlings with colchicine is traditionally employed for generating polyploids. However, low efficiency of polyploidy plant production and a high frequency of chimeras are often associated with this method. With the development of adventitious organ regeneration techniques, *in vitro* induction of polyploidy has become the main method in many plant species, including *Pyrus pyrifolia* (Kadota and Niimi, 2002), *Musa acuminata* (Van Duren et al., 1996), *Morus alba* (Chakraborti et al., 1998), *Bixa orellana* (Carvalho et al., 2005), etc.

Then, the basic method used for ploidy estimation was flow cytometry (FCM), which is a quick, reliable and widely used technique for identifying ploidy in plants (Urwin et al., 2007; Yang et al., 2006). The reliability of flow cytometry has been proved in cytological analysis (Gu et al., 2005). However, traits such as stomatal length and width, pollen grain diameter and spikelet length can be useful either in the identification of putative polyploids in preliminary scanning of obtaining material or as additional evidence for their induction.

Trigonella foenum-graecum is a diploid plant with $2n=16$ (Ahmad et al., 2000) not exhibiting aneuploid forms (Petropoulos, 2002). Polyploidy induction of this plant, to evaluate its morphological and chemical potentialities, was carried out by application of 0.5% colchicine solution on the shoot meristem (Marzougui et al., 2009). A comparative study between diploids and autotetraploids fenugreek populations was conducted to determine polyploidy effects on morphology and minerals contents (Marzougui et al., 2009), vitamins and protein reserves (Marzougui et al., 2010a), the physiological behavior (Marzougui et al., 2010b), salt stress tolerance

(Marzougui et al., 2010c) and molecular profile (Marzougui et al., 2010a).

The aim of the present study was to induce *in vivo*, the polyploidy by soaking fenugreek seeds in a solution of colchicine, identify cytological traits such as stomata and guard cell size, pollen grain diameter and estimation of nuclear DNA content of nuclei isolated from fresh leaf plants using spectrophotometric methods, fluorescence methods and flow cytometry.

MATERIALS AND METHODS

Plant source

Seeds of fenugreek (*Trigonella foenum-graecum* L.) were purchased from a supermarket. The seeds were germinated and the plants were grown under standard greenhouse condition in the experimental station of the Higher Institute of Agronomy of Chott Meriem, University of Sousse (Latitude 35°56'45.6"N, Longitude 10°33'57.6"E, coastal region, East of Tunisia, with a sub humid climate). Seeds were collected from these seed-grown plants and used in the present study.

In vivo colchicine treatment

Fenugreek seeds were surface sterilized by immersing in 0.525 g.L⁻¹ sodium hypochlorite for 15 min. Then, they were rinsed four times with deionised water, carefully blotted with a paper towel and immediately sown in a Petri dish. After 24 h, the seeds were divided into two groups: (i) the first group was left intact and continued germination for obtaining diploid plants; (ii) the second group was treated by colchicine to obtain mixoploid plants. Diploid fenugreek seeds were treated with colchicine solution at 0.05% for 4 h, rinsed three times with abundantly distilled water, and sown in a greenhouse under natural conditions during February 2011. The plants were irrigated daily with tap water.

Ploidy analysis

DNA contents

Flow cytometry analysis of polyploidy level: For flow cytometry, 100 mg fresh leaves was homogenized in 2 mL 15 mM Tris-HCl, pH 7.5; 80 mM KCl, 20 mM NaCl, 20 mM Na₂EDTA; 2% (v/v) β -mercaptoethanol and 0.05% (v/v) Triton X-100. The homogenate was passed through a nylon filter (pore size 300 mesh) and centrifuged at 1500 $\times g$ for 5 min at 4°C. 200 μ L (3000U mL⁻¹) RNAase, and 50 μ g.mL⁻¹ propidium iodide were added to the pellet and incubated for 30 min. Then, samples were analyzed by a flow cytometry. Leaves from a diploid plant were used as the control (Liu et al., 2011).

Spectrophotometric methods: DNA concentrations in the leaves of fenugreek plants were determined by spectrophotometer at 260 nm. A unit of absorbance (1DO) at 260 nm corresponds to a concentration of 50 μ g.mL⁻¹ of double stranded DNA or 40 μ g.mL⁻¹ RNA or single stranded DNA (Griffiths et al., 2002). To evaluate the purity of DNA in solution, it is necessary to read at 280 nm and to determine the ratio DO_{260}/DO_{280} , which must be range between

1.8 and 2.

