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Phenology and Reproductive biology of *Clerodendrum splendens* G. Don

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ABSTRACT

Phenology and floral biology of *Clerodendrum splendens* (Lamiaceae) commonly known as flaming glory bower and bleeding heart vine, was studied in different parts of Mathura city, India. This beautiful climber with bunches of crimson red flowers blooms from December to April, with optimum blooming in January and February. This species is highly self-incompatible. In order to avoid self-pollination, the flowers exhibit several morphological modifications in their reproductive parts (stamens and pistils). Black ants are the only floral visitors. There is no fruit formation either by self or cross-pollination, which may partly be due to cultivation by cuttings of the same clone. Only parthenocarpic fruitsdevelop in the months of March-May. The failure of fruit and seed set in *C. splendens* is likely to be related to its self-incompatible nature, absence of the nectar reward and the effective pollinators (butterflies, honey bees and humming birds).

Keywords : Lamiaceae, flaming glory, self-incompatible, floral contrivances, parthenocarpy.

Phenology is generally described as an art of observing the life cycle or the activities of organisms occurring throughout the year (Leith 1973).

Reproductive biology provides information on life forms, rate of flowering, type of breeding system, plant-pollinator interaction, breeding system, fruit and seed output, seed germination, seedling establishment, overall fitness and survival of the species. Studies on the biology of reproduction in endangered and threatened species are rare and useful for understanding the cause of their threatened status (Lavergne *et al*. 2004). Information obtained from such studies is helpful in the *in situ* and *ex situ* management strategies for their conservation and are integral feature of all conservation projects (Moza and Bhatnagar 2007).

Clerodendrum is a genus of subfamily Ajugoideae of Lamiaceae (Harley *et al.* 2004), being one of the most important and largest genera in this family, distributed worldwide, with over 400 species according to Harley *et al.* (2004), while Yao-Wu *et al*. (2010) reported only 150 species. *Clerodendrum* is native to tropical and warm temperate regions of the world, with most species occurring in tropical Africa and southern Asia, but with a few in temperate zone in eastern Australia and a few extending north into the temperate zone in eastern Asia (Mabberley 2008). Three species of the genus *Clerodendrum* (*C. phlomidis, C. inerme* and *C. splendens*) are found to grow both in wild and cultivated form in different parts of north India. *Clerodendrum splendens* G. Don is commonly known as flaming glory bower or bleeding heart vine. This large, sprawling, evergreen, ornamental climber of about 3-4 m in height is a native of western Africa, from Senegal to Angola. The leaves are oval, entire, coriaceous and arranged in opposite pairs. Flowers arranged in several corymbose spike, pentamerous, zygomorphic, hypogynous, four epipetalous stamens, gynoecium bicarpellary and syncarpous. The fruits blue, berry-like and parthenocarpic.

Reproductive biology of some *Clerodendrum* species has earlier been studied (Chauhan *et al.* 1986, Rohitash and Jain 2010, McMullen 2011, Rojas-Sandoval and Acevedo-Rodriguez 2012, Gautam and Rohitash 2012, Raju and Kumar 2016,Rohitash 2016, 2017). However, *C. splendens* has received little attention and there is some confusion about the formation of fruits and seeds. The present paper deals with phenology and floral biology, as well as changes in the floral morphology in order to prevent self- pollination in *C. spendens* plants growing at different sites of Mathura city.

MATERIALS AND METHODS

Present investigation was undertaken on ten plants of *Clerodendrum splendens* growing at five different places in Mathura city(27.28°N 77.41°E), Uttar Pradesh, India (Table 1).

Table 1-Study sites.

S. No.	Site in Mathura City
1.	B.S.A. College
2.	K.R. College
3.	Birla Mandir, Mathura-Vindraban, Road
4.	Krishna Janam Bhumi
5.	Dampier Nagar

Phenology—Phenological events on vegetative and reproductive parameters were recorded on the ten marked plants for three years (2010-13).Habit of the plant, leaf fall and leaf renewal, flowering and fruiting periods were recorded throughout the study period.

