Colchicine Induction of Polyploidy in Egyptian Clover Genotypes

Zeinab, M. Abd El-Naby[1]*, Nabila A. Mohamed[1], Khalid H. Radwan[2], and Dina A. El-Khishin[2]

[1]Field Crops Research Institute (FCRI). Agricultural Research Center (ARC) 9, Gamma St., Giza-12619, Giza, Egypt.
[2]Agricultural Genetic Engineering Research Institute (AGERI). Agricultural Research Center (ARC) 9, Gamma St., Giza-12619, Giza, Egypt.
zeina_bree@yahoo.com

Abstract: Induction of polyploidy and chromosome comparison of eight berseem clover (*Trifolium alexandrinum* L.*)* genotypes were performed. Different colchicine concentrations were applied to seeds and seedlings using two methods: immersion and sprinkling. Both methods were successful in polyploidy induction. Karyotyping of F_1 seeds and their parents were examined for polyploidy. Chromosome number was determined in root-tip cells 2-3d old from seed sowing. Tetraploids were evident in all populations (2n= 4X=32), whereas diploid parents had (2n=2X=16) chromosomes. Interactions between genotypes and colchicine treatments recorded high significant differences of no. inflorescences plant^{-1}, 1000-seed weight, no. floret inflorescence^{-1} and no. seeds floret^{-1} with significant differences of seed weight g m^{-2}, seed setting% and mortality%. Higher variation in leaflet shape and number, inflorescence size and shape and floret size was viewed over all colchicine treatments in tetraploid plants compared with the control. High positive significant correlation coefficient between number of seeds per floret and number of florets plant^{-1} (r=0.853**) and seed setting% (r=0.943**), mortality% was negatively correlated with seed setting% (-0.795**). Positive significant correlation was found between 1000-seed weight and number of florets plant-1 (r=0.711*) and seed setting% (r=0.744*). Polyploidy induction resulted in taller plants, faster re-growth after cutting, increased tillering and branching plant^{-1}, increases in 1000 seed weight and increased seed yield m^{-2}. This methodology can be used in plant breeding programs to increase forage production efficiency and select for more superior adaptive genotypes.

[Abd El-Naby, Zeinab, M, Nabila A. Mohamed, Khalid H. Radwan, and Dina A. El-Khishin **Colchicine Induction of Polyploidy in Egyptian Clover Genotypes** *J Am Sci* 2012;8(10):221-227].(ISSN: 1545-1003).http://www.americanscience.org. 33

Key words: *Berseem clover, Colchicine, Chromosomes, Correlation, Ploidy.*

1. Introduction

Forages consumed either directly or indirectly by livestock and poultry play a tremendously important role in animal production. Clover breeding aims to produce high quality fresh and dry matter combined with high seed yields. Berseem clover (*Trifolium alexandrinum* L.) (2n=2x=16) is considered the backbone of forage production, it is an annual forage legume well adapted to semi-arid conditions of the Mediterranean areas (Iannucci *et al.*, 1996 and 2001, Martiniello *et al*., 1999).

Colchicine extracted from the plant (*Colchicum autumnale* L.), is one of the most widely used chemicals for polyploidy induction. It inhibits the formation of spindle fibers and mitosis is arrested at the anaphase stage. At this phase the chromosomes have multiplied but cell division has not commenced, which results in polyploid cells. In contrast with their diploid counterparts polyploid cells have larger volumes which develop into thicker tissues, resulting in bigger plant organs (Franzke and Ross, 1952 and Downes and Marshall, 1983).

Polyploidy induction is an effective tool in plant breeding. Successful autoploidy has been reported in forage plants by many authors, (Evans, 1955; Stebbins, 1971 and Simmonds, 1980). Polyploidy breeding in species with low chromosome numbers were reported to be more successful (Poehlman and Sleper, 1995). Berseem clover has X=8 chromosomes which is ideal for polyploidy induction.

Polyploidy induction (e.g. tetraploids 2n= 4x = 32) in berseem clover has been used to obtain new genotypes with higher forage production when compared to diploids. Polyploids generally have bigger organs than their ancestors, are late in flowering and can be superior to the original diploids under cultivation with proper management. Since autopolyploids in particular were reported to be less fertile than diploids, best results obtained are those with induced allopolyploids, suppressing the hybrid sterility barrier Stebbins (1971).

