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Full Length Research Paper

Polyploidy induction of Tunisian *Trigonella foenum-greaum* L. populations

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Polyploidy plays an important role in plant evolution and constitutes an important mechanism of diversification and creation of genetic variability. Artificial polyploidy can be induced using the colchicine. The aim of this study was to determine the *Trigonella foenum-greacum* (2n= 16) population which resists better to colchicine treatment and which has the highest rate of polyploid formation among 38 populations collected from different Tunisian regions. The comparative study between treated and control plants showed significant increases ($p < 0.05$) for the parameters survival rate, rate of malformed leaves appearance, stem height and seeds number by pod. The 38 treated populations showed significant variations ($p < 0.05$) between them for the parameters survival rate, branches number, stem height and seeds number by pod. Results also showed that treatment of the shoot meristem by colchicine allowed to obtain diploid (2n= 16) and mixoploid plants having mixoploid branches (4n = 32 and 2n= 16) and branches entirely tetraploid (4n = 32). The obtained rate of mixoploidy was 65.79% of treated plants. Among the 38 analyzed fenugreek populations, the population 15 of Menzel Temime presented the highest rate of mixoploids formation and survival rate to colchicine treatment.

Key words: Fenugreek, induction of chromosome doubling, mixoploidy, colchicine, Tunisia.

INTRODUCTION

Plants of the genus *Trigonella* and particularly of the cultivated species *Trigonella foenum-graecum* L. (fenugreek) were known and used for different purposes in ancient times, especially in Greece and Egypt (Petropoulos, 2002). Actually, fenugreek is widely cultivated in Asia, in Europe, America and North Africa. In Tunisia, it is especially cultivated in the regions of the North (Marzougui et al., 2007). Fenugreek is a medicinal plant which is rich in proteins, vitamins and amino acids (Hidvegi et al., 1984). Its seeds and its leaves are used as forage.

T. foenum-graecum is a diploid plant with 2n= 16 (Ahmad et al., 2000) not exhibiting aneuploid forms (Petropoulos, 2002). The induced polyploidy can increase variability within species. Indeed, the polyploidy plays a very important role in evolution and constitutes an important mechanism of diversification and creation of genetic variability. Although, the first polyploid was discovered over a century ago, the genetic and evolutionary implications of polyploidy are still being elucidated (Bennett, 2004; Soltis et al., 2003) and artificial polyploids are still used as a valuable tool in plant breeding programs (Ranney, 2006). The importance of polyploidy in plants breeding arouses a considerable interest to induced polyploids development when the mitotic inhibitors were discovered for the first time in the thirties. At this time, the artificial polyploidy induction was discovered by using the colchicine which inhibits the formation of spindle fibers and temporarily arrests mitosis at the anaphase stage (Blakeslee and Avery, 1937). At this point, the chromosomes have replicated, but cell division has not yet taken place resulting in polyploid cells (Ranney, 2006). In later year, a number of other mitotic inhibitors including oryzalin, trifluralin, amiprophos-methyl and N_2O gas had been identified and used as doubling agents (Bouvier et al., 1994; van Tuyl et al., 1992; Taylor et al., 1976), but colchicine was preferred as polyploidi-

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Table 1. Location and main ecological traits for the 38 Tunisian *Trigonella foenum-graecum* populations.