Floral Biology—Flowering period, blooming intensity (number of open flowers/inflorescence, floral longevity and flowering cycle), floral dimensions of 50 flowers, anthesis, anther dehiscence and stigma receptivity on twenty five flowers on five inflorescences on marked plants were observed by various methods given by Kearns and Inuoye (1993).

Flower longevity—Flower longevity was determined by marking 50 buds on different branches on one plant (Gill *et al*. 1998). Time was recorded when new flowers opened i.e. when the petals reflexed to expose the androecium and gynoecium. The flowers were observed at regular intervals until the corolla withered. These changes were observed every day for a week.

Pollen Morphology—For light microscopic (LM) studies, the pollen grains were acetolyzed following the technique after Nair (1960). The morphology of pollen grains was studied using immersion oil 100X objective in Olympus Phase contrast microscope. The size of pollen grains was measured with an ocular micrometer under light microscope by the method after McKone and Webb (1988).

Scanning Electron Microscopic (SEM) studies—The pollen grains from fresh dehisced anthers and floral parts were fixed in 3% glutaraldehyde in 0.1M phosphate buffer at pH 6.8 for 8-20 hours at room temperature. Samples were washed in the same buffer by three changes. Post fixation was done in 1% osmic acid in the same buffer for four hours at 4°C. Samples were passed through the graded series of ethyl alcohol and isoamyl acetate (3-methyl butyl acetate). Samples were dried with liquid CO_2 in a HCP-2 Hitachi critical point dryer and coated with gold (20 mm coating) in a sputter coating unit (Polaron equipment Ltd, Walford, England). Observation and photographs were made in LEO-EM-SEM at All India Institute of Medical Sciences (AIIMS), New Delhi.

Pollen viability—Viability of pollen collected from fresh dehisced anthers was tested throughout flowering period by 0.2% TTC (2, 3, 5-tetrazolium chloride) test after Hauser and Morrison (1964) and by fluorochromatic procedure (FCR) test after Heslop-Harrison and Heslop-Harrison 1970). Pollen viability was also checked by Brewbaker and Kwack's (1963) *in vitro* pollen germination test by hanging drop culture method.

Pollen germination on stigma (*in vivo*)—*In vivo* pollen germination on stigmatic surface was observed by multiple staining technique after Alexander (1987). After 24 h pollinated pistils were fixed in Carnoy's fluid for 12 h. These were washed in ethanol and brought down to distilled water through descending series and finally kept in the staining mixture containing lactic acid, malachite green, acid fuchsin, aniline blue, orange G and chloral hydrate for 12 h. The stained pistils were kept in clearing and softening mixture containing lactic acid, phenol, choral hydrate and orange G. These pistils were washed twice in lactic acid and mounted in 1:1 lactic acid and glycerol. *In vivo* pollen germination was also carried out by fluorescence microscopic method after (Martin 1959). After 24 h pollinated pistils were fixed in Carnoy's fluid for 12 h, cleared in 4N NaOH and washed in distilled water. These pistils were mounted in 1:1 mixture of 0.005% decolorized aniline blue prepared in 0.05% M phosphate buffer at 11 pH and glycerin. The tissue was spread by applying gentle pressure on the cover glass. The preparation was observed under Olympus fluorescence microscope using UV filter.

Stigma receptivity— To check the receptivity of the stigma, cytochemical localization of non-specific esterases was conducted by hydrolysis of the substrate α-napthyl acetate as per Mattson *et al*. (1974).

Nectar—Volume of nectar from individual fresh open flowers at the time of flower opening (25 from each marked plant) was measured using 20 µl microcapillary tubes at 2 h intervals every day. Nectar volume was computed with the following equation after Cruden *et al.* (1983):

$$\frac{\text{mm of nectar in the capillary tube}}{\text{mm total length of tube}} \times \text{volume of calibrated capillary tube} = \text{volume of nectar}$$

Pollination Biology— Observations on type of flower foragers and their visitation rates were recorded. These were fixed in 70% alcohol and identified. Pollination efficiency of different pollinators was checked by observing the pollen load under microscope (Kearns and Inuoye 1993).

