FLORAL BIOLOGY OF 'WILD SNAKE ROOT' (*RAUVOLFIA TETRAPHYLLA* L.)

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Abstract : *Rauvolfia tetraphylla* L. (Apocynaceae) is a small perennial woody shrub with huge medicinal properties flowers more or less throughout the year. Flowers are small, white to creamy white born on apical portion of branches in umbellate cyme. They are hermaphrodite with small floral tube comprising nectar deep at the base of the corolla tube and introse anthers form cone around the apex of style head where pollens are deposited. Anthesis occurs during 06:30 to 08:30 h and anther dehisced between 07:30 to 09:00 h on the day of anthesis. Flowers survive for one day and are self-incompatible. Pollination is mainly brought about by insects (entomophily).

Keywords: Anthesis, self-incompatible, *Rauvolfia tetraphylla*

INTRODUCTION

Rauvolfia tetraphylla L. is commonly known as Wild snake root, Devil-pepper or Still tree belongs to family Apocynaceae, growing as a perennial woody shrub. It has been cultivated as an ornamental and medicinal plant in India (Farooqi and Sreeramu, 2001). The plant has various significances and it is extensively used by Indians as a substitute or adulterant of *R. serpentina*.

Rauvolfia tetraphylla L. have huge therapeutic properties due to presence of about 30 alkaloids in the root, stem, leaves and fruits. Out of them Reserpine is pharmacologically highly effective. Also reported alkaloids are ajmalicine, reserpitine, sarpagine, deserpidine, rescinnamine, serpentine, ajmalidine, alloyohimbine, chandrine, corynathine, iscajmaline, neo-ajmaline, papaverine, reserpoxidine, serpinine, thambine and yohimbine (Farooqi and Sreeramu 2001; Mukherjee 2004).

Due to the huge medicinal properties of the plants, it is important to study the floral biology which will be supportive for hybridization programme and the information of floral biology is a necessity in assessing overall reproductive potential of the species.

MATERIAL AND METHODS

Study site- Study was conducted in small field located in Sadak Arjuni of Gondia district of Maharashtra (21°.10'N 80°.15'E, 256m msl). Temperature ranges from 30 – 45°C in Summer and 12 – 30°C in winter. Sadak Arjuni receive 1296mm average annual rainfall and relative humidity highest during rainy season. A field was prepared by planting saplings of about 2- months old collected from nursery stock of Manas Ayurveda Nagpur.

Flower morphology- Position of flower in inflorescence, structure of flowers, structure of separate floral parts, position of nectary were studied. Twenty flowers (n=5 per plant) were used for this study and observed with Olympus stereoscopic microscope. They were dissected to locate anthers, stigma, ovary and ovules. Photomicrographs were taken using Canon Digital Camera.

Flowering phenology- The plant species under study were visited on each day. The flower phenology was determined by visual observations commenced at the beginning of flowering and continued until fruiting (Mark and Francoise, 2005). The initiation of anthesis, anther dehiscence, nectar production and termination of flower, flowering period were noted. Dates of mediocre and peak flowering were observed by visual observations. Longevity of flower determined by recognizing the time of opening and shedding.

Pollen Productivity- The total number of anthers per flower was counted and a single anther was randomly selected to estimate the total pollen number per stamen. To determine pollen productivity undehisced mature anthers from the flower buds were collected from different plants. Pollen productivity per flower was determined by simple method (Nair and Rastogi, 1963).

The undehisced mature anthers were crushed in 5ml of 50% glycerine in a graduated test tube. The plastic dropper was standardized and the pollen per drop were counted by adding one drop of suspension on a slide and covered by a cover glass. From this the mean pollen production per flower was calculated.

Pollen morphology- Pollen morphology studied by Light Microscopy (LM) and Scanning Electron Microscopy (SEM). The pollen grains were acetolysed by the method of Erdtman (1960). Acetolysed pollens for light microscopic observations were stored in glycerine and those for SEM examination were stored in absolute alcohol. For LM study acetolysed pollen grains mounted on glycerine jelly and observed under the light microscope. The size of the pollen grains was measured by using standard calibrated ocular micrometer.

