TAXONOMY AND REPRODUCTIVE BIOLOGY OF *NYMPHOIDES* SPECIES (MENYANTHACEAE) IN KERALA

Thesis submitted to the
University of Calicut in partial fulfilment of
the requirements for the Degree of

Doctor of Philosophy in Botany

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Under the guidance of

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2020

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CERTIFICATE

This is to certifiy that the thesis **titled "Taxonomy and Reproductive Biology of *Nymphoides* species (Menyanthaceae) in Kerala" submitted** to the University of Calicut by **Mrs. Pavisha P** in partial fulfillment for the award of the degree of Doctor of Philosophy in Botany is the bonafied record of research work done under my guidance and supervision. No part of this work has been presented elsewhere for any degree or diploma previously.

Dr. R. Prakashkumar
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DECLARATION

I hereby declare that the thesis **entitled "Taxonomy and Reproductive Biology of *Nymphoides* species (Menyanthaceae) in Kerala"** submitted by me in partial fulfilment of the requirements for the **Degree of Doctor of Philosophy in Botany, University of Calicut** is the bonafide work carried out by me in the Malabar Botanical Garden and Institute for Plant Sciences, Post Box No. 1, Kozhikode-673014, Kerala. No part of the work has formed the basis for the award of any other degree or diploma previously.

Pavisha P

Calicut

Date: 27/04/2021

Acknowledgements

Foremost, I express my heartfelt gratitude and indebtedness to my research guide to **Prof. P.V. Madhusoodanan**, Emeritus Scientist, MBGIPS, Kozhikode for his full support, understanding and consistent encouragement throughout the course of my work. His academic enthusiasm and energy have always been a source of inspiration during the entire course of my work.

I would like to thank **Dr. R, Prakashkumar**, my Co-Guide and Director, KSCSTE-Jawaharlal Nehru Tropical Botanical Garden and Research Institute, Palode, Thiruvanathapuram (former Director, MBGIPS) for providing valuable suggestions and support throughout my work.

I wish to place on record my sincere gratitude to **Dr. S. Pradeep Kumar**, Director in Charge, KSCSTE-Malabar Botanical Garden & Institute for Plant Sciences (MBGIPS), Kozhikode for providing the necessary facilities and infrastructure to carry out my work.

I wish to place on record my sincere gratitude to **Dr. N.S. Pradeep**, Senior Scientist, KSCSTE-MBGIPS for providing valuable suggestions and unfailing encouragement and support throughout my work.

I sincerely acknowledge the help and encouragement of **Dr. P.N. Krishnan**, Emeritus Scientist, KSCSTE-MBGIPS, Kozhikode.

I am indebted to **Dr. R, Ansari**, eminent taxonomist and former Managing Director of MBGIPS, Kozhikode for providing valuable suggestions and support throughout my work.

I am extremely thankful to **Dr. P. S. Udayan**, Asst. Professor, P.G. Department of Botany and Research Centre, Sree Krishna College, Guruvayur and **Dr. S.V. Pradeep**, S.V.R N.S.S College, Vazhoor, Kottayam for their help and support rendered during the study.

I wish to thank the Herbarium Curator and Librarian, Department of Botany University of Calicut for allowing herbarium consultation and their help in identification of specimens and providing literature during the course of the study.

I take this opportunity to thank Director and Staff, Botanical Survey of India, Southern Circle, Coimbatore (MH) and Northern Circle (CAL), Kolkata for their valuable help.

I have special mention of thanks to Mr. Rajilesh V. K. and Dr. Anoop. P. Balan (DST-SERB NPDF), MBGIPS, Kozhikode for their wholehearted support, help and guidance, especially during the time of field study and thesis preparation.

I extend my sincere thanks to Dr. R.B. Smitha (DST - SERB) MBGIPS, Kozhikode for her wholehearted support and suggestions especially during the time of field study and the thesis preparation.

My sincere and heartfelt thanks are due to Dr. Anoop K. P and Mrs. Prajitha B. for their timely help and encouragement throughout the period of the study.

The sincere assistance extended by my colleagues in MBGIPS, Kozhikode, Mr. Ajesh, P. P Mrs. Liji, M., Mrs. Jaseela, V. T, Mrs. Sinisha, E. K, Mrs. Rajisha T. K, Mr. MithunVenugopal, Mrs. Priya Andrews, Mr. Lijo Joseph, Ms. Aishwarya Srinivas, Mr. Prasobh, Mrs. Swetha Thilak and Dr. Saranya Babu C.P. at various stages of this work are gratefully acknowledged.

I wish to thank all the administrative and non-administrative staff of MBGIPS, Kozhikode for their valuable helps at various occasions during the course of this study.

I am deeply indebted to my beloved parents, my loving daughter and my husband for all their support, encouragement and blessings to accomplish this study.

Above all, I thank the Almighty for showering the unseen blessings on me with which I could complete my work successfully.

Pavisha P

CONTENTS

Abbreviations

ca.	-	Approximately
CBD	-	Convention on Biological Diversity
Diam.	-	Diameter
ie.	-	That is
IUCN	-	International Union for Conservation of Nature and Natural Resources
Mg	-	Milligram
$Na_2HPO_4\,12H_2O$	-	Dihydrogen sodium phosphate
$Na_2HPO_4\,7H_2O$	-	Dihydrogen sodium phosphate
NaH_2PO_4	-	Dihydrogen sodium phosphate
pH	-	Potential of Hydrogen
ppm	-	Parts per million
Species	-	Spp.
TTC	-	Tetrazolium chloride
UNCED	-	United Nations Conference on Environment and Development

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1

INTRODUCTION

1. INTRODUCTION

Plants are considered to be the invaluable gift that nature has endorsed to human beings, other animals and microorganisms to meet the basic requirements of life such as food, water, Oxygen for respiration, etc. They also play a pivotal role in the maintenance and sustainance of the subtle and fragile equilibrium (homeostasis) of all ecosystems and the nature as a whole. The diversity of life forms (biodiversity) is supposed to be the end product of the continuous natural processes of organic evolution such as competition, struggle for existence, variation for better adaptation and survival leading to speciation ever since life originated on earth millions of years ago. India is rich in biodiversity; having only 2.4 % of the total global land area it represents 12.5 % of floral diversity and has 18,664 taxa of flowering plants (15,000 to 17,000 species) with 5725 endemics (Nayar, 1996). Karthikeyan *et al.* (2009) reported 17,527 species of angiosperms under 2991 genera and 251 families in India. The Western Ghats in Kerala region alone harbours 5091 species of flowering plants (Sasidharan, 2013). However, in recent years the plant wealth of Kerala is facing a serious threat of destruction due to various reasons, mainly owing to the indiscriminate destruction of natural vegetation by injudicious anthropogenic activities. As a result, many species have became extinct and many are at the verge of extinction (IUCN, 2019). In this context, it is imperative to conserve the biodiversity either *ex situ* or *in situ* and record the biology of each and every plant or animal species which are facing threats of extinction. The Earth summit (UNCED) meeting held at Rio de janiero in 1995 (CBD) has emphasized the need for understanding and recording the local flora as a basic process of biodiversity conservation. Being the primary producers of natural ecosystem the plants possess the ability to determine the biota suited to a characteristic habitat and successional seres. In India, the biodiversity is high in the mountainous region of Western Ghats in South India and Himalayas in the North East region. These areas are now considered among the 24 ‘hotspots’ of the world indicating the serious threat to the very existence of many species which have already become rare,

endangered or endemic and many are at the verge of extinction. This is caused mainly owing to the influx of migrating people from lowland to high lands in search of land for agriculture and plantations. This is consequent to population explosion happened after 1950's in the post independent India. Earlier, the government has been promoting the conversion of virgin forests to agricultural lands in order to combat with the problem of food scarcity and famine withstanding the importance of forests in climate control, soil erosion and rainfall. Now being aware and deeply concerned and understood the importance of natural vegetation for the very existence of human population, the Government has taken steps for the conservation of the remaining nature and natural resources. In this context, it is pertinent to preserve the biodiversity and realised the potential of pharmaceutical and other economic prospets of each and every plant species. Moreover, the Government is interested in the data collection of our natural resources which are highly essential for undertaking any new developmental programmes or projects. The present research work was undertaken with the above perspective to understand record and utilize the information on individual genus and species of the local flora in detail encompassing all details on morphology, ecology, phenology, etc. of the aquatic plant genus *Nymphoides* Seguier in the Kerala state and developing a conservation strategy of rare species or taxa.

1.1. Taxonomy

The term taxonomy is derived from two Greek words 'Taxis' (means arrangements) and 'Nomos' (means science / discourse). So taxonomy is the exploration, description, naming and arrangements of organisms. It deals with collection, preservation, description, identification, nomenclature and classification of plants. Taxonomy is one of the oldest fields of biology, but undergone several vicissitudes from classical Taxonomy to Molecular systematics during the past 300 years. Taxonomic research has an important role in monitoring and managing the world's natural resources and it provides correct identity and information of species distribution.

Nymphoides species come under Menyanthaceae family which is one of the most diverse and widespread floating leaved species (Cook 1996; Kadereit 2007). Tournay and Lawalree (1952), included Menyanthaceae under the order Ligustrales. Stebbins (1974), classified it under Polemoniales. Cronquist (1981), treated this under the order Solanales. Dalhgren (1980), Morley and Toelken (1983) and Takhtajan (1987), classified this family under Gentianales.

In older works related with aquatic plants *Nymphoides* has been placed in the family Gentianaceae and has been considered under the tribe, Menyanthidae (Gilg, 1895). Based on the morphological and embryological study, Stolt (1921) recognized Menyanthaceae as a distinct family. Lindsey (1938) also identified it as a separate family based on the anatomical study. Later Hutchinson (1959), studied this family and removed this tribe from Gentianaceae and considered as a separate family Menyanthaceae, based on their wetland habitat and distinct morphological and embryological features.

Menyanthaceae come under the order Asterales, consists of 60–70 species worldwide (Tippery *et al*., 2008). It consits of five genera viz., *Menyanthes* L., *Nephrophyllidium* Gilg., *Liparophyllum* Hooker f., *Villarsia* Ventenat and *Nymphoides* Seguier. The first two are monotypic and found in Northern Hemisphere, *Liparophyllum*is also monotypic and *Villarsia*, consists of 18 species are found in Southern Hemisphere, and *Nymphoides* consists of 40 to 50 species is cosmopolitan in distribution and *Nymphoides* species might be confused with *Villarsia*.

Aston (1969, 1973), redefined two genera such as *Nymphoides and villarsia.* He reported that *Nymphoides* are true aquatics, flowers non-paniculate inflorescences and fruits maturing under water. But *Villarsia* spp. are mostly wetland herbs, inflorescence paniculate, fruits ripening above water. Tournefort (1700) identified the two genera *Menyanthes* and *Nymphoides*. Linnaeus (1753) mentioned these two genera in *Species Plantarum* and combined these under *Menyanthes*, an emergent wet land species.*Nymphoides* was validly published by Seguier (1754). The synonym of

the *Nymphoides* is *Limnanthemum*, which was considered as the accepted genus name for over 100 years (Gmelin, 1769). Grisebach (1845), included it under the tribe Menyanthideae of Gentianaceae under the name *Limnanthemum*. Several workers followed this tribal position of the genus.

1.2. Phenology

Phenology is the study of the timing of recurring biological events such as shoot growth, flowering, fruit setting, seed dispersal, leaf shedding, etc. through different times of the year. Knowing the periods of these phenological events which are helpful to understand the ecology and evolution of species and communities (Newstrom *et al.* 1994; Sakai 2001). Phenological studies are essential to understand the specific functions of plants in natural populations. According to Beatley (1974a, 1974b), Mooney (1983), Orshan (1989), the phenological pattern, *i.e*., the annual distribution of these events, is an important component of the plant's strategy to deal with seasonal climates and its knowledge contributes towards an understanding of the ecosystem function (Korner, 1994).

The term phenology comes from the Greek words ‘phaino’ and logos. Several workers studied the phenology of different plant species. According to Paramesan (2006), phenology of a species was the effect of climate change on that species. The flowering phenology is the study of recurring biological events that cause their timing with regard to the biotic and abiotic factors and their interrelationship among phases of the same or different species (Sivaraj & Krishnamurthy, 1989), abiotic components of the environment is very much influencing the life cycle of biotic components. The abiotic components include the seasonal changes such as, variations in day length, temperature and precipitation. Depending on the aim of the research, the type of plant and climatic conditions, various authors have developed different methods to study plant phenology. Some workers selected sampling as units may be a single branch (Baker *et al.*1982; Gill &Mahall 1986; Nilsen 1986; Negi & Singh 1992; Oliveira *et* al. 1994; Dhaila *et al*. 1995 and Nitta &Ohsawa 1997) or

selected whole plants (Frankie *et* al. 1974; Arroyo *et* al. 1981; Bertiller *et* al. 1991; Neeman 1993).

Phenology may be altered by changes in temperature, precipitation and weather and it is influenced by several factors. The main events in pheonological changes are the growth and developmental pattern of leaf, morphological evolution of leaf, flushing and bolting leaf fall and reproductive biology such as growth pattern of flower bud, anther dehiscence, pollination, fertilization, seed setting and seed germination.

1.3. Reproductive Biology

One of the major challenges in India and the World has to face in the coming decades are:

Substantial increase in the crop production to feed the growing millions of population. Indian population will grow from 1.3 billion (2010) to 1.5 billion (2030). Pollination is a prerequisite for seed and fruit development. Seed and fruit of the plants form the major parts of food of human beings and other animals. ca. 90% of fruit plants use a range ofanimals to achieve pollination

Reproduction in flowering plants is a series of complex procedures. The reproductive events start with floral induction and end with seed germination. Plants reproduce asexually by fragmentation, budding, spore formation, vegetative propagation, etc. and sexually by the fusion of gametes. Asexual reproduction is very important in aquatic plants for their establishment, growth and their population production. Cook (1993), Spencer and Bowes (1993) observed that weedy aquatic plants disperse by sexual propagules (seeds), whereas floating and submersed species disperse mainly through vegetative reproduction. Reproduction ensures the sustainability of plants life and the genetic diversity through sexual reproduction. *Nymphoides* can reproduce asexually and sexually; vegetative reproduction by means of fragmentation by which it perpetuates the exact copy of the parent genome. *Nymphoides* reproduces sexually through fusion of gametes (fertilization) to

produce seeds. Reproductive biology mainly focuses on pollen biology, mode of pollination, fertilization, pollinator interaction and breeding system.

1.4. Pollen biology

Pollen biology is one of the important areas in plant reproductive biology. Pollen biology deals with various aspects such as pollen morphology, pollen load, pollination, pollen germination, pollen tube growth and seed production. It plays an important role in the formation of seed and fruits and also in crop improvement programs. Pollination biology provides a framework to test a diverse array of paradigms in several subdisciplines of biology (Bawa et al. 1993). Pollination biology includes palynology, pollen viability, i*n-vitro* pollen germination and *in-vivo* pollen germination.

The term 'pollen' was coined by Linnaeus (Knox, 1979) and 'palynology' was suggested by Hyde and Williams (1945). Pollen grains are microscopic promicro (male) gametophytes of fine powdery substance, typically yellow, discharged from the male part (anther) of a flower or from a male cone. Erdtman is considered to be the foremost palynologist of the world, who mainly concentrated on experimental work on pollen and devised the Acetolysis technique for the preparation of pollen for microscopic studies. Erdtman (1952), Rudenko (1959), Erdtman *et al.*, (1961, 1963), Wodehouse (1928), Moore and Webb (1978) and Rowley (1981), have contributed much to the field of angiosperm palynology.

1.5. Objectives of the study

- Survey on the diversity of all *Nymphoides* species in Kerala
- Collection and *ex situ* conservation
- Detailed morphological and palynological studies on *Nymphoides* spp. in Kerala, and
- Reproductive biology and phenology of *Nymphoides* spp. in Kerala.

2

STUDY AREA

2. STUDY AREA

Area of the present study is Kerala state, a narrow strip of land situated on the South – West corner of the Indian Peninsula bounded on the north by the state of Karnataka, east and south by Tamil Nadu, and West by the Arabian Sea (Fig.1). Kerala lies between latitude 8^0 04 ´´ and 12^0 44´´N and longitudes 74^0 54´ and 77^0 12´ E. The state has an area of 38,863 km^2. It extends to a maximum length of 550 km between Kaliyikavila in the south and Kasaragod in the North, with a mean breadth of 70 km, narrowing towards both ends to about 12 km and widening at the central part to 121 km (Sasidharan, 2004).

2.1.Topography

The coastal region of Kerala is generally flat on the West; the surface suddenly becomes unequal roughening into slopes then gradually combines and swells into mountainonus Amphitheatre (Ward&Corner, 1863).

The state is naturally divided into three longitudinal zones based on physiography viz., Theerabhoomi(lowlands/coastal zones) bordering the seacoast below 30 m, the midland between 30-300m consist of hills and valleys east of the lowlands between 300 and 600 mIdanadu and Malanadu (Highlands) extreme east covered by dense forest above 600m. Anamalai is the highest peak with 1678m alt.

2.2. Rivers

Kerala is blessed with rich water resources like 44 main rivers, originating in the Western Ghats mountains, their tributaries and distributaries and a number of streams, lakes, ponds ,and backwater lagoons. Out of the 44 rivers, 41 are west flowing and 3 flow east (Nandakumaran, 2015).

2.3. Climate

The climate of Kerala is a warm humid tropical climate which varies little from season to season. The maximum mean daily temperature in the coastal region is 32^0C (in April-May) and minimum is 22^0C (Dec-Jan) .The maximum temperature may rise up to 40^0C (at Palakkad and Punalur) in some areas in the

plains and the minimum temperature drops to 7^0 C in mountains during December – January.

2.4. Rainfall and Temperature

The state receives rain from both south –west (June-Sep) and North –East monsoon (Oct-Nov.) The total annual rainfall in the state varies from 1800mm to 3800 mm (NBSAP,2005). During monsoon seasons extensive flooding occur in low lands and some of the midland areas which will bring a lot of silt from the highlands to lowlands. So the lowland areas become fertile (alluvial deposits).

2.5. Soil

Soil composition in an area is determined by the nature of rocks from which it is derived. Two types of soil are mainly seen in Kerala such as upland soil developed from bedrocks and low land soil developed from alluvial materials (Seth and Yadav, 1960). The major soil subtypes in Kerala are Laterite soil, derived from rocks rich in Aluminium, Red soil (red loam rich in Iron), derived from ancient crystalline and metamorphic rocks, Forest soil (medium black soil); derived from the decomposition of trap – rocks and Alluvial soil; including coastal alluvium and Peaty soil or kari soil; contain large quantities of organic matter.

2.6. Aquatic and wetland systems and vegetations of Kerala

Kerala is rich in wetlands owing to prolonged rainy season. The main aquatic or wetland systems in Kerala are rivers, backwaters, estuaries, lakes, canals, ponds, tanks, streams, ditches, marsh lands and paddy fields (Fig.2). In, Kerala seven types of aquatic vegetations are recognized viz., Riparian vegetation, Estuarine vegetation, Mangrove vegetation, Aquatic vegetation, Wetland vegetation, Myristica swamps and Wet – rock vegetation (Ansari *et al.*, 2016).

Based on the growth forms in relation to the substratum, water and air, the plants growing in aquatic systems can be classified into six types (Hutchinson, 1975; Sunil &Sivadasan, 2009); they are free floating hydrophytes, suspended

hydrophytes, submerged – anchored hydrophytes, anchored hydrophytes with free floating leaves, shoots and emergent–anchored hydrophytes. Of these, *Nymphoides*species are anchored hydrophytes with floating leaves found in paddy fields, ponds, canals and riverbanks.

2.7. Exotic aquatic plants

There are many incidents of invasion of aggressive alien aquatic weeds in the wetlands of Kerala. The floating fern, *Salvinia molesta* introduced from South America which is considered as the 'World's Worst Weed' has been prevalent in Kerala creating numerous problems to agriculture, irrigation, inland fisheries, water transport and electricity production.(Madhusoodanan*et al.*, 2014) *Eichhornia crassipes* (Pontederiaceae) commonly called "water hyacinth" is an another S. American weed creating problems in the wetlands. They compete with local flora and exterminate the latter. Though *Salvinia* is controlled through biological 'control'. *Eichhornia*remains insurmountable. (Madhusoodanan and Ajith Kumar, 1994).

