The International Journal of Plant Reproductive Biology 13(2) July, 2021, pp.103-108
DOI 10.14787/ijprb.2021132103108

Reproductive features and *in vitro* pollinia germination in *Holostemma ada-kodien* Schult, a RET species

D. Devipriya* and P. M. Radhamany

1. Department of Botany, Sree Narayana College for Women Kollam- 691001 Kerala India
2. Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram-695581, India
*e- mail : devipriyasnc@gmail.com

Received : 03.03.2021; Revised: 14.03. 2021

Holostemma ada-kodien Schult. belonging to the sub family Asclepiadoideae of family Apocynaceae was traditionally used for various ailments and to maintain vitality. The unscientific way of collection of root tubers and leaves for ayurvedic drug preparation leads to the population depletion in the wild. Self incompatibility was another problem which delimit its widespread. The present work is to analyze the reproductive behavior of plant by studying the structure and behavior of pollinarium in *in vitro* and *in vivo* conditions, stigma receptivity and *in vivo* pollen germination. The best medium for the germination of pollen was identified as Brewbakers medium with 25% sucrose. Macromorphology of fruits and seeds, micromorphology of ovary and ovule and the structure of pollinaria and arrangement of ovule in ovary using SEM were also studied. The data collected are very important for further breeding programmes.

Keywords : Asclepiadoideae, *Holostemma ada-kodien* Schult, pollinium, Brewbakers medium, pollen tube, abortive ovary.

Holostemma ada-kodien Schult.(Syn. *H. annulare* (Roxb.) K. Schum.) is a climbing herb with milky white latex belonging to the sub family Asclepiadoideae of family Apocynaceae. It is one of the potential, endemic medicinal plant distributed throughout the southern region of India especially in tropical forests (Ved and Goraya 2007). *Holostemma ada-kodien* is known in different vernacular names, such as Jivanti in Sanskrit, Jeeva haale in Kannada, Holostemma in English, Chirvel, Kanju in Hindi and Adapathian or Atapatiyan in Malayalam (Joy *et al*. 1998). Traditionally the plant is used as an astringent to the bowels; cures ulcers, diseases of the blood, itching, leucoderma, gonorrhea and it has ability to maintain vigour, strength and vitality (Gamble 1967, Kirtikar 1993, Irimpan 2011). The root and leaves are used in the form of powder and juice to treat spider-poisoning. The roots rubbed into a mash are used in cold milk as a curare to diabetes (Kirtikar and Basu 1975). All these reasons including other anthropogenic activities are the barriers in multiplying the species in wild, and thus the species became vulnerable (Nair *et al.* 1992).

For the sustainable utilization of our plant genetic resources, it is very important to conserve our endemic medicinal plants for the future. A detailed analysis on the reproductive behavior of the plants are inevitable for developing conservational strategies of this plant. So a thorough analysis on the reproductive characters of *Holostemma ada-kodien* was carried out in the present study.

MATERIALS AND METHODS

The material for the present study, *Holostemma ada-kodien* (Fig.1A), was collected from Peechi, Thrissur District Kerala, India (76º 18' East Longitude and 10º 28' North Latitude and Altitude 55.00 m) and was authenticated by Curator, Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, Kerala, India. Voucher specimen of plant (No: KUBH 6043) was deposited in the herbarium of Department of Botany,University of Kerala. Phenology of flowering, floral morphology, anthesis, stigma receptivity, pollinial morphology, *in vitro* and *in vivo* pollen germination, fruit morphology and seed morphology were studied.

Morphology of Pollinarium: The freshly collected pollinaria were placed on a clean slide and observed under an image analyzer (Leica DM 2000). Pollinarium dipped in distilled water for half an hour to identify the morphological variations after hydration. The colour and shape of pollinarium and corpusculum were observed and photomicrographed.

