American-Eurasian J. Agric. & Environ. Sci., 16 (3): 432-441, 2016
ISSN 1818-6769

DOI: 10.5829/idosi.aejaes.2016.16.3.12883

Fertility and Seed Setting of Ten Alfalfa Genotypes

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Abstract: Alfalfa (*Medicago sativa* L.) is among the most important forage crops worldwide. In order to know the reason of its low seed production, morphological and phenological experiments were conducted to investigate the fertility among eight local and tow exotic alfalfa genotypes and to follow floret development from budding stage till set seed. Results showed highly significant differences among alfalfa genotypes for plant height, number of tillers plant^{-1}, number of branches tiller^{-1}, number of inflorescences tiller^{-1}, number of floret inflorescence^{-1}, number of pods tiller^{-1}, number of seeds pod^{-1} and 100 seeds weight. The local genotype (Siwa) had the highest values of number of branches tiller^{-1} (14.0), number inflorescences tiller^{-1} (16.0), number of florets inflorescence^{-1} (27.0) and number of pods tiller^{-1} (60.33). Whereas, the exotic genotype (CUF-101) recorded superior values over all studied genotypes in plant height (71.97cm), number of tillers plant^{-1} (51.00), number of branches tiller^{-1} (13.00), number of inflorescences tiller^{-1} (15.67), number of florets inflorescence^{-1} (25.00), number of pods tiller^{-1} (57.00), number of seeds pod^{-1} (14.00) and 100 seeds weight (0.353 g). The results showed that the number of seeds pod^{-1} ranged from 7.00 to 15.00 with an average of 11.03 seeds. Variation in number of seed setting pod^{-1} between genotypes may be related to the difference of ovule size and number of ovules ovary^{-1} between genotypes. Alfalfa florets across genotypes were examined from budding stage to seed development and maturity. The study concluded that formation and successful seed set, do not only involve tripping but also the introduction of fertile pollen from other plants to induce pollination and fertile ovules to seed formation, i.e. cross pollination. Studied genotypes had fertile pollen grain, fertile ovules and compatible pollen stigma interaction under Giza conditions. Seed setting was not limited to pollination but presence of honey bees nurseries as an effective reason to increase seed setting of alfalfa field at blooming period.

Key words: *Medicago sativa* · Floret · Fertility · Pollination · Seed setting

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a cross pollinating crop (2n=4x=32). It is one of the most important perennial legume crops and a superior source of forage due to its high nutritional quality and herbage yield [1, 2, 3]. Seed yield of alfalfa is important concerning distribution of new cultivars to farmers [4, 5]. Alfalfa in Egypt is important because it plays a vital role of balancing crop production in three ways; by building soil fertility, by furnishing a high quality feed for livestock and by exporting seeds to other countries. Breeding and development of alfalfa varieties play an important role in alfalfa production in Egypt as well as in other countries around the world. Alfalfa is the best overall commonly grown hay crop because it is high in protein and minerals and it is a good source of vitamins [6]. In Egypt, an alfalfa seed is produced when the climate is conducive to the production of high yields with high quality seed alfalfa. Pedersen [7] used the variation in flower colors (white, cream, yellow and purple) among alfalfa varieties as a gene marker and measured the seed setting percentage. Alfalfa seed production is dependent on bees insect, it makes tripping, which is required for pod setting, both tripping and cross pollinating necessary for commercial seed production [8]. Effect of genetic factors and ecological conditions on

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alfalfa seed production has been extensively studied [4, 9, 10]. Tripping is the process whereby the staminal column is released from its attachment to the keel and wing petals and is permitted to strike against the standard petal [11, 12]. Number of ovules per ovary represents the potential ability of a floret to produce seeds. Thus, it appears that selection of plants with a high number of ovules per floret should result in maximum seeds per pod [13]. Wang *et al*. [14] recorded that the actual seed yield of alfalfa is low and the sterility of ovules is possibly just one of the limiting factors for maximum seed production. Lorenzetti [15] reported that the realized seed potential of alfalfa was only 4% of the potential seed yield. Many external and internal factors affected seed development in alfalfa plants, *i.e.* atmospheric humidity, soil moisture, climatic conditions specially temperatures, stresses, carbohydrates accumulated, definitely in the later stages as in the earlier stages of the flowering period, pollen sterility, ovule sterility, pollen stigma interaction and so in.

