Pollination of *Nigella sativa* L. (Ranunculaceae) in Jordan Valley to improve seed set

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Abstract: In Jordan, pollination is one of the problems faced by plants under plastic houses, in open fields and in off-season planting. Therefore this study was conducted in Jordan to investigate the role of pollinators and to investigate the systems of pollination in *Nigella sativa* species grown at two different altitudes, 150 m under sea level and 200 m above sea level. Up to now little attention has been paid to the events associated with pollination such as seed set, and to address this deficit, we examined six pollination treatments of the selected plant species. Field work was conducted, repeated and recorded from 2005 to 2007 in Jordan. Controlled pollinations were carried out in selected individual's plant at the time of maximum stigma receptivity and anthesis. *N. sativa* flowers had anthesis intervals which last for five days, then followed by stigmatic receptivity which last for few hours. Plants are pollinated trough outcrossing and complete selfing to insure the reproductive assurance. However, self-pollination was occurred due to style movement. The observations confirmed that a mixing mating including a combination of out-crossing and selfing is a better strategy than selfing alone.

1. Introduction

Black cumin, *Nigella sativa* (Ranunculaceae), is an annual herbaceous plant. The genus *Nigella* is represented in 20 species of Mediterranean-western Asian origin (Dantuono *et al*., 2002). Only *N. sativa, N. damascene* and *N. arvensis* are of interest in Jordan; *N. sativa* is the only species planted by farmers. There is no accurate data about planted area, but the annual production for the year 2005/2006 was 3-5 tons (personal communication). *N. sativa* is a hermaphroditic species with determined flowering patterns, starting with the flower terminating the main shoot and ending with the flowers on the lowermost branches. In the natural forms, flowers are delicate, and usually colored pale blue and white, with 5-10 petals and characterized by the presence of nectaries. The androecium comprises a large number of stamens, which shed their pollen as the filaments curve outward during the male phase. The gynoecium consists of up to five completely united follicles, each with a long, indehiscent style and composed of a variable number of multi ovule carpels, developing into a follicle after pollination, with single fruit partially connected to form a capsule-like structure. Seeds are generally small in size (1-5 mg) dark grey or black (Filippo *et al*., 2002). The fruit is large and its inflated capsule contains numerous seeds. *N. sativa* is extensively used in traditional medicine for healing various respiratory disorders from Morocco to Pakistan

and in southern Europe (Filippo *et al*., 2002). The seeds have been widely added as a spice to a variety of foods such as bread, yoghurt, pickles, sauces, and salads for flavoring. They are also used in Jordanian traditional folk medicine for some respiratory, gastrointestinal, rheumatic and inflammatory disorders (Nafisy, 1989; Zargari, 1990; Amin, 1991). *N. sativa* seeds have been reported to contain essential oil, fixed oil, flavonoids, saponins, alkaloids, and proteins (Zargari, 1990; Burits and Bucar, 2000; Al-Ghamdi, 2001). Pollination studies of *N. sativa* are very limited in the literature. Lloyd (1979) showed that *N. sativa* is self pollinated without mentioning the mechanism; Zohary (1983) showed that *N. sativa* is capable of setting seed without being cross pollinated. The flowers of *N. sativa* are visited by honeybees (Ricciardelli and Oddo, 1981).

2. Materials and Methods

The research considered specific plant species (landraces) of *N. sativa*, which were planted on-site at different elevations: location A, 150 m below sea level; and location B, 200 m above sea level. *N. sativa* was obtained from botanical gardens in Jordan (NCARTT). The seeds were planted in hills 30 cm apart on 5 November 2005. The rows were 20 m long, with 1 m between rows. Water was supplied daily by drip irrigation and extra fertilizers (N P K) were applied. Black plastic mulch was used. Each plant was represented by three rows per

location. Missing hills were replanted when necessary. The plants were thinned to two plants per hill when they were at two- to three-leaf stages. The two locations were kept weed-free by cultivation and hand weeding.

The time of stigmatic receptivity was determined with the aid of a dissecting microscope. The direct test of receptivity was an assay that detects the presence of stigmatic peroxides. To determine receptivity, the stigmas were treated with hydrogen peroxide 3%: small air bubbles that form by maturation of the stigma indicate that the flower is in the female phase (Dafni and Maues, 1998). To determine receptive periods, 50 flower buds per plant of each species were marked, 10 flower buds of the same age were bagged a day before the opening of flowers during the anthesis period. On the following day, 10 flower buds were taken to the laboratory in order to check for stigma receptivity.

The timing of anthesis was checked in the field using a hand magnifier. After bending, anther capsules were observed with the naked eye. The mechanism of pollen release is described based on direct observations in the field. Any rupturing of the capsule causes pollen to release where it is verified by anther dehiscence.

In order to observe pollinator visitation tour, the number of visits per bee was estimated by counting the number of visits with anther or stigma contact from the beginning of pollination to fertilization. The counts were conducted every 15 minutes for a period of eight hours on a daily basis during flowering period.

