Journal of Pharmacognosy and Phytotherapy Vol. 4(2), pp. 16-25, March 2012
Available online at http://www.academicjournals.org/JPP
DOI: 10.5897/JPP11.060
ISSN 2141-2502

Full Length Research Paper

Inbreeding and fertility in Egyptian clover, *Trifolium alexandrinum*

M. M. F. Abdalla[1] and M. Abd El-Naby Zeinab[2]*

[1]Department of Agronomy, Faculty of Agriculture, Cairo University, Egypt.
[2]Field Crops Research Institute, Agricultural Research Center (ARC), Egypt.

Accepted 27 September, 2011

Selection for manual tripping (MT) was effective in improving self-fertility (SF) in all populations and generations over original parents. Selection for MT was accompanied by improving fertility of open pollinated (OP) populations. Self-seed set (ST) was very poor. Some individual selections set seeds up to 88% in MT materials. The enigma of fertility, sterility and absence of inbreeding depressions in berseem was discussed. This was explained as inbreeding tolerance, due to selection for good vigor accompanying inbreeding. The results indicated that selection for high MT seed set could be accompanied by selection for good forage yield. An approach was suggested to develop new composite varieties characterized by high seed fertility and high forage yield. Improved selections for seed production was mainly due to profuse flowering and more flowers/ inflorescence, whereas improved forage yield was mainly due to more tillers / plant and to less degree of plant height. An approach was outlined to produce new composites with high seed setting and good forage. *T. alexandrinum* is self-compatible, but needs tripping to stimulate self seeds. This is mainly due to the relative position of male and female organs and the presence of bubbles on stigma. Molecular characterization revealed polymorphic differences between I_o and selected inbred.

Key words: *Trifolinm alexandrinum,* fertility, yield, tripping, compatibility, inbreeding.

INTRODUCTION

Fertility

Berseem clover, *Trifolium alexandrinum* L., is one of approximately 250 species and is one of about 20 species of agricultural value in the genus *Trifolium*. It is an annual species with 2n=16 chromosomes. Annual and perennial species are present in the genus. Perennial species are known to be mostly self-incompatible; (red clover T. pretense had a gametophytic system of incompatibility). However, annual species are self-compatible, but some may require tripping (Evans, 1976).

Berseem is a winter crop widely grown in Egypt (approximately 3 million acres annually) and other countries. The crop is a dilemma with regard to its self-fertility and infertility. Some authors regarded berseem as a self-sterile crop (infertile). Flower bagging, or excluding insects may result in no or very few seed set per head. (Zaher, 1947; Said, 1954; Tanash, 1970; Bakheit, 1989) and others consider the species as cross-fertilized (Latif et al., 1956; Roy et al., 2005).

On other side different authors reported berseem clover as a self-compatible (self-fertile). (khan and Bahati, 1953; Finn, 1964; Chowdhry et al., 1966; Beri et al., 1985a and b; Dixit et al., 1989; Rotili and Gnocchi, 1989; Rammah, 1969; Miawad, 1992; El-Shahawy and Gheit, 2001; Abd El-Naby, 2003; Abdalla et al., 2008).

All studies of berseem which reported on the effects of selfing and open field conditions recorded that seed set was high in open field and under tripping and was drastically reduced upon caging and selfing (without tripping). The introduction of honeybees or other pollinating insects to berseem grown plots improved seed set (Wafa and Ibrahim, 1960; Richards, 1995). This indicates that berseem clover, although self-compatible but needs "stimu lating agent" – insects or tripping- to set seed in high quantities.

The controversial results of self-fertility (SF) and self-sterility (SS) in berseem and the presence of self-sterile

*Corresponding author. E-mail: zeina_bre@yahoo.com.

(or self-incompatible) segregates (Mehta et al., 1963; Iannucci, 2001) raise the question concerning possibilities of improving fertility of this crop. Some authors reported that auto-fertility in berseem (expressed in better seed set) may be achieved through inbreeding and selection (Finn, 1964; Innucci, 2001; Abd El-Naby, 2003).

Vegetative characters and seed yield

The effect of inbreeding on Fahl variety of berseem was studied by Rammah (1969). The results indicated that the different inbred progenies did not respond similarly to inbreeding. Mahdy and Bakheit (1985) studied the effect of inbreeding on forage yield after two generations of selfing. They suggested that the inbreeding depression is caused by a decrease in heterozygosity. Rotili and Gnocchi (1989) demonstrated large variability in reaction to selfing. The dry matter yield was the most affected trait; the Italian populations were more depressed than the Egyptian populations. Sohoo et al. (1994) showed that inbreeding depression was absent in many yield components. El- Shahawy and Gheit (2001) reported that berseem populations did not respond similarly to inbreeding and extent of depression varied among populations and traits. Iannucci (2001) found that inbreeding resulted in an increase in the number of completely infertile plants. Abd El-Naby (2003) reported that the open-pollination and I_1 progenies of berseem plants were not significantly different in mean fresh yield. Populations developed by selfing among four inbred generations produced significantly greater dry matter yield than unselected progenies. Iannucci (2004) demonstrated severe depression in vigour and fertility from continuous self-pollination. Seed yield generally showed more depression from inbreeding than dry matter yield and its related traits. Selection for self-fertility during selfing did not prevent seed setting and seed yield from showing substantial depression. Abdalla et al (2008) demonstrated that selection for self-fertility based on manual tripping was effective in improving fertility of tripped, open pollination and self-set seed.

What would be the effect of selection for fertility in berseem? are there any flower structure associated with fertility? And could selection for high seed setting be accompanied by good forage yield? Answers to these questions are the objectives of this article.