For SEM analysis, acetolysed pollen grains were placed directly to stubs with double sided adhesive tape and sputter coated with gold, photomicrographs were taken using Scanning Electron Microscope (Carl Zeiss EVO 18).

Pollen-Ovule Ratio- The pollen ovule ratio was calculated dividing the average number of pollen grains produced per flower by the number of ovules in the flower (Cruden, 1977). Ovule number was achieved from dissection of ovaries under stereoscopic microscope.

Pollen Viability

Stainability in 1% Acetocarmine- Mature but undehisced anthers were squashed in a drop 1% Acetocarmine stain and incubated for 5 min. The slides were observed under microscope, all deeply/completely stained pollen grains were considered viable and unstained were non-viable (Qureshi *et al*., 2009). Duration of pollen viability determined by repeated the same procedure from 0600, 0800, 0900, 1000, 1200, 1400 and 1600hr.

***In-vitro* pollen germination-** We follow sitting drop method for *in-vitro* pollen germination study. Fresh pollen grains from undehisced but mature anthers were squashed in different concentration of Sucrose i.e. 1%, 2%, 3%, 4%, 5% to 20% along with 200 ppm of Boric acid (Shivanna and Rangaswamy, 1993). The prepared slides incubated for 24 hrs in humid chamber (made by keeping moist filter paper in covered petriplates) and observed for germinated pollen grains.

Stigma Receptivity- The receptive stigmas were appeared to be wet, shining and turgid when observed through hand lens (10X). Subsequently a definite period of time, it became dry and blackish in colour, representing the loss of receptivity. Also stigma receptivity was estimated through peroxidase activity by using a 3% H_2O_2 solution and examined under a stereoscope (Dafni and Maues, 1998; Etcheverry, 2005).

Further the receptivity and duration of stigma were determined by confining the activity of non-specific esterases (Mattsson *et al*., 1974), and peroxidases (Galen *et al*., 1985), at one day before and on the day of anthesis (flower opening).

Artificial pollination-Artificial pollinations viz, autogamy, geitnogamy and xenogamy were carried out to determine the type of pollination took place in plant. Experiment conducted at 07:00 to 08:00 h followed by bagging during peak flowering season. After time for fruit set passed, bags were opened and percentage fruit set and number of fruits and seeds recorded.

RESULTS

Plant morphology- Plant is perennial, small woody shrub belonging to family Apocyanaceae. It is grows nearly 2m (6 feet) in height. Stem is dichotomously branched. Leaves are sub-sessile, arranged in verticillate phyllotaxy having of 3–4 leaves at each node. The shape of leaf lamina is varying from ovate, narrowly

ovate to oblong. Leaves become membranous, base broadly cuneate to round with acute or obtuse apex (Fig. 1A).

Floral morphology and pollination mechanism- Inflorescence born on terminal and axillary position of branches and comprises of 4 - 10 flowers in umbellate cyme. Flowers are small, pedicillate, complete and hermaphrodite with small floral tube comprising nectar deep at the base of the corolla tube. Petals white in colour, corolla tube urceolate, 3–4 mm long, hairy inside at distal half and lobes ovate or suborbicular. Five epipetalous stamens inserted at corolla throat. Anther introse form cone around the apex of style head where pollens are deposited (Fig. 2-A,B). Anther dehisces longitudinally. The Ovary is superior with bicarpellary, syncarpous, bilocular with 2 ovules in each locule (Fig. 2C). Ovaries connate, style filiform, stigma is wet, papillate and dumbel shaped/capitat. Nectar secretion was started about one hour before the opening of flower and produces continuously throughout anthesis. Drupes subglobose, 5–10 mm in diameter, glabrous, connate. 2 Seeds per fruit. Flowering almost throughout the year but peak flowering occurs during the months of early March to late April and middle of June to middle of August.