Breeding behavior—Breeding system was tested by autogamy, geitonogamy and xenogamy through hand pollination tests.

a. **Natural/open Pollination:** Five inflorescences on each marked plant were tagged and left with the buds ready for anthesis for natural pollination, while all open flowers, young buds and fruits were removed. Number of mature fruits developed in the tagged inflorescence was recorded.
b. **Artificial/hand Pollination:** Hand pollinations were carried out at the time of maximum receptivity of the stigma, using fresh pollen from the same flower (self-pollination-autogamy), pollen from a different flower on same plant (geitonogamy), and pollen from the flowers of five plants of the same species (xenogamy).

RESULTS AND DISCUSSION

Phenology—Leaf fall and leaf renewal of *Clerodendrum splendens* takes place simultaneously, with maximum leaf fall in the months of December and January, and moderate in the months of March to September and minimum in the months of October-February. On the other hand, Raju and Kumar (2016) observed that in *C. inerme* leaves shed continually but leaf shedding is prominent during the dry months (March-May) and profuse leaf flushing was observed during rainy season (June-September) in Coringa mangrove ecosystem, Andhra Pradesh.

C. splendens blossoms from December to April. Flowering starts in the month of December and optimum flowering takes place in the month of February and declines in

March and only a limited number of flowers are left in April. Fruit formation starts in the month of March and lasts till May.

Floral morphology— There are 65±15 inflorescence/ plant and there are 25±10 flowers/ inflorescence. Thus there are 2800±600 flowers /plant. Every day 14-20 flowers open on each inflorescence and each flower lasts for 2-3 days. Flowers open between 0600-0630 h. The flowers are weakly protandrous and pollen is shed at 07:30–09:00 h within half an hour of flower opening and the stigma becomes receptive at 1100 - 1400 h.

The beautiful red flowers appear in several bunches and are arranged in corymbose spike (Fig. 1A). Flowers are pedicilate, bracteates, pentamerous, complete, hermaphrodite, zygomorphic and hypogynous. The calyx consisting of five green sepals, gamosepalous with valvate aestivation (Table 2). The corolla consisting of five petals is tubular, gamopetalous, red, The androecium is epipetalous, polyandrous and didynamous, alternate with the corolla lobes, comprising four red stamens (Fig.1B). The anthers are dithecous, introrse and dorsifixed with long filaments (Fig.3A). They turn brown on maturity and dehisce longitudinally.

The gynoecium is bicarpellary and syncarpous. The ovary is tetralocular, superior and four chambered with axile placentation. There are two ovules in each locule. The style is terminal and hollow throughout. The stigma is bifid like a fork (Fig. 3G) and covered with medium sized papillae (Fig. 3H). The papillate stigma is of the wet type and is receptive after anther dehiscence. The developing fruits are blue, berry-like and parthenocarpic (devoid of seeds). Only 1-3 chambers of the ovary were observed to develop into fruit (Fig.1D). They are supported by the star-shaped calyces and stay pretty for a long time.

Table 2- Floral morphology of *Clerodendrum splendens* (n=50)

S. No.	Parameters	Observations
1.	Flowering period	December to April
2.	Inflorescence	Corymbose spike
3.	Flower	Red, 3.04±0.42 cm in diameter.
4.	Calyx	Five, green, gamosepalous sepals; 0.61±0.11 cm long
6.	Corolla	Five, red, tubular, gamopetalous petals; 2.80±0.40 cm long
7.	Stamens	Four, red, epipetalous, 2.50± 0.12 cm long
	Anther	0.12 ± 0.06 cm long
	Filament	2.38±0.2 cm
	Pollen	5.9-55.3±0.53 µm in diameter. Colpi 53.31± 1.34 µm long. exine 3.02± 0.28 (-2.2) µm thick.
8.	Pistil	3.50 ± 0.52 cm long
	Style	Red, terminal; 2.92± 0.34 cm long
	Stigma	0.28± 0.13 cm broad, bifid
	Ovary	Superior, bicarpellary, syncarpous 0.30± 0.50 cm long