Autotetraploids have greater vegetative volume, higher seed weight, and more adaptability to wider ecological regions, but lower reproductive fertility than their diploid counterparts. At the cellular level, the cytoplasm and nucleus of polyploids are larger than those of normal plants (Swaminathan, 1970; Schulz-Schaeffer, 1980; Poehlman and Sleper, 1995).

Polyploidy is important in the evolutionary trends of plants, where around 30-50% of all

angiosperms are estimated to be polyploids, 70% of grasses, and 23% of legumes (Poehlman and Sleper, 1995). Moore *et al.*(1998) suggested that polyploids survive and flow in harsher environments where selection pressure is more intense. Similarly, Levan, (1942); Bingefors and Ellerstrom (1964) suggested that cross-pollinated tetraploid *Trifolium spp*. (red clover) had greater hybrid vigor than those of diploids.

Therefore, the objectives of the present work were to (1) induce polyploidy in *Trifolium alexandrinum* by colchicine treatment (2) identify tetraploids using karyotypic analysis (3) study and evaluate the effects of induced polyploidy on forage production. Such work aimed to produce new more adaptive genotypes of *T. alexandrinum* for cultivation in newly reclaimed lands and pastures and to increase fodder yield to secure and sustain more feed for Egyptian livestock.

2. Materials and Methods

Plant Material

A total of six berseem cultivars, five multi-cut (Helaly, Sakha-4, Gemmeza-1, Serw-1, Giza-6) and one mono-cut (Fahl) and two populations (Hatour and Narmer) (Abd El-Naby, 2003) were used in this study. This study was carried out in a greenhouse, field and genomics laboratory of the Agricultural Genetic Engineering Research Institute (AGERI), Giza, EGYPT.

Colchicine Treatment

One hundred seeds of each genotype were used for polyploidy induction. Two different methods of colchicine treatment (a) soaking of seeds in colchicine solution (0.05%) for 2.5 hrs then thoroughly washing with distilled water before sowing and (b) application of colchicine at three different concentrations (0.1, 0.2 and 0.3%), on the apical meristem of 7-d old seedlings every 3 hrs for three times at the same day. Colchicine treatments were applied in two replicates with 100 seeds in each pot per genotype per replication, whereas two pots per replication per genotype were treated with distilled water and used as control.

Pots were kept under greenhouse conditions. Numbers of surviving plants were counted and recorded for each treatment. The first cut was carried out 60 days after sowing in the pots. Then plants were transplanted into the field in three replicates for each treatment. All plants were transplanted individually in 4-m long rows 20 cm apart each among plants and rows.

Karyotypic Studies

Karyotypic studies were conducted on F_1 generation plants devised from the colchicine treated plants. Freshly grown root tips of ten young seedlings (2-3d from sowing) were excised and dipped in ice cold distilled water and placed at room temperature for a minimum of 12-24 hrs Squash technique. The somatic chromosome number and karyotypic details were examined metaphase plates, using 2% aqueous propionic carmine stane (Sheidai *et al.*, 2000; Sheidai and Rashid, 2007). Micrographs of good quality metaphase plates were taken using a Nikon Microscope and digital camera, using Karyo V. 3.0 and VIDEO TEST Image analysis Ltd., 2000.

Statistical Analysis

The experiment was arranged in a randomized complete block design in three replicates. Plant numbers were counted after each treatment and after transplanting into the field to determine the percentage of surviving plants. At least ten individual plants were evaluated for seven characteristics (no. inflorescences plant^{-1}, no. florets plant^{-1}, no. seeds floret^{-1}, seed weight g m^{-2}, weight of 1000 seeds g, percent seed set and percent mortality). Data from each replication were combined and subjected to analysis of variance using SAS statistical analysis system software version 9.1 for windows (SAS Institute, 2004), while means were compared using Duncan's multiple range test (Duncan, 1955).

3. Results and Discussion

Polyploidy Induction

Polyploidy induction using colchicine in berseem proved to be effective in all the genotypes studied. Overall efficiency varied among the different genotypes. As an example, somatic chromosome numbers and ploidy level of the berseem population Hatour is presented in (Fig.1). The somatic chromosome count of the diploid control was 2n=2x=16 vs. treated tetraploid 2n=4x=32 and hexaploid 2n=6x=48 (Figs. 1a-c), respectively. The karyotype of this species consists totally of eight chromosomes, no satellites were observed in the karyotype of this taxon. The present results are in accordance with those reported by Taylor (1987) and Abberton (2007).