Fluorescence method: Fluorescence intensity emitted by EtBr molecules intercalated between the DNA bases is proportional to the quantity of this last. Thus, the DNA can be quantified after electrophoresis on freezing agar (0.8%) and coloring with ethidium bromide, by comparison DNA known concentration fluorescence (marker of size).

Size and density of stomata: Estimate of suitability of the stomata size and density can be used as criteria for the determination of ploidy level in fenugreek. A fragment of limb was taken in the broadest zone of a leaf, for each sample with the same distance from a principal vein. Leaves underwent a treatment with 70% alcohol then 90% lactic acid until a transparency of tissue was obtained allowing a microscopic observation and a measurement using an ocular micrometer (Pacheco et al., 2008). 100 stomata were measured on leaves taken randomly from untreated and colchicine treated plants.

Pollen grains: Floral buttons were taken from colchicine treated at budding. The pollen morphological descriptions of untreated plants (diploid plants) were published by Haouala and Beji (2008) and these results were taken as reference. Pollen grains, for light optical microscopy (LO microscopy) examination, were prepared following the standard procedure of Erdtman (1960). Seven pollen morphological characters of polyploidy plants were measured with a Phywe optical microscope at magnification of 1000x, that is polar diameter (P), equatorial diameter (E), colpus length (Lc), porus length (Lp), mesocolpium (M), exine thickness (e) and P/E ratio. 200 measurements were made for each character. For SEM examination, acetolysed pollen grains were coated with gold. The microphotographs were obtained with PHILIPS XL30 SEM at magnification of 2800 to 13000x in the Faculty of Sciences of Sfax, University of Sfax – Tunisia. The pollen morphological descriptions follow the terminology of Reitsma (1970) and Punt et al. (1994).

Statistical analysis

All data were reported as mean \pm standard deviation (S.D.) of three replicates and analyzed using the program PASW Statistics 18. Differences between the means were established using one-way analysis of variance (ANOVA) followed by Duncan's test.

RESULTS

Characters of DNA content, stomata size, and pollen grains for the untreated and treated plants with colchicine were compared to confirm the identity of acquired polyploid plants.

DNA contents

Flow cytometry analysis of polyploidy level

Reconfirmation of ploidy level of colchicine-treated and untreated *T. foenum-graecum* was made by flow cytometric analysis. These plants were classified into exact diploid levels (2n) and chimera polyploids (2n+4n+6n+8n). The histogram results presented in

Figure 1 are a representative fluorescence profile for nuclei from plants of diploid in comparison to mixoploids from colchicine-treated plants. The diploid plants containing 2C DNA showed a peak at the position of channel 40 of relative fluorescent intensity, whereas chimeric plants with both 2C, 4C, 6C and 8C nuclei showed a peak at the channel 40, 80, 120 and 160 respectively.

Spectrophotometric methods

Quantification of DNA showed that the plants treated with colchicine presented higher contents of cellular DNA than the untreated ones. 371.12 $\mu\text{g/mL}$ was recorded for diploids and 627.86 $\mu\text{g/mL}$ for treating plants by colchicine (Table 1). The ratio $\text{DO}_{(260)}/\text{DO}_{(280)}$ was 1.82 and 2.01 for diploids and mixoploids plants, respectively, which indicated that the prepared DNA solutions were pure.

Fluorescence method

This quantification was checked by fluorescence method and comparison of the DNA profiles of diploid plants with those of mixoploid plants with colchicine showed a more significant thickness of the DNA band in the extract from mixoploids plants (Figure 2).

Size and density of stomata

100 stomata were measured on the lower epidermis of leaves taken randomly for each type of plants. Results show that the stomata size of treated colchicine plants was larger than that of diploid plants (Figure 3). On the other hand, the stomatal density in mixoploid was significantly lower than for the diploid (Figure 3). The stomata dimensions were 13.20/10.21 μm for diploid plants and 14.83/12.25 μm for mixoploid ones (Table 1).