SEM Analysis : Freshly collected pollinaria were fixed in 70 % ethyl alcohol and acetolysed according to the method of Erdtman (1960) and modified by Nair (2004). The length of corpusculum, translator arm and length and width of pollinium were recorded. The sculpture on the surface of translator arm was also photomicrographed using Carl Zeiss EVO 18 Research microscope.

***In vitro* pollen germination :** From the fresh flowers pollinaria were carefully collected at 7.00 am and placed in different concentrations of sucrose (5-40%) alone and in combination with Brewbaker and Kwack medium (Brewbaker and Kwack 1963). For every concentration of the medium five slides were prepared separately and also a control was maintained in distilled water. Intact pollinium and crushed pollinium were observed for *in vitro* germination. Pollen tube initiation and growth were monitored from 0 hours to 2 hours.

***In vivo* pollen germination:** Cross- pollinated pistils of *H. ada-kodien* were collected after 12 and 24 hrs and were fixed in Carnoy's fluid. Pollinated pistils were placed in 5ml of lactophenol solution supplemented with few drops of 1% cotton blue and this was kept in the oven at 50°C for 30 minutes. Pistils were then mounted in a drop of glycerine and pressed carefully. Pollen tube growth were observed under microscope and photomicrographs were taken.

Stigma receptivity: Stigma receptivity was studied from the very first day of anthesis. Ten fresh flowers at different stage were placed in 3% solution of hydrogen peroxide separately. The presence of bubbles on the stigmatic surface indicated its receptivity (Dafni *et al.* 2005).

Arrangement of ovule in ovary: Strctural and morphological details of pistils were studied using anatomical preparations stained using cotton blue and also SEM images.

RESULTS AND DISCUSSIONS

In *H. ada-kodien*, leaves are opposite, apex shape varied from acute cuspidate and base deeply heart shaped. Margin entire, hairless, lateral nerves about five and lower pairs arise from the base of the leaves. Leaf venations are distinct with green and purplish colour. (Kirtikar and Basu 1975, Irimpan *et al*. 2011). Cymose Inflorescence (Fig.1B), arising from the axil of leaf with large purple bisexual flowers about 10 to15 in number. The pedicel of flower is longer than peduncle. The peak flowering was observed during June to November and the fruits attained maturity during January-February. Corolla is gamopetalous, flower colour varying from creamish white to creamish white with pink streaks and pinkish. Five stamens united to form a fleshy hollow column enclosing the style. Anther adnate to pentangular stigma, pollen united into waxy pollinia, which are formed by half anthers, and are united by stalks called caudicles to gland called corpusculum, whole gynostegium is covered by thin membrane. The pollinarium of sub family Asclepiadoideae consists of corpusculum, retinacula and pollinium. Carpenter bees (*Xylocopa spp*.) was found to be the major pollinator. Pollen grains agglutinate to form two pollinia in each stamen, arranged on the lateral sides of stigma. Ovary consists of two free carpels with marginal placentation ,two styles are united at the apex to form a five angled, pentangular stigma. Natural selfing was inhibited by protogynous nature and artificial selfing failed to set fruit due to self -incompatibility but it is cross compatible (Kurien and Sankar , 2007). The fruit is a dry dehiscent follicle (Fig.1 C, D), seen as 1-2, the second one often suppressed (Tuppad 2017). It is green when young and exudes latex when streaks made on its surface and turns brown when ripened. Healthy fruits contain 325-350 seeds**.** The seeds are brown with tuft of white conspicuously silky hairs at its tip which favours dispersal by wind (Fig.1E). When seeds are stored for long time, its hairs withered off (Fig.1F).