Germination and Plant Survival

Germination percentages and plant survival varied significantly after colchicine treatment. After transplantation, a highly significant interaction between different colchicine treatments and genotypes were detected in the field. Results show no significant differences among genotypes and treatments. Figure 2 shows the results of different colchicine treatments for all genotypes. Control treatments across all genotypes recorded the best survival rate with a mean of 86.4%. Different colchicine treatments (0. 1, 0. 2 and 0. 3%) recorded a mean survival rate of 57.3, 61.5 and 54.6%, respectively. As mean survival rate of 58.1%was

observed after soaking treatment with 0.05% colchicine noted a.

The percentage of plant survival after transplantation under field conditions is shown in Table 1. Hatour population recorded the highest survival rate across all treatments with means of 24.5, 23.0, 21.0 and 14.0, respectively. Fahl genotype recorded a high survival rate with the 0.1% colchicine and with 0.05% soaking. Finally the lowest percentage of plant survival was recorded for Sakha-4 with the 0.3% colchicine treatment and the 0.05% soaking (Table 1). The highest mean percentages were observed in the genotypes Hatour and Fahl followed by Narmer (74.5, 67.5 and 66.5%) across all colchicine treatments respectively. Hatour population was the most promising genotype studied for plant survival after treatment. As for the treatments mean, Fahl genotype recorded the best performance across all colchicine treatments, followed by Hatour and Narmer (23.4, 20.6 and 20.1%) respectively. Helaly and Giza-6 genotypes gave good performance with significant differences in comparison to Fahl, Hatour and Narmer genotypes. Gemmiza-1 and Sakha-4 genotypes were extremely sensitive to colchicine treatments, although their controls were of average performance in regard to plant survival in the field (Table 1). Although this does not grantee its superiority in terms of polyploidy performance, it was found to have the highest performance in comparison with the other genotypes. These results confirm that control plants showed a much higher survival rate than the treated, while ploidy induction is genotype dependent, the best results were obtained with the lowest colchicine treatment of 0.1% across all genotypes.

Induction of polyploidy using colchicine resulted in reduced fertility expressed as presence of sterile plants across the genotypes, and also more vigorous plants across all genotypes, although numbers differed according to studied genotypes. Also, high seed-producing diploid parents did not necessarily produce highly fertile polyploids. Thus, identification of high seed-producing diploids would not necessarily result in high seed-producing polyploids. Colchicine treatments showed highly significant differences for no. inflorescences plant^{-1}, weight of seeds g m^{-2}, no. seeds floret^{-1}, seed setting % and mortality% while showing significant differences for 1000-seed weight and no. floret inflorescence^{-1}. Interaction between genotypes and colchicine treatments recorded high significant differences of no. inflorescences plant^{-1}, 1000-seed weight, no. floret inflorescence^{-1} and no. seeds floret^{-1} with significant differences of seed weight g m^{-2}, seed setting % and mortality%. Selection for high seed setting genotypes can help breeders produce new promising synthetic varieties.

Seed Characteristics

Seed characters of the eight genotypes using different treatments (control, T1, T2, T3 and soaking) are shown in Table (2). Narmer and Hatour populations recorded the best number of inflorescences (124.80 and 120.33 plant^{-1}), seed weight (59.3 and 60.75 g m^{-2}),1000 seeds (0.33 and 0.34 g^{-1}), no. florets plant^{-1}(104.2 and 107.1), no. seeds florets (77.6 and 80.4) and percent seed setting (74.4 and 75.3%) with lowest percent mortality across all genotypes (0.80 and 0.66%), respectively. Fahl, Helaly and Giza-6 genotypes showed good fertility characters followed by Narmer and Hatour populations. Serw-1 genotype recorded the poorest performance in comparison with others across all colchicine treatments except for number of florets inflorescence^{-1} (Table 2).

The mean seed weights across ploidy levels obtained in this experiment were similar to those reported by other authors (Bingefors and Ellerstrom, 1964; Anderson, 1971). The performance and adaptability of the selected tetraploids especially for seed production should be evaluated at different locations and years to determine the extent of the genotypic effect.