Controlled pollinations were carried out on selected individual plants at the time of maximum stigma receptivity and anthesis. Pollinated flowers were observed periodically for fruit set. The reproductive success of the studied species was assessed by performing a spontaneous self pollination, manual self and cross pollination treatments. Following the initiation of the first flower bud, flowers were selected randomly and tagged: 180 flower buds of *N. sativa* in each location. Thirty flower buds were marked for each pollination treatment. Pollination treatments were performed from February to March 2006 to determine the best pollination treatment in each locations. In order to conduct geitongamy and xenogamy pollinations, all stamen organs of each flower were removed using special scissors (emasculation). The flowers were pollinated using pollen from freshly dehisced anthers from male flowers (of the same plant) by using a fine brush for geitongamy pollination treatment and from another plant for xenogamy pollination treatment (cross pollination). The flowers were left exposed to any insect as occurs in nature for open pollination treatment. To test the bagged self pollination, flower buds, bagged till the end of pollination stage, were left untreated and uncovered again in order to avoid any negative impact on their germination. In order to check forced self pollination on the same hermaphrodite flower, flowers were bagged till the last day of the male stage. The flowers were pollinated using pollen from freshly dehisced anthers from male to female flowers on the same hermaphrodite flower by using a fine brush. With regard to emasculation, flower buds of nearly the same age were selected in order to remove male flowers to investigate the differences between the role of pollinator and the role of plant, by numbering of fruit set. The anthers were removed with a pair of tweezers and were left to pollinate by pollinator. If an emasculated flower sets fruit, then it must have received pollen from a pollinator. However, if an emasculated flower fails to set fruit, a pollinator will have had no role in fertilization.

Changes in the relative positions of anthers releasing pollen and the styles was also documented. A total of 30 flower buds were monitored during the study period using a hand magnifier. A single flower from this group was monitored from the morning to the end of the day. Each flower was scored for the number of anthers on the flower, the number of anthers dehisced, the position of the dehisced anthers and the positions of anthers relative to the stigma. The length of anther and style were measured using a special caliber. Representative photographs were taken of flowers at each stage.

Thirty marked flower buds were selected to count the number of ovules in order to determine the standard number of ovules in the stigma. The number of ovules per capsule were counted, averaged for both locations and the average number was used as a reference in the calculation.

Data were analyzed as complete randomized design with three replicates. Comparisons between means were made using least significant difference (LSD) at 0.05 probabilities lend (SPSS). For statistical data, standard descriptive statistics were performed for each of the following quantitative parameters: the number of produced fruits, the number of seed for each stigma, the number of ovules, the number of chambers per capsule, the number of non fecundated seeds and the total number of fecundated seeds. Mean number of buds and stigmas of plants, standard deviation, and differences between pollination treatments in terms of seed set per fruit were calculated. The statistical program package SPSS was used. Insect visits were standardized by calculating the number of visits per flower per plant. These data were summarized over the season by taking an average of the observations. Minimum and maximum value was observed and graphical analyses were applied.

3. Results

Anthesis and receptivity

Styles are the first floral organ to emerge and extend, followed by extension of the stamens. When the style has almost straightened, the anthers began to dehisce. After the dehiscence of anthers about half an hour when it is considered as the first day for pollen shedding till fifth day, the male stage activated between 8:30 AM to end of the day and anthers were sink down. The male

phase is initiated a few days before the stigmas become receptive and male stage lasted for five days. By the fifth day of the male stage, female stage started during this day, stigmatic peroxides tests indicate that receptivity occurred between 8:00-13:00 ᴘᴍ and for one day only. Male and female stages synchronized in the last day of the flowering period (Fig. 1). The weight of pollen was 0.064 mg/flower, whereas the volume of nectar was 0.13 µl. Affluent floral rewards (both nectar and pollen) during the male phase of the flowers.

(a)

(b)

(c)

7:00 8:00 9:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00
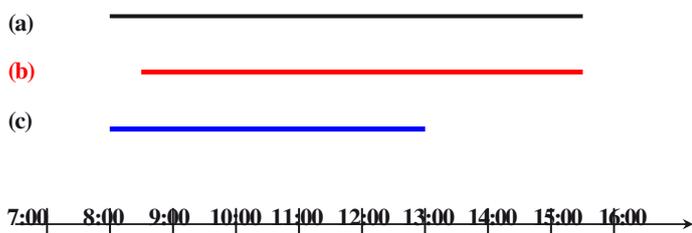
Fig. 1 - Blooming stages of *Nigella sativa*, a) flowers opening, b) anthesis and c) receptivity stage.

Movements of stigma and anthers

The male and female organs at bud stage are presented in (Fig. 2 a) At onset of the male stage, all the stamens stand erect (Fig. 2 b). They curve outwards one by one, roughly in whorls and strictly reflecting the order of initiation (Fig. 2 c). When the anthers reach a horizontal position, the pollen is released (Fig. 2 d). Then, the stamens sink down. An anther takes 4-7 hours to empty its contents. The stamen movement is not continuous, but it is divided into three phases. In the first phase (12-14 hours) the lower part of the filament inclines slightly, while the upper part curves more strongly, so that the anther is brought into a horizontal position. After reaching this position, movement comes to a standstill. The second phase - towards the ends of the male stage, the styles of the five carpels usually curve down (Fig. 2 e) and twist (Fig. 2 f). This ensures that in the female phase the stigmatic crests, whose bends were making an angle of 45°, continue to make a right angle with ovary to run down nearly the whole length of the style to touch the top of the anther at several points (Fig. 2 f).