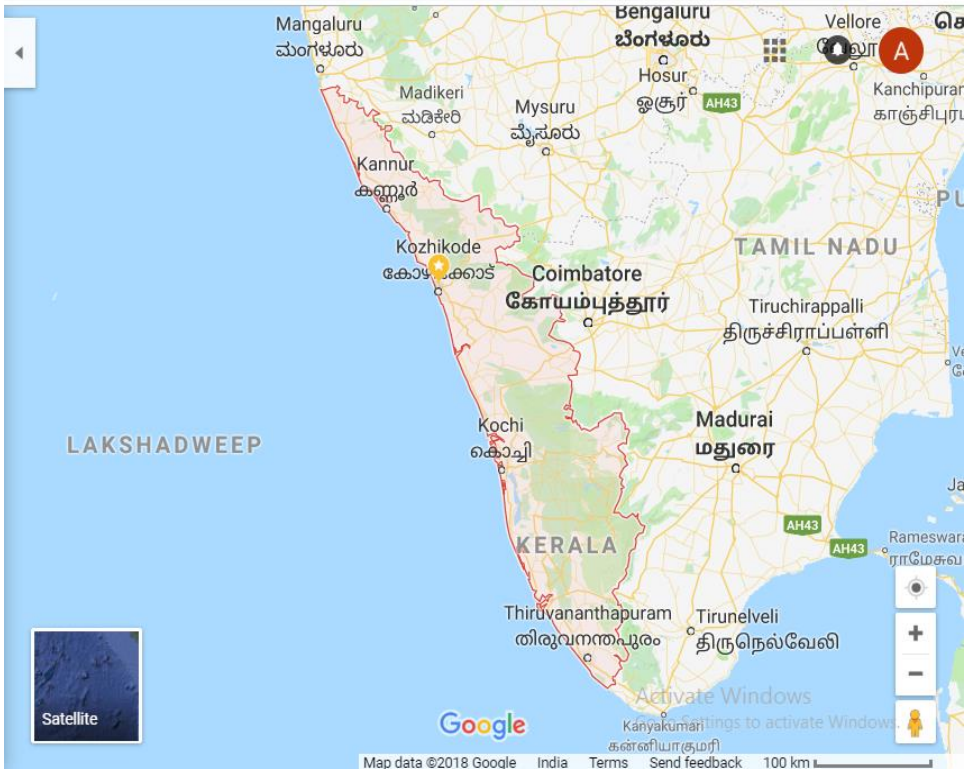

Fig. 1: Google map of South India showing the geographical position of the Kerala State.

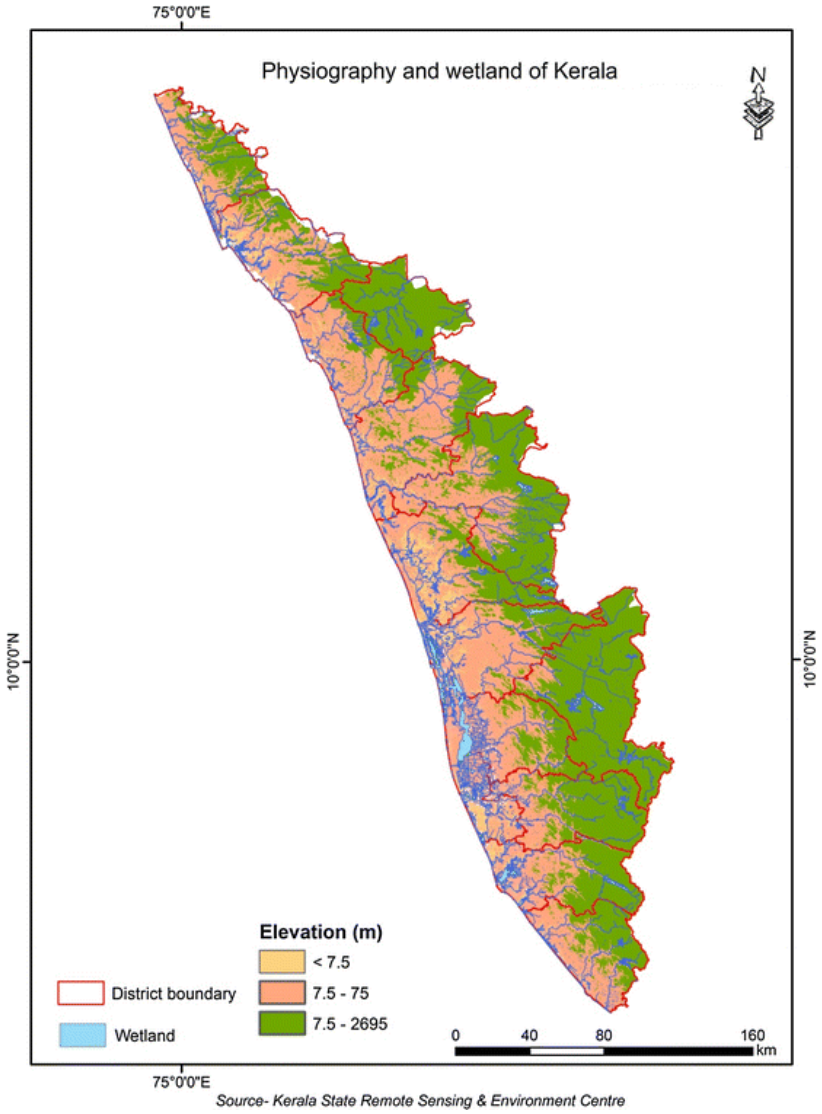

Fig. 2: Physiography and wetlands of Kerala

2.8. Research Problem

Nymphoides spp. are aquatic plants growing in shallow water and wetland (marshy) area. So its collection is very difficult. Plants grow in the field during rainy season and their life cycle is completed before summer during which the wetlands get dried. It is a soft plant and flowers are fragile, hence its flowers are poorly preserved in the herbaria. So identification is very difficult using herbaria specimens.

3

REVIEW OF LITERATURE

3. Review of Literature

3.1. Menyanthaceae

Menyanthaceae Dumort. comprises of aquatic or semi aquatic, monoecious or dioecious and rhizomatous herbs. Grisebach (1839), merged Menyanthaceae with Gentianaceae as a tribe. A comprehensive treatment of Menyanthaceae was done by Grisebach (1845), and listed species of both *Limnanthemum* and *Villarsia* in Australia and provided morphological characters to distinguish the genera, including epipetalous glands, capsule dehiscence, floating leaves, and inflorescence architecture.

Bentham & Mueller (1869), studied on Menyanthaceae and maintained the independence of *Liparophyllum* and established the boundary between *Limnanthemum* and *Villarsia* out of which current generic circumscriptions developed. They reported that capsule dehiscence and inflorescence morphology of these two genera were different. Von (1875), studied the Australian Menyanthaceae species and transferred every Menyanthaceous species in Australia to *Limnanthemum* Gilg (1895), treated this as a subfamily and also reported that dimorphic heterostyly in *Nymphoides.* But dioecism seems to have been previously unrecorded for this family.

Britton and Brown (1897), and Lindsey (1938), treated this as a separate family Menyanthaceae. Harada (1952), has done chromosome studies of few dicotyledonous aquatic plants and reported that *N. indica* is a diploid plant. Tournay and Lawalree (1952), reported the family Menyanthaceae under the order Ligustrales. Stebbins (1974), classified it under Polemoniales. Dahlgren (1980), classified Menyanthaceae under Gentianales. Cronquist (1981), treated this under the order Solanales. Morley and Toelken (1983) and Takhtajan (1987), classified Menyanthaceae under Gentianales.

Chuang and Ornduff (1992), and Cook (1996), reported Menyanthaceae as aquatic or semiaquatic, entemophilous, widespread herbs. Jacono (2002), observed the Florida's floating hearts and reported that introductions of some species of Menyanthaceae viz., *Menyanthes trifoliata*, *Nymphoides cristata, N.*

geminata, *N. indica,* and *N. peltata* have led to naturalized, often weedy populations in North America and New Zealand.

Li *et al.* (2002), made a revision work on the genus *Nymphoides* Seguier in Taiwan based on the comparative morphological, palynological, and cytological studies. Armstrong (2002), investigated on *Nymphoides* spp. (Menyanthaceae), and reported that the flowers are adapted to surface tension interactions and observed that reported that marginal corollar appendages of *Nymphoides* can be membranous, a fringe of trichomes, or a ruffle and this fringed corollar margin might function by generating a significant upward force through surface tension, an interaction adaptive in an aquatic environment. APG – II (2003), treated this family as a separate, monophyletic lineage within the order Asterales.

Aston (2003), investigated on seed morphology of Australian species of *Nymphoides* (Menyanthaceae) by light microscope and scanning electron microscope and reported the seed size, shape, ornamentation and caruncle. Shibayama and Kadono (2003), studied dimorphism in *Nymphoides indica* (L.) Kuntze in Japan. They reported the differences in the distance between staminate and carpellate parts, differences in pollen size in long styled and short styled flower, etc., and also observed the seed set in itermorph pollination. Many species are narrowly endemic, and some of viz., *N. indica* and *N. peltata* are considered to be locally endangered or threatened species. They studied the floral morph composition and pollen limitation in the seed set of *Nymphoides indica* populations.

Schmidt (2005), made a note on the genus *Nymphoides* (Menyanthaceae) of Colombia. Shibayama *et al.* (2006), studied the conservation of lake Kasumigaura (Japan) population of *Nymphoides indica* (L.)Kuntze based on genetic evaluation using microsatellite markers. Tippery *et al.*, (2008), studied a phylogenetic evaluation of the generic circumscription in Menyanthaceae. They investigated on phylogenetic analysis and resolved the relationship among the species based on the morphological and molecular data.

Tippery *et al*., (2008), reported that Menyanthaceae are a morphologically diverse family of aquatic and wetland plants in the order Asterales, consisting of 60 to 70 species that are distributed worldwide and also reported flowers in Menyanthaceae are yellow or white (rarely tinged with purple) and have either a dense covering of hair. Recently the family Menyanthaceae is put under the order Asterales (APG IV)

3.2. *Nymphoides* Seguier

Tournefort (1700), had recognized two genera *ie*., *Menyanthes* and *Nymphoides*. Linnaeus (1753), combined under *Menyanthes* included the emergent wetland species *M. trifoliata* L. and two floating leaved species that currently are circumscribed within *Nymphoides viz., N. indica* (L.) Kuntze and *N. peltata* (S.G.Gmelin) Kuntze. Seguier (1754), validly published *Nymphoides* subsequently; however, the later synonym *Limnanthemum* S. G. Gmelin (1769), remained the accepted genus name for over 100 years (Grisebach, 1845; Mueller, 1875; Bentham & Hooker, 1876).

Ornduff (1966), worked on the Menyanthaceae family and noticed that the heterostyly present in *Nymphoides.* Ornduff (1969, 1970), reported that *Nymphoides indica* is a distylous perennial plant.Velde *et al.* (1979), made a preliminary study on *N. peltata* and reported that the stolons (stem that form adventitious root) creep in and along the bottom layer and can be divided into long and short shoots, which morphologically differ only in the length of internodes and the short shoots are whitish in color, and serve to anchor the plant to the bottom with roots. Armstrong (2002), reported the fringed petals of *N. peltata* was an adaptation to fluctuating water levels by creating upward buoyancy through surface tension interactions These thickened roots hibernate during the winter, and form new leaves and long shoots in spring.

Takagawa *et al.* (2006), studied that self-fertilization in *N. peltata* as occur at low frequencies to avoid inbreeding depression. Larson (2007), studied on *N. petltata* and noted self-pollination, which occurs within a single flower or between different flowers on the same genetic individual. Darbyshire and Francis

(2008), studied on the biology of invasive alien plants in Canada and reported that each petals of *N. peltata* bears a broad membranous margin on both sides, which are wavy to slightly ruffled, creating a short, irregular fringe. Tippery *et al.* (2012), studied the evolution of inflorescence architecture in *Nymphoides* (Menyanthaceae) and reported three types of inflorescence shown in *Nymphoides* such as expanded type, condensed type and unique type.

3.3. Taxonomy

Menyanthaceae species in Australia initially were designated under *Villarsia* by Brown (1810), Don (1837), Endlicher et al., (1837) and Lehmann (1845). Hooker (1860), observed that some species of *Nymphoides* have carpellary disc glands (hypogynous glands) on gynoecium. Ornduff (1966), reported that heterostyly occurred in two species. Nair (1968), reported a new species of *Nymphiodes* (Menyanthaceae) named *N. macrospermum* Vasudev. from South India. Aston (1969), noted that several species of different genera of Menyanthaceaeare remarkably similar in their floral and seed morphology.

Ornduff, (1969), Worked on Neotropical *Nymphoides* and reported that the flowers of *Nymphoides* have conspicuous, attractive, UV-reflecting corollas that clearly function to attract insect pollinators. Ornduff (1970), worked on the cytogeography of *Nymphoides* (Menyanthaceae) and counted chromosome number in the aquatic genus *Nymphoides.* Ornduff reported that *N. indica* was tetraploid in the New World and diploid in the Old World. *N.peltata* was hexaploid. Self incompatibility, distyly, dioecism and homostyly present in different species of this genus.

Aston (1973), reported most *Nymphoides* spp. have floating leaves, some or all of which are integral to the inflorescence and serve to keep the flowers above water. He also studied and reported the flowers of Menyanthacea are sympetalous or synpetalous, calyx, corolla and stamens are five in almost all species and ovary unilocular with two parietal placentation. Raynal (1974a), reported that *Nymphoides* also have pleustonicstolons that originated from the inflorescence node associated with a floating leaf and also reported that the

leaves arise alternately from the rhizome; they are emergent in wetland species viz., *Liparophyllum*, *Menyanthes*, *Nephrophyllidium*, and most *Villarsia* but floating leaves in *Nymphoides* and also reported the leaves are simple in all taxa except *Menyanthes*, in which they are trifoliate. Raynal (1974b), reported that *N. indica* is widespread, broad, encompassing plant with diverse floral and seed morphologies that grow in Africa (*N. indica* subsp. *occidentalis*), Asia, and Australia

Cook *et al*., (1974), reported that the family Menyanthaceae is represented by about 20 species which are cosmopolitan. Nair (1975), investigated that gynodioecy has been reported in *N. cristata*, native to India. Reddy and Bahadur (1976), investigated some traits of heterostyly in populations of *N. indica* in India and Brazil and reported that intermorphic pollination was usually required for fruit and seed set and also reported *N. indica* is a heterostylous species with self incompatibility and the seeds were successfully produced only in case of inter - morph pollination. Qaiser (1977), reported two *Nymphoides* spp. viz., *N. cristatum* (= *N. cristata*) and *N. peltatum* (= *N. peltata)* are found in Pakistan. Hamashima (1979), observed fruit and seed set in *N. indica* in the Tokai region, Central Japan and reported that floral morph composition influenced seed set. Barrett (1980), noticed dimorphic incompatibility and gender in *N. indica.*

Velde and Heijden (1981), reported that floating seeds of *N. peltata* are disturbed by rain and forced under water, the seeds sink to the bottom, where the germination stage of the life cycle begins. Aston (1982, 1984, 1986& 1987), reported three new species of *Nymphoides* viz., *N. triangularis, N. elliptica* and *N. disperma* from Australia. Bohm *et al*., (1986), worked on the flavonoid chemistry of the Menyanthaceae included nine species of *Nymphoides*and 11 species of *Villarsia*. They reported a close relationship between *Nymphoides* and *Villarsia.*

Cook (1990), studied on the seeds of *N. peltata* (S.G. Gmelin) O. Kuntze (Menyanthaceae) and noted the method of seed dispersion and reported that the seeds can stay afloat on the surface due to a coating of a weak hydrophobic

substance and by the marginal hairs. He also reported *Nymphoides* is a cosmopolitan genus of 20 species of floating leaved aquatic plants and also observed that myrmecochory has been proposed for *Nymphoides* and *Villarsia*, seeds having enlarged, lipid-bearing caruncular cells. Josph and Sivarajan (1990), reported a new species of *Nymphoides* from India. They reported a new species *N. krishnakesara* Joseph and Sivarajan. Joseph (1991), reported *N. sivarajanii* Joseph, a new record from Malappuram district, Kerala, India, but none of later taxonomists could trace it out from the type locality and elsewhere.

Chuang and Ornduff (1992), studied in the Menyanthaceae seeds and reported that seeds are ornamented to varying degrees and range from smooth to tuberculate, with some bearing long trichome. They also reported seeds of many species are buoyant and hydrophobic and dispersed by water and water fowl and also noted the morphological similarities between the seeds of *Menyanthes* and *Nephrophyllidium*.

Barrett (1992), studied the evolution and function of heterostyly.Sivarajan and Joseph (1993), worked on the genus *Nymphoides* Seguier (Menyanthaceae) in India and found that most *Nymphoides* species have floating leaves, some or all of which are integral to the inflorescence and serve to keep the flowers above water and also reported some species of *Nymphoides* have disc glands (hypogynous), which is equal in number to the stamens located at the point of corolla insertion, opposite to the petal midline.

Chase *et al*., (1993) and Cosner *et al*., (1994), reported that Menyanthaceae are monophyletic and sister to the three families such as Goodeniaceae, Calyceraceae and Asteraceae. Kadono (1994), reported that *N. indica* was grown in meso – eutrophic lakes and ponds mainly in the South - West of Japan.Petals of many species have lateral wings, which also occur in a related family Goodeniaceae was reported by Gustafsson (1995). Ornduff (1966), postulated that the dioecious species arose from a heteromorphic ancestor within *Nymphoides*, following selection for increased self incompatibility. *Nymphoides spinulosperma* reported from south eastern Australia by Aston (1997).

Hydathodes, leaf hypodermis, scalariform vessel perforations and petal corona of Menyanthaceae were studied by Bremer *et al*., (2001). Aston (2002), reported *Nymphoides imulus,* a new record to Northern Australia. Li *et al.* (2002), worked on the genus *Nymphoides* Seguier in Taiwan.

Aston (2003), investigated on seed morphology of Australian species of *Nymphoides* (Menyanthaceae) by light microscope and scanning electron microscope and described the seed size, shape, ornamentation and caruncle. Boedeltje *et al.* (2003), studied the seeds buoyancy of *N. peltata.* Shibayama and Kadono (2003), studied dimorphism in *N. indica* (L.)Kuntze. in Japan. They reported the differences in the distance between staminate and carpellate parts, differences in pollen size in long styled and short styled flower, etc. and also observed the seed set in intermorph pollination. Shibayama *et al.* (2006), studied the conservation of *N. indica* (L.)Kuntze based on genetic evaluation using microsatellite markers.

Tippery *et al.,* (2008), studied a phylogenetic evaluation of the generic circumscription in Menyanthaceae. They investigated on phylogenetic analysis and resolved the relationship among the species based on the morphological and molecular data. They also reported that there are approximately 40 – 50 species of *Nymphoides,* the majority of which grow in tropical regions of Africa, Australia, the America, India, and South Eastern Asia. Molecular and morphological phylogenetic analyses of Menyanthaceae revealed that species circumscribed within *Villarsia* were paraphyletic (Tippery and Les, 2008).

Darbyshire and Francis (2008), studied on the seed dispersal of *N. peltaata* and reported seed dispersal can be mitigated by currents, digestion by amphibious animals or birds, or attachment to boats. Haddachi (2008), studied on the floral variation and breeding system in distylous and homostylous species of clonal aquatic *Nymphoides.* Aston (2009), worked on Australian taxa of *Nymphoides* (Menyanthaceae) and reported 20 species of *Nymphoides* occurred in Australia and provided information on their typification and nomenclature. Tippery *et al.* (2009), observed a new genus and new combinations in Australian

Villarsia (Menyanthaceae) and reported that *Nymphoides* Seguier is cosmopolitan in distribution.