Morphology of Pollinarium : The orientation of pollinia in *Holostemma ada-kodien* is pendulous and club shaped (Fig.3. A, B). The pollinial sac is yellow in colour (Fig.2.B) and smooth with an average length of 1.5520 ± 0.02821 mm while its average breadth is 31.8 ± 1.32 µm. The corpusculum is brown in colour with an average length of 426.6± 1.90 µm and its free end is seen as curved structure in SEM analysis (Fig 3.C). Average length of translator is 817.26 ± .874 µm and is brown in colour but the inner surface of retinaculm is white in colour and with special ornamentation (Fig 3.D). Pollinial morphology is also taxonomically significant like the pollen morphology, the size and shape of pollinial sacs, colour of pollinia, nature of corpusculum, position of pollinia, structure of caudicle or translator, etc are important features for phylogenetic studies. By SEM analysis, the arrangement of pollen in pollinium (Fig.3 E,F) and single pollen is observed (Fig.3 G). The pollination efficiency of pollinia was well studied by different authors (Wyatt 1976, Broyles and Wyatt 1993).

Behaviour of Pollinium on *in vitro* medium : In *H. ada-kodien*, stigma becomes receptive from the very first day of anthesis, but anther dehisce only on fourth day. At that time the stigma loses its receptivity . In 20% sucrose medium, an average number of 11.100±.9944 pollen tubes emerged from the pollinia followed by the liberation of pollen grains may be due to the hydration of pollen grains inside the pollinium . Highest pollen germination was observed in Brewbakers medium with 25% sucrose. In this concentration, the pollen grains were hydrated and swell within the pollinia (Fig.2.C). In the vicinity of bursting of pollinia, bubbling was noticed (Fig.2.D). The pollen liberated from the pollinium were found to be in germinated state (Fig. 2E, F). About 90% of pollen grains were liberated from pollinium in germinated condition when more medium was supplied. Gradually the length of pollen tubes increases to 21.100±1.100 followed by a rapid growth of pollen tube with average length 242.659 ±1.345 µm length (Fig. 2G, H).

Pal and Mondal (2018) reported that pollinial germination was effected by crushing the pollen with sterile needle. But in *Holostemma ada-kodien*, crushing the pollinium caused reduced pollen germination and pollen tube growth. Such crushing injured certain pollen and thus affect pollen tube formation. The medium with 35% sucrose concentration shows liberation of pollen and the germination rate of such liberated pollen was very less. Germination capability of pollens depend on various factors like nutritional conditions and environmental factors (Khan and Perveen 2008). The pollinial germination is also observed in distilled water, but the rate of germination was very less and pollen tubes were shorter, when compared to the growth of pollinia in culture medium. The effect of either sucrose or boric acid individually showed pollen germination but sucrose in combination with boric acid promoted pollen germination as