The third stage, in which the stamen sinks down, is much shorter than the previous ones (4-6 hours). Finally, the empty anthers curve up. This is a purely passive movement, apparently without any function. After uptaking the pollen, the stigma is pollinated (Fig. 2 g),

and then the stigma inclined upwardly erect as the order of initiation and makes an angle of 180° with the ovary (Fig. 2 h). The maximum style length reached 1.73 cm, whereas the maximum anther length was 1.72 cm. This indicates the equal length of style and anther.

Pollination

Season one. Location A. N. sativa' flowers produced a non significant number of ovules under all treatments conditions with an average of 96±0.5, as shown in (Table 1). Generally, all flowers under the different treatments produced seeds (Table 1). Open pollinated flowers produced significantly higher seeds as compared with other treatments 74.9±1.4. Hand cross, hand geitongamy and hand forced self ranked secondly in seed set and produced a nonsignificant differences between them with a seed set average of 82.9±1.6, 73.5±1.5 and 78.9±1.6 respectively. A non-fecundated seed production is also a common feature of *N. sativa'* flowers under the different treatments. In the first location, open pollination occupied the lowest average of non-fecundated seeds all over other treatments (Table 1). Hand cross, hand geitongamy and hand forced self ranked secondly in producing a non-significant fecundated seeds with an average of 12±1.7, 21.6±1.6 and 18±1.4 respectively. There were significant differences (P≤0.05) in the percentage of seed set between treatments (Table 1). Seed set percentage after open pollination (86.8% seed) was significantly higher than all other treatments (P≤0.05). Non significant differences were found between the average percentage of seed set when hand cross, hand geitongamy and hand forced self was used on flowers (79.8%, 75.4% and 81% respectively).

Location B. N. sativa' flowers produced a non significant number of ovules under all treatments conditions with an average of 91.1±0.5, as shown in (Table 2). Generally, all flowers under the different treatments produced seeds (Table 2). Open pollinated flowers produced significantly higher seeds as compared with other treatments 82.9±1.5. Hand cross, hand geitongamy and hand forced self ranked secondly in seed set and produced a nonsignificant differences between them with a seed set average of 72.4±1.4, 70±1.3, and 77.5±1.1 respectively for the first location. Characteristics of producing fecundated seeds in the second location were fairly constant in value and regulated mainly by treatments conditions. A non-fecundated seed production

Table 1 - Seeds set after different pollination treatments in *Nigella sativa*, location A. Season one

Treatment of pollination	No. of ovules/capsule	No. of fecundated seed/capsule	No. of non fecundated seed/capsule	Percentage of seed set/capsule
Open	93.3±0.5 A	74.9±1.4 a	19.0±1.5 A	86.8±1.2 A
Hand cross	95.6±0.7 B	82.9±1.6 b	12.0±1.7 B	79.8±1.3 B
Hand geitonogamy	95.1±0.6 B	73.5±1.5 b	21.6±1.6 b	75.4±1.2 B
Hand forced self	96.0±0.5 b	78.9±1.6 b	18.0±1.4 B	81.0±1.4 B

a and b are symbols related to difference in comparison.

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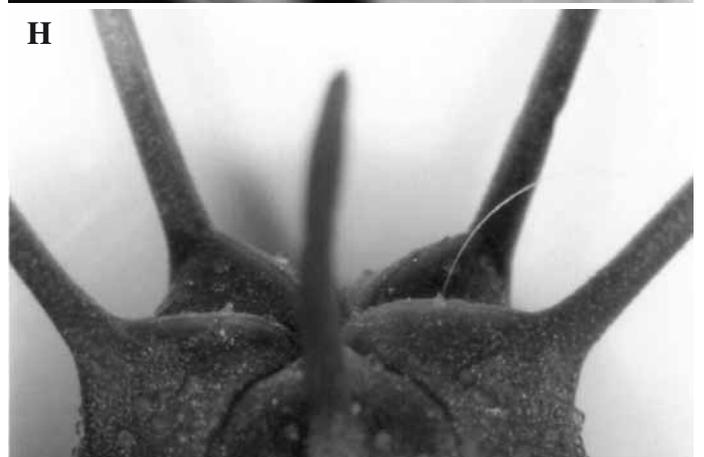
Fig. 2 - a) Plant at bud stage; b) The stamens stand erect; c) first phase of stames movement: The stamens curve outwardly in whorls; d) Pollens releasing; e) First phase of style movement: the styles of the usually five carpels curve down; f) Twisting point of style with anther; g) The stigma is pollinated; h) The stigma inclined upwardly erect.

is also a common feature of *N. sativa'* flowers under the different treatments. In the first location, open pollination occupied the lowest average of non-fecundated seeds (Table 2) all over other treatments. Hand cross, hand geitongamy and hand forced self ranked secondly in producing a non-significant fecundated seeds with an average of 18.7±1.3, 25±1.5 and 18.1±1, respectively.