Pollen grains

Pollen grains of fenugreek were 3-zonocolporate, radially symmetrical, and isopolar type. Significant differences in polar diameter, equatorial diameter, colpus length, and porus length were found between diploid and mixoploid (colchicine treated) plants (Table 2). Colpus membrane is covered by granules, pori is large, circular to slightly lologate, protruding in mesocolpium. Exine thickness was about 1.90 μm , ornamentation was suprareticulate (Table 2 and Figure 4D). MES showed a deformation of pollen grains of treated plants with colchicine (Figure 4M). In addition, dimension pollen grains of treated plants were weaker; an index in favor of an induced mixoploidy

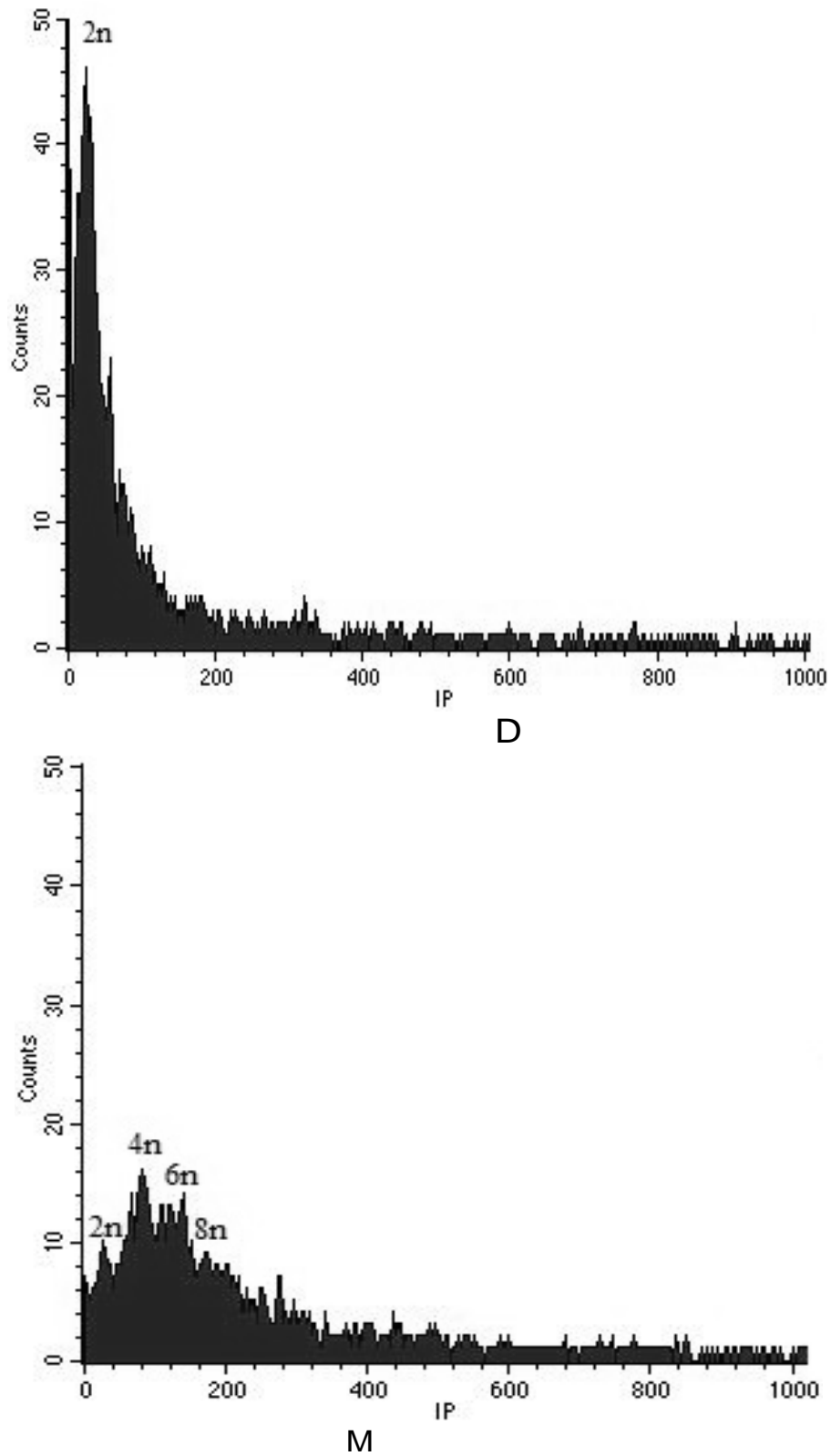


Figure 1. Representative flow cytometer histograms of the relative fluorescence intensity of nuclei isolated from fenugreek leaves. (D) Diploid profile showing diploid (2n) peak. (M) Mixoploid profile showing diploid (2n), tetraploid (4n), hexaploid (6n) and octaploid (8n) peaks