MATERIALS AND METHODS

Fertility and vegetative traits

These studies were conducted through 4 successive winter growing seasons (2003/2004, till 2006/2007). The two synthetic multi-cut varieties: Synthetic 79 (S79) and Ahaly of Berseem developed at the Agronomy Department, Faculty of Agriculture, Cairo University were employed in this study. Abd El-Naby (2003) practiced selection in the two varieties for seed set (female fertility) till I_4 (inbred plants in fourth generation). Only plants that set seeds above 25% (seeds per 100 florets) were selected.

In 2003/2004 season several populations in different inbreeding generations were evaluated compared to parents (I_o). Each population was constructed by blending seeds of 20 plants. Seeds were sown in dense planting in 4 m rows, after which plants were dug and transplanted into field after first cut. Selection was practiced for self-fertility above 40%.

In 2004/2005 season plants were selected also on the basis of high self fertility (>45% seed set). In 2005/2006, previous experiment of 2004/2005 season was repeated using seed fertility above 54%. Three cuts were harvested and plants were left for flowering and seed set. In 2006/2007 season, eight families selected from previous season were evaluated for fertility. Materials were sown under cage in RCBD with two replicates.

In all the seasons, data on agronomic traits were recorded on each plant per row. Also fertility was recorded on all plants in the row for bagged heads either manually tripped gently by fingers (MT) or for bagged heads left for spontaneous seed set (ST) and also for open-pollinated (OP) heads. The MT, ST and OP seeds were available on same plant but selection for self-fertility was based only on fertility of MT heads every season. For determinating forage yield, seeds were sown in dense planting (3×3 m plots), besides planting in 4 m rows with 25 cm between rows and hills. Selection was practiced (before transplanting) for good vegetative growth. All weak-performing plants were excluded and only healthy vigorous plants were transplanted.

Flower structure and molecular characterization

Flowering period generally extended from half May to late June. Macro images of floral features were captured using a Nikon Coolpix 950. Flowers in different stages were kept in wax to examine floral sex parts. To study anatomy, 5 inflorescences in full blooming and 5 in mid blooming stages from whorls No. 3 to No. 6 of each floret were killed and fixed for at least 48 h in F.A.A. (10 ml formalin, 5 ml glacial acetic acid, 85 ml ethyl alcohol 70%). Materials were washed in 50% ethyl alcohol and dehydrated in a normal butyl alcohol series, embedded in paraffin wax (melting point 52 to 54°C). Transverse sections, 20 jj thick, were cut using a rotary microtome and stained with crystal violet/erythrosine combination, and mounted in Canada balsam (Willey, 1971).

For flower characters, five inflorescences from each blooming stage and from each generation (about 100 plants) were assigned for morphology, floret form and length, floret parts shape and dimensions, nectar glands position, sexual organs forms and quantity and viability of pollen grains.

For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) protein extraction was used and electrophoresis was carried out according to Leammli (1970). After staining and distaining, the wet gel was photographed.

The random amplified polymorphic DNA (RAPD) analysis was done using genomic isolated DNA from the leaves of 10 to 15-day old seedlings. The polymerase chain reactions (PCR) for RAPD products were conducted using a set of 8 arbitrary 10-mer primers (Operon Technology, Inc., Alameda, CA, USA) according to procedure described by Williams et al. (1990). Names and sequences of primers that gave bands are shown in Table 1.

RESULTS

Inbreeding and selection for fertility

Data of fertility percentage (seed set per 100 florets) are

Table 1. Names and sequences of primers that gave bands.

Primer	Sequence (5'→3')	GC (%)
OPA-03	5`- AGG GAA CGA G -3`	60
OPB-07	5`- AGA TGC AGC C -3`	60
OPN-04	5`- GAC CGA CCC A -3`	70
OPC-05	5`- GAT GAC CGC C -3`	70

Data were analyzed according to Steel and Torrie (1980).

presented in Table 2. It is observed in season 2003/2004 that selection for fertility in MT resulted in improved fertility of not only advanced inbreeding generations (I_o through I_1, I_2, I_3, I_4, I_5) but also in OP and to minor extent in ST. Improvement was more pronounced when comparing MT selections with original populations of both varieties. Data of 2004/2005, 2005/2006 and 2006/2007 were essentially similar to those of first season.

Again a confirmation of the simultaneous improvement of seed set in MT and OP. Besides, selfed families were generally more fertile. Ranges were wide in both varieties in the two seasons. Ranges were wider in MT compared with OP in the two varieties and seasons. It is interesting to observe that individual plants set seeds that reached 88% in MT and 92% in OP (Table 2). Highest value for ST was 21%. Also in both varieties and seasons (Table 2), spontaneous seed set under bagging (ST) was still on the average very poor compared to OP and MT.

Forage yield of selections

Data presented in Table 3 indicated that forage yield was improved by both selection for fertility and selection for good vegetative growth (before transplanting). Yield of cuts 2, 3 and 4 and total green yield was significantly higher in all selected populations compared to original parent. Besides one observe that the lowest yield was that of first cut. Forage yield improved in following cuts and was highest in fourth cut.

Almost all selections were better yielders than parents in cuts 2, 3, 4 and total yield. As found in green yield, data of dry yield was lowest in first compared to cuts 2, 3, and 4. Selections also performed better than parents and significantly produced higher dry forage. This occurred in both MT and OP systems of pollination. The MT I_8 populations had more total yield than OP (I_{7-o}) ones, but differences were not significant. Data of Ahaly variety (not presented) were essentially similar to data of S79 variety.