There were significant differences (P≤0.05) in the percentage of seed set between treatments (Fig. 3). Seed set percentage after open pollination (87% seed) was significantly higher than all other treatments (P≤0.05). Non significant differences were found between the average percentage of seed set when cross, hand geitonogamy and hand forced self was used on flowers (79%, 73% and 80% respectively).
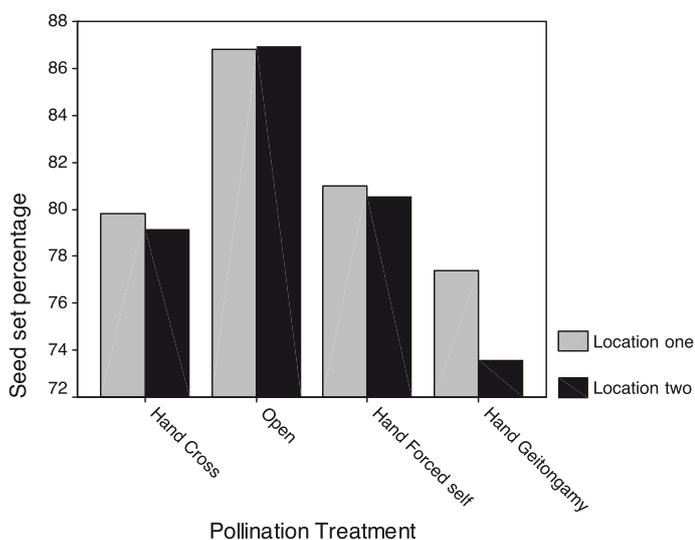
Fig. 3 - *Nigella sativa* seed set percentage upon pollination treatments in location A and B season one.

Season Two. Location A

N. sativa' flowers produced a non significant number of ovules under all treatments conditions with an average of 92.3±1.42, as shown in (Table 3). Generally, all flowers under the different treatments produced seeds (Table 3). Open pollinated flowers produced significantly higher seeds as compared with other treatments in both locations 83.4±0.67. Hand cross, hand geitongamy and hand forced self ranked secondly in seed set and produced a non-significant differences between them with a seed set average of 74.6±0.68, 73.6±0.67, and 79.7±0.32 respectively for the first location. Bagged self pollinated flowers ranked thirdly and produced 44.1±0.75 seeds. The lowest seed set was recorded in the case of emasculated flowers with an average seed production of 12.4±0.33. Hand cross, hand geitongamy and hand forced self ranked second. A non-fecundated seed production is also a common feature of *N. sativa'* flowers under the different treatments. In the first location, open pollination produced non-significant fecundated seeds with an average of 12±1.7, 21.6±1.6 and 18 ±1.4, respectively.

There were significant differences (P≤ 0.05) in the percentage of seed set between treatments (Fig. 4). Seed set percentage after open pollination (87% seed) was significantly higher than all other treatments (P≤ 0.05). Non significant differences were found between the average percentage of seed set when hand cross, hand geitonogamy and hand forced self was used on flowers (79%, 78% and 83%, respectively). Nearly half of the produced set seed in bagged flowers with an average of 47%. Emasculated flowers (13%) recorded the lowest seed set from other treatments with significant difference.

Location B. N. sativa' flowers produced a non significant number of ovules under all treatments conditions with an average of 97.2±1.67, as shown in (Table 4). Generally,

Table 2 - Seeds set after different pollination treatment in *Nigella sativa* location B. Season one

Treatment of pollination	No. of ovules/capsule	No. of fecundated seed/capsule	No. of non fecundated seed/capsule	Percentage of seed set/capsule
Open	95.6±0.48 a	82.9±1.5 a	12.8± 1.5 A	87.0±1.3 a
Hand cross	91.1±0.50 b	72.4±1.3 b	18.7± 1.3 b	79.0±1.2 b
Hand geitonogamy	95.0±0.55 b	70.0±1.3 b	25.0±1.5 B	73.6±1.0 b
Hand forced self	96.0±0.60 b	77.5±1.1 b	18.1±1.0 b	80.0±0.99 b

a and b are symbols related to difference in comparison.

Table 3 - Seeds set after different pollination treatment in *Nigella sativa* location A. Season two

Treatment of pollination	No. of ovules/capsule	No. of fecundated seed/capsule	No. of non fecundated seed/capsule	Percentage of seed set/capsule
Open	96.8±2.19 a	83.4±0.67 a	13.4±1.93 d	87±1.67 a
Hand Cross	96.0±2.31 a	74.6±0.68 b	21.4±2.39 c	79±1.98 b
Hand geitonogamy	93.0±2.81 a	73.6±0.67 b	21.1±1.81 c	78±1.46 b
Hand forced self	97.2±1.67 a	79.7±0.32 b	17.6±1.77 c	83±1.54 b
Bagged self	94.2±1.50 a	44.1±0.75 c	51.9±0.73 b	47±1.10 c
Emasculation	97.6±1.67 a	12.2±0.33 d	85.4±0.33 a	13±0.46 d