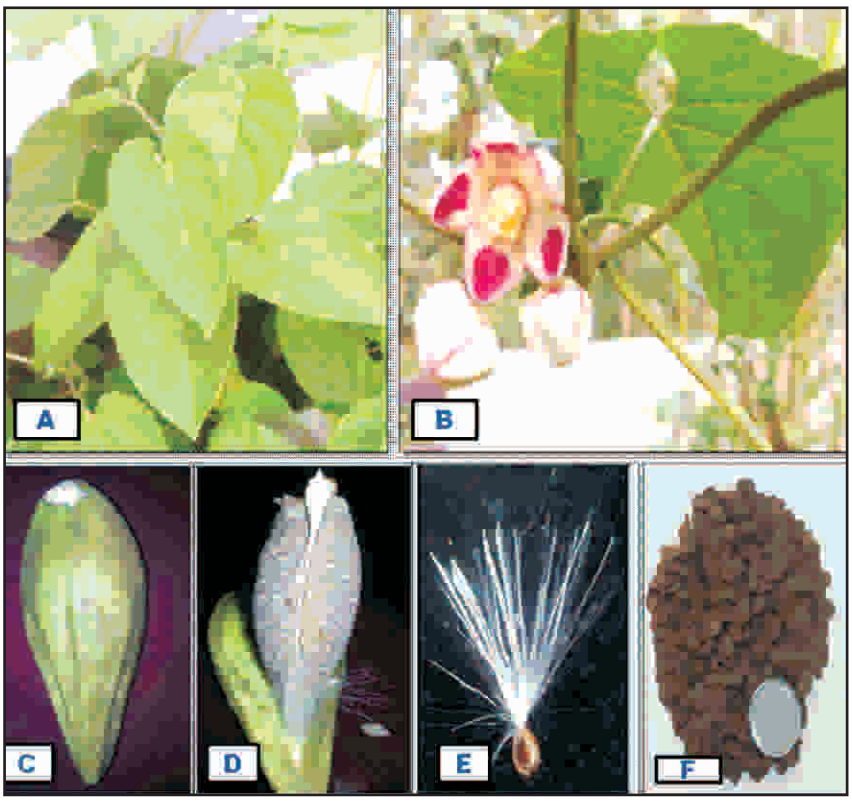

Fig. 1 A. *Holostemma ada- kodien* plant, B. An axil showing cymose inflorescence, C. Ripened fruit, D. Opened follicle, E. Seed with tuft of hairs, F. Dried seed

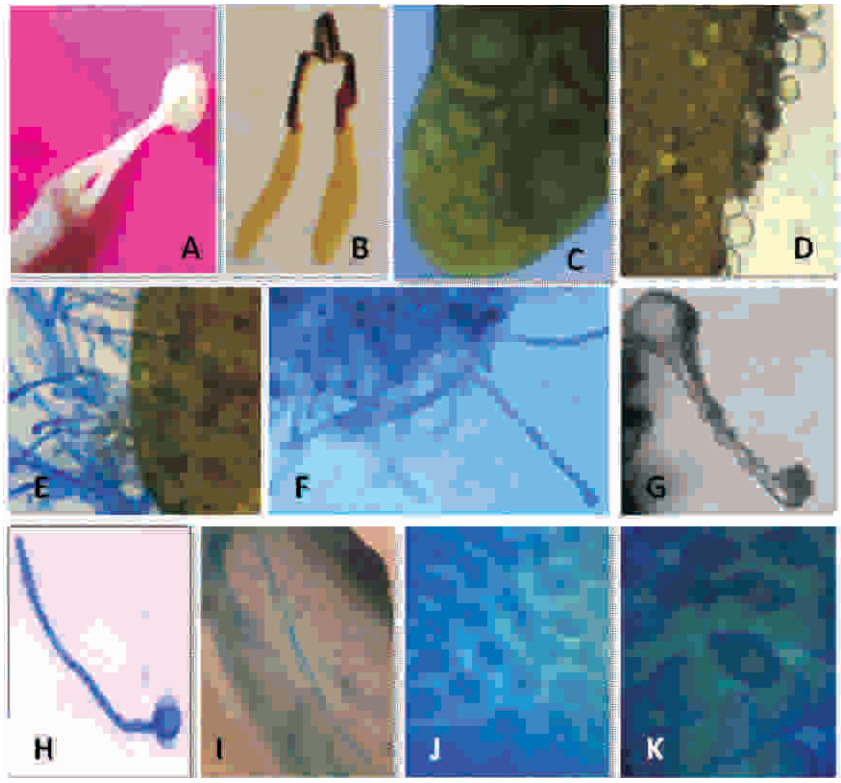

Fig. 2. A. Bicarpellary apocarpous ovary, B. Pollinium, C. Pollinium showing bulged pollen, D. Bubbling from bursting points of pollinium, E & F Germinated pollen from pollinium with long pollen tube G. Germinated pollen without staining H. Single pollen with long pollen tube, I. *In vivo* pollen germination, J&K. Mature ovule split apart.