Phylogenetic relationships and morphological evolution in *Nymphoides* (Menyanthaceae) were studied by Tippery and Les (2011). They studied 31 *Nymphoides* species, including all species native to Australia and reported the inflorescence architecture, vegetative, floral, and seed morphology.Tippery *et al.*, (2012), studied the evolution of inflorescence architecture in *Nymphoides* (Menyanthaceae) and reported three types of inflorescence shown in *Nymphoides* such as expanded type, condensed type and unique type. Pharmacognostical studies on the root and rhizome of *N. hydrophylla* (L.) O. Kuntze – an alternate source for *Tagara* drug was investigated by Madhavan *et al*., (2012) and found that β-sitosterol, betulinic, salicylic and tannic acids are present in *N. hydrophylla.*

Oyedeji and Abowei (2012), studied the Classification, Distribution, Control and Economic Importance of Aquatic Plants in Nigeria and reported several aquatic plants including *N. aquatica* and *N. peltata.* Kusuma *et al*., (2012), worked on the *in vitro* anthelmintic and anti – arthritic activity of alcoholic extract of *N. macrospermum* Vasud. They evaluated alcoholic extract of *N. macrospermum* Vasudev. against Indian earth worm *Pheritima postuma.* Huang et al. (2014), studied the seeds of *Nymphoides cristata* and revealed that undisturbed buoyant *N. peltata* seedlings could float for more than three months, enabling dispersal to other shallow areas within the same body of water. Huang *et al.* (2015), explained the contribution of seeds to the recruitment of a *Nymphoides peltata* population in Lake Taihu, China. They reported that low wind velocity had a slightly negative effect on seed buoyancy. Nowak *et al*. (2015), explained that heterostyly was one of the most effective mechanisms to avoid selfing and promote out crossing in flowering plants.

Biju *et al.* (2016), reported a new record *N. balakrishnanii* from the lateritic plateau of Southern Western Ghats. Biju *et al.* (2016), reported a new species of *Nymphoides* viz., *N. palyi* from the lateritic plateau of South India.

Biju *et al.* (2017), reported a new taxon viz., *N. krishnakesara* var.*bispinosa*, described and illustrated from ponds on the lateriticplateau of northern Kerala, India.

3.4. Distribution and Ecology

In Asia, *N. cristata* ranges from India (Clarke, 1885) and Srilankato Taiwan and North ward into China as far as Jiangsu.Ohwi (1965), commented that *N. cristata* was not known to occur in Japan. Gillet (1968), reported that two of the five Menyanthaceae genera are monotypic and restricted in distribution to the Northern hemisphere. Aston (1973), reported species of *Nymphoides* are absent in South-Western Australia. Kuo (1978), reported *N. cristata* in Taiwan. Sri Lankan distribution of *N. cristata* was reported by Cramer (1981). Brock *et al.*, (1983), studied the structure and annual biomass production of *N. peltata* growing in the back water of the river Waal, Netherland. Ho and Ornduff (1995), reported *N. cristata* distributed from Northward into China as far as Jiangsu province.They alsoreported the fringed water lily *N. peltata* (S. G. Gmelin) Kuntze is a typical floating-leaved plant with a widespread distribution in temperate and subtropical regions of Eurasia. According to Cook (1996), *Menyanthes* and *Nephrophyllidium* are restricted to the Northern Hemisphere.

Burks (2002), reported that *N. cristata,* are cent adventive expanding as a pest plant in Florida. *N. peltata* is an invasive species of North America and New Zealand and it is considered to be a nuisance in these areas (Champion and Clayton, 2003). Nikolic *et al.* (2007) studied *N. peltata, Myriophyllum spicatum* and *Ceratophyllum demersum* biomass dynamics in Lake Provala in Serbia. Patra (2008) evaluated the nutritional value and the possibilities of utilization of the aquatic weed *N. cristatum* which isused as a feed for an Indian major carp *Labeorohita.* Aston (2009), worked on Australian taxa of *Nymphoides* (Menyanthaceae). He reported 20 species of *Nymphoides* Seg. occurred in Australia and provided information on their typification and nomenclature. Nault and Mikulyuk (2009), reported that *N. peltata* is native to temperate Asia and Europe.

Marwat *et al.* (2009), reported that *N. indica* as a new record to Pakistan and a detailed taxonomic description of the plant has been prepared with the help of fresh specimen. Li *et al*. (2010), reported that *N. peltata* (Menyanthaceae) is a wide-spread aquatic species that occurs in a wide range of climates and habitats, especially in temperate regions of the northern hemisphere. Yu and Yu (2011) noted that the differential responses of the floating leaved aquatic plant *N. peltata* which is gradually increases in water levels. NGRP (2012), reported that *N. peltata* is native to temperate Asia and Europe and is present as a non-native species in Canada, New Zealand, and Ireland (CABI, 2012;).

Huang *et al.* (2015), reported seed bank characteristics of the *N. peltata* population in Lake Taihu and observed that *N. peltata* population has shown rapid expansion in Lake Taihu, China, in recent years due to high seed production, with a maximum seed yield of 1763 seeds per m^2. Zhu *et al.* (2019), studied response of aquatic plant and water quality to large scale *N. peltata* harvest in a shallow lake.

3.5. Phytochemistry and Physiology

Wang *et* al. (2004), studied effects of spermidine on resistance of *N. peltata* to Hg^{2+}. Wang *et* al. (2007), investigated in the leaves of *N. peltata* and reported that the protective effects of polyamines against Copper toxicity in the leaves. Qiao (2013), studied on the species *N. peltata* and reported lead induced oxidative damage in sterilized seedlings of *N. peltata.* Qiao (2014), studied on the biochemical defense strategies in sterilized seedlings of *N. peltatum* adapted to lead stress. Fu *et al.* (2014), studied bioaccumulation, subcellular and molecular localization and damage to physiology and ultra structure in *Nymphoides peltata* exposed to yttrium (Y). Shankar and Geetha (2014), studied *in vitro* anti oxidant activity and *in vivo* hepatoprotective activity of ethnolic whole plant extract of *Nymphoides hydrophylla* (Lour.) O.Ktze. in CCl_4 induced liver damage in Albino rats.

Phytochemical and pharmacological investigations on *N. indica* leaf extract were studied by Amin *et al*. (2016). Wu (2017), studied transcriptome

profiling of the floating leaved aquatic plant *N. peltata* in response to flooding stress. They investigated the genetic basis of stress adaptive response by the petiole transcriptomes of a floating leaved species *N. peltata* under normal and flooding condition. Khan *et al.* (2018), made a review work of the Asian *Nymphoides* species and explained the ethnomedicinal; pharmacological as well as phytochemical compounds extracted from different *Nymphoides* species. Bhattacharjee (2019), investigated *in-vitro* thrombolytic and *in-vivo* antipyretic, antidepressant potentiality and analgesic activity with phyto-chemical nature of methanolic extract of *N. hydrophylla*

3.6. Molecular

Barrett (1992), reported that the development of the two morph of flower is controlled by a single diallellic locus designated the S-locus that is a multigene complex. Uesugi (2004), reported allozyme polymorphism and conservation of the population of *N. peltata* in Lake Kasumigaura. The allozyme data indicated that the lake Kasumigaura population consist of a single sub population that includes several clones consisting of two type of heterostylous floral morphs and some subpopulations each consisting of a single clone.

Uesugi (2005), studied and reported the isolation and charectarization of highly polymorphic microsatellites in *N. peltata.* Ten microsatellite loci were described for conservation design of a threatened clonal plant of *N. peltata.* Shibayama (2007), explained the reproductive success and genetic structure of populations of the heterostylous aquatic plant *N. indica*. Zhonghua *et al*. (2007), reported the interference between the two floating leaved aquatic plants: *N. peltata* and *Trapa bispinosa.*

Larsen (2007) studied the growth of three submerged plants below different densities of *N. peltata.* Barrett and shore (2008), have been made to isolate and characterize the *S locus* in a number of distylous plant species. An integrated genetic/demographic study towards the restoration of a sustainable population of a threatened aquatic plant *N. peltata* was studied by Nishihiro *et al.*

(2009). Liao *et al*. (2013), investigated on the genotypic diversity and genetic structure of population of the distylous aquatic plant *N. peltata* in China.

Niranjan *et al.* (2010), reported hepatoprotective activity of *N. cristatum* on carbon tetrachloride induced acute hepatic damage in albino rats. Li *et al*. (2017), investigated the RNA sequence analysis of the distylous plant *N. peltata* and identified ortholog genes between long and short styled flowers. Middleton and Anemaet (2018), investigated on *Nymphoides* species in Florida and reported that certain morphologically-determined populations of *N. indica* in the U.S.A. may be incorrectly identified; such populations might be re-examined using DNA methods to avoid any management actions against *N. humboldtiana.*

3.7. Tissue Culture

Oh *et al.* (2010), explained High frequency plant regeneration system for *N. coreana* via somatic embryogenesis from zygotic embryo – derived embryogenic cell suspension culture. Regenerated plantlets were successfully transplanted into potting soil and achieved to full growth to an adult plant in a growth chamber. Thilak *et al.* (2018), studied *in vitro* conservation and propagation of the endemic species of floating hearts (*N. krishnakesara* Joseph and Sivar. - Menyanthaceae). They multiplied and propagated rare endangered species *N. krishnakesara* through *in vitro* and conserved *ex situ.*

3.8. Pollen Biology

Wodehouse (1936), investigated palynology of the Alismataceae family and classified pollen grains of some plants in this family. He reported that Alismataceae family are considered to be primitive monocotyledons based on the presence of many pored pollen grains. Rao and Rao (1961), studied the pollen morphology of the Pontederiadeae. Chanda (1965), worked on the pollen morphology of Droseraceae with special reference to its Taxonomy. Raj and Saxena (1966), worked on the aquatic angiosperm Pontederiaceae and reported pollen morphology of some species. Singh *et al.* (1969), investigated on the pollen biology of *Nymphaea* spp. and reported the morphological characters of *Nymphaea* spp.

Praglowski (1970), worked on Haloragaceae and reported the pollen morphology with reference to taxonomy. Nilsson (1973), investigated pollen morphology and recognized two general architectures: The *Menyanthes* type, which occur in both *Menyanthes* and *Nephrophyllidium* and *Villarsia* type consist of *Liparophyllum*, *Nymphoides* and *Villarsia.* Pollen of *Menyanthes* type have subprolate to prolate, tricolpate with each furrow with a single germ pore, and a striate to rugulose exine and that of the *Villarsia* type is oblate to suboblate, parasyncolpate with an isolated apocolpical area, and a spinulose, striate, rugulose or smooth exine.

Nilson (1973), worked on the pollen morphology of Menyanthaceae family. Pettitt and Jermy (1975), worked on the aquatic angiosperm and reported pollen types of some hydrophytes. Kupriyanova (1976), worked in the *Nymphaea* spp. growing in the European part of the USSR and noted its pollen morphology. Poole and Hunt (1979), investigated on the American Commelinaceae and reported pollen morphology and the taxonomy of the family. Christensen (1986), studied the pollen morphology of 120 species of Malvaceae using light and scanning electron microscopy. Simpson (1987), worked on the pollen ultrastructure of the Pontederiaceae.

Sorsa (1988), studied the pollen morphology of *Potamogeton* and *Groenalndia* (Potamogetonaceae) and reported its taxonomic significance. Tarasevich (1990), worked on the Palynological evidence on the position of the Lemnaceae family in the system of flowering plants. Takahashil (1994), worked on *Ottelia alismoides* (L.) Pers. and studied its pollen development. Khan (1995), worked on the palynological study of some Indian *Nymphaeaceae* with reference to Silent Valley, Kerala (India). Aizen (1995), scored the proportion of aborted pollen, age, and diameter at breast height of 78 male trees of the south-Andean dioecious conifer, Austrocedrus chilensis. He reported that aborted pollen was low and it increased significantly with tree age and size.

Murthy (2000), described the palynological features of six species of *Nymphaea* of India. Shivanna (2003) described what occurs when pollen is

transferred to a receptive stigma as a series of dialogues between the male gametophyte and the sporophytic tissues of the stigma and style. Several workers studied pollen biology of different angiosperms.Tanak a*et al*. (2004), investigated on Hydrocharitaceae of Japan and correlated the pollen morphology and pollination mechanism. Ansari *et al*. (2005), described the pollen morphology of *Nymphaea* in Kerala. Alwadie (2008), reported the pollen morphology of six aquatic angiosperms from Saudi Arabia. Arshid and Wani (2012), studied pollen biology and stigma receptivity in *Myriophyllum spicatum* L. an invasive species in Kashmir Himalayan aquatic ecosystems. During this study they observed that *M. spicatum* produces large amount of small, colpate pollen grains and reported pollen morphology, viability and stigma receptivity.

Saadi and Mayah (2012), studied the pollen morphological features of forty nine dicotyledonous aquatic and marsh species of Southern Iraq. Yadav and Kogje (2015), studied the microsporogenesis, structure and viability of pollen in *Canscora decurrens* Dalzell. They studied meiosis usingfluorescent microscopy and scanning electron microscopy and reported normal meiosis and pollen mitosis in *C. decurrens* and observed callose deposition around pollen mother cell.

Saklani and Mattu (2018), studied pollen analysis of honey samples from Hamirpur District, Himachal Pradesh, India. They recorded 84 pollen types belonging to 41 different families.

3.9. Pollination Biology

Conard (1905), narrated the history of hybridization of water lilies (*Nymphaea* sp.) initiated in the early 19th century; claims of successful crosses had been repeatedly challenged due to poor documentation or lack of compelling corroborative evidences. Prance and Arias (1975), studied the pollination of *Victoria amazonica;* which wasassociated with Cyclocephala beetles. Schneider and Chaney (1981), viewed this non-native bee as an ineffective pollinator of *N. odorata.* Bittrich and Amaral (1996), studied the pollination biology of

*Symphonia globulifera*in Central Amazonia, Brazil. They observed that major pollinator of this flower is hummingbirds and other visitors were butterflies, bees, other birds and also tamarin monkeys.

Schneider & Jeter (1982), reported the flowers of *Cabomba* are pollinated by Diptera and Hymenoptera. Thien *et al*. (2000), studied on *Cabomba* spp. and reported that flowers are bisexual and protogynic, produce floral odor and are pollinated mainly by beetles and flies. Shuang-Quan (2001), worked on the aquatic plant *Najas marina* and reported the mechanism of underwater pollination in *Najas marina* (Najadaceae).

Velde and Heijden (1981), observed on *N. peltata* and reported that the species is reproduced by different forms of reproduction, including insect and self-pollination, yellow petals are the insect attracted one and the pollinators are guided towards the basal nectaries, where they exchange the transport of pollen for glucose-rich nectar. Pollination biology of *Bombax ceiba* was studied by Bhattacharya and Mandal, (2000). They worked on the flower morphology, anthesis, pollen production, foraging nature of flower visitors, *in vitro* germination and stigma receptivity of *Bombax ceiba* and reported that each flower produced 8,863,000 tri – colporate pollen grains, pollen germination was maximum in 20% sucrose with 500 µg/ml H_3BO_3 and stigma receptivity maximum during the first day after anthesis.

Bhattacharya and Mandal (2004), examined the pollination, pollen germination and stigma receptivity in *Moringa oliefera* Lamk. They reported that the flowers of *M. oliefera* Lamk. shows delayed stigmatic receptivity. Because of this reason it favours cross pollination. Darbyshire and Francis (2008), studied on the pollination of *N. peltata* and he noted either insect or self-pollination occurs within a few hours of flower opening, after which the corolla (petals) begin to wither.

Sharanya *et al.,* (2014), worked on the pollination biology of *Callistemon citrinus* (Curtis) Skeels of Myrtaceae family. They studied flowering period, anthesis, anther dehiscence time, pollinators and breeding systems. Pollination

biology of critically endangered *Leucas sivadasaniana* was studied by Prasad and Sunojkumar (2014), from a single locality in the Kudachadri Hills of Udupi District in Karnataka. Detailed studies were carried out on the phenology, floral biology, pollination and breeding system of *L. sivadasaniana* by them.

Aswathi *et al* (2015), investigated on the pollination biology of *Costus woodsonii* Maas (Costaceae) and reported the life span of individual flower, anthesistime (05.00 – 06.00), Anther dehiscence time (3.00 and 03.30) and type (through the longitudinal slit), flower visitors, pollen germination and breeding systems. Simi and Sunil (2018), reported pollination biology of *Osbeckia wynadensis* and observed different pollinators visited on *O. wynadensis.* Aswani and Sabu (2019), studied the pollination ecology of selected taxa of Zingiberaceae and reported its pollen biology, pollinators and breeding systems.

3.10. Stigma receptivity

Shivanna and Johri (1985) reported that receptivity of stigma is maximum soon after anthesis; it depends upon temperature and humidity and varies among species. Joshirao and Saoji (1989), reported that stigma receptivity is a critical factor for the successful completion of the post – pollination events and stated that period of receptivity varies from species to species. They studied *in vivo* germination of pollen of some alkaloid bearing plants.

Thompson *et al.* (1998), studied the reproductive ecology of distylous *Menyanthus trifoliata* (Menyanthaceae) found in shallow bogs and river margins throughout the boreal ecosystem, including the island of Newfoundland.Tandon *et al*., (2003), studied the reproductive biology of *Butea monosperma* (Lam.) Taub. The study includes phenology, floral biology, pollination and breeding systems. They reported that the percentage of fruit set is higher in cross pollination than self pollination.

Pollination, pollen germination and stigma receptivity in *Moringa oleifera* Lamk. was studied by Bhattacharya and Mandal (2004). They observed that maximum receptivity (40%) with *in vivo* pollen germination (48.47%) was recorded in third day after anthesis.Choudhury *et al.* (2011), studied stigma

receptivity of *Rauvolfia serpentina* (L.) Benth. ex. Murz with reference to esterase and peroxidase activity and they reported that 69% of the receptivity with mean pollen tube length of 169 µm after 1 hour of anthesis.

Choudhury et al., (2012), worked on esterase and peroxidase activity of *Carissa carandas* L. in relation to stigma receptivity and observed that stigma showed maximum receptivity after 3 hrs of anthesis. Dey *et al.* (2016), worked on stigma receptivity of *Grewia asiatica* L. They reported that conspicuous presence of esterase, and peroxidase was observed during higher receptive period.

3.11. Floral Biology and Breeding System

Aparicio *et al.* (1995), worked on the reproductive biology of *Viscum cruciatum* (Viscaceae) in Southern Spain. They reported that male flower is 7.2 mm long and lemon-scented, produces ca. 58,000 pollen grains. Female Bowers, ca. 3 mm long, 2.7 mg dry mass, secrete small amounts of nectar (0.05 mg sugar/d).The reproductive success of a plant depends on its ability to pass through several phenological events such as germination, establishment, growth to adulthood and finally the production and dispersal of viable seed that occur during its life cycle was reported by Harper (1997).

Navarro and Guitian (2003), studied the role of floral biology and breeding system on the reproductive success of the narrow endemic *Petrocoptis viscosa* Rothm. They found that the flowers of *Petrocoptis viscosa* were well adapted to autogamy, inbreeding depression was negligible for fruit production but the germination percentage of seed was very low. Zhang *et al.* (2006), reported that phenological study has great significance because it provides knowledge about the plant growth pattern and also provides the idea on the effect of environment and selective pressure on flowering and fruiting behaviour.

Floral biology of *Rauvolfia micrantha* Hook. F., a rare and endemic medicinal plant of Western Ghats in India was studied by Ramasubbu *et al.* (2008). They studied the flowering time, peak flowering time, anthesis and stigma receptivity. They reported the maximum receptivity was found on the day

of anthesis. Nautiyal *et al.* (2009), made an investigation on the reproductive biology and breeding system of *Aconitum balfourii* (Benth) Mulk: a high altitude endangered medicinal plant in Himalaya, India. They observed protandry type of dichogamy and anti – selfing mechanism. They also studied nature of pollination, pollen germination, pollen tube growth and controlled pollination.