Plant height, tillering and flowering

The improvement occurring in fertility and forage yield drew our attention to search for reasons behind this improvement. Data in Table 4 showed that means of plant height and number of tillers were significantly improved for all selected populations compared to original parents. Similar trend was found for number of inflorescences per tiller. This indicated that not only, the basic tillering was improved, but also the high "branching" trait for each tiller. Number of inflorescences per plant indicated the significant improvements in this trait of all families compared to original parents (Table 4).

Compared to original S79 parent, the best family in each character expressed values that reached 110.3 in plant height, 197.8% in number of tillers per plant, 162.2% in inflorescences per tiller and 314.5% in inflorescences per plant. The corresponding values of the best family from Ahaly variety were 108.9, 171.2, 180.0 and 377.1%.

Flowers and seed setting

Frequency classes of number of flowers inflorescence^{-1} in the three pollination modes (OP, MT and ST) are presented in Table 5 for four growing seasons. None of the plants from any generation produced less than 60 flowers inflorescence^{-1}. Data of Ahaly variety (not presented) were essentially similar to those found in S79 variety; more flowers inflorescence^{-1} for OP system followed by MT and least were ST; also none of the plants produced less than 60 flowers inflorescence^{-1}. However, it was obvious that Ahaly variety produced less flowers inflorescence^{-1} than S79 variety. In both varieties too, it was clear that flowers inflorescence^{-1} were gradually increased by advanced generations. The three pollination modes had more flowers in 2006/2007 season than 2005/6 and last were more than 2004/2005 and 2003/2004.

Seed set is a reflection of numbers of inflorescences per plant and seeds per inflorescence. Data of 2006/2007 of both varieties presented in Table 6 showed that seed setting was greatly improved by selection for fertility. On average it reached to more 50% OP seeds per inflorescence. Due to high seed setting per inflorescence and the great improvement in number of inflorescences per plant (accompanying selfing and selection) seed set per head was improved by selection in advanced generations of inbreeding. Highest seed set head^{-1} was found in I_7 compared with earlier generations in the three modes of pollination. However best seed set was found in OP followed by MT and least was that of ST.

Table 2. Fertility means of selfed families selected for high fertility, their outcrossed sisters and the original parent (I_o) of S79 and Ahaly berseem varieties.

Season	Population	Families number	I_o parent			Selections			Range		
			OP	MT	ST	OP	MT	ST	OP	MT	ST
S79											
2003/2004		11	56.60	24.30	3.60	61.80**	30.40**	5.30	45.58-71.62	20.50-44.80	3.90-6.50
2004/2005	Selfed (I_6)	14	51.20	31.50	3.50	65.90**	57.40**	8.80**	28.00-88.00	24.00-88.00	3.00-21.00
	Outcrossed ($I_{5\text{-}o}$)	14				61.80**	55.50**	5.60**	28.00-88.00	18.00-88.00	1.00-20.00
2005/2006	Selfed (I_7)	14	53.75	27.00	5.70	66.04**	60.57**	8.68**	43.00-88.00	36.00-88.00	5.00-20.00
	Outcrossed ($I_{6\text{-}o}$)	14				64.07**	55.36**	8.00**	43.00-88.00	18.00-88.00	2.00-18.00
	Selfed (I_7)	8	54.00	31.50	4.00	73.80**	73.40**	11.60**	62.00-81.00	59.00-87.00	7.00-19.50
2006/2007	Selfed (I_8)	8	53.00	39.50	4.00	78.80**	71.30**	12.00**	74.00-84.00	63.00-80.00	9.50-17.00
	Outcrossed ($I_{7\text{-}o}$)	8	48.00	28.50	3.50	73.80**	71.60**	10.90**	62.00-81.00	62.00-87.00	7.00-14.50
Ahaly											
2003/2004	Outcrossed	11	54.70	16.00	3.30	61.60*	28.80*	4.50	52.10-76.00	24.10-36.50	3.70-5.50
2004/2005	Selfed (I_6)	14	42.00	24.80	3.50	69.60**	53.30**	6.80**	36.00-90.00	28.00- 81.00	2.00-12.00
	Outcrossed ($I_{5\text{-}o}$)	14				64.60**	49.50**	5.90 **	31.00-92.00	22.00-81.00	1.00-8.00
2005/2006	Selfed (I_7)	14	42.00	22.00	3.00	67.43**	54.96**	8.79**	35.00-85.00	18.00-82.00	3.00-16.00
	Outcrossed ($I_{6\text{-}o}$)	14				67.14**	56.79**	8.00**	28.00-88.00	28.00-77.00	2.00-18.00
	Selfed(I_7)	8	50.00	27.00	4.00	74.30**	68.40**	9.80**	62.00-81.00	59.00-87.00	7.00-19.50
2006/2007	Selfed (I_8)	8	50.00	23.00	3.00	73.60**	63.70**	10.40**	64.00-80.00	60.00-76.00	9.00-13.50
	Outcrossed ($I_{7\text{-}o}$)	8	48.00	27.00	4.00	73.80**	67.30**	9.80**	62.00-81.00	63.00-71.50	7.50-14.00

*,** Indicate significance at 5 and 1% levels of probability.

In last season both OP and MT were very close in seed set. Seed setting in I_8 selections reached more than 300% of seed setting of parents. Some selections in S79 gave 489.3% of parent and others of Ahaly 478.9%.