Bioclimatic stage and alternative	Locality	Population code	Edaphic substrate	Altitude (m)	Latitude (N)	Longitude (E)	Rainfall (mm/year)
Upper-semi arid[s]	Menzel Temime	2, 3, 4, 8, 9, 15, 17, 19, 26, 36	Argillaceous	22	36°47’	10°59’	390-630
	Bizerte	7	Limestone	4	37°16’	9°52’	300-800
	Mater	11, 12, 16, 29, 35		37	37°02’	9°41’	300-800
	Mhamdia	14, 32, 33	Clay/Limestone	64	36°40’	10°09’	275-515
	Sidi Hamed	18	Carbonated	143	36°29’	8°46’	450-1500
	Ben Bechir	20	Limestone	143	36°29’	8°46’	450-1500
	Nefza	30, 37	Marl/Limestone	213	36°43’	9°11’	350-1000
Upper-semi arid [m]	Sidi Khiar	1, 25	Marl/Clay	650	36°11’	8°43’	400-700
	Nebeur	5		524	36°17’	8°44’	400-700
	Elkef	6, 22		654	36°10’	8°42’	400-700
	Tel Elgozlan	21, 31		657	36°11’	8°43’	400-700
	Borj Berrzig	27		648	36°11’	8°43’	400-700
Sub humid[m]	Beja	10, 13, 28, 34, 38	Sandy	213	36°43’	9°11’	350-1000
Lower arid [m]	Menzel Habib	23, 24	Sandy loam	84	34°27’	9°44’	184,4

[s] The soft alternative; [m] the moderate alternative.

zation agent and was by far the substance with which Beji (1991) found the best results in the experimental induction of autopolyploids in the genus *Hedysarum*. Madon et al. (2005) obtained better results in their polyploidization tests of oil palm (*Elaeis guineensis* Jacq.) using colchicine. This substance allowed them to obtain nine tetraploids, two triploids and some mixoploids whereas treatment with oryzalin produced only four triploids and several mixoploids. Wei et al. (2007) also obtained satisfactory results using colchicine as a mitotic inhibitor for *Lespedeza Formosa* arriving at a polyploidy rate of 44.4% of treated plants.

To induce polyploidy in *T. foenum-graecum*, our team found that treatment of the shoot meristem by colchicine solution 0.5% provides a better survival percentage of polyploid plants than treating germinated seeds (Marzougui et al., 2009a). A comparative study between diploid and autotetraploid fenugreek populations was conducted to determine polyploidy effects on morphology and minerals contents (Marzougui et al., 2009a), 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). In this study, we were interested to determine the *T. foenum-greacum* population which resists better to colchicine treatment and which has the highest rate of polyploid formation among 38 populations collected from different Tunisian regions.

MATERIALS AND METHODS

Plant materials

Thirty eight (38) fenugreek local populations were collected from different Tunisian regions (Table 1).

Culture conditions

The seeds were sterilized with a 10% H_2O_2 solution for 20 min and then placed in germination on moistened filter paper in a conditioned room at 25°C, 70% humidity and photoperiod of 16 h. When the seedlings have reached a length of at least 2 cm, they were transplanted into pots of 20 cm in diameter in the same room. The pots were filled with sandy-loam calcareous soil, characteristic of the region of Elfejéh in south-east of Tunisia. The establishment of the pots was conducted in randomized block with four replications.

Polyploidy induction

For each population, maintained soaked cotton with aqueous solution of 0.5% colchicine was applied between the two cotyledons for three days to reach the greatest number of cells in prophase. This treatment concerned three seedlings per population and the total number of the treated plants belonging to the 38 considered populations reached 114. Ploidy levels of the treated plants were

Table 2. Examined parameters.

Abbreviation	Definition
S (%)	Survival rate to colchicine treatment
MA (%)	Rate of malformed leaves appearance at the flowering stage*
B	Branches number
H (cm)	Stem height at the flowering stage
D (cm)	Stem diameter at the flowering stage
A (cm^2)	Leaflet area
PN	Pods number by stem
SN	Seeds number by pod

*The malformations concern the number, color and size of leaflets.