Louis and Radhamany (2009), reported that pollen – pistil interactions in *Hamelia patens* Jacq. They reported that pollen tube growth on stigmatic surface was inhibited by various abnormalities after self pollination. Due to this reason interspecific pollinations overcome the incompatibility barriers and produced viable seeds. Raina *et al.* (2010), worked on reproductive biology and cytology of critically endangered plant *viz. Picrorhiza kurroa* Royle ex. Benth. The reproductive ecology of *Impatiens platyadena* Fischer, a critically endangered balsam of Western Ghats was studied by Ramasubbu *et al.* (2011). They studied floral phenology, pollination biology, pollen – pistil interactions and breeding systems.

Reproductive biology of *Withania ashwagandha* was worked by Mir *et al.* (2013). They studied on the floral biology, peak time of flowering, pollination behaviour, breeding system, stigma receptivity and recorded fruit set rate by different pollination experiments such as 90 % through passive autogamy, 72 % through assisted autogamy, 50.4 % through open pollination, 56.5 % through geitonogamy and 30.3 % through xenogamy.

Venugopal and Ahuja (2014), investigated on the reproductive biology of *Panax wangianus* of Araliaceae, a critically endangered medicinal plant. They studied various reproductive characters viz., number of pollen grains per flower, pollen ovule ratio, pollen germination, pollen tube growth, stigma receptivity, pollinators and breeding system. Pandey *et al.* (2016), presented an overview of reproductive biology data in plant systematics. They reported that flower morphology plays an important role in assessing relationships between reproductive biology and plant systematics. Floral biology of *Humboldtia sanjappae* Sasidh. & Sujanapalof Fabaceae was studied by Jayalakshmi *et al.*

(2016) and noticed anthesis, pollen biology, stigma receptivity and types of pollinators.

Chauhan and Nisha (2018), studied Reproductive biology of *Alstonia scholaris*(L.) R.Br. and reported pollinating insects, pollination, pollen : ovule ratio and hand pollination experiments. Reproductive biology of *Gynochthodes umbellata* (L.) Razafim. & B. Bremer was studied by Gangaprasad *et al.* (2018). They analysed flowering phenology, floral biology, pollen production per anther, pollen-ovule ratio, pollen viability, stigma receptivity, pollen visitors and pollination and breeding system of *Gynochthodes umbellata.* Kumari and Sharma (2018), investigated on foral anomalies in *Tephrosia purpurea* (Linn.) Pers., in response to fluctuating winter temperatures and reported pollen viability, flowering time and pods and seed production.

Reproductive Biology of *Eleutherina bulbosa* (Miller) Urban was studied by Manjula and Nair (2018) and recorded floral biology, breeding system and pollination mechanism of *E. Bulbosa.* They reported pollen viability was high (68.639 ± 2.619 %) and pollen germination percentage was moderate (57.684 ± 4.461%). The fruit set percentage was comparable in all the artificial crosses (28.33%-31.66%). Seed viability of *E. bulbosa*as tested by tetrazolium assay was found as 68.48% and seed germination was 31.66±0.957%. Maximum germination was observed between 20-30 days after sowing.Aswani and Sabu (2018) worked on the reproductive biology of dancing girl ginger, *Globbas chomburgkii* and reported its phenology, pollination system and fruit set under field condition.

Reproductive biology of malabar tamarind *Garcinia gummi-gutta* (L.) Rob. was studied by Aswathi *et al.* (2018). They observed flowering phenology, flower morphology, pollen viability, stigma receptivity and pollination mechanism and reported high percentage of fruit set i.e. 96% obtained by artificial pollination than natural pollination. Barman *et al.* (2018), reported reproductive biology of *Salvadora oleoides* Decne. and observed phenology, stigma receptivity and breeding mechanism.

Kar and Datta (2018), studied reproductive biology of *Crotalaria spectabilis* Roth. and recorded flowering phenology, floral morphology, and pollen biology. They reported anthesis and are protandorus, distylous, viability of pollen grains and the anthers dehisce before anthesis begins.

Phenology and population dynamics of six uncommon medicinal plants in the grasslands of Nilgiris was studied by Suresh and Paulsamy (2010). They observed the phonological varients such as leaf flushing, leaf expansion, flower initiation, peak flowering, fruit formation, seed formation and seed maturation.

Jeeshna and Paulsamy (2011), investigated on phenology of *Exacum bicolour* Roxb. They made a detailed study of phonological variants such as vegetative growth, leaf flushing and flowering with fruit development, fruiting and seed dispersal. Sihag and Wadhwa (2011), studied floral and reproductive biology of Sarpagandha, *Rauvolfia serpentina* in semi – arid environment of India. They observed the floral phenology, pollination mechanism and reproductive biolgy *of Rauvolfia serpentina.*

Sreekala *et al.* (2011), worked on the pollination biology of *Impatiens cuspidata* Wight and Arn. (Balsaminaceae), a rare and endemic balsam of the Western Ghats, India. They have been studied with phenology, pollination, pollen – pistil interaction, stigma receptivity and breeding programmes. They observed that stigma was more receptive on the first day of flower opening. It reproduced by cross pollination and seed set was 40 %. An artificial cross pollination was made on *Impatiens cuspidata* and noticed that the seed set was 80%.

3.12. Phenology

Barman *et al*. (2014), worked on various periodic behaviours of some medicinal plant species of Gopala District in Assam. Shivadas *et al.* (2014), investigated on reproductive phenology of *Lagerstroemia speciosa* in Southern Western Ghats, India.They observed that after leaf flushing the flowering was immediately initiated in *L. speciosa.* Borah and Devi (2014), made an attempt to unravel the major phenophases, seedling survival and growth of *Vatica lanceae*

Bl., a critically endangered species in Assam. Sivaraj *et al.* (2014), studied the floristic ecology and phonological observations on the medicinal flora of Southern Eastern Ghats, in India. They observed that the fruit formation in medicinal trees during dry season and in herbaceous medicinal plants during January to March. Phenology was greatly influenced by environmental factors such as temperature, rainfall, humidity and CO_2.

Sharavanan and Muthuchelian (2016), examined the relationship between the phenological pattern of *Nothopegia aureo–fulva* Beed. and climatic variables viz.,temperature and rainfall, in Agasthiyamalai Biosphere Reserve,Western Ghats, India, with the help of spearmans rank correlation coefficients . They reported that leaf initiation was negatively correlated with rainfall, leaf abscission was positively correlated with maximum and minimum temperature and flower initiation was negatively correlated with maximum and minimum temperature.

Rohitash (2018), investigated on phenology and reproductive biology of *Clerodendrum splendens* G. Don and reported only parthenocarpic fruits developed in the months of March-May. The failure of fruit and seed set in *C. splendens*is due to its self-incompatible nature. Phenological behaviour of *Piper sylvaticum* Roxb. (Piperaceae), a medicinally useful species occurring in Eastern Himalayan region was studied by Devi *et al.* (2018), and recorded different parameters like leaf initiation, leaf opening, initiation of flowers, peak flowering period, number of flowers, fruit formation and maturity, etc.

Jyothi and Sunil (2018), studied Floral Phenology and Breeding System of *Aponogeton appendiculatus* V. Bruggen and observed floral phenology and breeding system of *Aponogeton appendiculatus* a critically endangered aquatic species. They reported the highest percentage (3.2±0.6) of anthers dehisced between 09:00 to 11:00 h. The maximum percentage of stigma receptivity (95±0.1) and pollen viability (69±0.13) were observed on the day of anthesis.

4

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. Materials

Nymphoides Seguier is a cosmopolitan aquatic herb with submerged rhizome, roots and cordate floating leaves, under the family Menyanthaceae, which is widely distributed in tropical and temperate regions of the Old world and New World (Ho & Ornduff, 1995). Some species are found growing along with *Nymphaea* species. *Nymphoides* spp. can be easily distinguished from *Nymphaea* spp. by its floating cordate leaves, flowers arising from the region between the leaf petiole and the stem, small white or yellowflowers with petals with fimbriate margin born above the water surface.

The genus *Nymphoides* Seguier has approximately 50 species worldwide (Tippery *et al*. 2008). In India, Sivarajan and Joseph (1993), made a revision on *Nymphoides* spp. of Kerala and reported eight species. Recently two new species and one variety were reported from Kerala. Of these ten species, nine species and one variety are found in Kerala (Table 1). *N. aurantiacum* though reported from Alleppy District (Sunil and Sivadasan, 2009) we could not locate the plant. Sunil, who worked in the Alleppy flora, informed us that the plant is disappeared from the original locality (Thalavadi) and hence not included in this work (Pers. comm.). *N. sivarajanii* reported from Malappuram and *N. krishnakesara* var. *krishnakesara*, reported from Kasaragod. After extensive explorations in the previous known localities we could not relocate these plants, hence they were not included in the present study.

Sl. No.	Name	Habitat/frequency	Locality
1	*Nymphoides balakrishnanii* Biju, Josekutty, Haneef& Augustine	Seasonal pools of lateritic hillock 10% frequency	Kasaragod Endemic to ditches of lateritic hills of this area
2	*Nymphoides hydrophylla* (Lour.) Kuntze	Paddy fields, flooded lowlands, pools, ponds, river banks and canals. 50% frequency	All districts
3	*Nymphoides indica* (L.) Kuntze	Shallow ponds, canals, paddy fields and flooded lowlands. 70% frequency.	All Districts
4	*Nymphoides krishnakesara* K.T.Joseph&Sivar.	Seasonal pools on rocky hillocks. 20% frequency	Kannur (Korom&Madayipara)

5	*Nymphoides macrosperma* R.V.Nair	Paddy fields and ponds. 20% frequency	Kozhikode (Pantheerankavu) Malappuram (Aakode) Ernakulam
6	*Nymphoides palyi* Biju, Josekutty, Haneef& Augustine	Shallow ponds of lateritic hillocks. 10% frequency.	Kasaragod (Perla)
7	*Nymphoides parvifolia* Kuntze	Shallow ponds and flooded paddy fields. 20% frequency.	Kasaragod (Beemanadi)

Table.1. *Nymphoides* spp. in Kerala

Out of these, 3 species are endemic to Kasaragod District in Kerala. *Nymphoides peltata* (Gmel.) Kuntze.is also found in India but it is restricted to temperate Himalaya and Kashmir. *Nymphoides* spp. are found in shallow ponds, paddy fields, pools, slow–moving streams, lakes, flooded low lands, river banks and canals. It is commonly known as 'floating hearts' because of its heart shaped floating leaves which make a carpet on the surface of water bodies.

4.2. Significance of *Nymphoides* Seg.

Nymphoides spp. are cultivated as ornamentals in pools and ponds of garden for their elegant heart shaped leaves and small but bright white flowers. In Asia, *Nymphoides* spp. are widely used as a traditional medicinal plant in indigenous medical practices such as Ayurveda (Khan *et al*., 2018). Many studies have been carried out on the medicinal aspects of *Nymphoides* spp. Medicinal properties of *Nymphoides indica* and *N. hydrophylla* were mentioned in *Hortus Malabaricus*, the first classical well illustrated book on the flora of Kerala (1678 – 1702) published at Amsterdam in 12 volumes. An ayurvedic drug 'GranthikaTagara' is extracted from the rhizome of *Nymphoides macrosperma* (Yoganarasimhan *et al*., 1979). Recently Granthika Tagara is extracted from *Nymphoides macrosperma, N. hydrophylla, N. indica and N. parvifolia* (Jayasree *et al*., 2015; Ayyathan *et al*., 2015). It is used for the treatment of anaemia, epilepsy, fever, cough, jaundice and mental disorders. This drug is also used as a brain tonic (Mary *et al*., 1981; Murali *et al*., 2007) and an effective medicine against helminthiasis (Kusuma *et al.,* 2012).

4. 3. Methods

4.3.1. Field Exploration

Extensive survey has been conducted throughout the aquatic and wetland areas of Kerala State during different seasons from 2015 – 2018 based on the information from regional floras and other literatures. Fresh specimens were collected in blooming time as well as seedlings time and introduced in the Malabar Botanical Garden and Institute for Plant Sciences. The details of plants viz., local name, locality, habit, nature of leaf, flower colour and size, etc. were recorded in the field book at the time of specimen collection. Part of the collected plants was used to prepare herbaria and part of them was planted into the aquatic plant conservatory and also in the field conservatory for *ex–situ* conservation. Planted materials were regularly visited and maintained. Photographs of the specimens (phenological events) were taken using digital camera (Cannon C2).

4.3.2. Descriptions and Photoplates

Fresh specimens were brought to the Taxonomy laboratory and studied its morphological characters using stereo dissection microscope (Labomed CZM4). Measurements of plant and floral parts have been taken using a measuring scale. Detailed taxonomic descriptions of each species were prepared. Photographs of dissected plant materials were taken using by Leica MC 170 HD Camera attached stereo microscope MSV266 and Cannon EOS R Powershot Camera. Photoplates of each species were prepared, which contains the photographs of Habit, Rhizome, Flowers, Sepals, Petals, Stamens, Pistils, Fruits and Seeds.

4.3. 3. Identification of Specimens

Collected specimens were identified with the help of standard floras, books and other research articles dealing with Menyanthaceae family. Specimens housed at Central National Herbarium, Kolkata (CAL), Botanical Survey of India, Southern Regional Centre, Coimbatore (MH), Calicut University Herbarium, Malappuram (CALI) and Malabar Botanical Garden & Institute for Plant Sciences Herbarium, Calicut (MBGH) were studied.

4.3.4. Preparation of Herbarium

The herbarium specimens were prepared using conventional methods by pressing and drying the plant specimens after blotting with blotting papers in a herbarium press. Vegetative and reproductive parts of the collected plants were pressed in herbarium press. The dried specimens were mounted on standard herbarium sheets (28 x 42 cm). The specimens were labelled using standard herbarium labels (14.5 x 11 cm). The herbarium labels include the scientific name, family, altitude, date of collection, field notes and name of collector. The specimens were deposited in the MBGH, KSCSTE – MBGIPS, Kozhikode, Kerala.

4.3.5. Nomenclature and Citation

The nomenclature and citations of the *Nymphoides* spp. were corrected using the online database like, The Plant List Version 1.0 and 1.1 and also the standard literature Plant list (http://www.theplantlist.org), TROPICOS (http:// www.tropicos.org) and IPNI (http:// www.ipni.org).

4.3.6. Conservation Status and Endemism

The status of each species was assessed by using IUCN Red List Categories and Criteria Version 2 (2019) and India Biodiversity Portal (http://indiabiodiversity.org)

Endemism is the species restriction to a limited geographical area. Several studies were carried out of endemic plants in Kerala such as Ahmedullah and Nayar (1986), estimated 132 endemic species, Singh and Subramanyan (1991), enumerated 108 endemic species, Mohanan & Nair (1999), explored 4000 flowering plants in Kerala, out of these 165 species are found to be endemic. Ten *Nymphoides* species are distributed in Kerala. Of these, six species are endemic to Malabar region viz., *Nymphoides parvifolia*, *N. balakrishnanii*, *N. palyi*, *N. krishnakesara*, *N. krishnakesara* var. *bispinosa*are restricted in Kasaragod district and *N. sivarajanii* in Malappuram district. Endemism is assessed mainly based on the field observation data.

4.3.7. *Ex- situ* conservation of *Nymphoides* spp. in Kerala

The collected specimens were introduced and planted in the aquatic plant conservatory (Aquagene) of Malabar Botanical Garden and Institute for Plant Sciences. Six species could be successfully established here.

4.4. Dendrogram

Dendrogram was generated using the statistical package STATISTICA version 7.0

4.5. Reproductive Biology

4.5.1. Phenology

Phenological study is done based on field observations carried out for continuous three years during (2015 – 2017). Phenological events were recorded for three years according to the method suggested by Dafni *et al.* (2005) and Madhuri *et al.* (2016). It includes leaf initiation, bud initiation, bud development, flowering, duration of flowering, anthesis time, anther dehiscence time, fruit development, fruit dispersal; seed, seed dispersal and number of pollinators were obtained in the field. Anthesis and anther dehiscence were observed using hand lens, followed by the method of Reddi and Bai (1981), Mathur and Ram (1986) and Ramasubbu *et al.*, (2009). Regular field visits were made in the entire flowering time. The floral visitors were regularly observed during the flowering season. The Pollinators were caught and fixed in 70% alcohol for identification

Observation on flowering phenology include initiation of bud development, number of flowers/ fertile leaf/ plant, time of flower opening, flower longevity, anther dehiscence, peak period of flowering, fruit initiation, development, maturation and dehiscence.

4. 5. 2. Staminate – Carpellate Distance

To confirm dimorphism the length of stamen and pistil of heterostyled flowers were measured. Ten long styled and short styled flowers were selected randomly and fixed in FAA in the field. The length of stamens and pistils were measured from the base of the ovary to the top of them using a scale. The length

of stamen per flower was measured. The difference between these two lengths referred to as the staminate - carpellate distance

4.5.3. Pollen Biology

4.5.3. 1. Pollen morphology

Pollen morphology was studied by using the method of Erdtman (1952) and Nair (1960) and also studied with SEM photographs. The pollen grains were collected from the anthers of fully matured buds just before anther dehiscence and preserved in vials containing 70% ethanol. The materials were brought to the laboratory. Pollen morphology was studied by using light microscopy and scanning electron microscopy.

In light microscopy the pollen grains were subjected to acetolysis (Erdtman 1952) method.

4.5.3.1a. Acetolysis (Erdtman, 1952)

Mature flower buds of *Nymphoides* species were collected. The anthers from the bud were removed and transferred it to 70% alcohol taken in a watch glass. Then the anthers were crushed by tapping them with a scalpel or glass rod. The debris (anther wall, filament and connective) were removed using a needle. The pollen suspension was transferred in to a centrifuge tube and centrifuged at 900 rpm for 5 minutes and decant the alcohol. The sediment was washed with 5 ml of glacial acetic acid, and centrifuged at 900 rpm for 5 minutes and decanted the acetic acid. Acetolysis mixture was prepared by adding one volume of concentrated sulphuric acid to nine volume of acetic anhydride. 5 ml of freshly prepared acetolysis mixture was added to pollen sediment and kept the tube in a water bath at 70^0C and heated till the pollen became brown in colour. The tubes were cooled and centrifuged at 900 rpm for 5 minutes and decanted the mixture. 5 ml of glacial acetic acid was added into tube and mixed well, then centrifuged at 900 rpm for 5 minutes and decanted the solution. The material was washed repeatedly with distilled water and centrifuged at 900 rpm for 5 minutes and decanted the water completely. One drop of glycerine was added to the tube and mixed well. The suspension was transferred to a clean dry slide and placed a

cover glass. Then pollen grains were examined under Labomed Stereo microscope (LX 500). For permanent slides, the pollens were mounted in glycerine jelley and protected with paraffin wax on slides.

Scanning Electron Microscopic study

For scanning electron microscopic study, the pollen grains were collected from the mature anthers at the time of anther dehiscence and stored in 70% alcohol. Collected pollen grains were brought to the laboratory and transferred to 100% ethyl alcohol and kept in room temperature. The pollen grains were mounted on stubs using double sided adhesive tape and sputter coated with gold palladium. The coated pollen grains were observed under Scanning Electron Microscope.

4.5.3.2. Pollen – biochemical analysis

Mature pollen grains were collected from the mature anthers and brought to the laboratory. The pollen grains were stained in a drop of Iodine Potassium iodide (IKI) solution or Sudan black or Coomassie Brilliant Blue solution on a micro slide and examined under a microscope. A dark bluish black colour indicates the presence of starch when it stained with I – KI, black colour (stained with Sudan black) indicates the presence of lipids and dark bluish – black colour (stained with Coomassie Brilliant Blue solution) indicted the presence of protein

4.5.3. 3. Pollen production

Pollen production per flower was calculated by the method proposed by Cruden (1977) and Barret (1985). Ten mature flower buds before anther dehiscence were collected and removed one anther from ten flower buds, then kept in 1 ml centrifuge tube separately. Distilled water (0.5 ml) and 0.5 ml of glycerine was added instead of lactophenol glycerine with aniline blue. A known dilution of pollen solution was injected into the grid of haemocytometer and 10 replicate counts were made using haemocytometer (Barrett, 1985). The pollengrains were counted in each of the 0.1 mm^3 corner squares labelled A to E. The grains touching the top or left borders were counted. But do not count the grains touching the bottom or right boarders. The mean number of pollen grains

per flower was determined by the number of pollen grains per anther multiplied by the total number of anthers per flower. The pollen per anther was determined using the formula.

$$\text{Total pollen per anther } = \text{Total cell counted } \times \frac{\text{Dilution factor}}{\text{No. of squares}} \times 10000$$

Total pollen grains per flower were calculated by multiplying this value into number of anthers present in a flower.