The current study was a part of a program limit to assess internal factors affected alfalfa pollination and seed production under Egyptian conditions. The main question in this study: why alfalfa had a low seed production?. The aims of this study were to: (i) determine the agronomic and fertility variations between local and exotic genotypes and (ii) study the development and maturity stages of pollen grain, ovules and seeds from budding till seed set.

MATERIALS AND METHODS

Alfalfa Sources: Ten genetically divergent alfalfa (*Medicago sativa* L.) genotypes were investigated in the Alfalfa Breeding Program at Giza Research Agricultural Station, Agricultural Research Center (ARC), Giza, Egypt during 2011 till 2014 growing seasons. Eight local alfalfa genotypes, namely Siwa, Ismaelia-1, Ismaelia-94, Fixed-N, New Valley-1 and Valley-2 and two United States genotypes, namely CUF-101 and US Storm. On December 19th, 2010 season; the ten genotypes were planted in a randomized complete blocks design (RCBD) with three replications. Genotypes were sown in two 4-meter long rows and 0.25 cm apart, the distance between different genotypes was 1.0 m. Each genotype was seeded at the rate of 5 g/row (20 kg feddan^{-1}, one feddan = 0.42ha). Plots received 60 kg/fed of P_2O_5, 96 kg/fed of K_2O before emergence and 20 kg fed^{-1} nitrogen after emergence. Irrigations and fertilizers were applied according to the standard recommendation. The first cut was taken on February 24th, 2011 and ten cuts were taken. Ten random plants from each examined genotype were selected visually for vigorous growth. On March 5th 2012, the plants were transplanted in black cylinders constructed from polyvinylchloride (PVC) pipe. The design of the cylinders was described by Le Noble *et al*. [16] and modified by Vaughan *et al.* [17]. Each cylinder was 100 cm length and 10 cm in diameter, with a wall thickness of 3 mm. The soil used was a fine sand, clay and Peatmos mixture (1:1:1 v/v/v). Two alfalfa plants were placed in each cylinder. The cylinders were arranged in randomized complete block design (RCBD) with five replicates.

Plant Measurements: After two months of transplanting plants into cylinders plants were cut and examined for seed set and fertility characteristics in two growing seasons (2013 and 2014). Plant height, number of tillers plant^{-1}, number of inflorescences tiller^{-1}, number of florets inflorescence^{-1}, number of pods tiller^{-1}, seeds pod^{-1} and weight of 100 seeds for ten individual plants were recorded.

Morphology of Florets: At the flowering stage, ten random racemes genotype^{-1} were used for checking floret, stamens, pollen sac, anther dehiscence, pollen grain, stigma, ovary and ovules at different blooming times before and after pollination for the study of floret structure, development, fertility and setting seeds. Floret has been dissected for imaging its components. Images of floral features were captured using a Nikon Coolpix 950, Studio Microscope, digital camera and modified where necessary using Adobe Photoshop to enhance picture quality.

Anatomical Study: The present study counts the ovules and seeds number in alfalfa ovary of 30 florets plant^{-1}. Specimens of alfalfa floret ovary were taken at the mature stage. Specimens were killed and fixed for at least 48 hrs in F.A.A. (10 ml formalin, 5 ml glacial acetic acid and 85 ml ethyl alcohol 70%). The specimens selected materials were washed in 50% ethyl alcohol, dehydrated in a normal butyl alcohol series, embedded in paraffin wax of melting point 56°C, sectioned to a thickness of 20 microns, double stained with crystal violet-erythrosin, cleared in xylene and mounted in Canada balsam [18]. Slides were microscopically examined.

Statistical Analysis: Data analysis was performed using analysis of variance in SAS software [19].