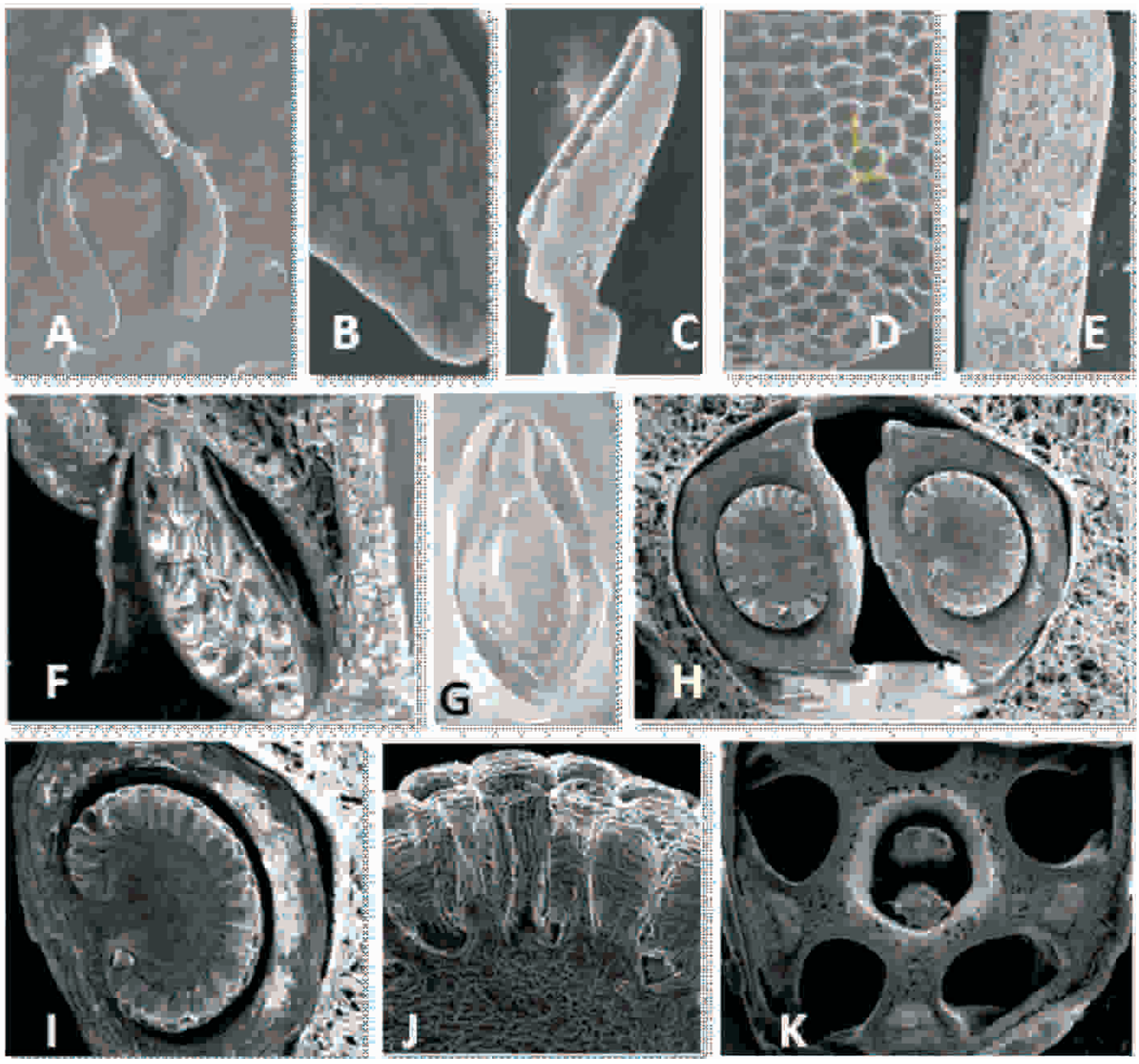

Fig. 3. A. Pollinium, B. Part of pollinium C. Ovule separated from ovary, C. rpusculum, D. Ornamentation on inner surface of retinaculum, E & F Pollen in pollinium, G. Single Pollen, H. Cross section of ovary, I. Single ovary showing arrangement of ovule, J. Cellular view under SEM, K. Abortive ovary.

well as tube development, because boron makes a complex with sugar and this sugar borate complex is known to be capable of better translocation (Sidhu and Malik 1986, Negalur and Lakshman 2017). The conspicuous role of sucrose and boric acid on *in vitro* with the views of Johri and Vasil (1961), Shivanna and Johri (1985), Pal *et al,* (2017). As reported by Pant *et al*. (1982) among *Cynanchum canescens* the pollinium of *H. ada-kodien* too exhibit unilateral germination.