4.5.3. 4. Pollen – Ovule Ratio

The pollen ovule ratio (P/O ratio) was determined by dissecting the ovaries first under dissection microscope and the number of ovules in the ovary was counted. The number of ovules per flower divided by the total number of pollen grains per flower (Anderson & Simson, 1989, Cruden, 1977, Shivanna & Rangaswamy, 1992). Flower buds just before anthesis were used to find out the pollen - ovule ratio.

The pollen ovule ratio was determined by the formula

$$\text{Pollen} - \text{Ovule ratio} = \frac{\text{Mean no. of pollen grains/flower}}{\text{Mean no. ofovules/flower}}$$

4.5.3. 5. Pollen Viability

Pollen viability was studied by different methods proposed by different authors

A. 1%Acetocarmine Method (Shivanna & Rangaswamy, 1992)

The pollen grains were collected from mature anthers at different intervals. Two drops of acetocarmine glycerine mixture (1:1) was placed on a clean micro slide and dusted few pollen grains from collected samples, thoroughly mixed. The number of plumped stained pollen grains was counted as viable and unstained pollen grains were counted as nonviable.

B. 0.1% TTC Solution Test (Shivanna & Rangaswamy, 1992)

A modified version of Shivanna and Rangaswamy (1992) was applied since the original preparation does not give results. A diluted version with 0.1 g of TTC and 5g of sucrose diluted in 100 ml distilled water. Take a drop of TTC

on a micro slide. Small amount of pollen was suspended in the TTC drop and distributed it uniformly in the drop. A cover glass was placed on the material. The preparation was transferred in dark field and observed it under stereo microscope after 30 minutes. The red stained pollen grains are considered as viable.

4.5.3. 6. Pollen Germination

Pollen germination was studied by *in vitro* as well as *in vivo* method

4.5.3. 6a. *In vitro* pollen germination

In vitro pollen germination were studied using Brewbaker & Kwack's medium (Brewbaker & Kwack's, 1964) and different concentrations of sucrose solutions such as 4%, 8%, 12%, 16% & 20%. Two drops of different concentrations of sucrose solutions were placed on a micro slide and dusted few pollen grains from collected samples then distributed uniformly and kept in petridishes lined with moist filter paper. After incubation period, the percentage of pollen germination and pollen tube length were calculated under microscope (Labomed LX 500). The percentage of pollen germination was calculated by using the following formula proposed by Shivanna and Rangaswamy (1992).

$$\text{Percentage of pollengermination} = \frac{\text{Number of pollen grains germinated}}{\text{Total number of pollen grains observed in the microscopic field}} \text{x } 100$$

4.5.3. 6b. *In vivo* pollen germination

Aniline Blue – Lactophenol Method (Hauser and Morrison, 1964)

To determine the *in vivo* pollen tube growth, pollination trials were conducted on the day of flower opening. Pollinated stigmas of *Nymphoides* spp. were fixed first in Carnoy's fluid (6 alcohol: 3 chloroform: 1 acetic acid) at different intervals (Pre - anthesis, Anthesis and Post anthesis) for 24 h and stored in 70 % ethanol. Fixed pistils were transferred to 1N NaOH for 12 hours to makes the tissue soft. After washed thoroughly in distilled water, the pistils were stained with 1 % aniline blue lactophenol solution and mounted on a drop of glycerine and squashed under a glass cover slip. The preparations were observed

under light microscope. The number of pollen tubes and the rate of pollen tube growth in the style were measured using camera attached microscope with progressive capture (Labomed LX 500).

4.6. Stigma Biology

4.6.1. Stigma Receptivity

The Stigma receptivity was studied using the following methods.

4.6.1a. Visual Observation (Teryokin *et al.*, 2002)

According to Teryokin *et al*. (2002) stigma receptivity was determined by visual observation. Stigmas which were transparent, shining and also adhere to $1mm^2$ piece of paper were considered as receptive..

4.6.1b. Hydrogen Peroxide Test (Joshirao & Saoji, 1989; Kearns and Inouye, 1993)

Hydrogen peroxide was used to determine the receptivity of stigma. Oxygen bubbles were produced from stigma by the addition of hydrogen peroxide, which is an indicator of the peroxidise enzyme present in the stigmatic surface. Fresh, mature, unpollinated flower buds were collected and the pistils were carefully excised from the flower. The pistils were kept on a dry micro slide. One drop of 3 % H_2O_2 was dropped on the stigmatic surface of the excised pistil kept on the micro slide. The stigma surface produced bubbles and counted the number of bubbles produced in 1 minute. Data was analysed using Kruskal-Wallis Test calculator for Independent Measures. The results were presented as mean ± standard deviation.

4.6.1c. Alpha – Naphthyl Acetate Test (Ghosh and Shivanna, 1884)

Alpha- Naphthyl acetate test was used to study the cytochemical localization of esterases on stigmatic surface. The receptive stigmas contain an important protein, esterase (extracellular protein). Esterases are present as a component of exudates in wet stigmas (Heslop – Harrison & Shivanna, 1977; Heslop – Harrison, 1981; Shivanna & Johri, 1985). Cytochemical localization of esterases is based on the hydrolysis of the substrate, ∞ - Naphthyl acetate and produced ∞ - naphthol (Ghosh & Shivanna, 1984).

Procedure

Fresh, mature, unpollinated flower buds and flowers were collected. The flowers were handled with forceps and without injuring the stigmas and style, carefully excised the pistil from the flower. The pistils were kept on a dry micro slide in a humid chamber (Petridishes lined with moist filter paper). Two types of solutions A and B (Table 2) were prepared for this experiment.

Sl. No.	**Chemicals**	**Solution A** **(With substrate)**	**Solution B** **(Control, without substrate)**
1	α- Naphthyl acetate	5 mg	0 mg
2	Phosphate buffer	10 ml	10 ml
3	Sucrose	10 – 15 %	10 – 15 %
4	Fast Blue B	25 mg	25 mg

Table 2.Cytochemical localization of esterase enzyme - solutions

a: ∞ - Naphthyl acetate is insoluble in phosphate buffer; therefore, first dissolved in few drops of acetone in a screw cap bottle, then added the buffer, sucrose and fast blue B and mixed thorough. b: Preparation of buffer: Solution X – 0.15 M NaH_2PO_4 (*ie*., 20.85 g/l), Solution Y- 0.15 M Na_2HPO_4 $7H_2O$ (*ie*.,40.24 g/l) or Na_2HPO_4 $12H_2O$ (*ie*., 53.78 g/l). Mix 51 ml solution of X with 49 ml of solution Y to get 100 ml of 0.15 M Phosphate buffer.

A few drops of solution A was taken on a micro slide and solution B on another micro slide. The excised pistils were dipped into solution A and B separately. The slides were then incubated at 25^0 C in a humidity chamber for ca. 20 min. The stigmas were removed after incubation period and rinsed thoroughly with phosphate buffer, pH 6.8. The stigmas were mounted with 50 % glycerine and observed under light microscope (Labomed LX500) attached with an image analyzer and studied the stigmatic surface.

4.6.2. Stigma – Biochemical analysis

4.6.2a. Starch test (Jensen, 1962)

The presence of starch on the stigmatic surface was tested by using Iodine – Potassium Iodide (I_2KI), prepared by dissolving 2g of potassium iodide

and 0.2 g of iodine in 100 ml of distilled water. Stigma was stained with I_2KI solution for 1 minute, mounted in the same solution or dilutes glycerine and observed under light microscope. Starch grains appear bluish black and newly formed starch appear red to purple.

4.6.2b. Protein test (Cawood *et al.*, 1978)

The presence of starch on the stigmatic surface was tested by using Coomassie Brilliant Blue R 250. Coomassie Brilliant Blue solution (0.02%) was prepared in Clarke's solution at pH 2. Clarke's solution was prepared by adding 1:1 acetic acid and absolute ethanol. Stigma was placed in 50% ethanol, stained with Coomassie Brilliant Blue for 10 minutes, then washed with Clarke's solution and mounted in dilute glycerine. Blue colour indicated the presence of protein.

4.6.2c. Lipid test (Bronner, 1975)

Lipids on the surface of stigma were tested by using Sudan Black. Sudan black was prepared by dissolving 0.4g of dye in 100 ml of 70% ethanol. The stain was stored in a closed container for 12 hour at 37^0 C and filtered. Stigmas were stained with 0.4 % Sudan black for 1 – 2 minutes and mounted in dilute glycerine. Lipids stained black.

4.7. Pollination Biology

4.7.1. Floral visitors and their behaviours

The floral visitors and their behaviour were observed in the field from anthesis to flower drooping time in three seasons. The basic requirements needed at the time of field visits were stopwatches, marking tape or flags, hand lenses, digital camera, data sheets, butter paper bag, 70% alcohol and marker pen. The number of floral visitors, visiting time, foraging nature, foraging hour,time spent in flower, stigma touch by insects, frequency of visit, mode of landing or entry, contact with the anthers and the stigma etc. were recorded.

After the visit of pollinators, observed the stigma by hand lens for the confirmation of pollen transfer to the stigma. The Butter paper bags used for trapping the insects at the time of foraging and were fixed in 70% of ethanol in a vials (10 ml).

The insects were identified with the help of an entomologist, at Kerala Forest Research Institute, Trichur.

4.7.2. Pollination Efficiency (Cruden *et al.*,1990)

Pollination efficiency can be calculated by total number of pollen on stigma devided by total number of pollen per flower

4.8. Breeding System

4.8.1. Natural pollination

Natural pollination was studied in the field. Flowers were labelled at the time of anthesis. The fruit and seed set were observed after two days.

4.8.2. Self pollination

The flower buds of one day before anthesis were bagged in butter paper bags.Theflower buds were pollinated on the next day using pollen grains from same flower or different flowers of same plant. The pollinated flowers were bagged and observed fruit and seed set.

4.8.3. Cross pollination

Mature flower buds of were bagged with butter paper bag the day before anthesis. The pollen grains collected from the anthers before anthesis were pollinated with the help of sterilized needle. Pollinated flowers were bagged after pollination and observed the fruit set, number of days taken for fruit development and seed development, etc.In incompatible species, inter specific and intra specific hybridization were tested.

4.9. Fruit and seed biology

4.9.1. Fruit morphology

The mature fruits were collected and studied its morphology under Leica MSV266 Stereo microscope.

4.9.2. Fruit and seed set

Fruit development was observed weekly from the day of pollination until maturation. The success of pollination was based on the formation of mature fruit. The mature fruits were collected before the capsule dehiscence. Fruit

dehiscence was also observed in the field. The percentage of fruit set was calculated by the formula:

$$\text{Percentage of fruit set} = \frac{\text{No.of fruits per leaf}}{\text{No.of flower per leaf}} \text{x } 100$$

The percentage of seed set was calculated by the following formula:

$$\text{Percentage of seed set} = \frac{\text{No.of seeds per fruits}}{\text{Number of ovules per pistil}} \text{x } 100$$

4.9.3. Flower - fruit ratio and ovule – seed ratio

Total number of flowers formed at the leaf base and the number of mature fruits developed from these flowers was scored to determine the flower – fruit ratio. The ovule – seed ratio was determined by the total number of ovules formed in an ovary and the number of mature seeds developed from these ovules.

4.9.4. Fruit and Seed dispersal

Fruit and seed dispersal mechanisms were studied by direct observation in the field.

4.9.5. Seed germination

Seed germination observed in naturally and in lab condition.The mature seeds were immediately collected after natural dispersal. The collected seeds brought to the laboratory. The seeds are pre-soaked in water for 2 days. After settled in the bottom the seeds were taken for experimentation. The seeds were sown in a small jar filled with 1 inch of soil taken as control, soil with 50 ppm and 100 ppm Gibbberellic acid and dung – soil mixture taken as separate jar. The jars were covered with lid and kept for germination with proper sunlight available. Observed the days taken for germination of seeds in various jars andcalculate the percentage of germination using the following formula.

$$\text{Percentage of seed germination } = \frac{\text{Number of Seeds germinated}}{\text{Total number of seeds sown}} \text{x } 100$$

5

RESULTS

5. RESULTS

5.1. Systematic Treatment

5. 1. 1. Menyanthaceae Dumort.

Anal.Fam. Pl. 20, 25. 1829.

Aquatic or semi aquatic, annual or perennial herb, monoecious or dioecious, rhizomatous or with tufted root stock. Leaves simple or trifoliate, monomorphic or dimorphic, alternate, petiolate; basal leaves spathulate and rosulate, Floating leaves fertile. Petioles sheathing at the base. Inflorescence simple or branched racemes or cymes or umbellate cluster. Flowers white or yellow or rarely pink, sympetalous and synsepalous, bisexual or unisexual, radially symmetrical, heterostylous or homostylous. Sepals and petals 3 – 8, united at the base; petals often fimbriate or crests. Stamens as many as petals.Ovary hypogynous, hypogynous glands present or absent, unilocular 2 parietal placentas. Fruit a capsule or berry like.

5.1. 2. Nymphoides Seg.

Aquatic, annual or perennial, monoecious or gynodioecious or dioecious, rhizomatous or stoloniferous with monomorphic or dimorphic shoots.Leaves alternate, spiral, petiolate monomorphic or dimorphic. Basal leaves are rosulate, submerged and vegetative, floating leaves are fertile with primary or secondary shoots. Flowers unisexual or bisexual, homostylous or heterostylous, in umbellate inflorescence arising between the junctions of the stem and leaves. Calyx 3 – 8 lobes, united at the base. Corolla white or yellow, 3 – 8, united at the base ornamented with fringes or crests or fimbriate. Stamens as many as corolla lobes, united to the petals or reduced to staminodes in the female flowers. Interstaminal glands presents opposite to the stamen. Pistil reduced to pistillode in male flowers, bottle shaped with or without hypogynous disc glands in bisexual or female flower. Ovary unilocular, few to many ovules with parietal placentation; ovule variable in number in same species. Style short, stout; stigma variously lobed or lacerate. Fruit a capsule, submerged, rupturing irregularly. Seeds variable in number, shape, size, tuberculate or smooth, brown – black or yellowish.

5.1.3.Key to the Species of *Nymphoides*in Kerala

1a	Flowers distylous; corolla upto 40mm in diam.,totally covered with dense hairs.....	*N. indica*
1b	Flowershomostylous; corolla upto22mm in diam., partially or not covered with hairs.....	(2)
2a	Plants dioecious; stigma bilobed with a whorl of radiating glandular hairs.....	(3)
2b	Plants monoecious or gynodioecious; stigma bilobed without a whorl of radiating glandular hairs.....	(4)
3a	Shoot dimorphic; leaves monomorphic; corolla lobes without median wings; hypogynous glands hairy	*N.macrosperma*
3b	Shoot monomorphic; leaves dimorphic; corolla lobes with median wings; hypogynous glands not hair	*N. krishnakesara*
4a	Flowers unisexual (female) or bisexual; corolla lobes not fimbriate, undulate margine with upper median wing; seeds 2 mm across.....	*N.hydrophylla*
4b	Flowers bisexual; corolla lobes fimbriate without median wing; Seeds small, less than 2 mm across.....	(5)
5a	Floating leaves obovate – orbicular; petals 4 in numbercalyx exceeds the fruit; petals 4 in number.....	*N.balakrishnanii*
5b	Floating leaves ovate – orbicular; calyx not exceeds the fruit; Petals 3 or 4 in number.....	(6)
6a	Floating leaves pale green – brownish green with pinkish tinge; hypogynous gland present; seed surface tuberculated.....	*N. parvifolia*
6b	Floating leaves dark green; hypogynous gland not distinct; seed surface smooth.....	*N. palyi.*

1.*Nymphoidesindica*(L.) Kuntze. Revis. Gen. Pl. 2: 429. 1891. Manilal& Sivar, Fl. Calicut 175. 1982; C.N.Mohanan, Fl. Quilon 265. 1984; R.Ansari, Fl. Kasargod 273. 1985;V.T.Antony, Fl. Kottayam 254, 1989; A.Babu, Fl. Malappuram 453. 1990; Vajr., Fl. Palghat 297. 1990; K.T.Joseph, Observ. Aquatic. Ang. Malabar 136. 1991; Sivar. & K.T. Joseph in Aquat. Bot.45: 156. 1993; M.Mohanan&A.N.Henry, Fl. Thiruvananthapuram 308. 1994; C.D.K.Cook, Aquat. Wetland pl. India 265. 1996; Sasidh., Flow. Pl. 300. 2004; Anil Kumar *et al.*, Fl. Pathanamthitta 224. 2005; Sunil &Sivad., Fl. Alappuzha 451. 2009. *Menyanthesindica*L., Sp. Pl. 145. 1753. *Limnanthemumindicum* (L.) Griseb., Gen. Sp. Gent. 343. 1838; Hook, f., Fl.Brit. India 4: 131. 1883; Gamble, Fl. Pres. Madras 883 (621), 1923. *Nedel – ambel.* Rheede, Hort. Malab. 11: 55 – 56, t. 28. 1692. **Fig. 3, 4 and 5.**

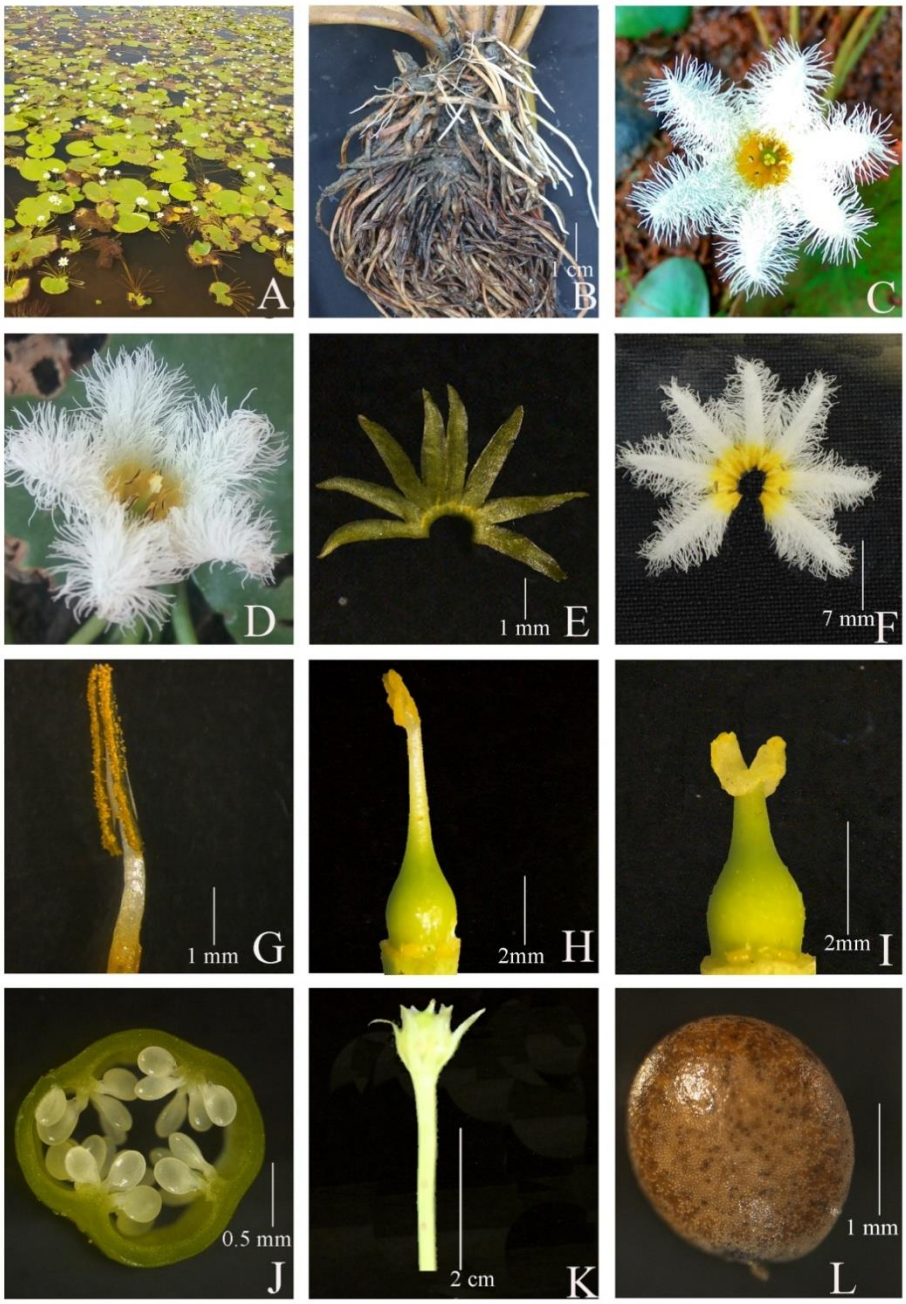

Fig. 3: *Nymphoides indica* (L.) Kuntze. **A.** Habit, **B.** Rhizome, **C.** Short styled flower, **D.**Long styled flower, **E.** Calyx, **F.** Corolla,**G.** Stamen, **H.** Pistil - long styled, **I.** Pistil - short styled, **J.** Ovary C. S, **K.** Fruit, **L.** Seed.