RESULTS AND DISCUSSION

Fertility Traits: The analysis of variance (Table 1) revealed highly significant difference (P= 0.01) among the studied genotypes for plant height and No. tillers plant^{-1}, No. branches tiller^{-1}, No. inflorescences tiller^{-} ,^{1}No. florets inflorescence^{-1}, No. pods tiller^{-1} and No. seeds pod^{-1} and in addition to 100 seeds weight. Also, year x genotypes interaction was high significant difference for all studied traits. This result confirms the variation among alfalfa genotypes used in the present study. Plant height ranged between 55.67 and 77.30cm with an average of 67.02cm. Ismaelia-1 genotype was the tallest one (77.30 cm) followed by Siwa and CUF-101 (75.50 and 71.97cm, respectively), whereas New Valley-2 (55.67 cm) was the shortest one. Genotypes (CUF-101, US Storm and Siwa) had more tillers number (51.00, 45.00 and 40.00, respectively), while Nubaria has the lowest number of tillers (14.33). No. of branches varied from 7.33 to 14.00 with an average of 10.87 across all genotypes. Siwa genotype had the largest one (14.00) followed by both of CUF-101 (13), US storm (13.00) and both of Ismaelia-1(12.00), Ismaelia-94 (12.00), respectively, whereas Nubaria genotype had the lowest number (7.33) of branches (Table 2). Studied genotypes varied in number of inflorescences tiller $^{-1}$ and ranged from 16.00 for Siwa genotype to 7.00 for Nubaria genotype with an average of 11.93 inflorescences tiller^{-1}. Genotypes confirmed wide variation of the number of florets inflorescence^{-1} and ranged from 10.67 for Nubaria genotype to 27.00 for Siwa genotype with an average of 19.40 floret. These results are in harmony with those of Boulus [20]. Bodzon [21] reported seed yield plant^{-1} was positively associated with flower number raceme^{-1}.

New Valley-2 genotype had low values of number of pods tiller^{-1} (34.33) may be due to the early blooming. Siwa, CUF-101 and Ismaelia-1 genotypes had large number of pods tiller^{-1} (60.33, 57.00 and 56.00, respectively). Number of seeds pod^{-1} ranged between 7.33 for Nubaria genotype to 14.00 for CUF-101 genotype with an average of 11.03 seeds. Variation in 100 seed weight ranged from 0.270 for both of Nubaria and New valley-2 genotypes to 0.353 for CUF-101 genotype with an average of 0.310g across all studied genotypes. Variation in 100 seeds weight may be related to seed size whereas; variation in number of seeds pod^{-1} may be related to ovary size, number of fertile ovules ovary^{-1} and number of aborted ovules. Ovary size and number of ovules ovary^{-1} depend on genetic variation between studied genotypes. Seed size in herbage legumes mainly depends upon genetic factors mentioned by Sengul and Sağsöz [22], whereas Lorenzetti [15] reported that variation among alfalfa genotypes is generally small. Siwa genotype gave the highest value of No. of branches tiller^{-1} (14.00), No. of inflorescences tiller^{-1} (16.0), No. of florets inflorescence^{-1} (27.0), No. of pods tiller^{-1} (56.33) and No. of seeds pod^{-1} (13.00), also Ismaelia-1 recorded superior values among the studied genotypes in plant height (77.30 cm) and number of; tillers plant^{-1} (29.00), branches tiller^{-1} (12.00), inflorescences tiller^{-1} (14.33), florets inflorescence^{-1} (22.00), pods tiller^{-1} (57.00), seeds pod^{-1} (12.33) in addition to 100 seeds weight (0.320g). The local selected genotypes Siwa, Ismaelia-1 and Ismaelia-94 were considered as local promising higher seed yield genotypes (Table 2).

Floret Development, Structure and Sexual Organs: The inflorescence is an oval or rounded raceme, (Fig. 1), with purple or violet and light-purple color [23]. In the present study, the flowering time of alfalfa was from mid-May to late-June and pods became ripe in July [24]. McGregor [25] concluded that blooming period takes a week from budding to full-blooming. The stages of alfalfa floral development were divided into three vital stages: (a) budding calyx exposed petals, (b) mid -blooming and (c) full-blooming thus permitting the floret to open (Fig. 2). The keel then bends with a spring and strikes the bundle of stamens, causing the pollen to fall down from mid-blooming stage till pre full-blooming stage and each of these stages was passed on two steps, pre and post (Fig. 2). Wang *et al.* [14] demonstrated that the flower bud developing period were divided into five stages according to Liu's method [26]: (1) calyx enwraps the petals, (2) petal appears between calyxs, but do not yet exceed sepal, (3) the length of petals exceed calyx more than 2 mm, but the keel is still enwrapped by vexillas, (4) keel has appeared in the vexilla, but the vexilla has not yet turned up and (5) flowers open, the vexilla turned up and the keel has been exposed between the wings. The present results indicated Liu's method [26], that these stages of floret development period were take about 48-72 hr according to temperature, humidity and other environmental effects, which agreed with Liu's method [26]. In other words at the fourth stage (post mid blooming) keel has appeared in the vexilla, but the vexilla has turned up, dehisces beginning with higher chance of self-pollination and florets ready to receipt bees for tripping. At the fifth stage (pre-full blooming) (Figs. 2 and 3), floret was opened and the keel has been exposed