For estimation of the amount of nectar, flowers were randomly selected from different plants and bagged just before opening to prevent floral visits. They were excised at hourly intervals (N=15) and the amount of nectar was determined using calibrated microcapillary tubes. Anthesis and anther dehiscence was observed in the field using hand lens, following the method of Reddi & Janaki Bai (1981) and Mathur & Mohan Ram (1986).

As the corolla tube short, the flowers of *R. tetraphylla* offers both nectar and pollens to the pollinators it shows generalized mode of pollination and took place by insects (Entomophily).

Floral phenology- Flowering in *R. tetraphylla* occurred almost throughout the year under climatic conditions of Sadak Arjuni. But peak flowering occurred during early March to late April and middle of June to middle of August when ambient temperature ranges from 31.4^{o}C to 39.7^{o}C maximum and 20.2^{o}C to 27.8^{o}C minimum. The flower buds take 10-15 days from initiation to full bloom. Mediocre flowering persisted for about four weeks from last week of June to last week of July when maximum and minimum temperatures ranged between 29.2^{o}C - 37.3^{o}C and 20.5^{o}C - 28.9^{o}C, respectively. From the month of June rainy season starts in study area, plants gets sufficient moisture and humidity so it is most favorable period for sprouting of new buds as well as flowering of *R. tetraphylla*. But due to the heavy rainfall during July and August, soil becomes more moistened, that affects the plant and leads to the decline of flowering which was started from end of August. During this period plants were seen with an abundant number of fruit. Number of flowers per plant reduced greatly after September.

Flowers started opening in the early morning during 06:30-08:30 h when ambient temperature fluctuated between 21.2^{o} – 30.4^{o}C (Table 1). Anthers dehiscence occurred just after the anthesis at 07:30 – 09:00 h. The longevity of flower is 1-day.

Pollen:Ovule ratio- A flower of *R. tetraphylla*, have 5 anthers and single ovary. Each anther contained an average number of 460 pollen grains, therefore single flower has an average number of pollen is 2300 (n = 20). Pollens are round and circular/spherical in shape having tricolpate aperture (Fig. 1-E,F,G). They are smaller in size with 41.83µm in diameter. The ovary has average 4 ovules (n = 20) (Fig. 2C), so the pollen-ovule ratio is 575:1.

Pollen viability- The maximum viability of pollen grains of *R. tetraphylla* observed at the time of anthesis to six hours after anthesis (ranges from 81 to 86%). After that it declined and virtually low after 8 hours and very low after 12 hours (Fig.1B-C).

***In-Vitro* pollen germination-** *In vitro* pollen germination (Fig.1D) was detected from 5% to 10% sucrose supplemented with 200 ppm boric acid. But maximum germination found in 6% sucrose + 200 ppm boric acid. Although the percentage of pollen grain germination was very low.

Stigma receptivity- Stigma remained receptive almost 1-hour before the opening of flowers and became fully receptive for about six hours after the anthesis. Thereafter, receptivity of stigma declined. Stigmatic receptivity also determined by location of enzyme activity on surface of stigma – 1. Before anthesis (one day before opening of flower) and 2. After anthesis (on the day of opening of flower).

I. Esterase activity- Esterase activity was confined by the presence of a brown precipitate uniformly spread across the surface of stigmas of pistils excised from flowers before anthesis (Fig. 2E). It also detected in style. Enzyme esterase activity found to be more in stigma and style after anthesis produced more intense dark brown colour (Fig. 2E-F).

II. Peroxidase activity- Peroxidase activity was detected as a yellowish-orange precipitate in the papillar cells of stigmas of flowers. Result indicated that Peroxidase activity found more in stigma after anthesis. No reaction was detected either in the sub-papillar cells or in the styles of these flowers (Fig. 2G-H).