High positive significant correlation coefficient was found between number of seeds floret $^{-1}$ and number of florets plant^{-1} (r=0.853**) and seed setting % (r=0.943**). Mortality % was negatively correlated with seed setting% (-0.795**). Positive significant correlation was found between 1000-seed weight and number of floret plant^{-1} (r=0.711*) and seed setting % (r=0.744*) Table (3).

Significant relationships were recorded between seed setting % and the number of florets plant^{-1} (r=0.790*). Negative correlations were observed between mortality% and seed setting% (r=-0.785*) and 1000-seed weight (r=-0.757*). However, there was no correlation between seed weight and seed number. Results indicate that high seed setting percentage is related to the number of florets inflorescences^{-1}. 1000 seed weight and seed setting % were positively correlated with number of seeds florets^{-1}. Selection for number of seeds florets^{-1} can be considered as an indicator for future seed yield.

Morphological Characteristics

Morphological changes were observed in the polyploid-induced genotypes when compared to control. Figure (3 a, b, c and d) shows higher variation in inflorescence size and shape of polyploid plants in comparison with the diploid control. Polyploid plants had a larger inflorescence size and number per plant with larger size florets, which improves pollination efficiency of different

pollinators. This increment will consequently increase seed yield per unit area.

A great number of variations after colchicine treatments were observed in plant vigor in comparison to control (Fig. 4). Figure (4) shows the differences between the control (Fig.4-a) and treatments in terms of increase in tillers, branches sub branches and number of inflorescence (Fig.'s 4 b, c and d) leading to an increase in forage production. In addition, variations in leaflet size, shape and number Figure (5 b, c and d) were observed across all genotypes starting from the first cut in comparison to control (Fig. 5 a). It is also noteworthy to mention that fourth and fifth leaflets were observed from the first cut, after treatment and remained until harvest in some plants; this observation is a subject of further study.

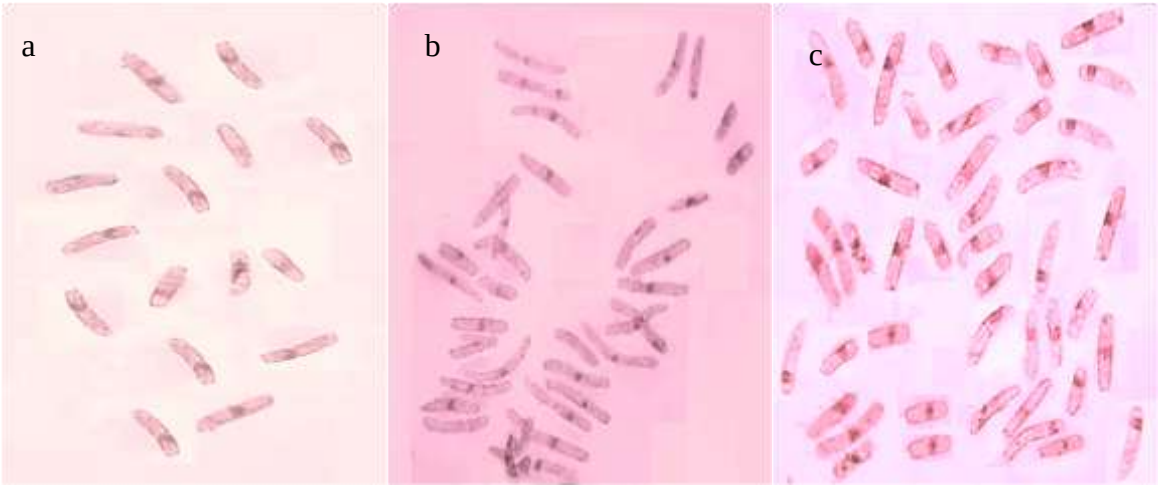

Figure (1): Mitotic chromosomes of Hatour population taken from root tips: (a) diploid control, (b) tetraploid, and (c) hexaploid.