Table 1: Analysis of variance for the studied characters of ten alfalfa genotypes over two seasons

		MS							
SOV	df	Plant height plant^{-1}	No. of tiller plant^{-1}	No. of branches plan^{-1}	No. of inflorescences tiller^{-1}	No of florets inflo.$^{-1}$	No. of pods tiller^{-1}	No. of seed pod^{-1}	100 seeds weight
Year (Y)	1	*	**	NS	**	**	**	**	**
Rep.	2	NS	NS	NS	NS	NS	NS	NS	NS
Genotypes (G)	9	**	**	**	**	**	**	**	**
Y x G	9	**	**	**	**	**	**	**	**
Error	18	-	-	-	-	-	-	-	-

NS: not significant. *, **: indicate significance at 0.05 and 0.01 levels, respectively.

Table 2: Means of vegetative and fertility traits of eight local and two exotic alfalfa genotypes over two seasons

	Vegetative traits			Fertility traits				
Genotypes	Plant height plant^{-1}	No. of tiller plant^{-1}	No. of branches plan^{-1}	No. of inflorescences tiller^{-1}	No of florets inflo.$^{-1}$	No. of pods tiller^{-1}	No. of seed pod^{-1}	100 seeds weight
Siwa	75.50b	40.00c	14.00a	16.00a	27.00a	60.33a	13.00ab	0.337b
Nubaria	57.60i	14.33 j	7.33g	7.00g	10.67h	38.00f	7.33f	0.270e
Balady	64.53g	22.67 h	10.67c	9.00e	16.33f	45.00d	11.00c	0.310c
Ismaelia-1	77.30a	29.00d	12.00b	14.33c	22.00d	56.00b	12.33b	0.320c
Ismaelia-94	67.63f	28.00e	12.00b	14.33c	21.67de	46.67d	12.33b	0.313c
Fixed-N	69.03d	24.67 f	9.00e	9.33e	16.00f	44.00e	9.00e	0.280d
New valley-1	62.30h	24.00 g	9.67d	11.00d	20.33e	44.00e	10.00d	0.280d
New valley-2	55.67j	20.00i	8.00f	8.00f	11.33g	34.33g	9.00e	0.270e
CUF-101	71.97c	51.00a	13.00ab	15.67b	25.00b	57.00b	14.00a	0.353a
US storm	68.70e	45.00b	13.00ab	14.67c	23.67c	52.33c	12.33b	0.317c
Mean	67.02	29.87	10.87	11.93	19.40	47.77	11.03	0.310

Means in each column followed by similar letters are not significantly different at 5% level

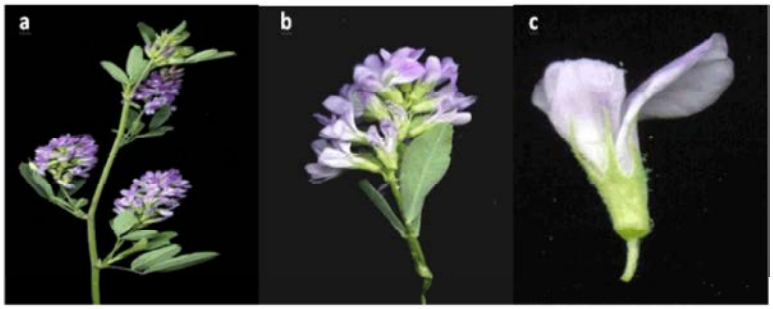

Fig. 1: Alfalfa inflorescences floral arrangement (a) racemes in plant branch, (b) florets arrangement on inflorescence and (c) full blooming floret