± *Standard deviation*

Floral contrivances—The flowers exhibit several interesting floral modifications (herkogamy) to avoid self-pollination (Figs.2A-I). The following different types of modifications in the position of stamens and style were observed in the flowers of the same inflorescence :

1. In nearly 20-35 % flowers/inflorescence, the stigma remains straight but closed, while two stamens bend on one side, while the other two bend to different side and remain away from the stigma (Fig. 2A).
2. In some other flowers, the stigma bends down while all the four stamens remain upright (Fig. 2B).
3. In some other flowers, two stamens remain upright, while the other two bend down and the bifid stigma remains straight, away from the stamens (Fig.2C).
4. In some other flowers, the filaments of all the four stamens bend down in one direction while the open bifid stigma is upright (Figs.2D).
5. In some other flowers, the filaments of two stamens bend in different directions, while the filaments of the other two are curled and reduced to touch the corolla surface and bend in different directions (Fig.2E).
6. In a limited number of flowers of an inflorescence, the filaments of all the four stamens are reduced and they curl and touch the corolla surface (Figs.2F, G, H).
7. In a few flowers, the filaments of all the four stamens are reduced in size, become much coiled and bend down towards the corolla tube (Fig. 2I).

Raju and Kumar (2016) observed only three forms of flowers in *C. inerme* in respect to the position of sex organs. In the first form the stamens were elongated and the style occurred in close proximity to the stamens. In the second type, the flowers exhibited scattered position of stamens and style. In the third type of flowers, the stamens were fully extended while the style was curved away from them. According to Rohitash (2017), the flowers of *C. inerme* are protandrous and show four types of sex organ positions in order to avoid self-pollination. While in presently studied *C. splendens* seven different types of the relative position of stigma and stamens have been recorded.

Trichomes—Both glandular and non-glandular trichomes are present on different floral parts viz. calyx, corolla and ovary. Peltate scales and unicellular trichomes are present on the inner surface of corolla tube (Fig. 3F).

Nectar— The nectar was not available in the measurable quanitiy, it may be in traces at the time of anthesis.

Pollen morphology— The pollen grains are spherical or sub-spherical, tricolporate, prolate or sub-prolate (Figs.3B,C,D). The tectum is reticulate with minute spines distributed all over the surface (Fig. 3B). Exine reticulate, thick, sexine and nexine are more or less same thickness. Rohitash (2016, 2017) observed tricolpate, sub-prolate, circular, echinate pollen grains *C. phlomidis* and *C. inerme*. Parveen and Quiser (2007) examined the pollen morphology of 13 species representing 9