a, b, c, and d are symbols related to difference in comparison.
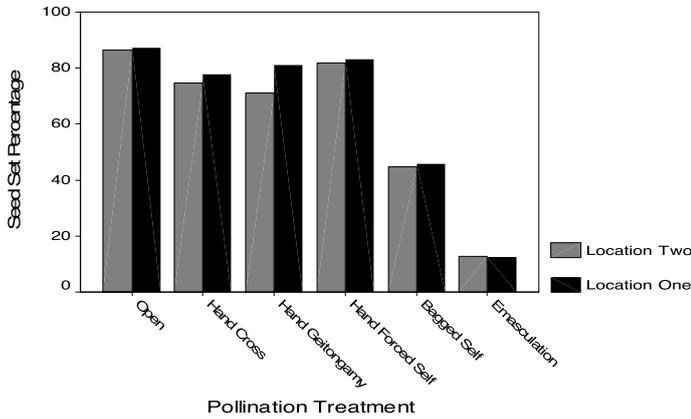
Fig. 4 - *Nigella sativa* seed set percentage upon pollination treatments in both locations, season two.
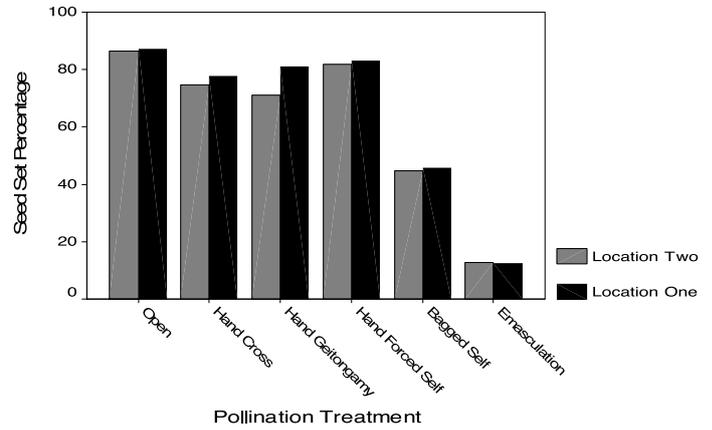
Fig. 5 - *Nigella sativa* seed set percentage upon pollination treatments in both locations, season two.

all flowers under the different treatments produced seeds (Table 4). Open pollinated flowers produced significantly higher seeds as compared with other treatments in both locations 82.4±0.57. Hand cross, hand geitongamy and hand forced self ranked secondly in seed set and produced non-significant differences between them with a seed set average of 71.8±0.57, 67.9±0.62, and 78.6±0.5, respectively. Bagged self pollinated flowers ranked third and produced 43±0.74 seeds. The lowest seed set was recorded in the case of emasculated flowers with an average seed production of 12.4±0.5. Open pollination occupied the lowest average of non-fecundated seeds 13.7±1.76 (Table 4) all over other treatments. Hand cross, hand geitongamy and hand forced self ranked second, a non fecundated seed production is also a common feature of *N. sativa'* flowers under the different treatments in producing a non-significant fecundated seeds with an average of 20.2±1.57, 25.1±1.62 and 14.5±1.6, respectively. There were significant differences (P≤0.05) in the percentage of seed set between treatments (Fig. 5).

Seed set percentage after open pollination (87% seed) was significantly higher than all other treatments (P≤0.05). Non significant differences were found between the average percentage of seed set when hand cross, hand geiton-

gamy and hand forced self was used on flowers (78%, 73% and 85% respectively). Nearly half of the produced set seed in bagged flowers with an average of 46%. Emasculated flowers (13%) recorded the lowest seed set from other treatments with significant difference.

Behavior of honey bee visitors

During our observation, honey bees were the only visitor and pollinator that visited *N. sativa* in the morning around 7:00 A.M. Every flower had one bee at least. Each bee spent different time with an average of 12.5 s for nectar collecting, 8 s for pollen collectors. The only diurnal visitor and pollinator were honey bees. Honey bees were frequent visitors to *N. sativa* in the Jordan Valley. The honey bee had same behavior in the two locations. In the evening, no pollinators were found in the flowers in both sites. The major pollinator was honey bees. *N. sativa'* flowers' mean visit rates for the three replicates in both locations were 14.9 and 14.6 daily visiting tours, respectively. The ultimate activity during the three replicates was approximately from 9:30 to 12:30 in both locations. 33% of the total bees observed were pollen collectors, while the rest 67% were nectar collectors (Table 5). Honey bees

Table 4 - Seeds set after different pollination treatments in *Nigella sativa* location B. Season two

Treatment of pollination	No. of ovules/capsule	No. of fecundated seed/capsule	No. of non fecundated seed/capsule	Percentage of seed set/ capsule
Open	96.1±1.76 a	82.4±0.57 a	13.7±1.76 d	87±1.56 a
Hand cross	92.3±1.42 a	71.8±0.57 b	20.2±1.57 c	78±1.28 b
Hand geitonogamy	93.1±1.68 a	67.9±0.62 b	25.1±1.62 c	73±1.22 b
Hand forced self	93.1±1.53 a	78.6±0.50 b	14.5±1.60 c	85±1.39 b
Bagged self	93.6±1.50 a	43.0±0.74 c	50.6±1.71 b	46±1.10 c
Emasculation	94.9±1.9 0a	12.4±0.50 d	82.5±2.01 a	13±0.62 d

a, b, c, and d; are symbols related to difference in comparison.

Table 5 - Behavior of honey bees and their bearings for *Nigella sativa*

Behaviors of bees	Average spending time/flower (second)	Landing on			Departure of			Percent of bees according to their bearings
		Petals	Anthers	Twisting	Petals	Anthers	Twisting	
Nectar collector were observed	12.5	•			•			67%
Pollen collector were observed	8.0			•			•	33%

visiting tours were conducted in two stages during five days. Anthesis period took place in the first four days, and anthesis and receptivity periods were in the fifth day. Visiting tours were for functional nectar collecting. On the first day, honey bees landed on petals and then collected nectar during circular stepping upon petals, without getting directly exposed to anthers. On the second till the fourth day, the same behavior occurred. The pollen grains fell down upon bees back from the horizontal anthers during circular motion. On the fifth day, receptivity period began, in which the styles inclined towards anthers, and then the styles twisted themselves around the anthers. Honey bees were landing directly on this synapse (not on petals). After that, they left and flew to another flower.