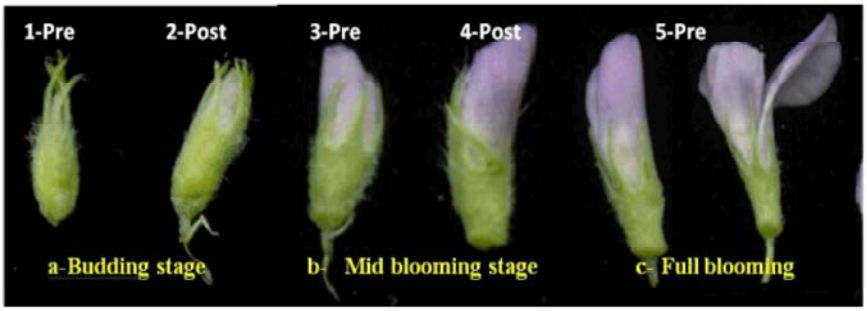

Fig. 2: a- Budding stage: 1 and 2 calyx enwraps the petals, b- mid-blooming stage: 3 and 4 petal appears between calyxs and the calyx taller than petals, c- full-blooming stage: 5 florets open, the keel exposed between the wings

between the wings and pistil to receipt more fertile pollen grains from other florets of the same plant or from different plants by bees (open pollination). Then in the post blooming stage seeds were developed during 60 to 70 days, after fertilization, then mature seeds were produced. These results are in agreement with those obtained by Aurelija *et al.* [27]. The staminal tube and anthers observed in Fig. 3 show that, the ten filaments not fused into a tube, which surrounds the pistil. The filament and the pistil surrounds by thin membrane. The cells on both filaments are of about the same shape and dimension, being oblong and united by oblique ends. When the flower is tripped, the staminal tube is instantly released and assumes a rigidly curved form.

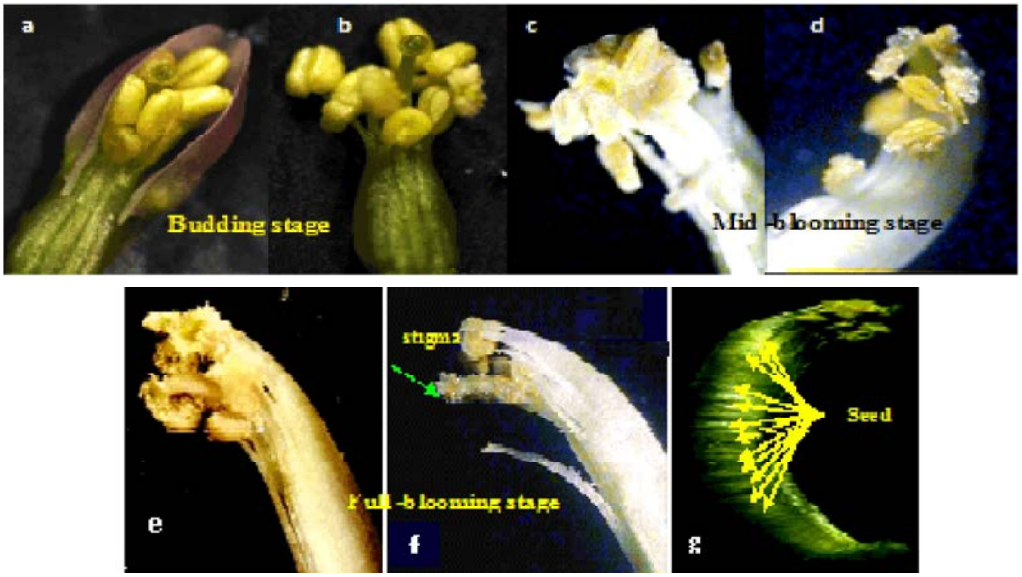

Fig. 3: Sexual parts of alfalfa floret at: (a and b) budding stage, (c and d) mid-blooming stage and (e and f) pollen grain penetrate the stigma and (g) seeds development within ovary

When once curved, it is impossible, without causing a transverse fracture, to straighten the tube so as to make it assume its original position, however, whether the curvature resulted from the contraction of the cells on the concave side or the distention of those on the convex side. The explosion was due to tension of the upper portion of the filaments in the staminal tube. Bosch and Kemp [28] refer to the possibility of pollination accomplished on younger florets, which is agreed with the present study. Pollen grains were transferred from a separate floret to other florets. It is very improbable that the pollen can blow from one flower to the other by dehisces pressure, bees and other insects. Pollen grains in alfalfa are tricolporate, the structure of a pollen grain showed dense cytoplasm surrounded by thick wall and three thin wall regions (germ pores: G1, G2 and G3, illustrated in Fig. 4, d-2 and d-3).