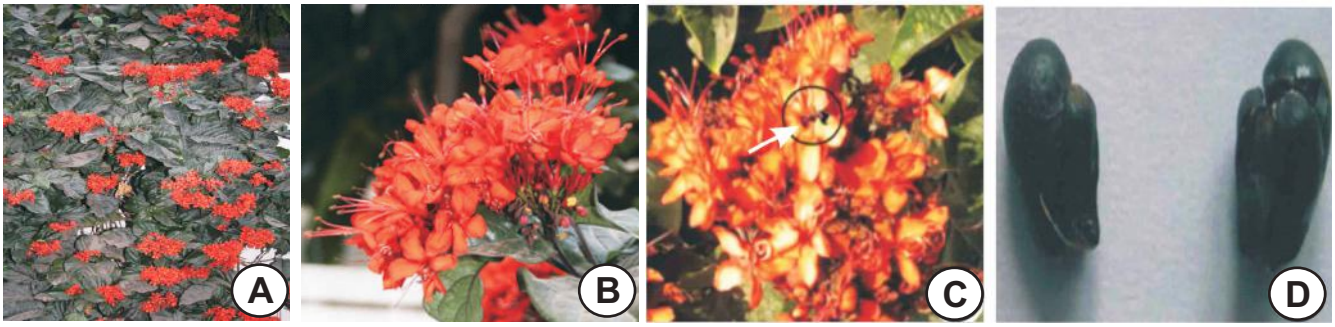

Fig. 1A. Plant in full bloom; B. Inflorescence; C. A black ant on the flowers of a mature inflorescence (circle); D. Parthenocarpic fruits.