Flower organs in S79 variety and descendant selections

In 2006/2007 growing season, measurements of flower length and flower organs were studied at different stages for the variety S79 (I_o original and 10 selected descendants). Only the data of the "full blooming" stage will be presented. Measured inflorescences were chosen from top ones on the basis of equal age "visual judgment" and specific blooming" stage will be presented. Flowers possess five petals, two of them are united (keel) which is enclosed by two wings and fifth petal is standard. Data of measurement of floret and floret organs are presented in Table 7. When comparing data of I_o and selections, one observes that all floret measurements were increased in selections. Such increase was in favour of selections without single exception. It reached 146.6% in floret length, 165.5% in calyx, 116.7% in holder, 147.9% in standard petal, 127.2% in wing, 127.9% in keel, 127.6% in pistil and 134.5% in stamens in selections compared to I_o parent. In addition lower and upper limits of organs were higher in selection compared to I_o parent.

The data presented in Table 7 showed that the relative length of pistil and stamens (relative length of female and male sex organs) differed in the selections from I_o parent. Length ratio of female to male organs was 110.8% in I_o parent but this ratio decreased to 104.8%in selections. This reduction in the relative length means that I_7 plants had stigmas that were more close to the length of the stamens than I_o. This might have contributed to better fertility and seed set in I_7 plants compared to the original population.

Figure 1 shows the flowers of S79 variety in different stages. The figure shows the stucked pollen grains in the bud stage (before dehiscence) and after dehiscence. The relative length between female and male sex organs is observed. It is clear that female organ is longer than male one (see also data of Table 7). Such characteristic will

Table 3. Means of fresh (FW) and dry yield (DW) t fed^{-1} of selections and their outcrossed sisters as a percentage of I_o (original parent of S79 berseem variety.

Season	Selection	Cut 1		Cut 2		Cut 3		Cut 4		Total	
		FW	DW	FW	DW	FW	DW	FW	DW	FW	DW
2003/04	(I_{1-o}–I_{4-o})	130.30	136.99*	111.43	100.26	117.17	109.41	127.92	123.58	119.79	119.80
	(I_1 – I_5)	123.75	123.53	120.38*	131.15*	109.43	94.52	-	-	126.23	108.80
2004/05	(I_{1-o} – I_{5-o})	159.24**	122.16**	129.64**	132.48**	119.50**	137.01**	103.46**	117.24**	114.48**	126.89
	I_6	129.08*	180.73**	243.04**	260.39**	-	-	-	-	196.11**	183.19**
	I_{5-o}	122.06*	170.87**	199.29**	213.54**	-	-	-	-	167.47**	156.41**
2005/06	I_7	159.62**	164.42**	284.14**	212.32**	244.50**	244.57**	-	-	239.73**	225.16**
	I_{6-o}	153.05*	157.67*	258.00**	205.57**	226.89**	226.90**	-	-	222.07**	211.94**
2006/07	I_8	150.73*	151.72*	177.22**	182.89**	181.22**	176.75**	198.91**	189.11**	176.79**	184.85**
	I_{7-o}	160.20**	164.94**	192.76**	204.29**	205.29**	196.30**	226.75**	214.81**	195.14**	204.21**

*,** Indicate significance at 5 and 1% levels of probability respectively.

Table 4. Effects of inbreeding (I_8) and selection on plant height, tillering and flowering plant^{-1} of both varieties (2006/2007).

Family	Plant height	No. of tillers	No. of inflorescences tiller^{-1}	No. of inflorescences^{-1}
			S79	
Original	75.0	20.8	4.5	93.9
Family mean	82.7**	33.3**	6.1**	209.8**
Range	79.8-85.0	31.1- 41.2	5.2-7.3	181.4-295.3
			Ahaly	
Original	73.0	24.0	4.0	95.4
Family mean	79.5**	35.6**	6.4**	234.5**
Range	77.5-83.5	33.7-41.1	5.9-7.2	204.5-268.3

** Indicate significance at 1% level of probability; values are means of two replications.

favour cross pollination.

Pollen grains, ovary and fertilization

Pollen grains were counted in different flowers of I_o and selected descendants of variety S79. Number of pollen grains per sac ranged from 300 to 420 in I_o florets. In selected plants number of pollen grains per sac ranged from 460 to 500. This indicated that total pollen grain production was improved in each sac by about 20-30 % in I_7 compared to I_o plants. Pollen grains stain ability was more than 96%. When pollen grains reach styles, they germinate and pollen tubes traverse styles in bundles to the ovary to fertilize the ovules resulting in the successful fertilization of fruits possessing two seeds each (Figure 2).

Table 5. Number of florets inflorescence^{-1} in 3 modes of pollinatios of S79 variety in 4 growing seasons.

Season	Modes of pollination	*Classes of flowers/number inflorescence						Mean
		<80	<100	<120	<140	<160	>160	
	OP	16	38	31	12	3	-	92
2003/2004	MT	17	38	35	10	-	-	89
	ST	20	45	26	9	-	-	86
	OP	15	37	30	8	10	-	104
2004/2005	MT	10	48	22	12	4	4	98
	ST	18	40	32	8	1	-	90
	OP	18	32	22	10	11	7	122
2005/2006	MT	14	30	22	14	14	6	108
	ST	17	44	20	19	-	-	94
	OP	8	18	21	25	20	13	136
2006/2007	MT	5	22	35	14	18	6	117
	ST	7	29	36	17	8	3	106

*100 plants were tested from each of OP, MT and ST in each season.

Table 6. Effects of inbreeding (I_8) and selection on seed set of selected families of both varieties (2006/2007).