Figure 5 shows some stages of seed development from stigma receipt for pollen grain in pre-full blooming stage (Fig. 5-a), pollen sac was empty after dehiscence (5-b), mature ovules within ovary (Fig. 5-c) till (Fig. 5-d) and to produce mature seeds. Under the conditions of this study alfalfa genotypes had two blooming periods, the first was from Mid-April till late-June and pods became ripe in July whereas, the second was from Mid-August till Mid-October and pods became ripe in November. Number of ovules ovary^{-1} was varied between and within genotypes among blooming period. Earliness or delay in flowering produced lower number of ovules ovary^{-1} ranging from 3 to 7 (Fig. 6-2c, d and e). Whereas, in activate flowering (≥50%) ovary produced the highest number of ovules ranging from 6 to 20. The mean number of seeds (fertile ovules) pod^{-1} across all studied genotypes recorded 11.37 seeds (Table 1). Wang *et al.* [14] counted the number of ovules per ovary in nine alfalfa varieties and found that it ranged from 7 to 13, whereas Martin [29] reported that number of ovules per ovary in tetra alfalfa ranged from 12 to 18. Muschler [30] reported that alfalfa superficially resembles clover, with clusters of small purple florets followed by pods spiraled in 2 to 3 turns containing 10-20 seeds. Genetic variation was identified for pollen fertility [31], ovule fertility [32] and eases tripping [33] as for traits influencing seed yield.

Fig. 6-1 of longitudinal section of alfalfa floret ovary shows the pollen tubes penetrate and fertilize ovules within ovary whereas Fig. 6-2 view longitudinal section of low fertile plants (Nubaria) and ovules abortion within ovary. Number of seeds (8-2 c, d and e) was (6, 4 and 3 seeds, respectively. Fig. 6-2 b and c show little number of seed set within ovary which may be related to ovule sterility, environmental conditions. Variation in number of seed setting pod^{-1} between studied genotypes may be related to the differences in ovule size and number of ovules ovary^{-1} according to the differences between genotypes in genetic structure.

Lower floret has the biggest number of seeds pod^{-1} and pod turns, whereas the top florets produced a little number of seeds pod^{-1} and pod cycles (Fig. 7a, b and c). No difference between the pods turns of primary and secondary branches plant^{-1}. Number of pods turns varied

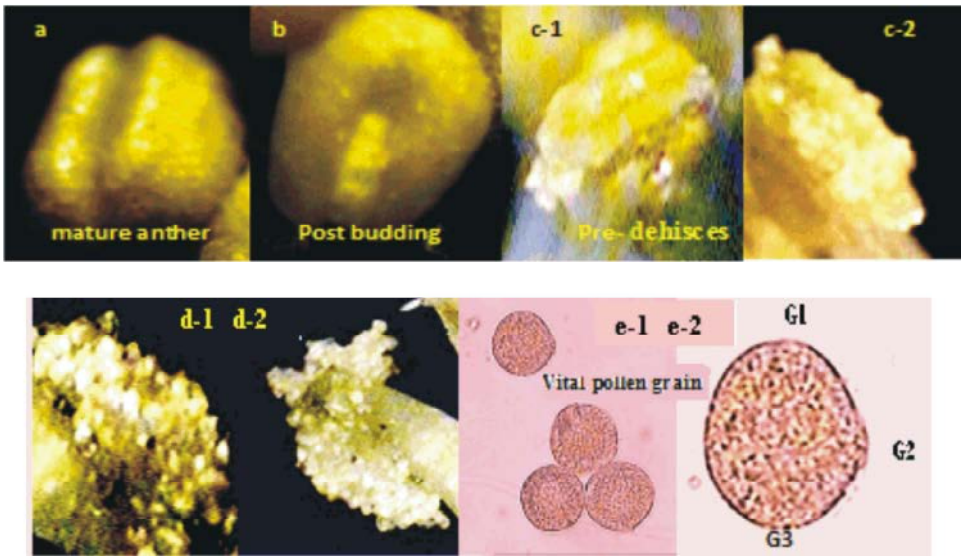

Fig. 4: Pollen grain maturity among floret development stages: (a) budding, (b) mid blooming, (c) post mid blooming stage, (d) full blooming and (e) vital pollen grain