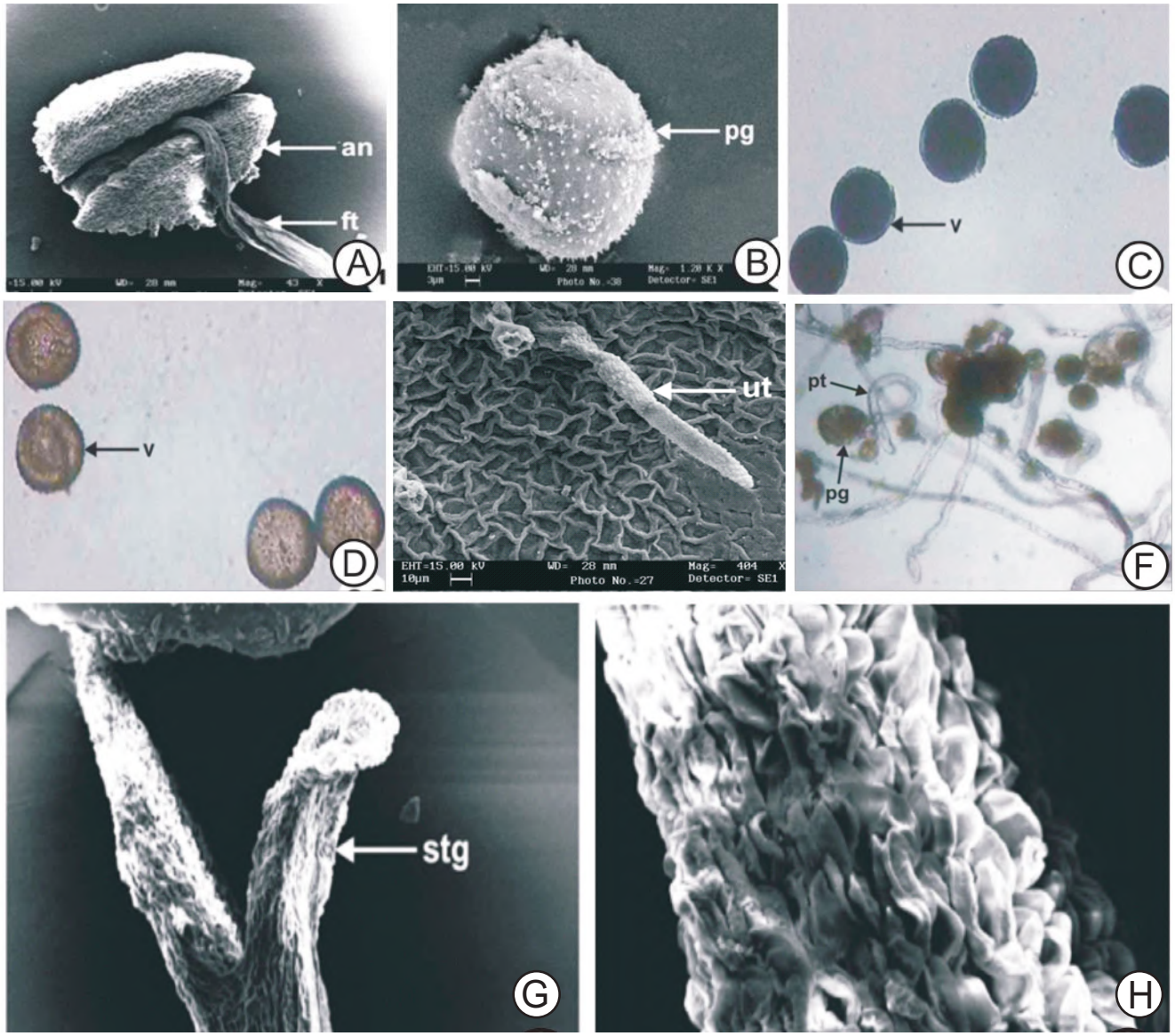

Fig. 2-A & B. SEM pictures showing dorsifixed anther and curved filament; B. mature pollen grain; C & D. viable pollen grains; E Pollen germinating in vitro; F. Peltate scales and unicellular trichomes on corolla surface; G. bifid stigma; H. magnified view of stigmatic surface with small compactly arranged papillae (an: anther, ft: filament, pg: pollen grain; pt: pollen tube, stg: stigma, ut; unicellular trochome; v: viable).