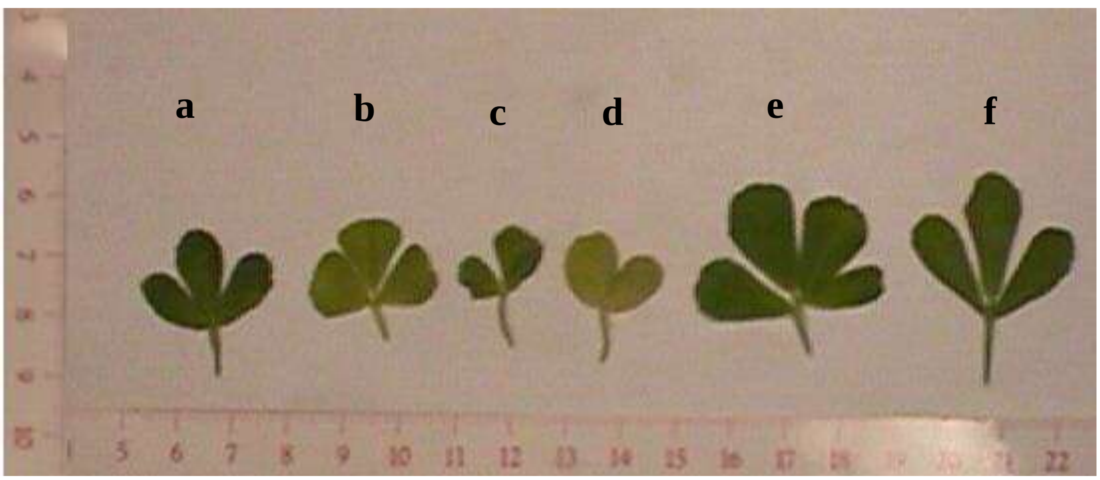

Figure 1. Changes in size, color and number of leaflets of colchicine treated *Trigonella foenum-graecum*. a: leaf of a control plant, b, c, d, e and f : leaves of treated plants.

verified by chromosome count made by the method described by Ferchichi (1997) for the observation of meiosis in the anthers of young flower buds. For each population, the counting was concerned at least twenty cells and was verified at various meiosis stages (mainly prophase 1, metaphase 1 and anaphase 1). Treated and control diploid plants of each population were compared on the base of the parameters presented in Table 2.

Statistical analysis

Variations of the different studied parameters between treated and control fenugreek plants and between the 38 collected populations were subjected to one-way variance analysis using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Variations of the mixoploidy rate between the treated populations were also subjected to the same test.

RESULTS

Effects of colchicine treatment

The colchicine treatment allowed a polyploidisation which entailed several visible changes. The early effects of colchicine treatment were manifested by hypocotyl swelling, changes in dimensions, thickness, stiffness and number of leaflets per leaf, in addition to a slow plasto-chron of treated plants compared with controls. Some plants did not survive the treatment, others presented deep morphological malformations (no branches, many leaves with two leaflets and four leaflets (Figure 1), leaflets with wrinkled and thick limbs), followed by a slow growth and flowering lack, while other plants have survived until flowering stage.

The comparative study showed increases in measured values of all analyzed parameters in treated populations compared with control populations (Figure 2). These variations are significant for the parameters survival rate ($p < 0.05$), rate of malformed leaves appearance ($p < 0.05$), stem height ($p < 0.05$) and seeds number by pod ($p < 0.05$). The not significant variations were observed for the parameters branches number (p= 0.97), stem diameter (p= 0.92), leaflet area (p= 0.99) and pods number by stem (p= 0.97).