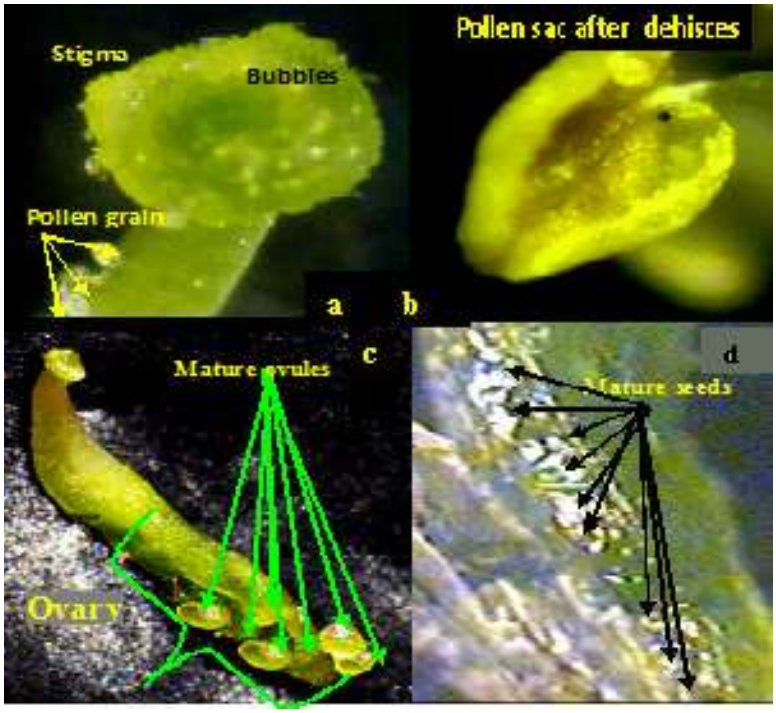

Fig. 5: Seeds development from pollination, fertilization till fertile seed: a- stigma in receipt of wilt pollen grain, b-empty pollen sac, c- mature ovules within ovary and d-seeds within pod

according to the position of florets in inflorescence whorls. The Bottom floret had the biggest number of pod turns ranging from 4 to 6, whereas the upper floret of in the top of inflorescence has the lowest number of pod turns ranging from 1-2 only. The other inflorescences on primary and secondary branches are different in number of pod turns according to number of fertile ovules per ovary. Regulation of the number of seeds per pod depended on the position of the inflorescence as indicated by Hole and Scott [34]. The opening up of florets (tripping) occurs when an insect enters in and presses the top large petal (vexillum). This pressure activates mechanism that opens lower, basic petal (naviculum), freeing up the reproductive system and

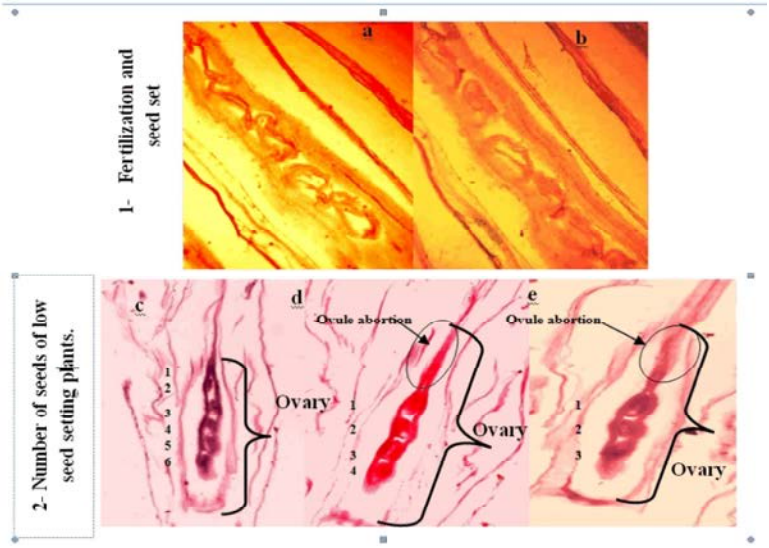

Fig. 6: Longitudinal section of alfalfa floret ovary showing the ovule fertilization and seed set of low seed set genotypes. (a) pollen tube penetration ovule and (b) set seeds, (c, d and e) seed set and ovule abortion within ovary (l=40x)