Table 1. DNA contents and stomata size of *T. foenum-graecum* diploid and mixoploid plants obtained by immersion of germinated seeds in 0.05% colchicine solution.

| Parameter | Diploid | Mixoploid |
|--|----------------------|-------------------------------|
| DNA content ($\mu\text{g/ml}$) | 371.117 ^a | 627.855 ^b |
| DO ₍₂₆₀₎ /DO ₍₂₈₀₎ ratio | 1.82 | 2.01 |
| Stomata size (μm) | | |
| Length | 13.20 \pm 1.01 | 14.83 \pm 0.94 ^a |
| Width | 10.21 \pm 0.70 | 12.25 \pm 0.73 ^a |

All analyses are the mean of triplicate measurements \pm standard deviation. Means followed by at least one same letter are not significantly different at $P < 0.05$.

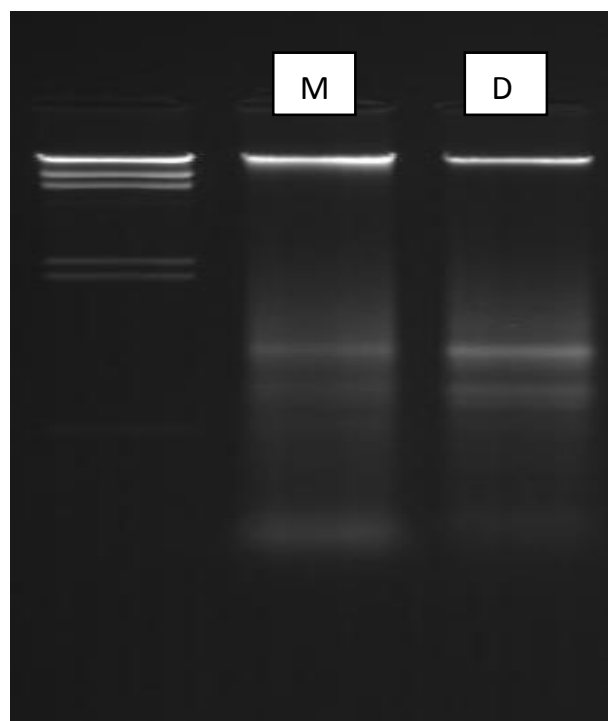


Figure 2. Migration profile of DNA extracted from leaves of diploid (D) and mixoploid (M) plants obtained by immersion in 0.05% colchicine solution of *T. foenum-graecum* germinated seeds.

by colchicine.

DISCUSSION

Colchicine, acts as antimitotic agent and, as such, has been widely used to induce polyploidy in plant breeding (Nigel et al., 2007). Beji (1991) considered that the

purpose of a colchicine treatment is to obtain a large number of surviving plants with a high percentage of polyploids. Seeds of *T. foenum-graecum* were used in this study as the initial targets of colchicine application, and this follows the techniques previously applied in many other plant species, such as *Platanus acerifolia* (Liu et al., 2007) and *Rosa chinensis minima* (Zlesak et al., 2005).

Table 2. Dimension of pollen-morphological characters of diploid (Haouala and Beji, 2008) and mixoploid plants of *Trigonella foenum-graecum*.

| Parameter | Diploid | | Mixoploid | |
|-----------|------------------|------|------------------|------|
| P (µm) | 30.2 (36.8) | 41.3 | 26.2 (33.2) | 40.2 |
| E (µm) | 16.5 (19.4) | 22.7 | 14.5 (17.5) | 20.5 |
| Lc (µm) | 22.8 (26.3) | 29.4 | 19.6 (22.9) | 26.3 |
| Lp (µm) | 4.7 (6.8) | 7.9 | 3.9 (5.4) | 6.9 |
| M (µm) | 15.3 (17.3) | 21.3 | 13.9 (17.3) | 20.6 |
| e (µm) | 1.43 – 2.57 | | 1.45 – 2.59 | |
| P/E | 1.83 (1.90) 1.82 | | 1.81 (1.89) 1.96 | |

P, Polar diameter; E, equatorial diameter; Lc, colpus length; Lp, porus length; M, mesocolpium; e, exine thickness.