Floral rewards- The flowers of *R. tetraphylla* (Wild Snake Root) offered both nectar and pollen to the visitors. Nectar secretion started 1-hour before the anthesis (05:30 - 06:30 h) and it oozes continuously in minute quantity all over a day.

Artificial pollination- Various artificial pollination experiments were performed for determination of type of pollination (Cross/Self-pollination) favoured in flowers (Table 2). Three types of pollination treatment given to flowers n = 50 in each case. 50 marked open/natural pollinated flowers carefully observed for percent fruit set. 74% fruit set was detected in natural pollination (open-pollination), however 58% fruit set was observed through manual geitonogamy and 82% fruit set from xenogamous pollinations and there is only 4% fruit-set by autogamous self-pollination.

The fruit development took 22-28 days for attaining ripeness after fertilization. Each fruit commonly contains two seeds. The seed germination percentage in natural habitat was approximately 25-30%.

DISSCUSSION AND CONCLUSION

The flowering in *Rauvolfia tetraphylla* occurred throughout the year but timing of blooming and anthesis were varied in summer and winter season. This due to factors like photoperiod, light intensity, temperature, moisture supply including ambient humidity, soil moisture and nutrient supply. Similar findings also reported by Sihag (1982).

The pollen production and availability of pollen to the receptive stigma is an indispensable necessity for achievement of pollination. We observed average number of 2300 pollen grains per flower. But our result fluctuated from the previous study on *R. tetraphylla* done by Subbu *et al*., (2008) they reported 9280 pollen grain per flower. Single ovary contained 2 ovules per flower under the climatic conditions of Sadak Arjuni (Gondia district). But there were initially 4 ovules found in flower, 2 in each locule (Ovary bilocular). 2 ovules get aborted (1 from each locule) during the developmental stages by unknown cause and hence ovules number reduces to 2 (1 in each locule) (Fig. 2C-D). Abortion of ovules started after 3-4 days of fertilization. Similar result also found in Sarpagandha (*R. serpentina*) by Sihag and Wadhawa (2011).

Pollen germination is the first significant morphogenetic event in the pollen in the direction of accomplishing its release of male gametes in the embryo sac. The stigma provides an appropriate site for pollen germination, however studies on in vivo are not easily possible because of the obstacles involving in pistillate tissue (Biswas and Mondal, 2014). In *R. tetraphylla* maximum pollen germination occurred in 6% sucrose + 200 ppm boric acid solution.

Stigma receptivity is the capability of stigma to support the viable and compatible pollen to germinate. The time and duration of stigma receptivity should be accompanied for successful breeding of crops (Stone *et al*., 1995). In general at the time of anthesis, the stigma is receptive and may lose for one, two or several days (Shivanna *et al*., 1997; Kalinganire *et al*., 2000). Here highest receptivity of stigma was found from an anthesis time to 6 hrs after the anthesis, afterwards it became declined. It is also confirmed by

peroxidase and esterase enzyme activity. Both peroxidase and esterase enzyme activity higher at the day of anthesis which indicates that stigma become most receptive.

Flowers of *R. tetraphylla* offered pollen and nectar to pollinators. Nectar secretion started one hour before the time of anthesis and oozed in minute quantity throughout a day. Generally, the nectar secretion depends on the physiological state of the plant (Huber, 1956). But even in healthy and well-nourished plants, nectar production shows a marked autonomous rhythm that corresponds to the periodicity of the pollination process.

Viability of pollen grains and stigmatic receptivity were highest at the time of anthesis i.e, both pollen grains and stigma mature at the same time. But Autogamy could not take place because self-pollen unable to fertilized ovary. This indicated that there is self-incompatibility found in flower and due to this self-pollination prevented and cross pollination permitted. Similar results also reported in *R. micrantha* (Kulloli and Sreekala, 2009). From the observation and results it noticed that the plants favour geitonogamy but autogamy occurred very rarely. While observing of stigma of bagged flowers it seemed that pollen germination was very less on the surface of stigma. So this may be one of reason for inhibiting self-pollination.