The 38 c olchicine treated populations showed

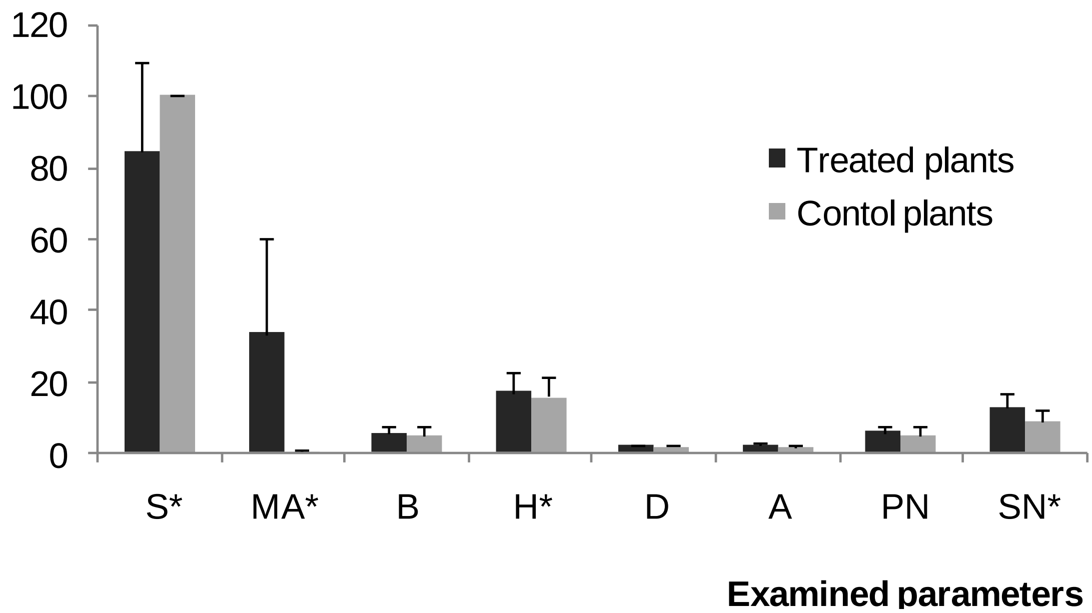

Figure 2. Variations between treated and control populations groups of *Trigonella foenum-graecum*. *Significant variation (ANOVA). Standard deviation corresponds to 38 populations for each group.

significant variations between them (Table 3) for the parameters survival rate ($p < 0.05$), branches number ($p < 0.05$), stem height ($p < 0.05$) and seeds number by pod ($p < 0.05$). The not significant variations were observed for the parameters rate of malformed leaves appearance ($p= 0.06$), stem diameter ($p= 0.96$), leaflet area ($p= 0.41$) and pods number by stem ($p= 0.53$). The maxima rates of survival (100%) and appearance of malformations (100%) were observed at population 15 of Menzel Temime (Table 3). In this population, 22% of the treated plants experienced deep morphological deformations, slow growth and flowering lack, whereas 78% of the plants of this population survived until the flowering stage.

Meiotic configurations

For all populations, young floral buds of the treated plants were collected from each branch. The chromosomes counting of these buds revealed that several treated plants were mixoploids. In fact, they had certain branches with two levels of ploidy: a tetraploid level (4n = 32; Figure 3a, c) and a diploid level (2n = 16; Figure 3b, d), and had other branches with only one tetraploid level (4n = 32). The floral buds of all branches of other treated plants were diploids (2n = 16) like those of the control plants.

The results showed also, after repeated treatments at three different plants within the same population, that the rate of mixoploidy varied significantly ($p < 0.05$) between the different considered fenugreek populations (Figure 4). A total of 75 mixoploid plants were obtained among the 114 treated ones belonging to the 38 studied populations, giving a mixoploidy rate of 65.79% of treated plants. The populations 9, 15, 26 and 36 of Menzel Temime, 14 of Mhamdia, 21 and 31 of Tel Elgozlan, 30 of Nefza and 35 of Mater showed the highest rate of mixoploidy formation (100%).

DISCUSSION

The thirty eight (38) *T. foenum-graecum* collected populations were treated with colchicine in an objective of improvement by the polyploidy induction. This technique has been used in breeding programs to produce new varieties (Koutoulis et al., 2005) and has contributed to the improvement of many plants such as red clover (Berthaut, 1965), kale (Chevre et al., 1989), sulla (Beji, 1991) and rice (Bouharmont, 1963).