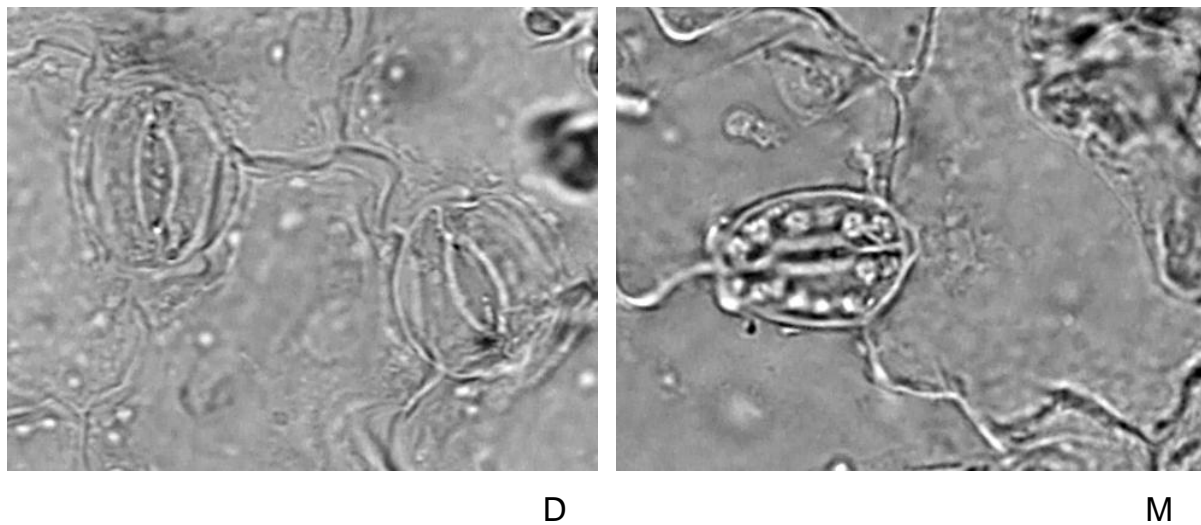


Figure 3. Stomata in abaxial leaf epidermis under optical microscope of diploid (D) and mixoploid (M) plants of fenugreek (x100).

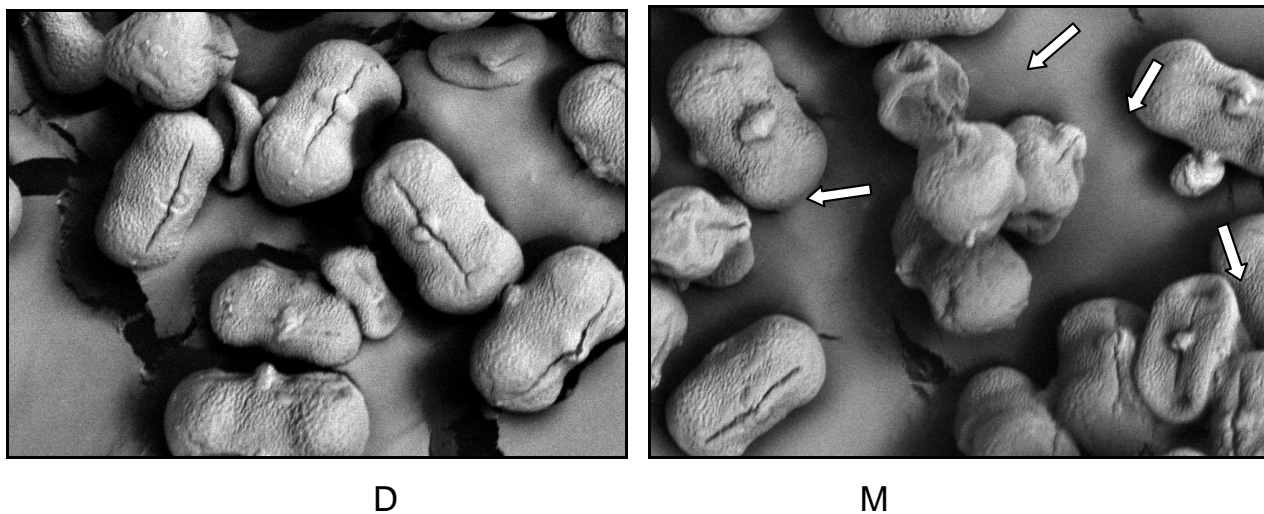


Figure 4. Pollen grains of diploid (D) and mixoploid (M) plants of fenugreek (x750).

The first visible effect of colchicine was the delayed growth of treated explants. The slow growth may be due to a physiological disturbance caused by colchicine, resulting in a reduced rate of cell division (Swanson, 1957).