Fig. 7: Pods position and turns in inflorescence whorls: (a) topic pods of topical florets (1-2 turns), (b) middle pods of middle florets in whorl (2-3 turns), (c) bottom pods of lower florets in primary whorls (4-6 turns) and (d) mature seeds

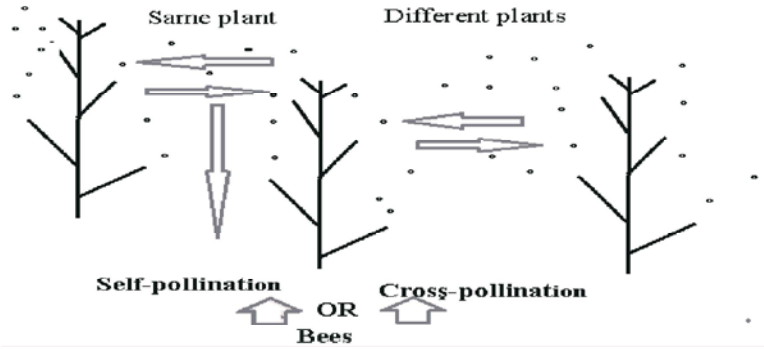

Fig 8: Pollen grain movement from florets to other in different whorls in the same plant (self-pollination) and from florets in different plants (cross pollination)

causing anthers dehisces. When pollen grain moved from top to bottom whorls and between florets by honeybees at the same plant, the possibility of self-pollination was the choice; also movement of fertile pollen grain from plant to others by honey bees or other pollinators, cross-pollination was the best choice. It is necessary for pollinator to trip the florets, release the stylus and thus enable foreign pollen to set onto the stigma. In alfalfa, some of the pollen that lands on a stigma may be from the newly tripped anthers of the same

floret, but if out-crossing also occurs, the self-pollen will fertilize few ovules [35, 36]. Zonjiæ *et al*. [37] claimed that alfalfa is self-fertile to a lesser extent and its complete and high fertility is manifested in uncontrolled free pollination.

In an alfalfa field this tripping and cross pollination can come from the beneficial bees, moving from floret to another causing tripping. Presence of bees nursery was an effective reason to increase seed setting of alfalfa field at blooming period. Bosch and Kemp [28] pointed that smaller bee populations whose nesting was better timed with peak bloom.

The low yield of seed production in alfalfa field has been suggested that this is due to the fact that only 20% of florets are pollinated under field conditions, a problem that is partly due to the lack of appropriate insect pollinators [38]. Seed yield is further reduced by pod abortion [39]. To reduce the amount of bee-mediated gene movement between varieties during seed production, alfalfa varietal purity is achieved by maintaining adequate isolation distances between alfalfa seed production fields. In Canada, an alfalfa crop grown for the production of certified seed must be isolated by a minimum distance of 50 meters from a different variety or non-pedigreed crop of the same kind [40]. For the production of foundation seed grown from Breeder seed, this isolation distance is increased to 200 meters for fields exceeding 5 acres or to 300 meters for fields that are 5 acres or less. The following points need to be studied in alfalfa seed production program such as:

- Ovules fertility and sterility
- Male sterility,
- Self- incompatibility
- Selection for high self-fertile plants between and within genotypes.

CONCLUSION

Alfalfa genotypes varied in most of studied traits. All genotypes under study were similar in floret structure but different in the blooming dates where the local genotypes were earlier in blooming than exotic genotypes. Low seed setting in alfalfa pods among studied genotypes may be related to differences in ovary's size, number of fertile and number of aborted ovules within ovary. These anatomical differences of ovary may be attributed to genetic variation among genotypes. Local selected genotypes Siwa, Ismaelia-1 and Ismaelia-94 proved as promising high seed yield genotypes.

ACKNOWLEDGEMENTS

The researchers are highly thankful to Prof. Dr. Adel Khatab, Faculty of Agriculture, Cairo University, for providing the necessary facilities and support.

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