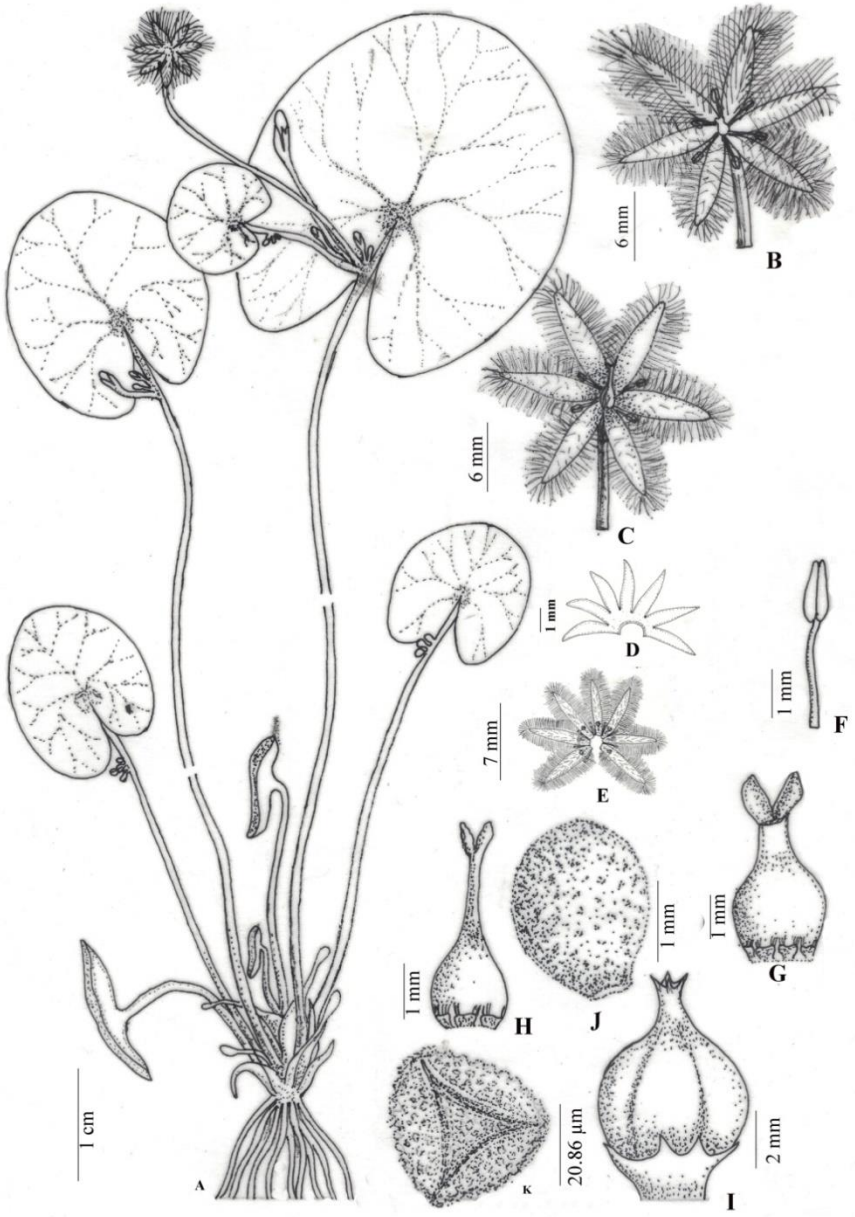

Fig 4: *Nymphoides indica* (L.) O. Kuntze - **A.** Habit, **B.** Short styled flower, **C.** Long styled flower, **D.** sepal, **E.** Petal, **F.** Stamen, **G.** Pistil - Short stled, **H.** Pistil - Long styled, **I.** Fruit, **J.** Seed, **K.** Pollen.

Fig. 5: Number of petals in *N. indica.* **A - E:** Short styled flower. **A.** 4 petal flower, **B.** 5 petal flower, **C.** 6 petal flower**, D.** 7 petal flower, **E.** 8 petal flower. **F - I:** Long styled flower. **F.** 5 petal flower, **G.** 6 petal flower, **H.** 7 petal flower, **I.** 8 Petal flower

Annual or perennial herb. Rhizome 4cm long and 1.3cm thick with scars and scale leaves. Primary shoots many, fertile, length depends up on the water depth; secondary shoots arising from the nodes of primary shoots, all are fertile, sympodial, zig – zag and ends in a single leaf. Leaves all cauline, fertile, floating, upper surface glossy green, lower surface light pinkish – green, gland dotted, ovate – orbicular, rounded, entire, cordate at base, prominently veined, up to 27 cm x 30 cm. Petiole short, up to 1.5 cm long. Flowers bisexual, distylous, hypogynous, bracteate, umbellate inflorescence, 15 – 45 flowers born from the junction of petiole and shoot; bract ovate or oblong, up to 8mm x 5mm length. Pedicel 12 cm long. Calyx 4 – 8 partite; lobes oblong – acute, greenish brown with hyaline margins, 5 mm x 2 mm. Corolla upto 40cmm in diam., white with yellow throat; corolla tube ca. 2mm; lobes 4 -8, oblong, acute, up to 16 mm x 4mm, covered with long white hairs. Stamens as many as corolla lobes, dimorphic, 8 mm in short styled flower, 6 mm in long styled flower; Pistil bottle shaped, 4 mm x 2 mm in short style 7 mm x 2 mm in long style; style stout, about1 mm long in short styled, 3mm in long styled flowers. Stigma sinuately 2 – 4 lobed; hypogynous disc glands as many as stamens, hairy, orbicular; ovary unilocular with parietal placentation.Capsules ellipsoid, torulose when ripe.Seeds 14–32 per capsule, discoid, brown, 1- 1.5 mm across, smooth.

Flowering and Fruiting: August - March

Ecology: Growing in shallow ponds, paddy fields and flooded lowlands with *Nymphaea nouchali*.Plants with short styled flowers and long styled flowers are grown in same locality.

Distribution

Kerala: All districts

World: Pantropical

Status: Least concern (IUCN)

Specimens examined: Kottayam, Vaikom. 21. 4. 1988.Swaminathan M. S. 88274 (CAL). Trichur, Paliappara, Chalakkudy. 22. 9.1982. Ramamurthy. 74703, 74863 (CAL).Alappuzha, Kanichukulangara junction. Sunil C. N.2624(CALI). Kasaragod, Peelikode. 16. 02.2007. Suresh K. K. 03152 (MBGH). Kasaragod, Peelikode. 16. 02.2007. Suresh K. K.

03317(MBGH).Kasaragod, Kayyur. 05. 02.2007. Suresh K. K &Jaris P. K. 04405(MBGH). Kasaragod, Kayyur. 05. 02.2007. Suresh K. K &Jaris P. K. 03181(MBGH). Kozhikode, Olavanna. 25. 09. 1999. Krishnan P. N. 825 (MBGH).Kozhikode, Oorkadavu. 27.06. 2008. Suresh K. K. 03994 (MBGH). Kozhikode, Olavanna. 27. 11. 15. Pavisha P. 12370(MBGH). Kozhikode, Pantheerankavu. 21. 12. 2016. Pavisha P. 12375 (MBGH).

2. Nymphoides macrospermaR.V.Nair. Kew Bull. 22: 101 1968. K.T.Joseph, Observ. Aquat. Ang. Malabar 141. 1991; Sivar. &K.T.Joseph , Aquat. Bot. 45: 162. 1993; C.D.K.Cook, Aquat. Wetland Pl. India. 265. 1996; Sasidh., Flow. Pl. 300. 2004. Sunil & Sivad., Fl. Alappuzha 454. 2009. R.Ansari *et al*. Aqut. and Wetland flora of Kerala: Flow. Pl. 583 – 584. 2016. **Fig. 6 and 7.**

Annual or perennial, herbs. Dioecious, rhizomatous; rhizome pale orange brown, vertical, 7 cm long and 8 mm thick, stoloniferous, tapering to tips. Roots many, thick, spongy. Shoots all fertile, dimorphic, length varies depends on the depth of water. Secondary shoots arise from the nodes of primary branches, zig – zag, many jointed, single small floating leaves at the apex, produce flowers and roots at the nodes. Leaves alternate, simple, ligulate sheathing base, ovate - orbicular, cordate at base, rounded apex, ca.18 cm across, thick, upper surface glossy green, lower surface purplish green, membranous, gland dotted. Petiole up to 5cm long, 5mm width. Inflorescence an umbellate clusters of 15–22 flowers at the nodes on the axillary branches. Flowers unisexual, gamopetalous, actinomorphic, bracteate, pedicellate, hypogynous, 5 mm x 10 mm in diam. Bract transparent, ovate – obtuse, 2 mm x 1 mm length, gland dotted; pedicel, pink, 2 – 3 cm long. Male flower: Calyx 5, pink, oblong – obtuse, 2 mm x 1 mm long. Corolla petals 5, white, gamopetalous up to 3mm in diam., tube very short; lobes 5, oblong, acute, up to 5 mm x 3 mm, hairy at base and centre, fimbriate toothed wings on the margins and crest. Stamen 5, inserted at the throat of corolla, filaments 2mm long, anthers ca. 1mm long, pale purple in colour, pollen yellow. Pistillode bottle shaped stigmatic hairs short. Female flower: Calyx and corolla same as in the male flower, staminode 5, small, white and non – polleniferous, 1.5 mm long. Pistil bottle shaped, 3 mm x 1 mm, hypogynous glands Surrounded by the base of the ovary; glands hairy at the tip, 1 mm width; Ovary unilocular with parietal

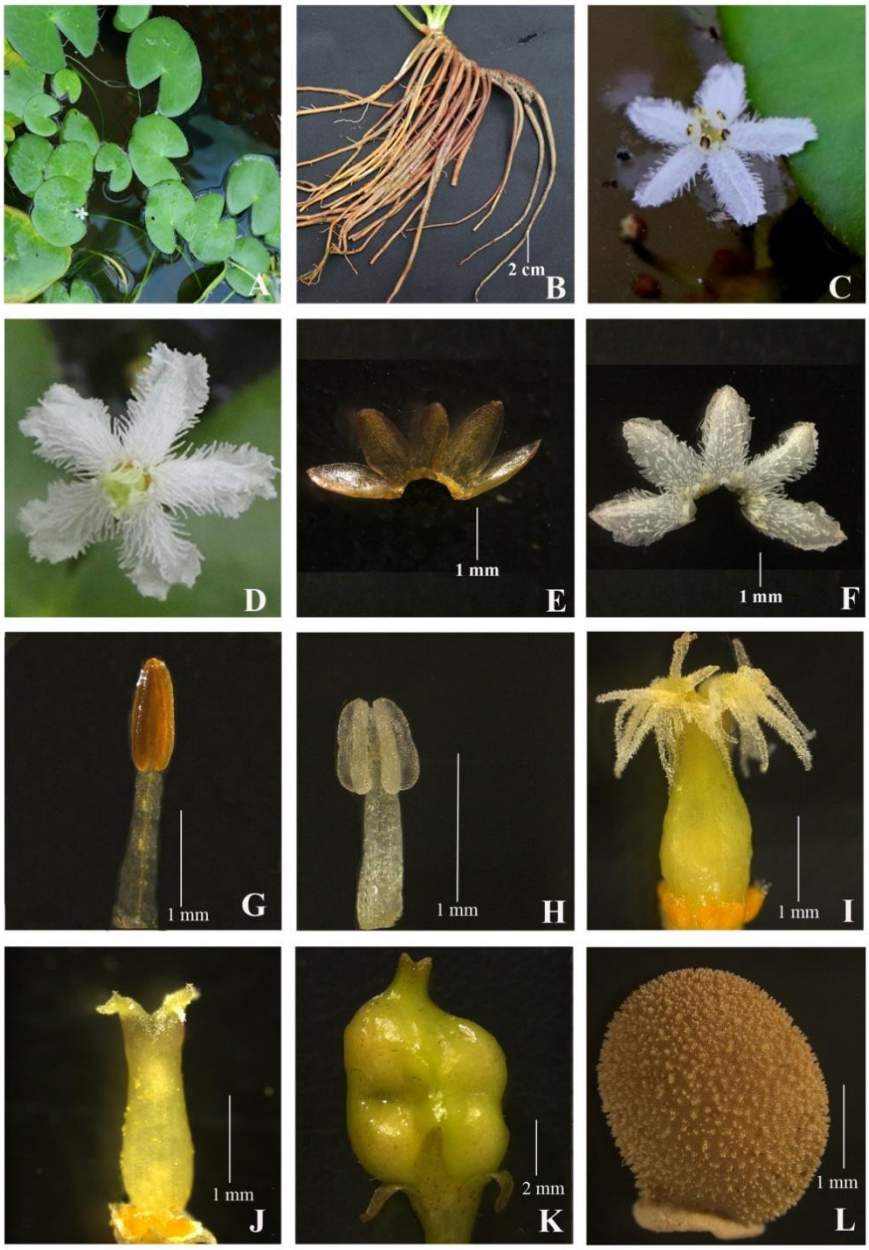

Fig. 6: *Nymphoides macrosperma* R.V. Nair. **A.** Habit, **B.** Rhizome, **C.** Male flower, **D.** Female flower, **E.** Sepal, **F.** Petal, **G.** Stamen, **H**. Staminode, **I.** Pistil, **J.** Pistillode, **K.** Fruit, **L.** Seed.

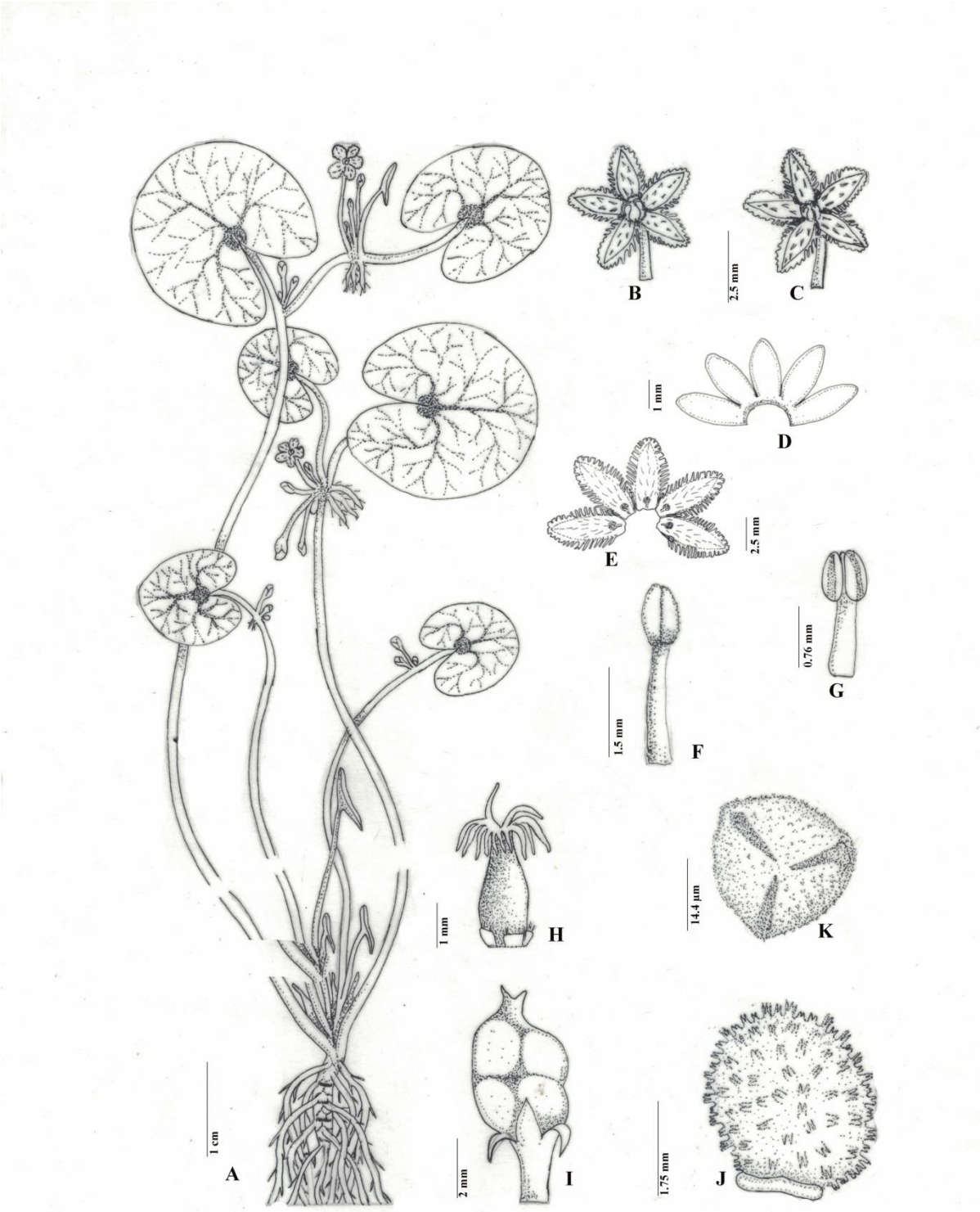

Fig 7: *Nymphoides macrosperma* R. V. Nair. **A**. Habit, **B.** Male flower, **C.** Female flower, **D.** Sepal, **E.** Petal, **F.** Stamen, **G.** Staminode, **H.** Pistil, **I.** Fruit, **J.** Seed, **K.** Pollen.

placentation. Style very short. Stigma yellowish white, bifid and long multicellular, minutely papillate stigmatic hairs. Fruit sub globose, 7 mm x 5mm length, green, persistent calyx. Seeds light brown or creamy, 2 – 4, obovate or ellipsoid, 3.5mm x 4.5 mm long.

Flowering and Fruiting: August – February

Ecology: Growing in paddy fields and ponds, associated with *Nymphoides indica*, *Eichhornia crassipes* and *Nymphaea nouchali.* Male plant and female plant are found growing in different localities as distinct population.

Distribution

Kerala: Kozhikode (Pantheerankaavu), Malappuram (Aakode), Alappuzha (Angadikal), Kollam (Kaithakodu).

World: Southern Western Ghats

Status: Critically Endangered (IUCN)

Specimens examined: Kozhikode, Olavanna. 14.07.2006. Krishnan P. N. 2064 (MBGH).Kozhikode, Pantheerankavu. 22.2.2016.Pavisha P**.**12372(MBGH). Malappuram, Akkode. 15. 3. 2018. Pavisha P. (MBGH).

3. Nymphoides krishnakesaraK.T.Joseph &Sivar. Nordic. J. Bot. 10(3): 281 – 284. 1990; Sivarajan& Joseph , Aquat. Bot. 45: 159 – 162. 1993; C.D.K.Cook, Aquat. Wetland Pl. India. 265. 1996; Sasidh., Flow. Pl. 300. 2004 (Fig. 252; pl. 8h). R.Ansari *et al.* Aqut. and Wetland flora of Kerala: Flow. Pl. 583 – 584. 2016. **Fig. 8and 9.**

Plants annual or perennial herbs, dioecious, rhizomatous and endemic species. Rhizome 2 –5 cm long, 1–2 cm thick and produce many shoots. Shoots are monomorphic, fertile, petiole-like, variable in length depends on the depth of water bodies; secondary Shoots absent. Leaves dimorphic; basal leaves submerged and sterile, rosulate, spatulate up to 4 cm long. Floating fertile leaves ovate – orbicular, apex rounded, deeply cordate below up to 5.1cm x 5 cm long, creamy green below and shiny green above, petiole short, 3 mm long. Flowersunisexual,20 – 40arising from the junction of petiole and shoot of fertile leaves, inflorescencevumbellate. Male flower: calyx gamosepalous, 5 lobed, oblong, sub acute, 4 mm x 1 mm long. Corolla gamopetalous up to 11mm in diam., white, 5 lobed; lobes up to 6mm x 2 mm, marginal and median wings fimbriate, median wings extend from the apex to the middle. Stamens 5, inserted

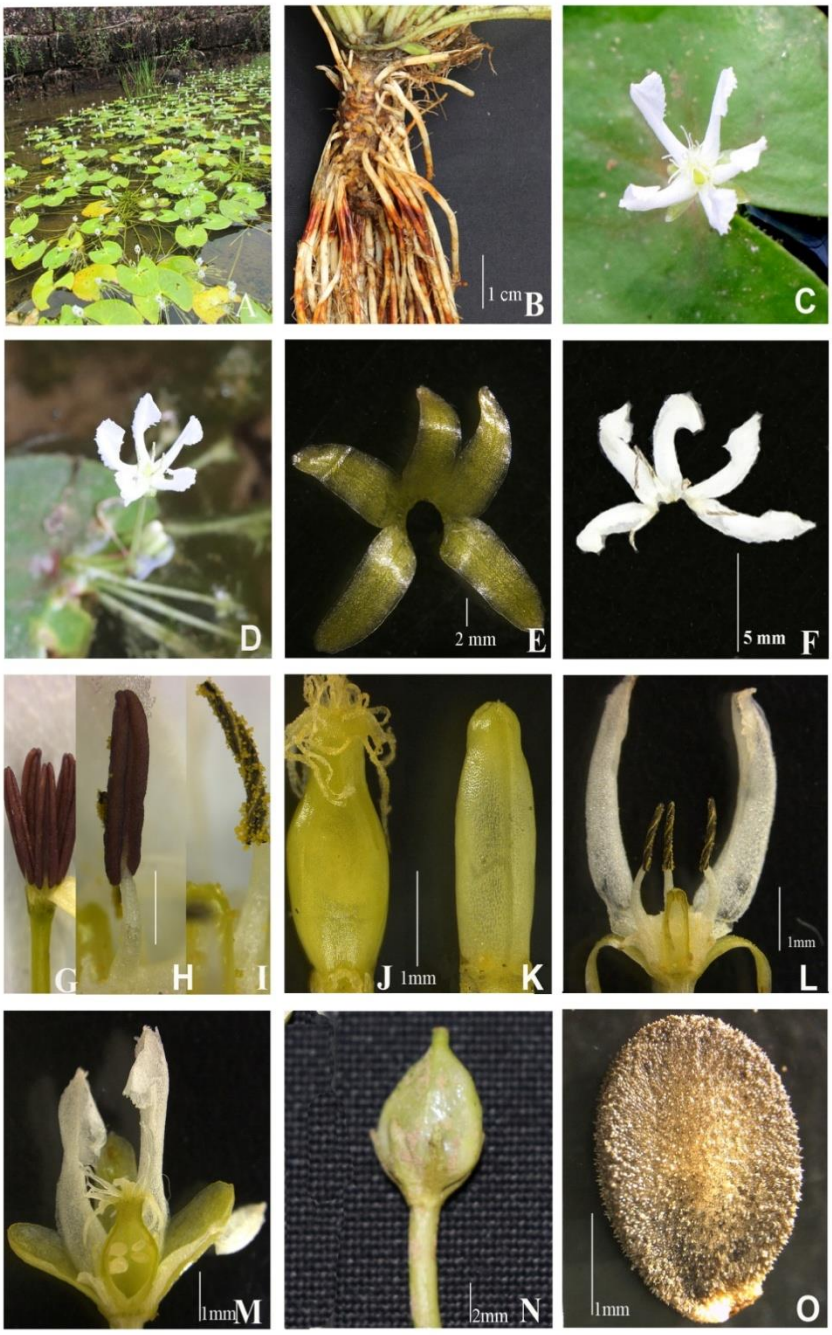

Fig. 8: *Nymphoides krishnakesara* K. T. Joseph & Sivar. **A**. habit, **B**. Rhizome, **C**. female flower, **D**. male flower, **E**. sepal, **F**. petal, **G - I.** stamen, **J.** pistil,K. pistillode, **L.** L.S. of male flower, **M.** L. S. of female flower, **N**. fruit, **O.** seed.

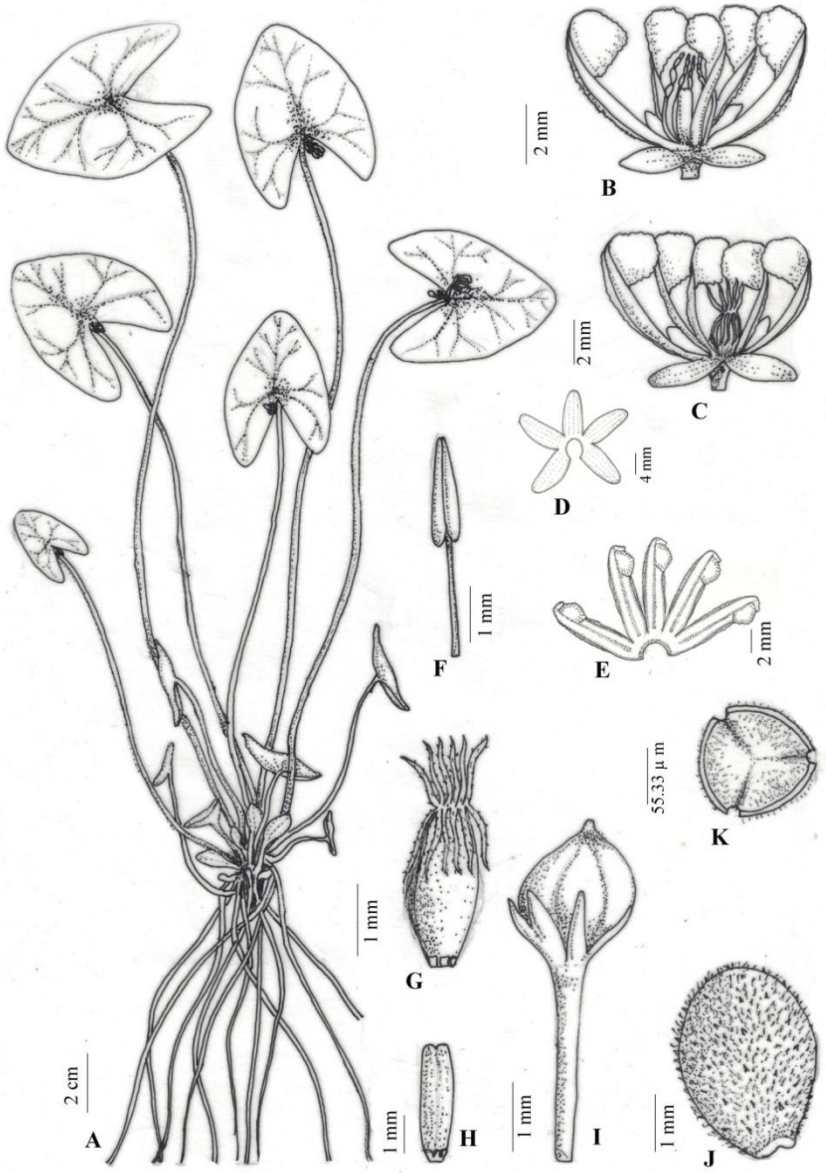

Fig 9 : *Nymphoides krishnakesara* K. T. Joseph & Sivar. **A**. Habit, **B**. Male flower, **C**. Female flower, **D**. Sepal, **E**. Petal, **F**. Stamen, **G**. Pistil, **H**. Pistillode, **I**. Fruit, **J**. Seed, **K**. Pollen

at the throat of the corolla and alternating with the petals, filaments ca. 2 mm long; anthers bluish - black in colour, 2 mm long, spirally twisted when dehisced; pollen cream – yellow. Pistillode oblong, 3mm long, slightly emarginated at apex, stigmatic hairs absent, abortive ovule on each placenta. Hypogynous disc glands present fleshy and entire. Female flower: Calyx, corolla and hypogynous glands same as in male flower. Staminods 5, smaller, white. Pistil bottle – shaped, up to 3 mm; stigma sessile, 2 seriate, cream coloured, radiating, minutely papillose long hairs; ovary unilocular with parietal placentation; capsules obovoid, ca. 9 mm long. Seeds 4 – 9 in each capsule, obovoid, rounded or obtuse at distal end, subacute at proximal end, testa brown in colour, surface spiny, 3.5 mm long and 2.5 mm wide, bilateral surface minutely spinulose.

Flowering and Fruiting: August – February

Ecology: Plant specimens growing in seasonal pools on lateritic hillocks of Kannur (Madayipara and Korom). It grows in association with *Eriocaulon truncatum* Mart., *Rotala malampuzhensis* Vasudev. Ex Cook, *Blyxa octandra* (Roxb.) Thw, *Dopatrium junceum* and *Elaeochari sdulcis*, etc.

Distribution

Kerala: Endemic to Northern Kerala (Kannur Dts.).

World: Kerala (Endemic)

Status: Endangered – IUCN.

Specimens examined: Kannur, Madayippara. 25.10.1988. Joseph K. T. 43001 (MH). Kannur, Madayippara, 20. 09. 2005, Krishnan P. N., and Ansari R., 2563 (MBGH); Kannur, Payyannur, 07. 10. 2008, Suresh K. K, 04288 (MBGH); Kannur, Madayippara, 04. 09. 2008, Suresh K. K., 4004 (MBGH); Kannur, Payyannur, 04. 09. 2008, Suresh K. K. &Jaris P. K, 3317 (MBGH); Kannur, Payyannur, 29. 07. 2009, Suresh K. K., 4964 (MBGH); Kannur, Madayippara, 12. 12. 2016,Pavisha P, Rajilesh V. K. &Ajesh P. P., 12373(MBGH); Kannur, Korom, 15. 1. 19,Pavisha P, Jaseela V. T &Sinisha P., 17748(MBGH).

4.*Nymphoides hydrophylla*(Lour.) Kuntze.Revis. Gen. Pl. 2: 429. 1891; R.Ansari, Fl. of Kasargod 237. 1985; Vv.s.Ramach.&v.j.Nair, Fl. Cannannore 293. 1988; Hort. Malab. 181. 1988; V.T.Antony, Fl. Kottayam 253. 1989; Vajr., Fl. Palghat 297. 1990; Sivar. & K.T.Joseph in Aquat. Bot. 45: 153. 1993;

M.Mohanan and &A.N.Henry, Fl. Thiruvananthapuram 308. 1994; C.D.K.Cook, Aquat. Wetland Pl. India 264. 1996. SasidH., Flow. Pl. 300. 2004; Sunil &Sivad., Fl. Alappuzha452. 2009. **Fig. 10 and Fig. 11**

Type - Birma, S.Kurz. 2264 (Holotype) -CAL

Menyanthes hydrophylla Lour., Fl. Cochinch. 105. 1790.

Villarsia hydrophyllum (Lour.) Roem. &Schult., Syst. 4: 181. 1819.

Limnanthemum hydrophyllum (Lour.) Griseb., Gen. Sp. Gent. 348. 1838.

Menyanthes cristata Roxb., Pl. Corom. 2: 3, t. 105. 1798.

Limnanthemum cristatum (Roxb.) Griseb., Gen. Sp. Gent. 342. 1838; Hook. f., Fl. Brit. India 4: 131. 1883; Gamble, Fl. Madras 2: 883. 1923.

Nymphoides cristatum (Roxb.) Kunze, Hook. f., Fl. Brit. India 4: 131. 1883; Gamble, Fl. Pres. Madras 620.1923.

Tsjeroea – chitambelRheede, Hort. Malab. 11: 57, t. 29. 1692.

Annual or perennial herbs, rhizomatous, monoecious or gynodioecious. Rhizome 2 – 3 cm long and 1 - 2 cm. Primary and secondary shoots present; primary fertile shoots many, arising from the rhizome, length variable depends upon the depth of water and producing secondary shoots, many jointed shoots, zig – zag manner, sympodial. Leaves all floating, ovate - orbicular, 9.6 x 8.8 cm across, cordate at base, green, sometimes with purplish bloaches above, gland dotted below. Petiole up to 8 mm long. Flowers umbellate clusters of 10 – 20, bisexual and female, bracteate. Bract ovate. Pedicels 3.8 cm x 1mm. Calyx gamosepalous, 5 partite; lobes linear - lanceolate, acute, ca. 5 x 1.5mm. Corolla gamopetalous, upto 22mm in diam.; petals 5, white with yellow throat; tube 2mm with glandular hairs; lobes 5, oblong, obtuse, 8 x 5 mm, with flexuous membraneous wings on the margins and a median longitudinal crest within. Stamens 5, yellow, 2mm length, reduced in female flowers. Pistil bottle – shaped, 3 x 2 mm, with 5 orbicular disc glands at the base, glands hairy on the upper side; Style short; ovary unilocular with parietal placentation; Stigma 2 lobed. Capsule oblong, up to 5 mm long; pedicel 3.1 cm. Seeds 4 – 6, brown, discoid, up to 2mm across; surface tuberculated.

Flowering and Fruiting: August - March

Ecology: Growing in paddy fields, flooded lowlands, pools, ponds, river banks and canals. Bisexual flower and female flowers are born on different plants.

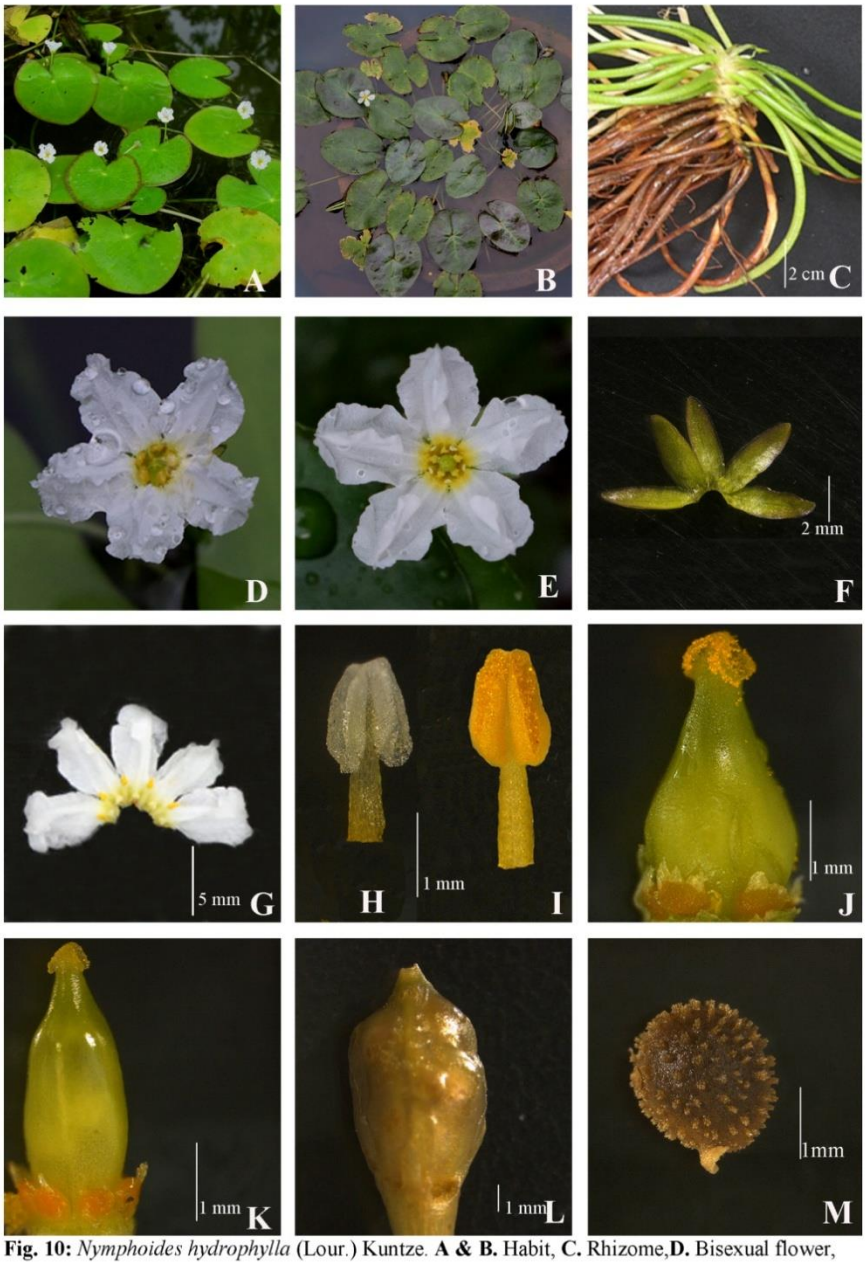

Fig. 10: *Nymphoides hydrophylla* (Lour.) Kuntze. **A & B.** Habit, **C.** Rhizome,**D.** Bisexual flower, **E.** Female flower, **F.** Sepal, **G.** Petal, **H.** Staminode, **I.** Stamen, **J.** Pistil of bisexual flower, **K.** Pistil of female flower, **L.** Fruit, **M.** Seed.

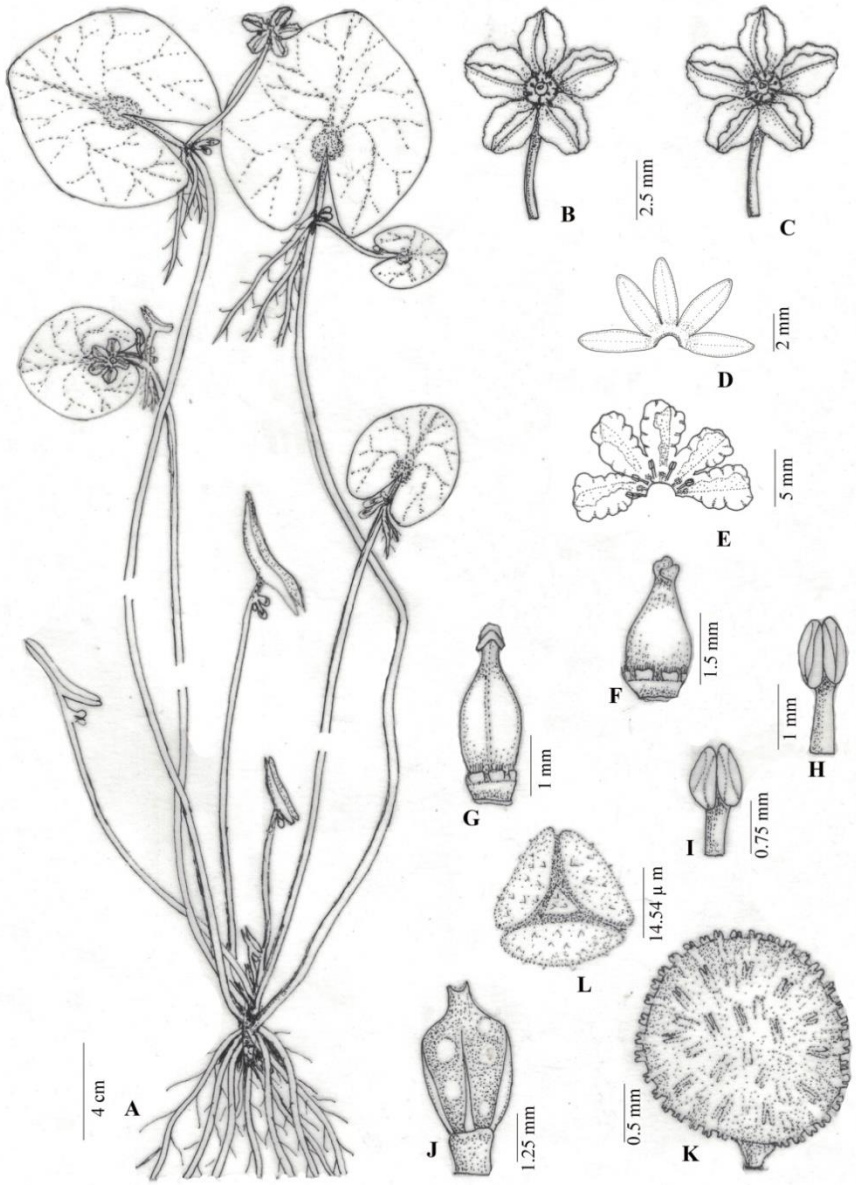

Fig. 11 : *Nymphoides hydrophylla* (Lour.) Kuntze **A**. Habit, **B**. Female flower, **C.** Bisexual flower, **D.** Sepal, **E.** Petal, **F.** Pistil - Bisexual flower, **G.** Pistil - Female Flower,**H.** Stamen, **I.** Staminode, **J.** Fruit, **K.** Seed, **L.** Pollen

Distribution

Kerala: All districts

World: India, Sri Lanka, Malaysia and Southern China.

Status: Least Concern (IUCN)

Notes: *N. hydrophylla* (Lour.) O. Kuntze is a gynodioecious plant *ie*, bisexual flowers and female flowers. In female flowers stamens reduced to staminodes. Pistil bottle shaped, 3mm x 1mm. Two type of flowers bearing on different plants. Based on leaf morphology *N. hydrophylla* is of two types. One with green leaves and shoots; leaves ovate - orbicular, cordate at base with rounded apex. The other types the leaves are purple, ovate – orbicular, apex obtuse.

Specimens examined: Thiruvananthapuram, Pulimath, 2. 12. 1977. Mohanan. 52675.Idukki, Moolamattam. 17. 12.2008. Suresh K. K. 04436 (MBGH). Kollam. 16.02.2007. Suresh K. K. 03229 (MBGH).Palghat, Elavanchery. 2. 11. 1976. Thrissur, Chalakudi, 15. 9. 1976. Ramamurthy. 48535.Vajravelu M. S. 48836, Cannanore, Ezhuvamalai, 17. 12. 1979. Ramachandran. 65266, 20. 7. 1981. Ansari R. 70966. Kasaragod, Peelikode. 06. 02. 2007. Suresh K. K. &Jaris P. K. 03162 (MBGH). Suresh K. K. &Jaris P. K. 03162 (MBGH). Kasaragod, Kayyur. 06.02.2007. Suresh K. K. &Jaris P. K.03188 (MBGH). Malappuram, Koottumoochi. 18. 11 2011. Anoop K. P &Hareesh K. T. 6537(MBGH). Kozhikode, Palazhi. 22.2.16. Pavisha P. 12371(MBGH). Kozhiode, Mavoor. 22. 2. 16. Pavisha P. 12384 (MBGH).

5.*Nymphoides parvifolia* Kuntze. Revis. Gen. Pl. 2: 429. 1891; Subram., Aquat. Ang. 24. 1962; Cramer in Dassan. &Fosb., Rev. Handb. Fl. Ceylon 3: 210. 1981. Manilal & Sivar., Fl. Calicut 175. 1982.**Fig. 12. and 13.**

Limnanthemum parvifolium Griseb. in DC., Prod. 9: 141. 1845; Clarke, J. Linn. Soc. Bot. 14: 450. 1875 & in Hook. f., Fl. Brit. India 4: 132. 1883; Trimen, Handb. Fl. Ceylon 8: 189. 1895; Gamble, Fl. Madras 2: 883. 1923. Type: Wallich Num. List. 4351. Burma, Tavoy (holotype, K – Wall.)

Limnanthemum moonii Thw., Enum. Pl. Zeyl. 205. 1860. Type: Moon, C. P. 2842. Ceylon, Kalutara.

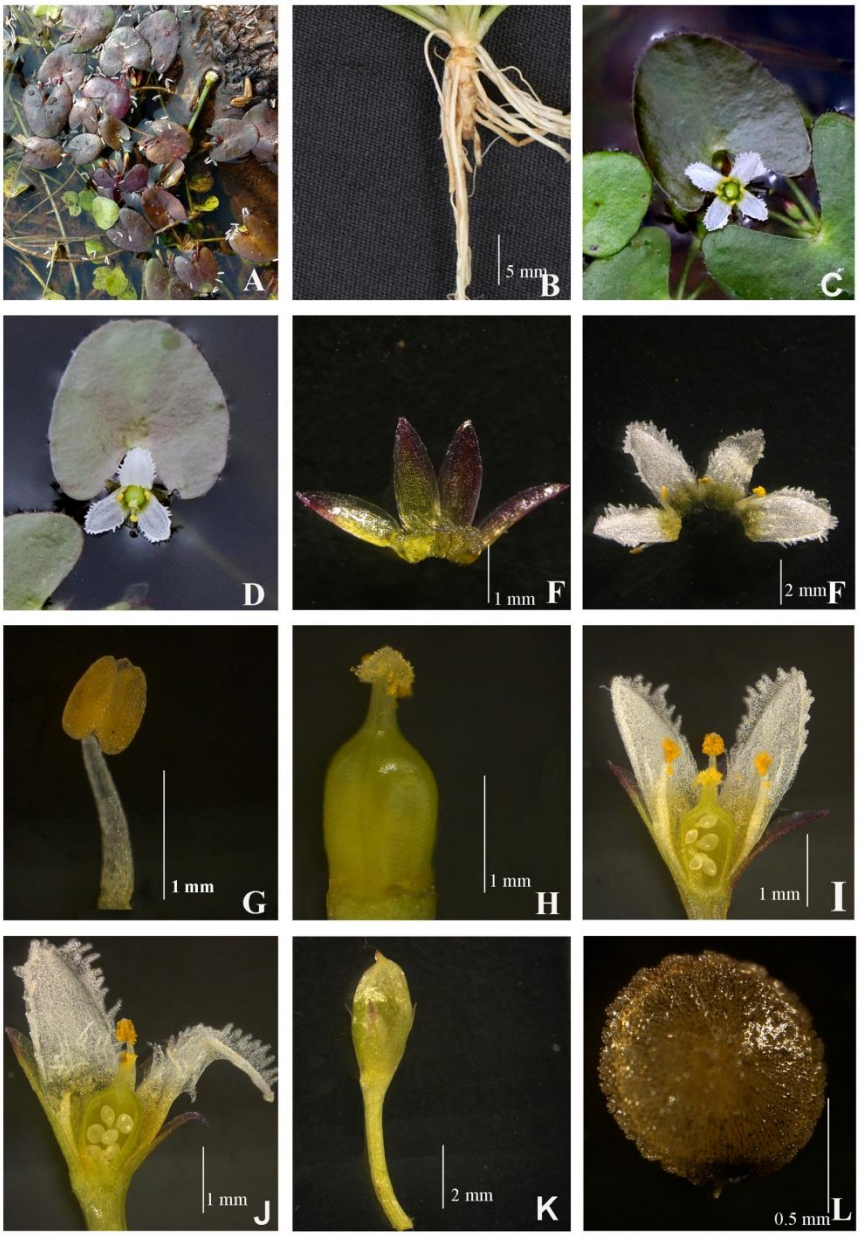

Fig. 12: *Nymphoides parvifolia* Kuntze. **A.**Habit, **B.** Rhizome, **C.** Single flower, **D.** Single flower, **E.** Sepal, **F.** Petal, **G.** Stamen, **H.** Pistil, **I.** Flower L. S, **J.** Flower L. S, **K.** Friut, **L.** Seed.

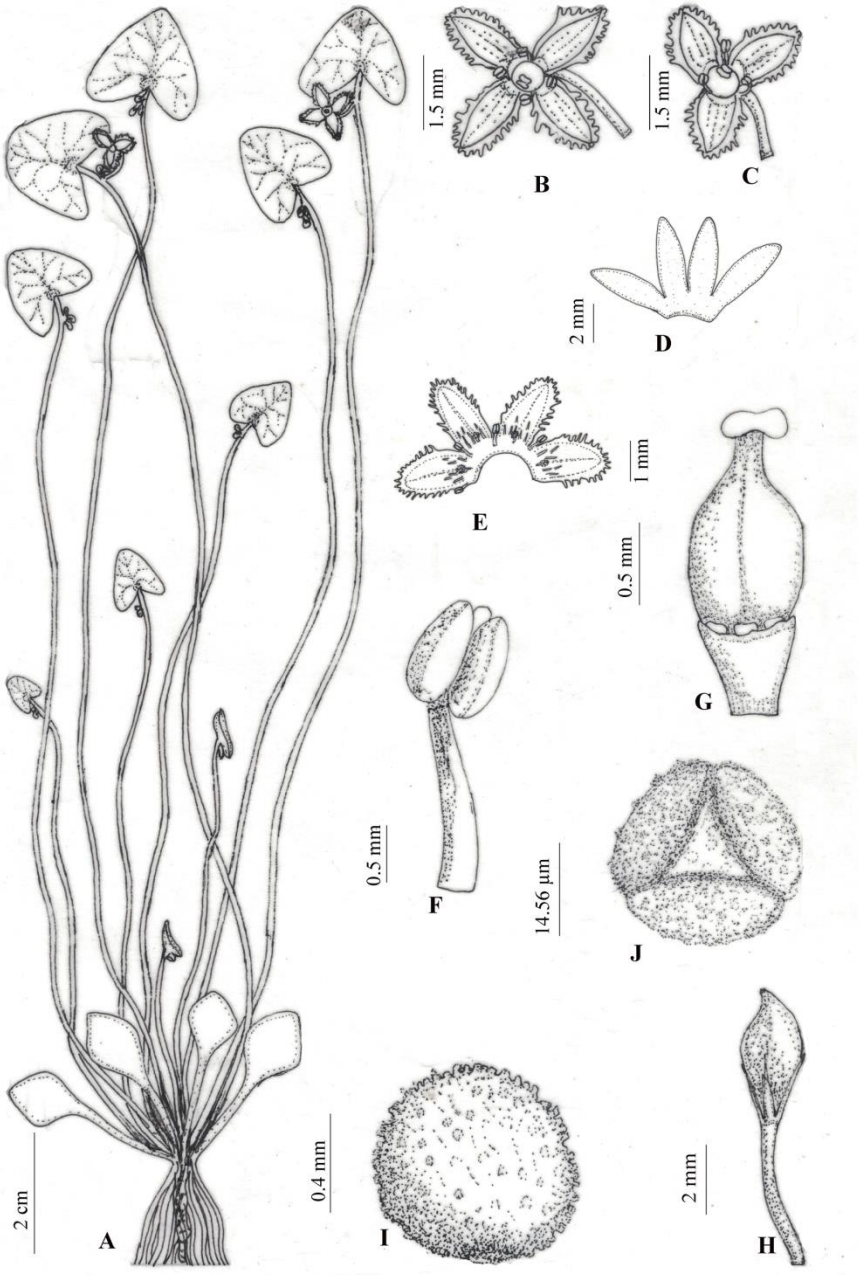

Fig 13 : *Nymphoides parvifolia* Kuntze **A.** Habit, **B & C** Single flower, **D.** Sepal, **E.** Petal, **F.** Stamen, **G.** Pistil, **H.** Fruit, **I.** Seed, **J.** Pollen

Limnanthemum parvifolium var. *moonii* (Thw.) Clarke in Hook. f. , Fl. Brit. India 4: 132. 1883.

Annual or perennial herb. Rhizome short, obconical 1 cm long and 3 mm wide. Shoots slender, all fertile, uniphyllous, 8 – 22 cm long. Leaves dimorphic, small; Sterile leaves submerged, rosette, up to 3 cm x 1.5 cm spatulate, winged at base; petiole 2–4 cm long; fertile leaves floating, solitary on the apices of primary shoots, ovate – orbicular, base deeply cordate, apex obtuse or rounded with narrow sinuses, entire, green to light brown coloured, up to 2 cm x 3 cm; petiole short, 1 mm length. Flowers bisexual, in umbellate clusters of 4 – 8. Calyx gamosepalous, 3 or 4 lobed; lobes oblong – acute, pinkish at apex, hyaline at margine, up to 2mm x 1mm. Corolla gamopetalousupto 3mm in diam.; petals 3 - 4, white with yellow throat and a ring of hyaline hairs at the throat, corolla tube 2 mm long, oblong or elliptic, 4 mm x 2mm long, margin fimbriately toothed towards the apex. Stamens 3 or 4, epipetalous, inserted below the throat between two corolla lobes, bithecous; filaments 1.5mm, alternate with white glandular hairs. Gynoecium bottle – shaped, 2mm x 1mm, disc glands present below the ovary; ovary unilocular with parietal placentation; style short; stigma bilobed. Capsule ellipsoid, torulose when ripe, up to 4mm x 2mm. Seeds 8 – 12 in each capsule, 1 mm across, discoid, tuberculate, light brown in colour.

Flowering and Fruiting: August - February

Ecology: The plant is growing in the shallow ponds and flooded paddy fields along with *Rotala malampuzhensis*, *Eriocaulon* spp., *Nymphaea* nouchali and *Nymphoides indica.*

Distribution

Kerala: Kasaragod, Alappuzha, Kozhikode

World: Southern India, Sri Lanka, Malaysia and Tropical Australia.

Status: Least Concern (IUCN)

Specimens examined: Kasaragod, Beemanadi. 27. 9. 1982. Ansari R. 74328 (CAL).Kasaragod, Beemanadi. 18.12.2008. Suresh K. K. 4954(MBGH). Kasaragod, Beemanadi. 7. 12. 17. Pavisha P, Hridhya P &Ajesh P. P. 15813(MBGH).

6. ***Nymphoides balakrishnanii*** Biju, Josekutty, Haneef & Augustine.Int. J. Advanced Res. 4(7): 799 – 803. 2016. **Fig. 14 and Fig. 15.**

Rhizomatous annual or perennial herb; Rhizome cylindrical, covered by roots, 2 cm long and 7 mm wide. Leaves dimorphic; submerged leaves sterile, rosettes and spatulate; petiole spongy, 4 – 5 cm; lamina deltate – ovate, 10 x 8 mm, obtuse at apex and truncate at base. Fertile shoots arise from the axils of sterile leaves, 6 – 30 cm long; floating leaves 3.5 – 5 x 3 – 4.5 cm, orbicular – ovate with cordate base. Petiole *ca.*, 3 mm long. Flowers arise from the junction between the fertile shoot and petiole, 4 – 10, bisexual, 6 x 7 mm across, pedicellate, bracteate; pedicel 4 mm; bracts triangular. Calyx gamosepalous, 4 lobed, green; lobes linear – lanceolate with hyaline margins, 4 x 1 mm long. Corolla gamopetalous,upto 4mm in diam., white with yellow throat; petals 4, hairy with fimbriately toothed wings on the margins; wings 1mm length. Stamens 4, epipetalous, obliquely oriented 1mm long; filaments 0.5 – 0.6 mm, hyaline; anther black along the dorsal side and yellow along the ventral side with broad connective; clusters of glandular hairs alternate with the stamens. Pistil bottle shaped; style 0.4 – 0.6 mm; stigma bifid, hypogynous glands with hairs; ovary obovoid, 2.5 x 1 mm long, unilocular with parietal placentation. Capsule green, light yellow at meature, oblong – obovoid, equal or slightly shorter than the fruiting calyx, 4 x 2 mm, stalk 2 cm long. Seeds brownish black, 5 - 13 in capsule, 1 mm across, faveolate, tuberculate.

Flowering and Fruiting: August –January

Ecology: Grows in the seasonal ponds of the lateritic hillocks associated with *Eriocaulon* spp.

Distribution

Kerala: Kasaragod

World: Kerala (Endemic)

Notes: *N. balakrishnanii* is a highly endemic species, restricted to few seasonal pools in Kasaragod district. The plant is facing severe threat due to anthrapogenic activities. The type locality is situated near a KSEB substation. KSEB installed solar panels throughout the area. Local people are using these seasonal pools for washing cloth and other purposes.

Fig. 14: *N. balakrishnanii* Biju, Josekutty, Haneef & Augustine. **A.** Habit, **B.** Rhizome, **C.** Flower, **D.** Sepal, **E.** Petal, **F.** Stamen, **G.** Pistil, **H.** Ovary C. S, **I.** Flower L. S. **J.** Vegetative propagated plant, **K.** Fruit, **L.** Seed.

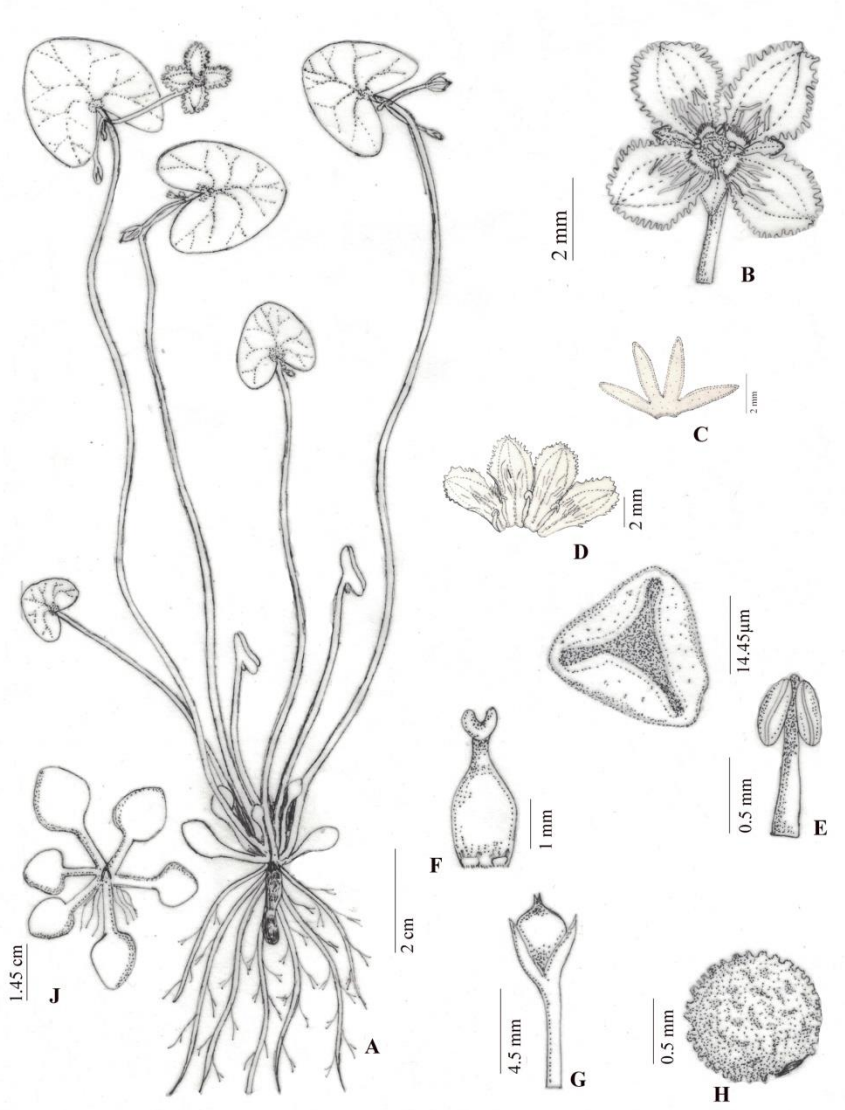

Fig. 15: *Nymphoides balakrishnanii* Biju, Josekutty, Haneef & Augustine. **A.** Habit, **B.** Single flower, **C.** Sepal, **D.** Corolla, **E.** Stamen, **F**. Pistil, **G.** Fruit, **H.** Seed, **I.** Pollen, **J.** New saplings from existing rhizome.

Specimens examined: Kasaragod, Koovappara. 24. 01. 2019. Pavisha P. 17747(MBGH). Holotype not found at CAL.

7. *Nymphoides palyi* Biju, