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Floral biology and reproductive behaviour of *Nigella sativa* L. var. Ajmer Nigella-1

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Abstract
Nigella sativa L. is an annual herbaceous plant of the family Ranunculaceae. Seed being the economic part is the spice of commerce, composed of therapeutically potent metabolites. Owing to its wider application, the plants of *Nigella sativa* L. are widespread in Mediterranean, Middle European and South Asian countries. The present study is aimed at laying foundation for crop improvement and germplasm conservation programmes in *Nigella sativa* L. The results indicate that, plants upon germination takes 58 to 62 days for initiation of reproductive phase. Pollination activities starts 20 to 25 days later, upon anthesis an individual flower take one day for onset of male phase and on fifth day of anthesis the female part will be receptive. About seventy per cent of the pollens showed viability in terms of *invitro* germination in media composition of 45 % sucrose + 0.04 % boric acid. The phenotypic indices strongly reflecting the chronology of flowering events have been identified and illustrated, enabling the breeders and curators to assess and carryout the key activities of pollination.

Keywords: *Nigella sativa* L.; Ajmer Nigella-1; floral biology; reproductive behaviour; stigma receptivity

Introduction
Nigella sativa L. provincially known as kalonji, is an annual diploid (2n=12) minor seed spice crop of South and South West Asian origin. The genus *Nigella* of family Ranunculaceae includes about 20 different species distributed from Mediterranean to West Asia (Hegnauer, R. 1973) [7], *viz.*, *Nigella sativa* L., *Nigella damascena* L. and *Nigella arvensis* L. Among these, *Nigella sativa* L. is the only species being widespread and found cultivated in farmers field as a seed spice. Seed resembling the shape of onion seed, is the spice of commerce and therapeutically potent economic part being employed in many Middle Eastern and Far Eastern countries as a natural source of drug for past 2000 years (Gamze *et al*. 2006) [5]. The plant being native to Southern Europe, North Africa and South West Asia is found cultivated in India, Pakistan, Bangladesh, Syria, Israel, Lebanon, Turkey and Saudi Arabia (Khare, C. P. 2004) [8]. In India it is distributed all over, majorly in Punjab, Jammu & Kashmir, Himachal Pradesh, Madhya Pradesh, Bihar, West Bengal, Assam, Rajasthan and Maharashtra. It is apparent from the pharmacological studies that *Nigella sativa* L. possess important therapeutical properties like Antioxidant, Hepatoprotective, Anti-nephrotoxic, Anti-cancerous, Anti-diabetic, Anti-microbial, Anti-parasitic, Anti-malarial, Anti-ulcer, Anti-histamanic, Anti-inflammatory, Analgesic, Anti-hypertensive, Anti-hyperlepidemic and other respiratory, gastro intestinal and reproductive problems (Ahmad, A. *et al*. 2013) [3]. Seeds of *Nigella sativa* L. on distillation yields fixed oil ranging from 28 - 36 % and 0.4 - 2.5 % essential oil (Diwakar, Y. *et al*. 2015) [4] each composed of an array of bio-chemical constituents like fatty acids, flavanoids, phenolics (Rathore, S. S. *et al*. 2016) and other active constituents responsible for diverse therapeutic attributes. The important ones among them are thymoquinone, dithymoquinone, nigellone, thymol, dithymohy droquinone, carvacrol, anethole, 4- terpeniole, nigellicine, nigellidine, nigellimine and alpha-heredin apart from carbohydrates, proteins, fats, vitamins, minerals and essential amino acids.

Owing to its rich biochemical composition, the crop is highly accustomed in the field of medicine as well as food industry. Since there exists a greater demand for seeds both at domestic as well as global level, to make the venture attractive and remunerative, it is imperative to improve productivity through high yielding improved quality genotypes tolerant/resistant to biotic and abiotic stresses.

Genetic diversity study conducted in past have resulted in narrow genetic base among almost all the selections for most of the yield characters except in few Egypt and Oman collections. Hence the present study on floral biology and reproductive behaviour of *Nigella sativa* L. var. Ajmer Nigella - 1 was conducted with breeders and ecologists perspective to understand the basic floral biology and reproductive nature for future population development and conservation programmes, respectively.