Family	No. of OP seeds inflorescence^{-1}	No. of seeds plant^{-1}
	S79	
Original	47.2	4571.0
Fam. mean	71.0**	15363.0**
Range	66.3-79.7	12045-22366
	Ahaly	
Original	49.2	4762.0
Fam. mean	71.9**	17306.0**
Range	69.2-83.2	13691-22808

*, ** Indicate significant at 5% and 1% level of probability respectiveyl; Values are means of two replications of 15 plants plot^{-1}.

Table 7. Ranges and averages (± standard error – SE) of floret and floret organs (mm) of full blooming flowers of the original and 10 seletions descendent of S79 variety (2006/2007).

Floret parts	*I_o			*Selections (I_8)		
	Range	Average	SE	Range	Average	SE
Holder	5.0-6.0	5.43	± 0.09	5.0-7.0	6.30	±0.120
Floret	8.0-17.0	11.83	± 0.35	14.0-20.0	17.30	±0.330
Calyx	5.0-6.0	5.57	±0.09	8.6-10.0	9.19	±0.100
Standard	8.0-17.0	11.7	± 0.38	14.0-20.0	17.30	±0.330
Wing	3.3-5.1	4.01	±0.07	4.7-5.4	5.10	±0.040
Keel	3.2-5.0	3.91	±0.07	4.6-5.4	5.00	± 0.040
Pistil	3.4-5.0	4.10	±0.06	4.8-5.5	5.23	±0.040
Stamen	3.0-4.2	3.71	±0.06	4.4-5.3	4.99	±0.050

*3 flowers were tested from OP pollination mode from each of 10 plants per Io and selection-1.

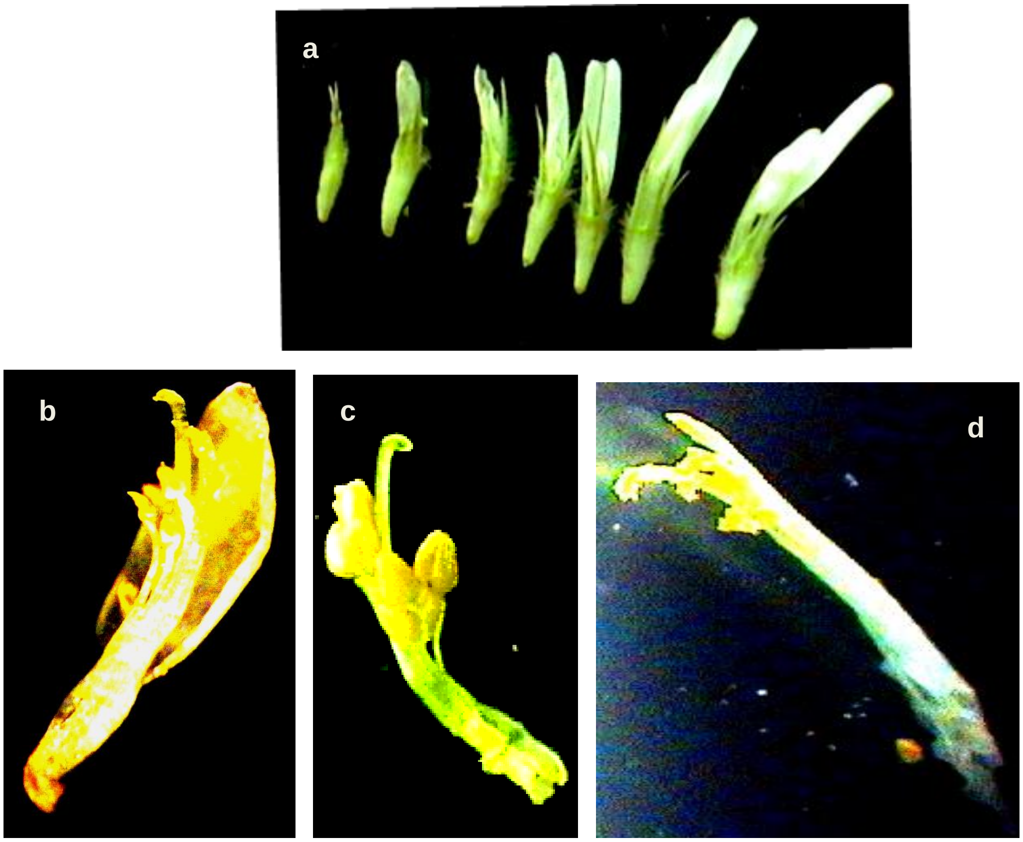

Figure. 1. Flowers of S79 variety at different stages a) seven flowers at three stages (bud, mid and full blooming), b) sex organs within keel of Io floret (pre-bud stage), c) sex organs of Io floret (post-bud stage) and d) I4 floret after dehiscence.