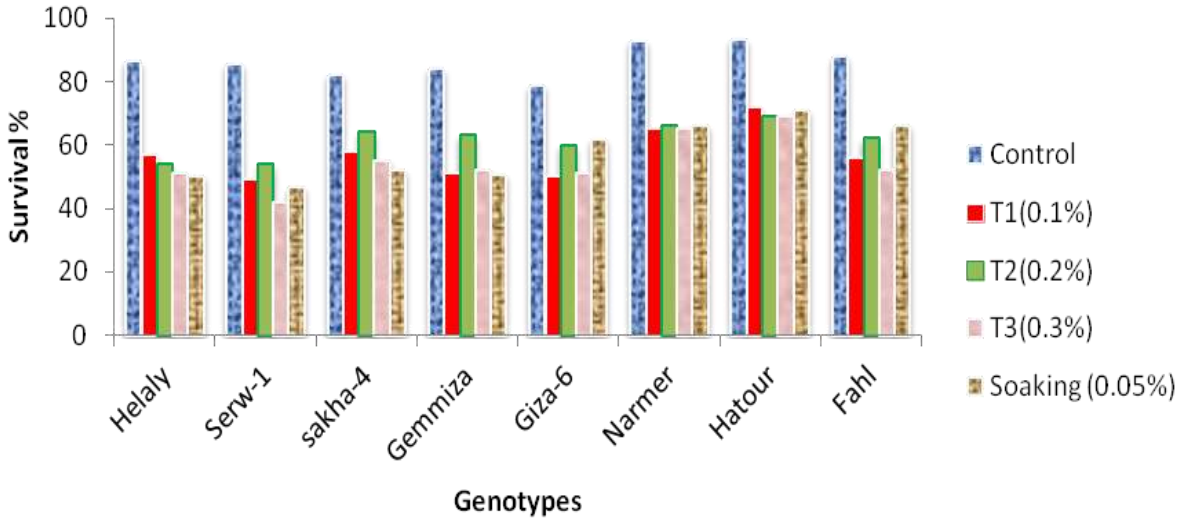

Figure (2): Plant survival percentages after colchicine treatments. *T=colchicine treatment*

Figure (3): Variation in inflorescences size after colchicine treatment (b, c, and c) and diploid control (a).

Figure (4): Variations in morphological characteristics (inflorescence) in treated plants (b, c, and d) vs. control (a).

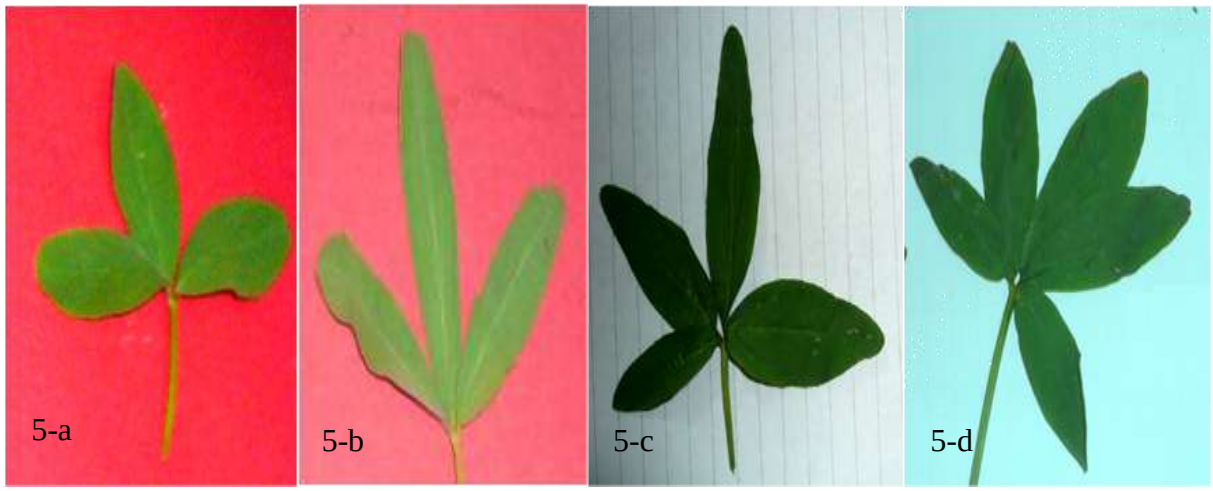

Figure (5): Variations in morphological leaflet characteristics in treated plants (b, c, and d) vs. control (a).

Table 1. Mean percentages of plant survival after transplanting in field.

Genotypes	Germination after transplanting					Treatment Mean
	Control	Colchicine Treatments				
		0.1%	0.2%	0.3%	Soaking 0.05%	
Helaly	43.0	23.0^{c}	20.0^{b}	18.0^{b}	15.0^{a}	**19.0B**
Serw-1	39.5^{d}	22.5^{cd}	17.5^{bc}	17.5^{b}	14.0^{ab}	**17.9C**
Sakha-4	49.0^{c}	23.5^{c}	16.0^{c}	13.5^{c}	9.0^{c}	**15.5D**
Gemmiza-1	45.0^{c}	20.5^{d}	16.0^{c}	16.0^{bc}	12.0^{b}	**16.1C**
Giza-6	64.5^{b}	25.5^{b}	19.0^{b}	19.0^{a}	12.0^{b}	**18.9B**
Narmer	66.5^{ab}	28.0^{b}	20.0^{b}	19.5^{a}	13.0^{b}	**20.1A**
Hatour	74.5^{a}	24.5^{b}	23.0^{a}	21.0^{a}	14.0^{ab}	**20.6A**
Fahl	67.5^{ab}	37.5^{a}	21.5^{a}	18.5^{ab}	16.0^{a}	**23.4A**
Mean	**56.19A**	**25.6B**	**19.0B**	**17.9C**	**13.1D**	**18.9**

Values within a column for each principal factor not followed by the same letter are significantly different at $p \leq 0.05$.

Table (2): Seed character means with different colchicine treatments.

		No. inflorescences plant^{-1}	Seed weight m^{-2} g	1000 seeds g^{-1}	No. floret plant^{-1}	No. seeds floret^{-1}	Seed set%	Mortality %
Treatments across genotypes	Control	58.42d	38.90c	0.28c	46.50b	50.08c	57.35c	3.10b
	T1	82.37b	46.40ab	0.32a	90.15a	54.17bc	58.42bc	3.46b
	T2	89.71ab	49.56a	0.31a	97.54a	61.75a	62.71ab	7.95a
	T3	95.83a	47.28a	0.30b	90.54a	54.37bc	59.97abc	8.07a
	Soaking	70.92c	45.60b	0.31b	90.46a	59.79ab	64.80a	9.92a
Genotypes across treatments	Helaly	73.27b	41.60cbd	0.31b	89.27b	54.27b	60.19cd	4.46cb
	Serw-1	50.80e	37.39d	0.25d	90.00b	44.00c	48.79e	11.97a
	Sakha-4	63.13cd	38.81cd	0.30b	89.13b	48.13bc	53.56cde	12.61a
	Gemmiza-1	60.60d	39.56cd	0.28c	82.67bc	43.93c	52.63de	9.77a
	Giza-6	66.13c	42.42bc	0.31b	87.53b	50.20bc	57.12cbd	8.93ab
	Narmer	120.33a	59.31a	0.33a	104.20a	77.60a	74.40a	0.80c
	Hatour	124.80a	60.75a	0.34a	107.07a	80.40a	75.30a	0.66c
	Fahl	76.53b	44.53b	0.31b	85.73bc	54.53b	63.22b	2.81c
Mean		79.45	45.55	0.31	91.95	65.63	60.65	6.50

Values within a column for each principal factor not followed by the same letter are significantly different at $p \leq 0.05$.

Table 3: Correlation Coefficient among different seed characteristics

	X1†	X2‡	X3§	X4¶	X5$^{\#}$	X6††
X2‡	0.372					
X3§	-0.023	0.853**				
X4¶	0.699	0.699	0.524			
X5$^{\#}$	0.062	0.711*	0.801*	0.425		
X6††	-0.288	0.632	0.0943**	0.321	0.744*	
X7‡‡	0.397	-0.621	-0.785*	-0.164	-0.757*	-0.795**

* Significant at the 0.05 probability level. ** Significant at the 0.01 probability level.

†X1=No. inflorescences plant^{-1}, ‡X2= No. florets plant^{-1}, §X3=No. seeds floret^{-1}, ¶X4= Seed weight plant^{-1}g, $^{\#}$X5= 1000-seed weight g, ††X6= Seed set% and ‡‡X7= Mortality% .

Conclusion:

Induction of polyploidy in berseem *(T. alexandrinum)* using colchicine treatment can increase the variability in plant morphology, fertility and yield potential. Such methods of improvement are relatively fast and cheap. However, because distinguishing between tetraploids and hexaploids in the field is rather difficult, other methods of screening such as molecular markers should be employed for tetraploid selection. Results show that polyploidy induction resulted in: taller plants, faster re-growth after cutting, increased tillering and branching per plant, increase in 1000 seed weight and increased seed yield m^{-2}. This methodology can be used in plant breeding programs to select for more superior adaptive genotypes.