There are various approaches for the identification of polyploid plants (Yang et al., 2006) such as morphological, cytological, chemical and physiological parameters. Earlier studies of induced and spontaneous polyploid plants have frequently observed that the size and number of stomata and the number of chloroplasts within the guard cells change significantly in the event of chromosome doubling, compared with the diploid state (Nigel et al., 2007). In many of the plant species, there are correlation between ploidy level and cytogenetic characteristics such as chloroplast number in guard cells, size of stomata cells, stomata density and pollen grain diameter. Thus in pepper, stomata density and especially the number of chloroplast in guard cells seemed to be reliable for the estimation of ploidy level (Abak et al., 1998).

In our study, traits such as the stomata length and width and the pollen grain diameter were evaluated to determine whether they could be used to identify or provide supporting evidence for putative polyploids in a preliminary scanning. Therefore, mixoploid plants had significantly greater dimensions for stomata and pollen grain compared to controls. Stomata size and changes in plant morphology were found useful indicators in the primary screening for new ploidy level an M1-generation of *Viola x Wittrockiana* Gams (Ajalín et al., 2002). Furthermore, in using colchicines, the pollen size offers a quick and fairly accurate way to locate polyploid plants (Blakslee and Avery, 1937). Majority of pollen grain in tetraploids were bigger than those in diploids; comparable results have been reported for *Helianthus annuus* L. Var. Morden (Strivastava and Strivastava, 2002), and *Carum carvi* L. (Dijkstra and Speckmann, 1980). The utility of stomata size in distinguishing plants with different ploidy has been demonstrated in other plant types (Campos et al., 2009), that increase in stomata size in tetraploid plants as compared to diploid plants supported by the findings of several researcher such as Gao et al. (2002) in *Scutellaria baicalensis* and Thao et al. (2003) in ornamental *Alocasia* and so comparable results that stomata diameter, and guard cell length increase with higher ploidy level, has been reported by Yetisir and Sari (2003) in *Cucumis melo* L.

For fenugreek, the autotetraploid cultivar has a larger leaf area, higher stem height, more seed number by pod and branch number and has larger productivity compared to the diploids (Marzougui et al., 2011). Further, its leaves are richer in potassium, sodium, calcium and phosphorus (Marzougui et al., 2009). Then, the autotetraploids presented mean heterozygosity values higher than those of the diploids for 50% of the studied primers.

They presented higher vitamins B6 and B9 and higher albumin, prolamin and glutelin seed contents compared to the diploids resulting from the treated plants and the diploid parents (Marzougui et al., 2010a). According to the study of Marzougui et al. (2010b), polyploid plants are distinguished from diploids by an improved photosynthetic capacity related to the increase of the photosynthetic enzymes and pigments quantities. Total chlorophylls content, photosynthesis rate, transpiration rate and stomatal conductance were measured for each genotype of *Trigonella foenum graecum*. Results show highly significant variations ($p < 0.01$) between the different genotypes for the studied physiological characters and salt stress tolerance (Marzougui et al., 2010c).

The polyploidy status confirmation by flow cytometry proved to be a rapid, efficient and economical way of identifying polyploid plants; indeed ploidy determination by examining of stomatal size and pollen grain diameter or spikelet length, are relatively laborious methods, mainly because of the high number of required replications. Flow cytometry is now routinely used for ploidy analysis and is regarded as the most accurate tool for ploidy determination (Loureiro et al., 2005). Advantages of using flow cytometry to estimate the ploidy level include the ease of plant sample preparation, requiring just a few milligrams of tissue, and multiple samples can be analyzed in one working day (Doležel, 1997). In the present study, mixoploid plants were confirmed by flow cytometry, and this indicates that the use of morphological markers as a preliminary screening method had been successful.

Conclusion

The results reveal that morphological changes in treated plants were not reliable and accurate indicators for identification of polyploid plants but selection on pollen size, stomata and guard cell size, stomata density and flow cytometry, proved to be an effective way to identify the mixoploid plant and these methods are suitable. Although there was significant differences ($P < 0.05$) in the stomata length and diameter, stomata density and pollen diameter between diploids and mixoploids, but for confirming the ploidy level of mixoploid plants, flow cytometric analysis was being required. Therefore, estimation of morphological changes (abnormality of grain pollen), pollen diameter and stomata counting, is an effective method in primary screening of mixoploid *T. foenum-graecum* plants in a polyploidisation breeding program and it is the recommended flow cytometry to be used for accurate identification of ploidy level in mixoploid plants of fenugreek.

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