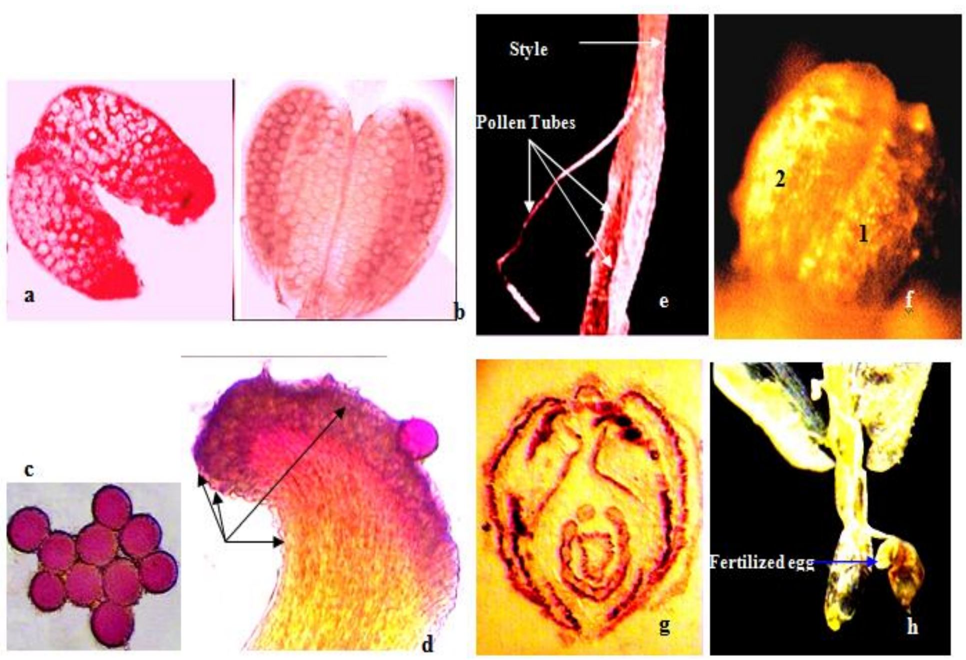

Figure 2. Flower structure and fertilization: a) pollen sacs (external view), b) internal view, c) stainable pollen grains, d) bubbles on stigma, e) pollen tubes traversing style, f) ovary with two ovules, g) trans section I7 floret and h) fertilized egg within ovary.

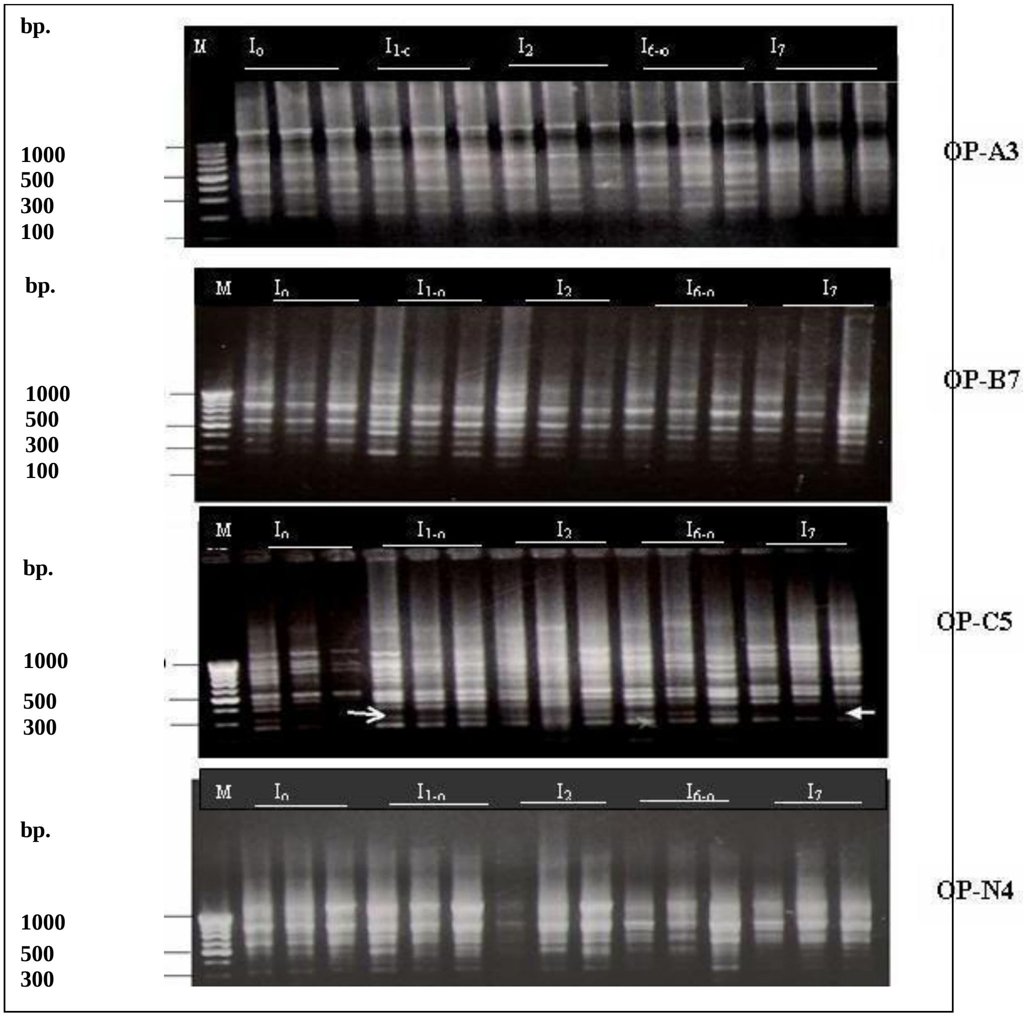

Figure 3. RAPD profiles of 5 generations after selection for self fertility, Io (lanes 2-4), I1-o (lanes 5-7), I2 (lanes 8-10), I6-o (lanes 11-13) and I7 (lanes 14-16) as generated by RAPD-PCR using different primers, M is a 100 bp DNA ladder.

Figure 2 shows the ovary at different stages. The I_7 floret ovary has two lobules with two ovules spread by incomplete (false) septum. The figure shows stainable pollen grains, bubbles on pistil and stigma and germinating pollen tubes. The transaction in floret showing all flower organs showed I_7 plants to have 10 stamens closely connected surrounding pistil. The presence of bubbles and suprastaminal pistil are structures that favours cross pollination and hinder self-pollination. These parries have to be removed to effect pollination. This may occur due to insect visits and tripping.