***In vivo* pollen germination:** *In vivo* pollen germination on stigmatic surface was observed using cotton blue method confirmed that stigma support the pollen germination (Fig.2.I).

Stigma receptivity, Pollinators and Fruit set: The stigma become receptive before the flower bloom. The viscous and sticky exudates was observed on the entire stigmatic surface for two days after the flower blooms. The receptivity of the stigma gradually decreased from the third day so that the

Table 1. Observations noted for the behavior of Pollinium during various concentration of *in vitro* medium

Conc. of *in vitro* medium	Observations after 30	Observations after 1 hr minutes	Observations after after 2 hr.
Distilled water	Pollinium swells	Bursting of pollinium not occur	Bursting of pollinium but not significant pollentube noted
5% Sucrose	Pollinium swells	Bursting of pollinium not occur	Bursting of pollinium but not significant pollentube noted
10% Sucrose	Pollinium swells and point of bursting can be identifiable	Pollen tube formation not noticed	Bursting of pollinium but not significant pollen tube noted
15% Sucrose	Pollinium swells and point of bursting can be identifiable	Short pollen tube with an average length of 184.85±1.119µm	Few pollen with short pollen tube were liberated from pollinium
20% Sucrose	Pollinium swells and at the point of bursting pollen noticed as swelled	Long Pollen tube is generated from pollinium.	Germinated pollen is released from pollinium but slowly.
25% Sucrose	Pollinium swells and at the point of bursting pollen noticed as swelled	Long pollen tubes are emerged from pollinium with an average length of 242.659 ±1.345 µm	Germinated pollen are released continuously from pollinium
30% Sucrose	Pollinium swells and at the point of bursting pollen noticed as swelled	Two or three pollens released and a few pollens within the pollinium release pollen tube	Liberated pollens do not show pollen tube formation
35% Sucrose	Pollinium swells and more than one bursting region at inner side noticed	Four to six pollens are liberated	Liberated pollens do not show pollen tube formation
40% Sucrose	Pollinium swells and more than one bursting region at inner side noticed	Six or Eight pollens are liberated	Liberated pollens do not show pollen tube formation

Table 2. Length and colour of pollinarium and pollen tube

Structure	Length	Colour
Pollinium	1.55±0.02821 mm	Yellow
Corpusculum	426.6±1.90 µm	Brown
Translator	817.26 ± .874 µm	Outer surface brown and inner surface is white colour

stigmatic lobes became more creamish and completely dry, indicating complete loss of receptivity. Ovary is bicarpillary apocarpous (Fig.2A) and the ovule could be seen as separated from each other (Fig.2 J), in cone shape (Fig.2 K). The structure of ovary, position of ovule and its arrangement can be observed clearly by SEM analysis (Fig.3 H,I,J). The change in intensity and period of rainfall affects the rate of flowering in *Holostemma ada-kodien* which indirectly affect the visit of pollinators. Due to lack of effective fertilization, abortive ovules were observed in the ovary in such flowers (Fig.3K). All these adversely affect on fruit set.

CONCLUSION

The information on pollinial germination and pollen structure of *Holostemma ada-kodien*. are megre. The result of stigma receptivity, pollinial behavior in *in vitro* studies observed has significant role in flower selection for breeding programme. In *H. ada-kodien* self-incompatibility and reduced number of natural pollinators affects the fruit setting. These constrains can be overcome by careful selection of flowers for artificial pollination.

Acknowledgement : We acknowledge the Head, Department of Botany, University of Kerala, Karyavattom, for providing the facilities to carry out the research work.

REFERENCES

Brewbaker JL and Kwack BH 1963. The essential role of calcium ion in pollen germination and pollen tube growth. *Am. J. Bot.* **50** 859-865.