Acknowledgments

The authors would like to express their gratitude to Prof. Fawzia Ismaiel Mohamed and Ms. Enas Mohamed Abdel Ghany, National Research Center-Egypt, for their help in the karyotyping work.

Corresponding authors
Abd El-Naby, Zeinab, M
Field Crops Research Institute (FCRI). Agricultural Research Center (ARC) 9, Gamma St., Giza-12619, Giza, Egypt.
zeina_bree@yahoo.com

References

Abberton, M. T. 2007. Interspecific hybridization in the genus Trifolium. Plant Breeding 126: 337–342.

Abd El-Naby, M. Z. 2003. Breeding Studies in Berseem (Egyptian clover) Populations. M.Sc. Thesis, Faculty of Agriculture, Cairo Univ., Egypt.

Abd El-Naby, M. Zeinab (2009). Inbreeding, selection, fertility and flower structure in the Egyptian Clover (*Trifolium alexandrinum*). Ph.D. Thesis, Faculty of Agriculture, Cairo Univ., Egypt.

Anderson, L. B. 1971. A study of some seedling characters and the effects of competition on seedlings in diploid and tetraploid red clover (Trifolium pratense L.). N. Z. J. Agric. Res. 14: 563-571.

Bingefors, S., and S. Ellerstrom. 1964. Polyploidy breeding in red clover. The tetraploid variety *Svalof.s Ulvuna* compared with some diploid and tetraploid varieties. Z.Pflanzenzuecht. 51: 315-334.

Downes, R. W., D. R. Marshall. 1983. Colchicine induced variants in sunflower. Euphytica 32: 757-766.

Duncan, D. B. 1955 . Multiple range and multiple F test. Biometrics 11:1-42.

Evans, A. M. 1955. The production and identification of polyploids in red clover, white clover and lucerne. New Phytol. 54: 149-162.

Franzke, C. J., J. G. Ross. 1952. Colchicine induced variants in sorghum. J. Heredity 43: 107-115.

Iannucci, A. 2001. Effects of inbreeding and pollination modes on self-fertility and seed weight in berseem populations. J Genet & Breed 55: 165–171.

Iannucci, A., N. Di Fonzo and P. Martiniello. 1996 . Effects of the developmental stage at harvest on dry matter and chemical component partitioning in berseem. J. Agron. Crop Sci., 176: 165-172.

Levan, A. 1942. Plant breeding by induction of polyploidy and some results in clover. Hereditas. 28: 245-246.

Martiniello, P. 1999. Effects of irrigation and harvest management on dry matter yield and seed yield of annual clover grown in pure stand and in mixtures with graminaceous species in a Mediterranean environment. Grass Forage Sci., 54: 52–61.

Moore, R., W. D. Clark and D. S. Vodopich. 1998. Botany, 2*nd* ed. WCB/McGraw Hill, Boston, Massachusetts, USA.

Taylor, N. L. 1987. Chromosome numbers of Trifolium species from Romania. Notulae Botanicae Horti Agrobotanici 17: 39–45.

Poehlman, J. M. and D.A. Sleper. 1995. Breeding field crops. 4th ed. Iowa State Univ. Press, Ames.

SAS Institute. 2004. SAS system for windows. Version 9.1. SAS Inst., Cary, NC.

Schulz-Schaeffer, J. 1980. Cytogenetics-Plants, Animals, and Humans. Springer-Verlag, New York.

Sheidai, M. and S. Rashid. 2007. Cytogenetic study of some Hordeum L. species in Iran. Acta Biol. Szegediensis, 51 (2): 107-112.

Sheidai, M., A. Nasirzadeh and M. Kheradnam. 2000. Karyotypic study of Echinops (Asteraceae) in Fars Province of Iran. Bot. J. Lin. Soc., 134: 453-463.

Simmonds, N. W. 1980. Polyploidy in plant breeding. Span.23:73-75.

Stebbins, G. L. 1971. Chromosomal evolution in higher plants. Addison-Wesley, London, p. 216

Swaminathan, M. S. 1970. The significance of polyploidy in the origin of species and species groups. p. 87-96. In O. H. Frankel and E. Bennett (ed.) Genetic resources in plants-their exploration and conservation. International Biological Programme Handbook No. 11. Blackwell Scientific, Oxford.