MOLECULAR CHARACTERIZATION

Molecular characterization was tried to differentiate between I_7 high MT fertile and OP populations using different techniques. Total number of bands generated by SDS-PAGE technique was seventeen which were not necessarily present in all genotypes. Two bands (Nos. 5 and 12) were found only in the electrophoregram of I_o population and absent from the electrophoregrams of high fertile I_7 plants.

Polymorphism was apparent in peroxidase isozyme banding patterns of different genotypes. I_6-$_o$ population characterized by high fertility two anodal and one cathodal band which was identical to I_o parent, whereas two cathodal and one anodal band were present in the zymogram of the I7 generation (high MT-low ST) which were not originally found in the open pollinated populations. These bands may be related to high fertility in selfed materials. The RAPD-PCR technique was also adopted. Four random primers (OP-A3, OP-B7, OP-N4 and OP-C5) used to develop banding pattern were successfully able to amplify scrabble bands (Figure 3). All OP plants in all generations exhibited the same RAPD banding patterns using all primers as shown in Figure 3 where all bands were monomprphic. When comparing the RAPD profiles obtained from selfed generations (I_2-I_7), polymorphic differences were noticed in the absence of a RAPD fragment of about 350 bp when plants were amplified using OP-C5 primer. This fragment may be used to distinguish between self-fertile and non-fertile plants.

DISCUSSION

Differences in fertility-incompatibility which affects seed set in berseem led to controversial data of several authors. Some considers the species as self-incompatible (SI) or self-sterile (SS) without direct documentation to self-incompatibility (Zaher, 1947; Said, 1954; Latif et al., 1956; Tanash, 1970; Bakhiet, 1989) and others as self-compatible (SC) (khan and Bahati, 1953; Finn, 1964; Chowdhury et al., 1966; Rammah, 1969; Dixit et al., 1989; Rotili and Gnocchi, 1989; Miawad, 1992; El-Shahawy and Gheit, 2001; Abd El-Naby, 2003; Roy et al., 2005; Abdalla et al., 2008; Beri et al., 1985a) assured self-fertilization of the crop, but Abberton (2007) mistaken *T. alexandrinum* as self-incompatible perennial. In the present studied materials which were handled for four seasons (after I_4), it was apparent that seed set rarely occurred when flowers were bagged. After manual tripping bagged inflorescences set seed freely. Also under open pollination seed set was good indicating the materials to be self-compatible but of cross-fertilizing nature, it needs a tripping agent to result in self- set seed. The enigma and controversial data publishedon *T. alexandrinum* concerning fertility, selfcompatibility and self-incompatibility were explained by Abdalla et al. (2008) assuming the species (or its progenitor) was self-incompatible that has been forced to inbreeding. However remnant characteristics that favour cross pollination may be still functioning in different populations. These may be associated with flower structure, self-incompatibility genes (with gametophytic or sporophytic function), unilateral incompatibility genes, female incompatibility genes, male sterility genes. Structures assumed to favour cross-pollination are relative position of pistils and stamens and presence of bubbles on style and stigma as shown in Figure 2. Improved seed set that occur from tripping may be due to dispersal of pollen grains from sacs, rupture of bubbles and or pollination in bud stage. That is why the MT system results in more seed set than ST system. It was apparent from data obtained that selection for high self-fertility through inbreeding and MT was accompanied by good vegetative growth. This means that berseem breeders will be able to select for high seed setting without loosing good vegetative growth. Comparing between selfed MT and OP stocks showed MT to have better vegetative performance. This indicated that reducing heterozygosity (expected in MT inbreds) did not result in noticeable inbreeding depression. Reasons to explain this enigma may be: a) inbreeding and selection for high SF was also accompanied by selection for good vegetative growth. In other words excluding deleterious inbred segregants, b) both fertility and vegetative characters are controlled by dominant genes that could be fixed in inbreds and OP populations, c) slow inbreeding effects (selfing tolerance) as found when comparing MT-MT with OP-OP which showed good performance of MT and, (d) deleterious characteristics are simply inherited and were greatly wiped out in early generations (I_1- I_3) of inbreeding during earlier selection for SF using MT mode of pollination.

An approach was therefore suggested by Abdalla et al. (2009) to develop improving composite populations. It is based on (a) screening and selection in adapted germplasm for high fertility and good forage yield till I3 generation, (b) selection for combining ability from data of OP sisters, (c) selection from I4 (and OP) for both self-fertility, and vigorous forage yield and (d) best performing materials with high combining ability are blended to provide first composite. It is apparent from the data that inbreeding coupled with selection improved seed setting. This was mainly due to production of more tillers / plant and more inflorescences per plant (Table 4) besides more flowers per inflorescence (Table 5). This profuse flowering will ultimately result in more seed setting per plant (Table 6). The profuse flowering, the abundance of pollen grains production, the viability of pollen and greater florets extraction and composition (data not presented) in addition to the increase of flower organs (Table 7), will introduce more insect visits. This will result in improving self-fertilization (due to tripping) and cross-fertilization, result of which will be more seed set per plant.

REFERENCES

Abberton MT (2007). Interspecific hybridization in the genus *Trifolium*. Plant Breed. 126: 337-342.

Abd El-Naby ZM (2003). Breeding Studies in Berseem (Egyptian clover) Populations. M.Sc. Thesis, Fac. Agric. Cairo Univ.

Abdalla MMF, Hoballah AA, Radwan MS, Soliman AM, Rammah AM, Abd El-Naby ZM (2008). Inbreeding and fertility in Egyptian clover, *Trifolium alexandrinum.* 1- Enigma of compatibility and selection for fertility. Egypt. J. Plant Breed., 12(2): 207-223.

Abdalla MMF, Hoballah AA, Radwan MS, Soli-man AM, Rammah AM, Abd El-Naby ZM (2009). Inbreeding and fertility in Egyptian clover, *Trifolium alexandrinum.* 2. Forage yield of high self-fertile selections. 9th International, Plant Breed Conference, Ismalia, Egypt. May 3-5, pp. 484-513.

Bakheit BR (1989). Pollination and seed setting in different genotypes of Egyptian clover (Berseem, *Trifolium alexandrinum* L.). Assiut J. Agric. Sci., 20: 199-208.

Beri SM, Sohoo MS, Sharma HL (1985a). Estimates of natural cross pollination in Egyptian clover. J. Euphytica, 34: 147-151.

Beri SM, Sohoo MS, Sharma HL (1985b). Mode of pollination and seed setting in Egyptian clover. J. Euphytica, 34: 745-750.

Chowdhury JB, Mehta RK, Joshi AB (1966). Pollination in berseem. Ind. J. Genet., 26: 118-120.

Dixit OP, Singh UP, Gupta JN (1989). Significance of bee pollination in seed setting efficiency of berseem (*Trifolium alexandrinum* L.). J. Agron. Crop Sci., 162: 93-96.

El-Shahawy AE, Gheit S (2001). Inbreeding effect on Egyptian clover (Berseem), *Trifolium alexandrinum* L. J. Agric. Sci. Mansoura Univ. 26 (1): 51-62.

Evans AM (1976). Clovers. In Evolution of Crop Plants. N. W. Simmonds (ed). Longman, London and New York, pp. 175-179.

Finn KH (1964). A contribution to the biology of flowering of Egyptian clover (*Trifolium alexandrinum* L.). Z. Aker-und-Pflanzenbau. 119: 227-240.

Iannucci A (2004). Effect of generation of inbreeding, cutting treatment and year on agronomic traits in berseem populations. J. Euphytica, 136: 103-113.

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

Khan A, Bhatti AG (1953). Floral development, mode of pollinaton and seed setting studies in Berseem. Proc. 5th Pakis., Sci., Conf., Lahore. Part III, p. 208.

Latif A, Manzoon-ul-Hag , Quyyum A (1956). Role of *Apis indica* in the pollination of Egyptian clover (*Trifolium alexandrinum* L.). Pak. J. Sci. Res., 8: 48-50.

Leammli UK (1970). Cleavage of structural proteins during the assembly of the head bacteriophage T4. J. Nat., 227: 680-685.

Mahdy EE, Bakheit BR (1985). The inheritance of forage yield in Egyptian clover (*Trifolium alexandrinum* L.). Alex. Sci. Exchange, 6: 114-127.

Mehta RK, Suramanyam KN, Swaminathan MS (1963). Studies on induced polyploids in forage crops.III. Growth, cytology behaviour and seed fertility of C_1, C_2 and C_3 cultures of berseem. Ind. J. Genet. Plant Breed, 23(1): 67-81.

Miawad NS (1992). Studies on The Pollination of Some Varieties of Egyptian Clover (*Trifolium alexandrinum* L.) by Honey Bees in Middle Egypt. M.Sc. Thesis. Fac. Agric., Minia Univ.

Rammah AM (1969). Breeding Studies on Berseem Clover (*Trifolium alexandrinum* L.) M.Sc. Thesis, Fac. Agric. Cairo Univ.

Richards KW (1995). The alfalfa leafcutter bee, *Megachile rotundata*: A potential pollinator for some annual forage clovers. Res. Center, Agric. and Agric. Food Canda, Lethbridge, Alberta, Canada, pp. 307-308.

Rotili P, Gnocchi G (1989).The effect of selfing on *Trifolium alexandrinum* measured in conditions of interference among the plants. Proc. of the XVI Int. Grass. Cong., Nice, France, pp. 307-308.

Roy AK, Kaushal P, Malaviya DR (2005). Pollination behavior among different breeding populations of Egyptian clover. J. Plant Breed., 124: 171-175.

Said E (1954). A Study of Flowering and Factors Influencing Seed Setting in Berseem or Egyptian Clover (*Trifolium alexandrinum* L.). M. Sc. Thesis, Fac. Agric., Cairo Univ.

Sohoo MS, Sandhu JS, Beri SM (1994). Inbreeding depression in Egyptian clover. Crop Improv., 21: 81-83.

Steel RGD, Torrie JH (1980). Principals and Procedures of Statistics, 2nd Edition Mc Graw-Hill Book Company, Inc., New York, New York, U.S.A.

Tanash LF (1970). Self-Incompatibility in Egyptian Clover (*Trifolium alexandrinum* L.) Preliminary studies in var. Fahl. M.Sc. Thesis. Fac. Agric. Cairo Univ.

Wafa AK, Ibrahim SH (1960). The effect of honey bees as a pollinating agent on the yield of clover and cotton. Bull. Fac. Agric., Cairo Univ. 206: 3-44.

Willey RL (1971). Microtechniques, A Laboratory Guide Mcmillan Publishing Co., Inc., New York, p. 99.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531–6535.

Zaher A (1947). Morphological Studies on *Trifolium alexandrinum* L. M.Sc. Thesis, Fac. Agric. Cairo Univ.