Beji (1991) considered that the purpose of a colchicine treatment is to obtain a large number of surviving plants with a high percentage of polyploïds. Our results showed that treatment of the fenugreek shoot meristem by colchicine allowed to generate mixoploid plants having mixoploid branches (4n = 32 and 2n= 16) and branches entirely tetraploid (4n = 32) and that among the 38 analyzed fenugreek populations, the population 15 of Menzel Temime presented the highest survival rate (100%) and rate of mixoploids formation (100%). The difficulties met by the doubling of chromosome number by colchicine treatment are situated in the choice of timing and duration of treatment (Baudoin et al., 2002). In an apical meristem, the most active cells and most easily doubled by colchicine are located at certain distance from

Table 3. Variations between the 38 treated populations of *Trigonella foenum-graecum* for the different parameters.

Pop	S*	MA	B*	H*	D	A	PN	SN*
1	99.3±1.2	33.3±0	5.3±1.2	21±1	0.2±0	1.7±0.2	3.7±1.5	17.3±2.1
2	30.1±4.5	33.3±0	4±1	24.7±1.5	0.3±0.1	1.9±0.3	5±3	16.3±1.5
3	37.8±6.3	0	3.3±0.6	17.7±2.5	0.3±0.1	1.7±0.3	5.3±2.5	5.3±2.1
4	92.3±7.5	33.3±0	2±1	11.7±1.5	0.3±0.1	1.8±0.3	2.3±0.6	10±5.6
5	68.9±5.4	0	5.3±1.2	24.7±1.5	0.3±0.1	1.8±0.3	5.7±2.1	8.3±8.5
6	98.3±2.9	33.3±0	4.7±1.5	16.3±1.5	0.3±0.1	1.7±0.5	4.3±3.2	16.3±1.5
7	99.3±1.2	0	2.3±1.5	16.7±1.5	0.3±0.1	1.7±0.5	3±1.7	16±1.7
8	72.2±7	33.3±0	3±1	13±1	0.3±0.1	1.9±0.2	4.7±3.1	10.7±5.1
9	98.3±2.9	66.7±0	5.3±0.6	10.3±0.6	0.3±0.1	1.7±0.6	6±1.7	14±4.6
10	76.2±18.9	33.3±0	3±0	10.3±1.5	0.3±0	1.8±0.4	4.3±3.2	9±5.3
11	94±8.7	33.3±0	4.7±0.6	10.7±0.6	0.3±0	1.7±0.5	3.7±1.5	6.3±2.3