From above observation it is concluded that, cross-pollination (xenogamy) boosted the quantity of fruit-set in *Rauvolfia tetraphylla*. Also this study has provided valuable information on the floral biology and mode of pollination of *R. tetraphylla*.

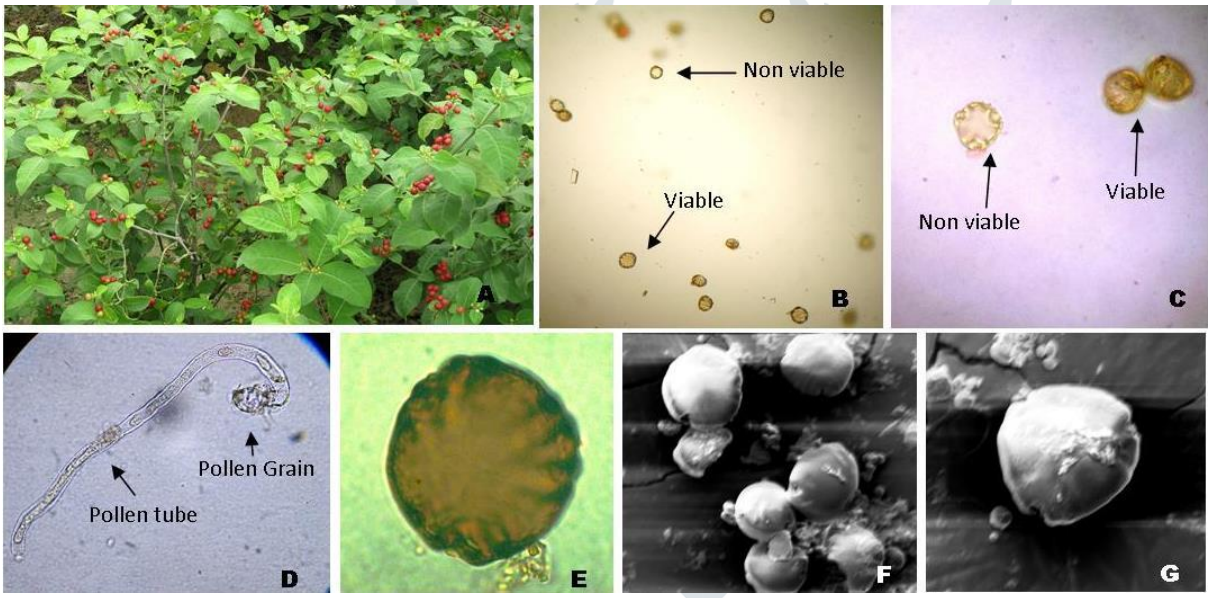

Fig.1-A. Plants with full bloom and fruits, B-C. Pollen viability by 1% Acetocarmine stain, dark red stained pollens are viable, poorly stained (light coloured) - Nonviable D. Germinated pollen grain, E. Acetolised pollen grain, F-G. Scanning Electron Micrograph of pollen grain

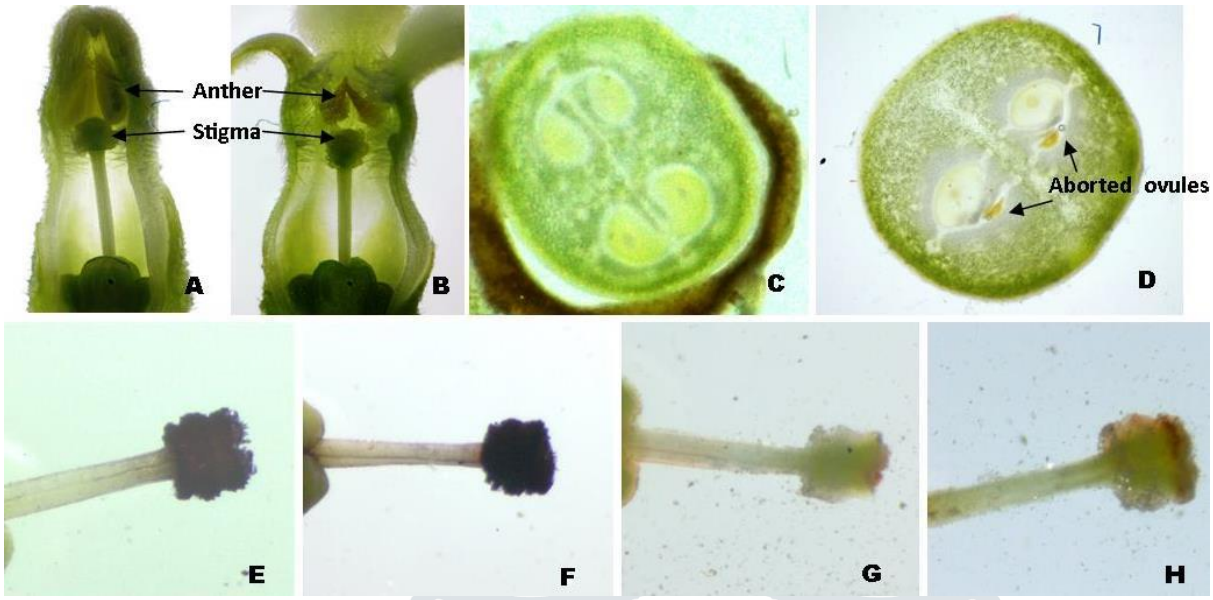

Fig. 2- A-B. Longitudinal Section of flower showing position of anthers and stigma - A. Unopened, B. Opened flower, **C-D.** Transverse Section of Ovary - C. Ovary showing 4 ovules D. Ovary showing 2 healthy and 2 aborted ovules, **E-H.** Enzyme activity on stigma - Esterase activity on stigma: E. One day before anthesis, F. On the day of Anthesis, Peroxidase activity on stigma: G. One day before anthesis, H. On the day of Anthesis

Table 1- Floral characters of *Rauvolfia tetraphylla*

S.N.	Floral Characters	Observations
1	Flowering period	Throughout the year but peak during - March to April and middle of June to middle of August
2	Number of inflorescences on a plant	30 - 94
3	Number of flowers in an inflorescence	4 - 10
4	Flower type	Pentamerous, hermaphrodite, actinomorphic
5	Flower colour	White to Creamy White
6	Flower opening time (Anthesis)	06:30 - 08:30hr
7	Nectar	2 - 3µl
8	Nectar secretion	Started 1 hour before the time of anthesis and secreted continuously in minute quantity throughout a day
9	Number of anthers/flower	5
10	Anther dehiscence mode	Longitudinal slit
11	Anther dehiscence time	07:30 - 09:00 hr
12	Average no of pollens/anther	460
13	Mean no. of pollen grains/flower	2300
14	Number of ovaries/flower	1
15	Mean no. of ovules/flower	4
16	Pollen /ovule ratio	575:1
17	Pollen shape	Round and circular/spherical
18	Pollen aperture	Tricolpate

19	Pollen size	41.83 µm in diameter
20	Stigma type	Wet, calyptriform and papillate type
21	Stigma receptivity	78 - 83 %
22	Pollen viability	81 - 86%
23	Fruit type	Drupe
24	Longevity of flower	1 day

Table 2- Details of pollination treatments, percentage of fruit set and number of seeds per fruit

S.N.	Treatment	Total Number of fruit set	Fruit set percent	No. of seeds per fruit
1	Autogamous self pollination*	2	4%	2
2	Geitonogamy (pollen of different flower of same plant)*	29	58%	2
3	Xenogamy (pollen of different flower of another plant)*	41	82%	2
4	Open (Natural) pollination*	37	74%	2

* (n = 50)

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