4. Conclusions

The male phase is initiated a few days before the stigmas become receptive, where the anthesis duration remains for five days

Full flowering started with the appearance of bright blue petals. Male stage started as the anthers started to shed their pollen, since the first day till fifth day, the male stage activated between 8:30 A.M. to the end of day. The viability of one anther remained during one day then started to sink down. It is interesting to point out that anthers remain active for five days, which leads to synchronize the receptivity period in the fifth day. Because the flowering period for *N. sativa* coincides with good temperature in April in Jordan, this may lead to an increase of the interval of anthesis since the pollen responds to temperature. It is surprising for pollen of *N. sativa* to continue for five days. Another reason for this long period of anthesis is the large number of anthers in staminate. Climatic factors affect the anthesis intervals in *N. sativa*, there is evidence that high temperature had a direct effect on pollen performance since the pollen responds to temperature. However, at the same time they are advantageous for the pollen by hastening its tube growth rate. On the other hand, low temperatures may act against the pollen by reducing its germination and growth rate, which could limit the fertilization success (Thompson and Liu, 1973; Jakobsen and Martens, 1994)

The duration of stigmatic receptivity in Nigella sativa *was approximately hours*

In angiosperms, the stigma is the first female structure, the pollen grains and pollen tubes have to face on their way to the female gametophyte. The stigma provides an adequate environment for pollen grain germination (Knox, 1984; Helsop-Harrison and Shivanna, 1997). One of the most important features of stigmas is stigmatic receptivity, defined as the ability of the stigma to support pollen germination, which is a decisive stage in fertilization success and has a large variability among plant species (Helsop-Harrison, 2000). At the end of the fifth day on the male stage, the female stage started to be active during 8:00 A.M.

to 13:00 P.M. and then ended up. It is interesting to point out that the stigma of *N. sativa* is receptive throughout anthesis. Inspite of the flowering period in April when we don't have high temperature which may hurt the plant; the stigma receptive only for hours. The explanation for that is that the stigma is exposed in direct way to the sun which may increase the exposed area. In addition, the receptivity of stigma occurred after the stigma lost most of the anthers that surrounded the stigma so that the whole stigma is exposed to the sun which may also increase the exposed area to sun. That means high temperature affects stigma receptivity and reduces receptivity interval. There is evidence that ensures stigma responds to high temperature. High temperatures are detrimental for the female part by reducing the length of stigmatic receptivity and accelerating ovule degeneration (Postweiler *et al.*, 1985).

It is well documented that the reproductive phase, especially from pollination to fertilization, is highly vulnerable to the prevailing environmental conditions including temperature (Hall, 1992; Stephenson *et al.,* 1992). The duration of stigmatic receptivity is variable depending on the species, and it is also variable within genus. There is evidence that indicates duration of stigmatic receptivity is variable, that the duration of stigmatic receptivity is variable depending on the species and is usually greater in wind-pollinated than in insect-pollinated species (Khadari *et al.*, 1995). Thus, the stigma can be receptive for not much more than an hour or so, as in *Avena* or *Dactylis*, to as long as several days, as in other grass species (*Pennisetum* or *Zea*) or *Eucalyptus* in which it can remain receptive for more than a week, particularly in hostile environments (Helsop-Harrison, 2000).

From an agricultural perspective, stigmatic receptivity has also a clear practical implication as it limits floral receptivity, the effective pollination period (Guerrero-Prieto *et al.*, 1985) and hence fruit set (reviewed in Sanzol and Herrero, 2001). Moreover, in an ecologist context, by altering stigmatic receptivity, flowering plants may influence the likelihood of fertilization by indirectly controlling the number and the quality of mating through the control of the number of pollen grains deposited and the time of germination (Cruden *et al.*, 1984; Primack, 1985; Galen *et al.*, 1986).

Autonomous pollination

First of all, I would like to define the Autonomous phrase for the reader to understand. As Lloyd, 1992 defines it: Prior self pollination within-flower: self-pollination that occurs before the opportunity for outcross-pollen receipt for that flower has occurred, competing self pollination within-flower; self-pollination that occurs during the opportunity for outcross-pollen receipt for that flower has occurred, and delayed selfing pollination within-flower; self-pollination that occurs after the opportunity for outcross-pollen receipt for that flower has occurred. One of these three types of self pollination occurred in our research in *N. sativa*, which is delayed selfing pollination. Automonous delayed selfing late in *N. sativa* flower's life

is favored when honey bees service and thus outcross-pollen receipt is unpredictable. *N. sativa* flowers attract honey bees but they can also autonomously perform delayed self pollination, which provides reproductive assurance if pollinators fail to visit. The delayed self pollination occurred in our research because the synchronization between male and female occurred in the end of flowering period. I agreed with Darwin (1877), Muller (1883), Baker (1955, 1965) and Lloyd (1979, 1992) that pollinator absence or low pollinator abundance during some periods within or among flowering seasons favor shifts from outcrossing to autonomous self-fertilization because self-pollinated seeds provide reproductive assurance. Some authors support the research result that absence of pollinators can shift to delayed self pollination; the extinction of pollinators or range expansion in a plant lineage can favor shifts to biotic modes of pollination, including wind pollination and autonomous self fertilization (Baker, 1955; Stebbins, 1957; Regal, 1982; Cox, 1991; Weller *et al.*, 1998).

The results agreed with Barrett and Harder (1996), and Ramsey and Vaughton (1996) that pollinator scarcity and reduced pollinator services may result in high selfing rates. Cross pollination and bagged self pollinations occur; approved by seed set achieved by all treatments applied on the research where bagged selfing and outcrossing boosted seed production means of 45% and 77% respectively. The results agreed with Zohary (1983) as he found that *N. sativa* are capable of setting seed without being cross-pollinated, but he didn't mention the mechanism for such a result. The results also agreed with Faegri and Van Der Pijl (1971) who reported: There are a few flowers that can self-pollinate by their own, but this limits them to in breeding. The results agreed with Goodwillie (1999) in believing the ability of self pollination to provide some insurance against pollination failure.

In addition to the reproductive assurance benefits, prior selfing could be favored since it reduces the costs associated with the longer floral maintenance time required for outcrossing, and sets the stage for the evolution of reduced investment in cues for pollinators and the amount of pollen per flower. In contrast with early selfing, later-selfing species will retain floral traits and costs associated with outcrossing (i.e., cues to attract pollinators, pollinator rewards, and prolonging floral maintenance relative to prior selfing species. At one extreme, selfing early in a flower's life (prior) is favored when a population requires pollinators are chronically absent (Lloyd, 1992), or when population size is so low as to be undetectable by pollinators (Lloyd, 1992; Fausto *et al*., 2001; Goodwillie, 2001), or when a population experiences high levels of interspecific pollen flow (Fishman and Wyatt, 1999). Many authors are interested in common type of pollination as cross, open and self pollination, but through our research I have been devoted all our efforts to point out some thing out of traditional efforts such as delayed self pollination. Thus, delayed selfing may be achieved by either a partial overlap in timing of male phase with female phase or changes in the relative position of anther and stigma during development.

For example, delayed selfing in *Hibiscus laevis* (Klips and Snow, 1979) and Campanula species (Faegri and Van der Pijl, 1979) is characterized by a progressive downward curling of the stigmatic area towards the style where anthers or pollen are located. Conversely, in the protogynous *Aquilegia canadensis* (Eckhert and Schaeffer, 1998) the stamens progressively elongate towards the exerted stigma. In *Kalmia latifolia* (Lyon, 1992), anthers collapse into the stigma on the final day of floral development; thereby achieving self pollination. Others have found in self pollination late in floral life without changes in morphology. The breakdown of self incompatibility as the flower ages in both Lilium and Longifolium (Ascher and Peloquin, 1966) is attributed to degradation of the proteins that control self incompatibility and can be viewed as another form of delayed selfing. Faegri and Van der Pijl (1971) used the term ''self pollination'' or "autogamy" when pollination takes place within one flower (idiogamy), and "allogamy" or "cross pollination" when pollen from one flower is carried out to the stigma of another one. Allogamy may further be divided into "geitonogamy" if the flowers are on the same plant and "xenogamy" if they are from different plants. However, it is that geitonogamy that has the ecological properties of cross-fertilizer but the genetic properties of self fertilization. Thus, geitonogamy appears to be equivalent to autogamy (Lloyd and Schoen, 1992).

Style movement acts towards promoting self-pollination and leads Nigella sativa *to delayed self-pollination*

Weber (1995) has produced a presentation film showing the pollination mechanism for *Nigella arvensis.* He concisely presented the mechanism in written steps. The mechanism was demonstrating style movement in *N. arvensis* which exactly resembled our observations on style movement of *N. sativa*; through pictures shown above. I used his written description has quotation for its meaningful. I have measured the style and anther length and style twisting angle. Hence the equal length of anther and stamen demonstrate the style twisting, whereas Weber (1995) did not mention the length. Weber (1995) mentioned that insects bear pollen on their thorax after touching the horizontal anthers. Our observations showed that honey bees are landing on the horizontal anthers and twisting point of style and anther to bear pollen grains on their legs. So, how could insect carry pollen to another flower if the pollen is on their thorax. The beginning of receptivity caused a strong twist for stamens and style that leads to self pollination. This was observed as the end of male stage and the beginning of the receptivity stage. Style movement acts towards promoting self-pollination as in *N. sativa*. In another plant, style movement leads to avoiding self-pollination and promoting cross-pollination as Verma and Magotra (2004) reported for *Eremurus himalaicus* where they observed the mechanism of the stigma movement away from the dehiscing anthers, hence, it avoided receiving any left over pollen, and so self pollination is impossible. It is interesting to point out that *N. sativa* plant relies solely on animal vectors to move pollen among individuals, and if

pollinators are absent or in low numbers at certain times or years, individuals of *N. sativa,* that can self pollinate if not previously out crossed, will be at a selective advantage. This reproductive assurance process has been termed delayed selfing.

N. sativa *mixed mating is a better strategy than selfing alone*

Mixed mating is a better strategy; that means open pollination system is better to seed setting than other pollination treatment. This open system leaves the plant exposed to biotic and abiotic factor. The plant will be without any restriction which may cause any reduction in seed setting. The open system includes the role of honey bees and role of plant to pollinate itself by delayed self pollinated flowers. The manual pollination, which included: hand cross, hand geitongamy and hand forced self, ranked second after open pollination, and this significant difference is attributed to human performance which is not like natural performance. Excluding the biotic and abiotic factor from the plant by bagged self, that means plant will be restricted without honey bees, and the plant depends on itself to develop its style to reach the maximum length to catch the anthers in order to twist. In spite of the style movement towards the anthers, it gained half of the seed setting from open pollination, and this attributed to the fact that the style's movement occurred once the stigma was receptive and at the final stage of anthesis when there is small number of anthers, and then sink down, this may not be enough to get high percent of seed setting as there isn't enough quantity of pollen.

Honey bees are pollinator to N. sativa *which is considered unattractive to wild bees*

The only diurnal visitor and pollinator were honey bees. Honey bees frequently visited *N. sativa* in the Jordan Valley. The honey bee had similar behavior in the two locations. In the evening no pollinators were found in the flowers in both sites and seasons. Flower visitors can only be considered pollinators if four pollination conditions have been met: pollen transfer to the vector is observed; pollen transport by the vector is observed, pollen transfer from vector to stigma is observed; and pollen deposited by the vector is shown to result in fertilization of the value (Cox and Knox, 1988). The flowers of *N. sativa* were unattractive to wild bees' visitors. An important aspect used in many pollination studies is the number of visits made by a pollinator (Proctor *et al.*, 1996). *Apis mellifera* engaged in pollen and nectar collection as a pollinator of *N. sativa* flowers with low frequency. The unattractively of *N. sativa* flowers to wilds bees may be attributed to several factors such as the presence of other floral resources. During our research, *N. sativa* flowering coincided with that of other species such as *Centurea syriaca* and *S. arevensis* which are important for apiculture in Jordan due to their abundant nectar and the large floral patches through out the area. The attractiveness of any species is a function such as favor, color, nectar volume, sugar concentration (Frisch,

1967), and the bees fly to plant species that yield the greatest nectar and pollen (Gary, 1979).

The role of honey bees in the pollination of N. sativa *is too small*

Honey bees' role as pollinator in fertilizing *N. sativa* flower buds was very small compared to the role of plant itself and the role of open natural conditions in pollination. The emasculated buds were let exposed to the pollinators in order to fulfill the pollination where it sets up 12% of seed formation percent, while the natural conditions and self pollination conditions gave 87% and 45%, respectively. It is necessary to ask whether the removal of stamens affected subsequent flower development, e.g. the growth of the perianths, a factor that would make it difficult to distinguish between the costs of stamens or pistils and the costs of structures associated with display and reward (Andersson, 2003). Such effects seem likely considering the work of Andersson (2000), who detected a cost of producing and maintaining sepals and petals in a related species (*N. degenii*), and Plack (1957), who found a negative effect of emasculation on corolla size in hermaphroditic plants of the gynodioecious *Glechoma hederacea* (Lamiaceae). In the present study of *N. sativa*, stamen removal caused significant reduction in the mean of seed set. The results agreed with Andersson's study (2003) where he observed the stamen removal produced reduction in total seed number. As a furthermore for *N. sativa,* Andersson (2003) carried out removal of styles from *N. sativa* flowers and he found that; style-less plants initiated almost three times more flowers and invested 57% more biomass in stamens, than plants whose flowers were permitted to set fruit.

He found also stamen-less plants produced significantly heavier seeds after hand-pollination. These observations indicate that stamens draw upon the same pool of resources as the other floral organs and that the removal of immature stamens therefore influences patterns of resource allocation. Furthermore, Andersson and Jorgensen (2005) carried out removal of perianth from *N. sativa* flowers and found that; perianth removal produced 12.5% heavier seeds and allocated 15.8% more biomass to seed production than plants on which all perianths were left intact, whereas differences in flower production and total seed number were not significant. Perianth removal did not significantly affect the proportion of seeds that germinated, but caused a shift toward earlier germination dates.

The ultimate visitation rates for *N. sativa* flower in both Locations was diurnal visitation type especially at early morning

The ultimate visitation rates for flower in both locations were during 9:30 A.M. to 12:30 P.M., because the bees' activity is limited by environmental factors; the radiation rate and the daily temperature. Visitation rate was estimated by counting the number of visiting tours, those with anther or stigma contact. Counts were made for one hour periods during (8) hours a day, while plant species flowers were open. Pollinators may accidentally take place without any relationship existing between blossom and agent. Even

with concept of definite relationship in mind, it is not always easy to draw the line between pollinators and accidental visitors. The quantity of pollen transferred from anthers to stigmas, visit frequency to flower, pollinator forage pattern during anthesis, and floral rewards availability are parameters that can adequately explain the pollination efficiency of floral visitors (Primack and Silander, 1975; Herrera, 1987; 1989). It is generally thought the more visits made, the more efficient is the pollinator, though this also depends on the per visit pollen contribution to the pistillate flower part (Primack and Silander, 1975; Herrera, 1989).

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