Materials and Methods

Field and lab experiments were conducted during rabi season at ICAR-National Research Centre on Seed Spices, Tabiji, Ajmer, Rajasthan, India to determine the floral biology and reproductive phenology of *Nigella sativa* L. by using variety Ajmer Nigella-1 (AN-1). The mean of 25 plants each with three flowers were selected to record observations. The methodology used is as follows

- Days for bud initiation was recorded by observing number of days taken by a plant to produce flower bud (appear under naked eye) from the date of sowing.
- Days for anthesis was calculated by recording the number of days taken by a plant from the date of sowing to flower opening (unfolding of all the sepals, revealing male and female reproductive organs)
- Days for 50 per cent flower anthesis was assessed by recording the number of days taken for 50 per cent of the flowers in a plant to open from the date of sowing
- Days for 50 per cent flowering was calculated by recording the number of days required for 50 percent of the flowers in a plant to open the date of sowing
- Days for male phase was calculated by recording the number of days taken for anther dehiscence (splitting of anther sac and subsequent pollen release) from the date of sowing, from flower bud initiation and from flower anthesis with the help of a hand lens (10 x magnification)
- Phenotypic indices of male phase were identified by observing and recording the cycle of morphological modifications and movements in floral parts during male phase
- Pollen viability was determined through staining and germination study

a. Pollen Collection: Before the day of anthesis (at partial bud stage or balloon stage) the flowers were collected from 7 am to 5 pm at hourly interval from five flowers and stored it in small vials with water. The pollens from these flowers were used for pollen viability study through *invitro* staining and pollen germination study.

b. Pollen Staining: Cotton blue (in lacto phenol) stain was used for pollen viability study by following methodology given by Hauser and Morrison, 1964. Pollens were stained by adding few drops of above said stain on pollens mounted on clean dried glass slide. A clean dry cover slip was then gently lowered onto the drop of stain bearing the pollens. The preparation was left undisturbed for 30 minutes to allow the pollens to absorb enough stain. Percentage pollen viability was determined by recording the staining ability and shape of the pollens. Pollens which stained blue (Mione and Anderson, 1992; Nyman 1992) [9, 10] and had round shapes were considered to be viable.

c. *Invitro* Pollen Germination: Since viability may not directly reflect the germination ability of pollen grains (Vitagliano and Viti, 1989) [13]. Protocol for preparing germination medium was followed as per Acar *et al*. 2010. Initially a media standardization study was conducted, varied concentrations were tried in three replications by adding 5, 10, 15, 20, 25 and 30 g of sucrose in 100 ml of distilled water to produce 5, 10, 15, 20, 25 and 30 % of sucrose solution with varying concentrations of Boric acid (0.01, 0.025, 0.03, 0.05 and 0.1 per cent) in each of the above sucrose media. The lead from media standardization study *i.e*., 45 % sucrose + 0.04 % boric acid was used as a standard media for *invitro* pollen germination. The sucrose boric acid mixture thus prepared by adding 45 g of sucrose (Sigma Aldrich) and 0.04 g of analytical grade boric acid (Sigma Aldrich) was used after proper stirring using glass rod. *In-vitro* germination of pollens was observed on above media in borosil bi-concavity glass slides with five replications per observation. Pollens from matured flowers were then transferred to media using paint brush as well as needle. The concavity glass slides were later incubated on a glass slide platform in petri dish, bottom lined with wet blotting paper for germination.

d. Microscopic observation: The slides were observed under luminous fluorescent microscope for pollen germination and pollen tube length. Pollens were considered to have germinated if the length of pollen tubes were equal to, or more than, the diameter of pollen grains (Vizintin and Bohanec 2004) [14]. Germinated pollens was counted and expressed in percentage. Average of 100 pollens per microscopic field and three such fields was scored to determine germination percentage.

- Duration of male phase: Duration of male phase was determined by recording the number of days during which the anther dehiscence and subsequent pollen release lasted
- Days for initiation of female phase/stigma receptivity: Determined by recording the number of days taken by a plant for the onset of female phase by determining the stigma receptivity.
- Phenotypic indices of female phase: Phenotypic indices were assessed by observing and recording the floral morphology and other visual indices during the time of female phase.
- Stigma receptivity: Stigma receptivity was determined by Hydrogen peroxide method. The stigma from selected flowers was immersed in a drop of Hydrogen Peroxide (Paarakh, P. M. 2010) [11]. Occurrence of pure effervescence producing bubbles around the stigma revealed catalase (peroxidase) enzyme activity and thereby receptivity.
- Length of flowering period: Length of flowering period was determined by recording the duration of flowering period in an individual plant.

A corresponding field experiment was also carried out to validate the results of *invitro* stigma receptivity and its duration. About 100 plants each with three flowers of equal maturity (uniform bud stage) were emasculated and covered with transparent poly bags. Among the 100 plants, each day 3 plants with emasculated flowers were selected, tagged and manually pollinated by using freshly dehisced pollens, and the same procedure was continued till a month. Similarly, in another field experiment about 100 flowers of equal maturity were covered with a transparent plastic cover without emasculation. Here, each day about 3 flowers were emasculated by allowing them to naturally pollinate until

emasculation, and the same procedure was continued for 15 days. Finally at crop harvesting stage, the pollinated flowers and emasculated flowers with respective dates of pollination and emasculation were examined for any seed set to determine the time of stigma receptivity. The day of overlapping between emasculated, pollinated and naturally pollinated, emasculated flowers with respect to seed set was used to identify the day of stigma receptivity and similarly duration was assessed by observing the number of days during which seed set has been found in emasculated and pollinated flowers.

Results and Discussion

Plants of *Nigella sativa* L. takes 58 to 62 days for flower bud initiation from the day of sowing. The buds upon formation with pale green in colour, takes 20 - 25 days for complete flower opening *i.e.*, 78 to 87 days from the day of sowing. Initially the sepals with pale green colour are found tightly positioned upright engulfing all major floral parts (Fig. 2). Upon advancement of maturity, the sepals unfurl revealing floral parts (Fig. 3) and turns greenish white to white colour (Fig. 4). Small tongue like paired petals that are initially held upright and tightly packed, with the advancement of maturity inclines downward loosening the anther bundles. Anther dehiscence in the lower most whorls of stamens starts a day after anthesis (Fig. 5). With the onset of anther dehiscence, a light blue tinge appears in the whitish sepals and correspondingly reflective blue colour develops in the basal portion of stamens as well as petiole region of paired petals containing nectarines (Fig. 6). The fluorescent colour development strictly with the onset of male phase may occur to attract the insects for assisting in pollination.

Phenotypic indices of male phase could be ascertained easily by visualizing the movement of stamens. Stamens initially found upright in clusters with symmetrical arrangement around siliqua, after anthesis projects the distal end of filaments in outermost whorled stamens slightly outward following the downward movement of petals with sparse separation from the succeeding stamens in the respective clusters. The anthers of thus inclined filaments each containing two anther sacs on either side reaches near horizontal position and dehisces. Initially anther dehiscence occurs in one anther sac, followed by its other counterpart. The onset of anther dehiscence could be assessed easily with the outward bending of filaments (Fig. 7). The movement of stamens and subsequent anther dehiscence strictly occurs in whorls starting with the lower most stamens and terminating with the upper most ones in respective clusters. The process of downward inclination continues in succession and terminates with upper most whorled stamens. The duration of male phase lasts for four days, and its termination could be assessed by observing the empty anther sacs in last whorled anthers (Fig. 8). Freshly dehisced pollens and pollens stored up to two hours had 70 per cent *in-vitro* germination in media with 45 % sucrose and 0.04 % Boric Acid (Fig. 9 and 11). Female phase was initiated on fifth day of anthesis *i.e.*, stigma receptivity overlapped on fourth day of male phase (Fig. 10) and remained receptive for one day with peak time ranging between 8.00 am to 1.00 pm (Fig. 10 a). It is observed that, the period of stigma receptivity strongly overlapped with peak availability of pollens. The results thus obtained are in close agreement with the findings of Abu-Hammour, K. 2008 [1].

Similarly the key phenotypic indices of female phase could be assessed by observing the movements of style from its near horizontal position during onset of male phase to downward inclination with severe twisting at distal end during stigma receptivity. The downward movement of style with severe twisting thus found on the fourth day of male phase strongly coincides with the availability of pollens (Fig. 11 b). Because at this stage majority of anthers confined to third and fourth whorl will be at peak stage of pollen release, thereby enhancing the probability of sticking more number of pollens on to the stigma of twisted style, thus ensuring effective pollination and seed set. Laboratory experiment and corresponding field experiments involving emasculation and hand pollination conducted to determine the stigma receptivity revealed that stigma was receptive on fifth day of anthesis. In the field experiments, seed set was observed only in those emasculated and bagged flowers that were manually pollinated on fifth day of anthesis (Fig. 11), reflecting stigma receptivity positively coinciding on fifth day *i.e.*, fourth day of male phase (Fig. 12). The results are in agreement with Abu-Hammour, K. 2008 [1]. An individual flower cycle lasts for 29 days and with the completion of pollination process the inclined stigma reverts back to its horizontal position with tip projecting upward through inward bending and rests in upright position as shown in figure 1(f).

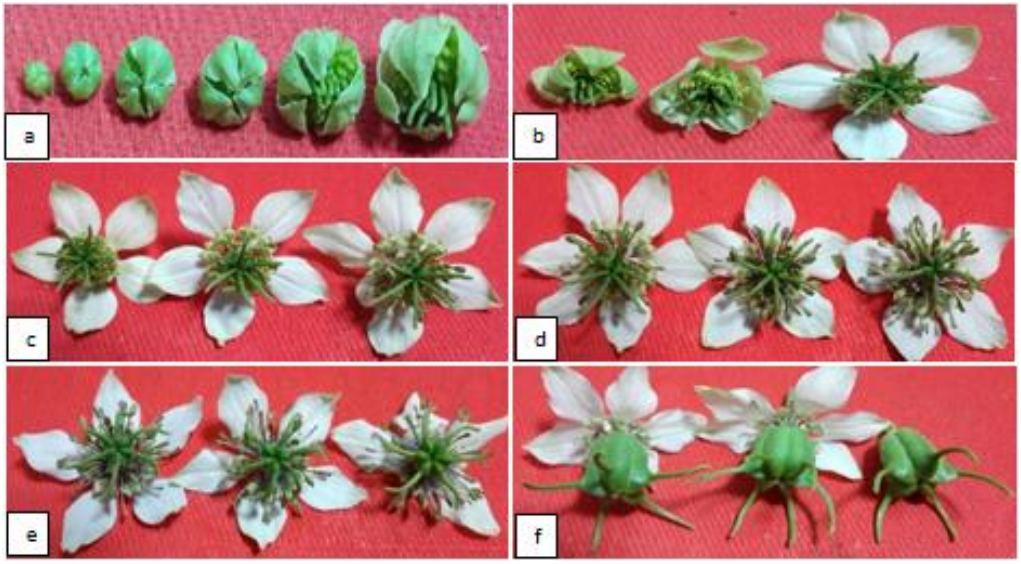

Fig 1: Chronology of flowering events in *Nigella sativa* L.

Fig.2 – Bud

Fig.3 – Unfurling of sepals

Fig.4 – Flower Anthesis

Fig.5 – Male phase

Fig.6 – a. Bluish tinge on filament and petals with nectarines; b. Insect aiding in pollination

Fig.7 – Phenotypic indices of male phase

Fig.8- Last day of male phase

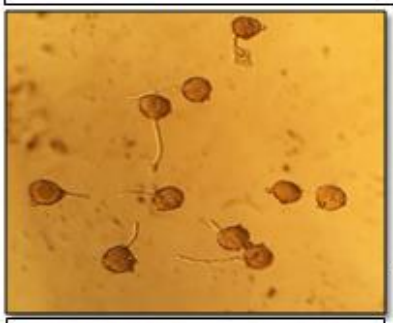

Fig.9 – *In vitro* pollen germination

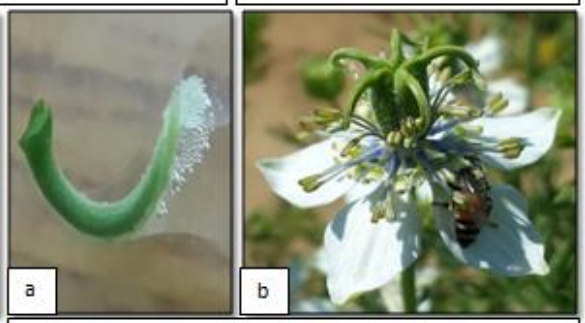

Fig. 10 – Female Phase (a - Effervescence in H_2O_2; b. Receptive Stigma)

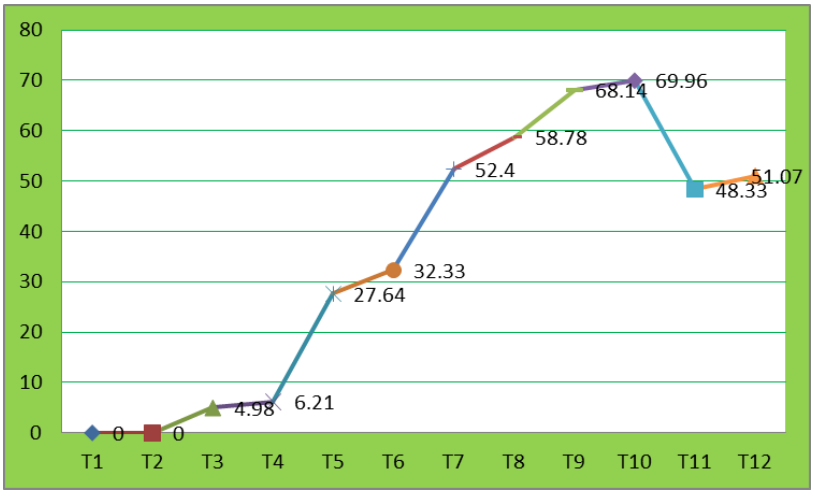

Fig11: *In vitro* germination of *Nigella sativa* L. pollens

Fig 12: Stigma receptivity as indicated by seed set in flowers pollinated on fifth day after anthesis

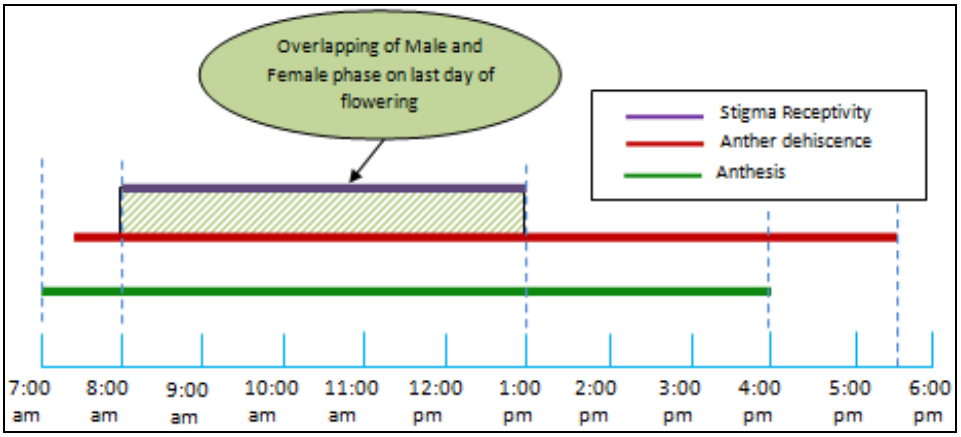

Fig 13: Diagrammatic representation of flowering events in *Nigella sativa* L. var. AN-1

Conclusion

It is apparent from the study that, flowers of *Nigella sativa* L. are protandrous, solitary in habit, often cross pollinated and decorated with 6±2 sepals, 8 pairs of petals, 6±1,2 indehiscent styles originating from siliqua and 8 clusters of 5±1 stamens arranged in whorls. During rabi season of arid and semi arid climate plants of *Nigella sativa* L. var. Ajmer Nigella -1 take 58 to 62 days for flower bud initiation, 78 to 87 days for flower opening from the day of sowing, one day after anthesis for onset of male phase and female phase on fifth day of anthesis with peak time ranging from 08.00 am to 01.00 pm. Availability of good amount of pollens and appealing phenotypic indices like movement of stamens from upright to horizontal position during male phase and correspondingly gradual downward inclination of stigma from upright to below horizontal position with severe twisting could be effectively used as key events for assisted pollination in hybridization programme for evolving high yielding superior quality hybrids in *Nigella sativa* L.

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