Broyles SB and Wyatt R 1993. The consequences of self-pollination in *Asclepias exaltata*, a self-incompatible milkweed. *Am. J. Bot.* **80** 41–44.

Dafni A, Keavan PG and Husband BC 2005. *Practical pollination biology.* Enviroquest, Cambridge

Erdtman G 1960.The acetolysis method, a revised description. *Svensk Botanisk Tidskrift* **54** 561- 564.

Gamble JS 1967. *Flora of the presidency of Madras*, 2nd edition. Botanical Survey of India. 1286.

Johri BM and Vasil IK 1961. Physiology of pollen. *Bot. Rev.* **27** 318-381.

Irimpan M T, Jolly CI and Sheela D 2011. A study of the phytochemical composition and antibacterial activity of *Holostemmaada-kodien* Schultes. *Int. J. Pharm Tech Res*.**3** 1208-1210.

Joy P, Thomas J, Mathew S and Skaria PB 1998. *Medicinal Plants,* Kerala Agricultural University. Aromatic and Medicinal Plants Research Station.

Khan SA and Perveen A 2008. Germination capacity of stored pollen of *Ficus carica* (Moraceae) and their maintenance. *Pakistan J. of Botany.* **40** 2251-2254.

Kirtikar KR and Basu BD 1975. *Indian Medicinal Plants.* M/S Bishen Sigh Mahen drapal, New Delhi, India, Pp. 162.

Kirtikar KR 1993. *Indian medicinal plants* 2nd ed. Periodical Experts Book Agency; *India*. **2** 894-895.

Kurien A and Sankar AM 2007. *Medicinal plants*. Peter.K.V(ed).New Delhi: New India Publishing agency. Pp. 130-134.

Nair KV, Nair AR and Nair CP 1992. Techno economic data on cultivation, preservation of some south Indian medicinal plants. *Aryavaidyan* **5** 238–240.

Nair PKK 2004. Plant taxonomy. *Cur. Sci.*. **86**(5) 665-667

Negalur CB and Lakshman HC 2017. Effect of sucrose and Boric acid on invitro pollen germination of *Guaiacum officianale* L. *Inter. J. Curr. Research*. **9**(2) 46490 -46493.

Pal S, Ghanta R and Mondal S 2017. Effect of sucrose and Boric acid on *in vitro* pollen germination of *Asparagus racemosus* Wild. *Advances in Bioresearch* **8** 190-195.

Pal S and Mondal S 2018. Studies on in vitro pollinium germination of *Oxystelma esculentum* (L.F.) SM. *J Palynology* **54**: 77-84.

Pant DD, Nautiyal DD and Chaturvedi S K 1982. Pollination ecology of some Indian Asclepiads. *Phytomorphology*. **32**:302-313.

Sidhu RJK and Mali CP 1986. Metabolic role of boron in germinating pollen and growing pollen tubes. In : Mulcahy *et.al.* (edi.) *Biotechnology and Ecology of Pollen.* Springer, New York.Pp. 373-378.

Shivanna KR and Johri BM 1985. *The angiosperm pollen structure and function*. Wiley Eastern Ltd, New Delhi.

Tuppad S, Shetty GR, Mastiholi L, Sandesh MS, Souravi K and Rajasekharan PE 2017. A. Review on the Pharmacology of *Holostemma ada-kodien* – A Vulnerable Medicinal Plant. *Int. J. Curr. Microbiol. App. Sci* . ***6***(11) 1532-1537 1532.

Ved DK and Goraya GS 2007. *Demand and Supply of Medicinal Plants in India.* NMPB, New Delhi & FRLHT, Bangalore, India.

Wang Q, Lu L, Wu X, Li, Y and Lin J 2003. Boron influences pollen germination and pollen tube growth in *Picea meyeri*. *Tree Physiology* **23** 345-351.

Wyatt R 1976. Pollination and fruit set in Asclepias: A reappraisal. *Am. J. Bot,* **63** 645-885.