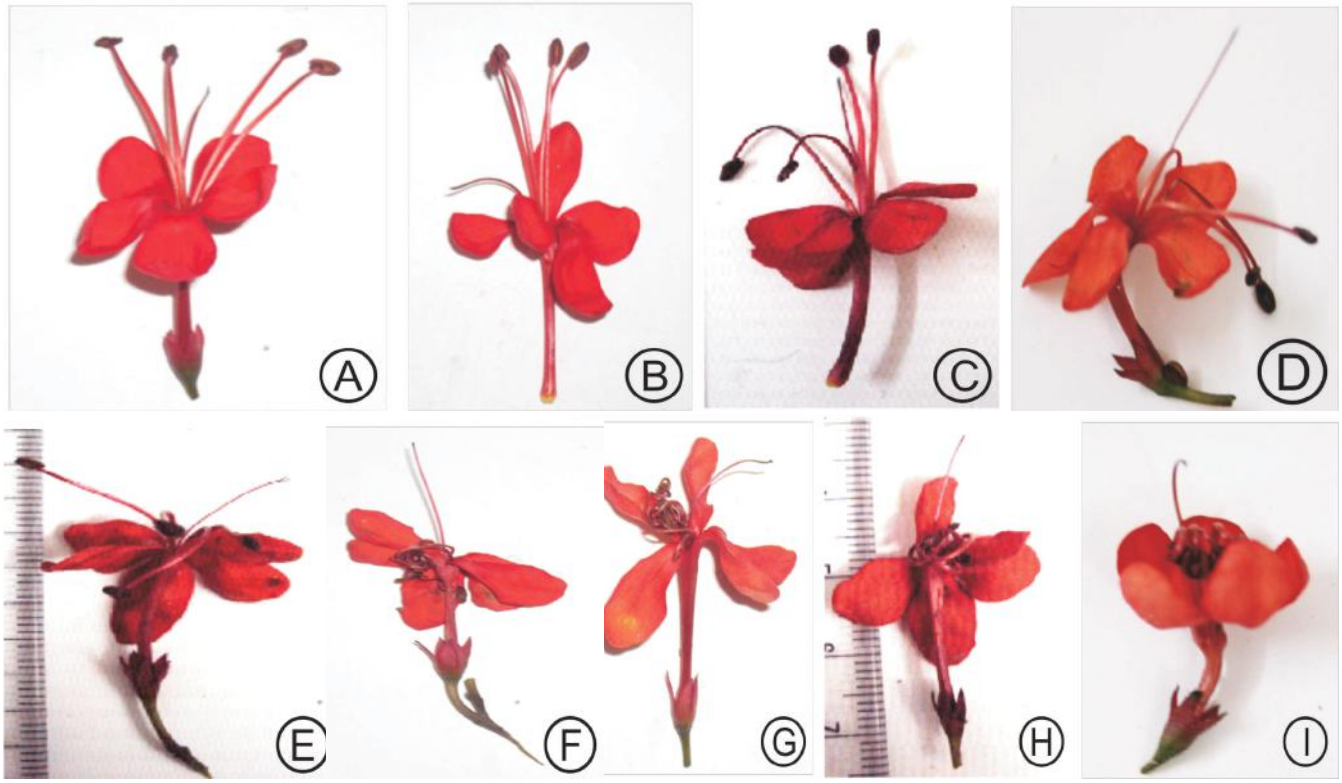

Fig. 3A-I Flowers showing contrivances.

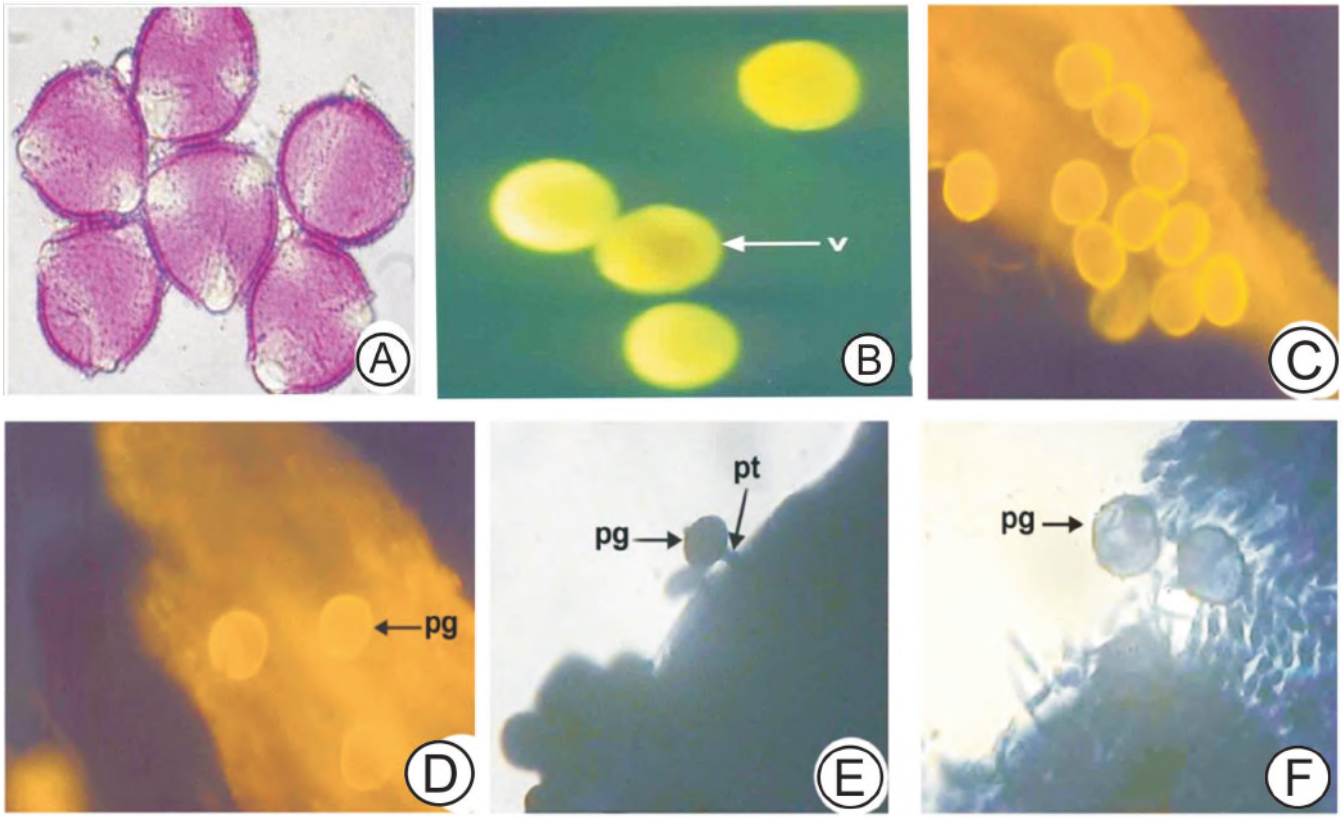

Fig. 4A & B. Viable pollen grains (v); C & D. pollen grain (pg) on stigmatic surface (fluorescence test); E & F. Pollen (pg) germinating on stigmatic surface with small pollen tubes (pg: pollen grain; pt: pollen tube; v: viable).

genera of the family Verbenaceae now Lamiaceae from Pakistan by light and scanning electron microscope. According to them, the pollen grains were usually tricolporate, rarely tricolpate.

Pollen viability—Average pollen viability as tested by FCR, and 1% TTC was 83% (Figs. 3C,D). There was 58% *in vitro* pollen germination with 763.65± 34 mm long pollen tubes in Brewbaker and Kwack's medium (Fig. 3E).

***In vivo* pollen germination—**Pollen germination on the stigmatic surface as tested by Alexander's multiple staining method and aniline blue fluorescence methods was only 5 and 8% respectively (Figs.4C,D,E,F).

Pollination biology— The beautiful and attractive flowers of *C. splendens* are visited by black ants only (Fig.1C). These ants go deep in the corolla tubes which are devoid of nectar and they fail to contact the anthers and stigma. Other floral visitors, including honey bees and butter flies were not seen. It may be because honey bees cannot see red colour (Riddle 2016). According to the same author, honey bees see colours that human beings cannot, way up in the ultraviolet end of the spectrum and they cannot see colours in the red range too well. Red flowers would appear dark and featureless to them. Absence of butterflies may be due to absence of the reward, the nectar.

Primack *et al.* (1989) observed bird pollination in *C. inerme* in Queensland. Keng (1990) reported that *C. laevifolium* is pollinated by bees and butterflies. According to Reddy and Reddy (1995), papilinoid butterflies (*Papilio polytes, P. polymnestor* and *Atrophaneura hector* and *Atrophaneura hector*) are the exclusive pollinators of *C. infortunatum* and effect pterogotribic pollination by striking the anthers and stigma with their wings. McMullen (2011) conducted some pollination experiments in *C.molle*, a widespread member of the Galαpagos flora. According to him, this species exhibited incomplete protandry and set fruit via autonomous autogamy as a result of natural selection in an environment with few faithful pollinators. Nocturnal visitors were ants, spiders, hawk moths, and roaches, whereas diurnal visitors were carpenter bees and ants. In *C. inerme* with creamish white flowers, Rohitash (2017) observed hawk-moth (*Macroglossum* sp.), bees (*Apis cerana*, *A. dorsata*, bumble bees (*Bumbus lapidarius*) and butterflies (*Danaus genutia*, *Neptishylas papaja* and *Eurema hecabe*) as floral foragers and it was observed that honey bees and bumble bees were more active as compared to butterflies. Humming birds would most probably feed on this vine's flowers as they usually find red flowers most attractive. However, there are no humming birds in this part of the world.

Breeding system— There was no fruit formation in the begged flowers indicative of self-incompatibility. Formation of fruits in open pollinated flowers in *C. splendens* was only 2%. However, these fruits also show some interesting features. Only 1-3 chambers and very rarely all the four chambers of the ovary swelled into fruit like structures, while the other chambers remain undeveloped (Fig.1D). The swollen structures consisted of rudimentary seeds which failed to germinate when sown on the moist filter paper. These features indicated that these fruits are parthenocarpic. Formation of parthenocarpic furits has been reported earlier in *C. phlomidis* (Rohitash 2016).

Thus, the self-incompatible nature, absence of the important reward, the nectar and the effective pollinators (butterflies, honey bees and humming birds) are the causes of fruitlessness in *C. splendens.*

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