12	34.4±10	0	6.3±0.6	15.7±0.6	0.3±0.1	1.7±0.5	5±3	15.7±2.1
13	93.3±6.5	33.3±0	4±1.7	11±1	0.3±0.1	2±0.3	6±1.7	11.3±5.9
14	98±2.6	66.7±0	6.3±0.6	22.2±0.8	0.3±0.1	2±0.2	4.7±3.1	13.7±5.1
15	100±0	100±0	6.7±0.6	16.3±2.1	0.3±0.1	2.4±0.2	8.3±0.6	18.3±0.6
16	92.3±7	33.3±0	2±00.6	11.7±0.5	0.3±0.1	1.8±0.2	2.3±1.7	10±5.5
17	68.9±0.7	0	5.3±1	24.7±1.6	0.3±0.1	1.8±0.4	5.7±0	8.3±4.9
18	98.3±6.5	33.3±0	4.7±0.6	16.3±0.6	0.3±0.1	1.7±0.5	4.3±3.1	16.3±5
19	99.3±5.2	0	2.3±1.5	16.7±0.8	0.3±0.1	1.7±0.4	3±1.5	16±5.5
20	72.2±3	33.3±0	3±2.6	13±1	0.3±0.1	1.9±0.4	4.7±3	10.7±2.5
21	98.3±7	66.7±0	5.3±1.5	10.3±0.6	0.3±0.1	1.7±0.4	6±2.1	14±4
22	76.2±1.9	33.3±0	3±1.2	10.3±1	0.3±0	1.8±0.6	4.3±2.1	9±2.5
23	94±24.3	33.3±0	4.7±2.1	10.7±1.5	0.3±0	1.7±0.3	3.7±3.2	6.3±1
24	34.4±7	0	6.3±1.5	15.7±1.3	0.3±0.1	1.7±0.7	5±2.6	15.7±2.5
25	93.3±3.7	33.3±0	4±1.5	11±0.8	0.3±0.1	2±0.5	6±3.5	11.3±4
26	98±5.5	66.7±0	6.3±1	22.2±0.6	0.3±0.1	2±0.4	4.7±2.6	13.7±2
27	93.7±8.5	33.3±0	4±1	18±1	0.3±0.1	1.9±0.7	6.3±2.3	16.7±1.2
28	85.7±12.7	33.3±0	4.7±0.6	14±1	0.4±0.1	2.1±0.3	5±3.6	14.3±3.2
29	90.3±9.1	33.3±0	4.3±2.1	14.3±1.2	0.3±0.1	2.1±0.2	5±3.6	15±2.6
30	96.7±3.5	66.7±0	4±1	11±1	0.3±0.1	2.1±0.3	5.7±3.1	15.3±4.6
31	67.9±1.9	100±0	5.3±0.6	25.7±0.6	0.3±0.1	2.2±0.1	8±1	16±2
32	94±6.6	33.3±0	5.7±1.5	13.7±0.6	0.3±0.1	2.1±0.3	5±3	12.7±0.6
33	64.2±2.9	0	6±3.6	29.7±1.5	0.3±0	2.3±0.1	9±1	17±2.6
34	92.7±9.5	0	5.7±2.1	13.3±0.6	0.3±0.1	2.4±0.3	5.3±3.5	16.7±2.3
35	95.7±4.5	66.7±0	3.7±3.6	9.3±1.5	0.4±0.1	2.3±0.3	4±1.7	12±5.6
36	96.3±4	66.7±0	5±0.6	22.3±2.5	0.3±0.1	2.3±0.2	6.7±4.2	17.3±1.2
37	95.7±4.5	0	4.3±0.6	19.7±2.1	0.3±0.1	2.1±0.1	7.7±1.5	18±1
38	32.1±1.2	33.3±0	6.3±0.6	21±1	0.4±0.1	2.2±0.1	6.7±3.2	15.7±2.1

Pop: population, *: Significant variation (ANOVA). Standard deviation corresponds to three replicates for each population.

the top; these are the cells responsible of stem elongation and leaf production. And for that the treatment is effective, it is necessary that the sub-epidermal layer, which is the origin of reproductive cells in the ovule and anther, is affected (Baudoin et al., 2002). Many adaptations were proposed to improve treatments by colchicine such as increase of the mitosis frequency by the addition of growth substances (Badoc et al., 1998), increase of the soaking duration in the colchicine solution (Aalders, 1963), prolonged treatment, but limited to apical meristems (Ghaffari, 2006), choice of stages where there are numerous divisions like seed germination (Hassen et al., 2001; Wei et al., 2007), floral induction (Bhattacharya, 1974), meiosis (Baudoin et al., 2002) and embryogenesis (Grzebelus and Adamus, 2004). All the mentioned techniques were allowed to obtain polyploids and mixoploids. In our study, the obtaining of mixoploids can be justified by the fact that the antimitotic agent can not reach all meristematic cells at the time of treatment. And even if so, the agent can not penetrate to the deepest

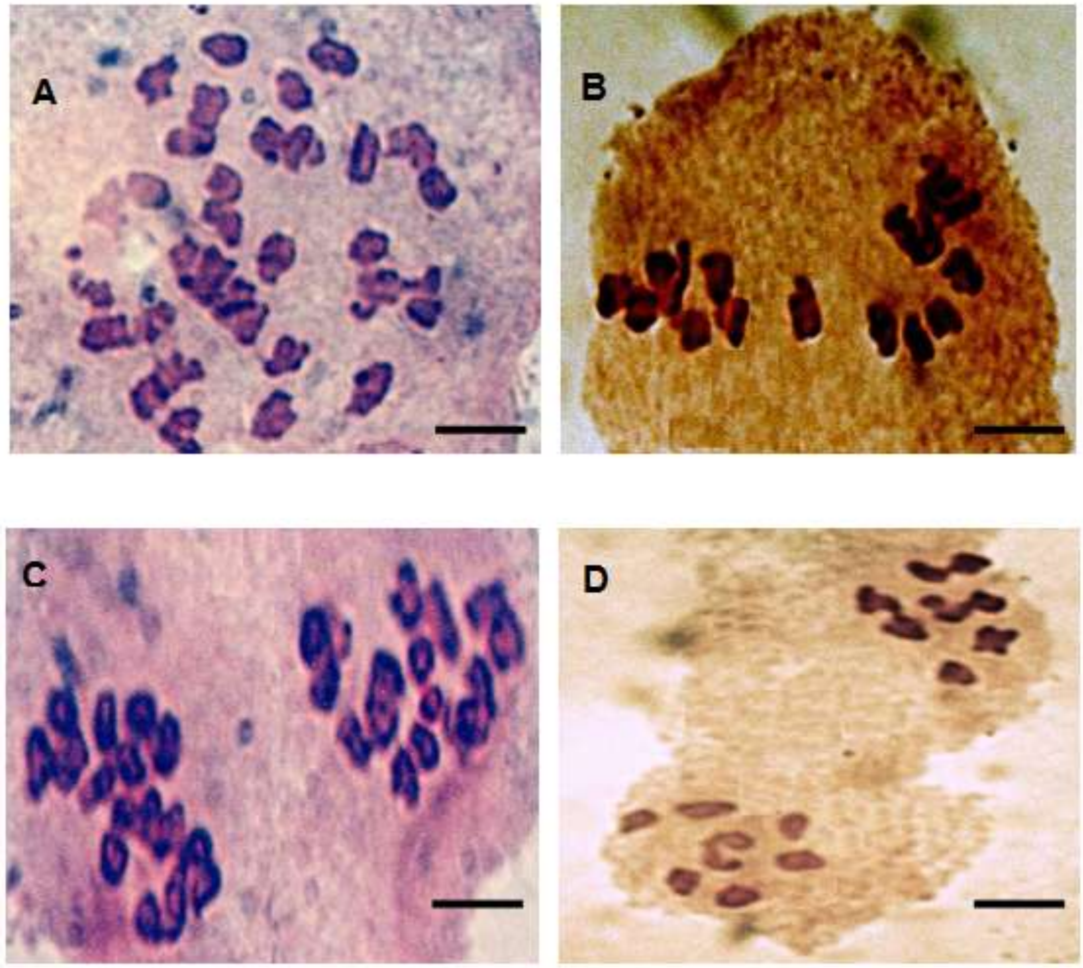

Figure 3. (A and B) metaphase 1 and (C and D) late anaphase 1 of mixoploid *Trigonella foenum-graecum* L. A and C tetraploid level (4n = 32); B and D diploid level (2n= 16). Bars= 10 µm.

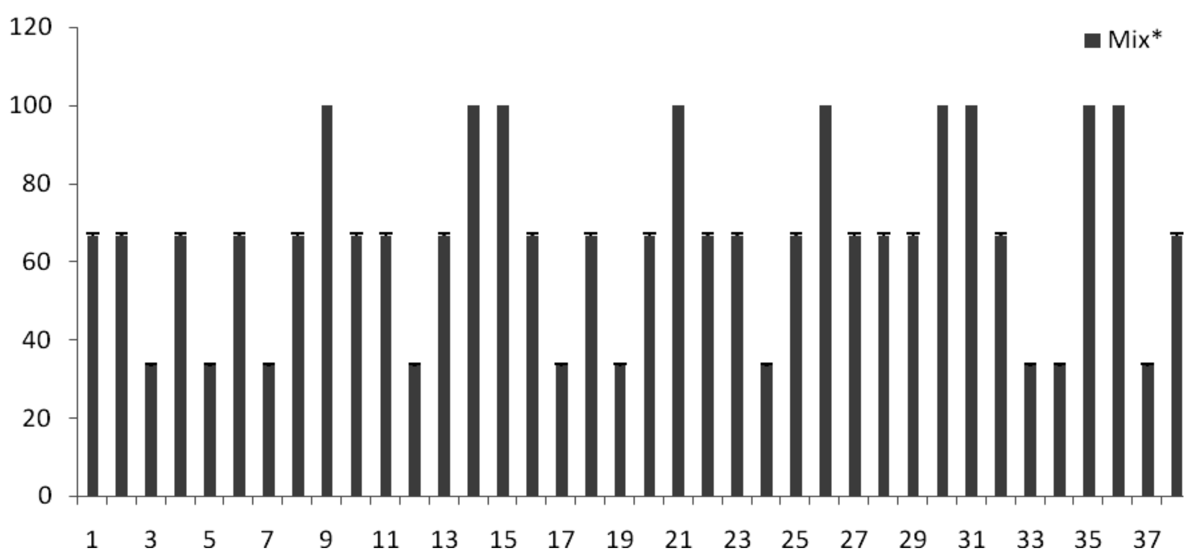

Figure 4. Mixoploidy rate variations between the 38 studied populations of *Trigonella foenum-graecum*. *: significant at $p < 0.05$. Standard deviation corresponds to three replicates for each population.

meristematic cell layer only after a relatively long application time during which the cells already affected have been destroyed (Raney, 2006; Beji, 1991). Furthermore, the meristematic cells can not be all at the same time, at the same stage of division (Beji, 1991). Arriving at a mixoploidy rate of 65.79% of treated *T. foenum-graecum*, we can consider that our treatment was successful because mixoploid plants could end in the obtaining of autotetraploid ones. And this was demonstrated in later work, in which entirely autotetraploid population of *T. foenum-graecum* was obtained by the culture of seeds stemming from tetraploid branches of mixoploid plant (Marzougui et al., 2009a). Hill and Myerst (1944) were also able to isolate diploid and tetraploid Rayegrass (*Lolium perenne* L.) from mixoploid rayegrass produced by treatment of germinating seeds with colchicine. Rugini et al. (1996) isolated triploid and tetraploid olive plants (*Olea europaea* L.) from two mixoploid mutants obtained earlier by treating plantlets with gamma radiation. Recently, Huang et al. (2010) were as well able to generate tetraploid plants of *Dioscorea zingiberensis* from mixoploid ones.

Our results also showed significant variations in the rate of mixoploidy between the different studied populations of fenugreek. This can be justified by the fact that these populations were collected from areas with large differences in edaphic substrate, altitude, longitude, latitude and rainfall. As well as, our previous studies demonstrated that these populations present variations between them on the morphological, chemical (Marzougui et al., 2007), biochemical (Marzougui et al., 2009b), physiological (Marzougui et al., 2009c) and molecular (Marzougui et al., 2009d) levels.

Conclusion

The treatment of the fenugreek shoot meristem by colchicine allowed generation of diploid and mixoploid plants with a mixoploidy rate of 65.79% of treated plants. Among the 38 treated populations, population 15 of Menzel Temime presented the highest rate of mixoploids formation and survival rate to colchicine treatment. This population will be a good material for the isolation of tetraploid seeds, resulting in the formation of autotetraploid *T. foenum-graecum*. The production of such population will increase the variability within the species and may have effects on morphology, yield, chemical and biochemical compositions or resistance to biotic and abiotic stresses, allowing it to be preferentially used in agriculture.

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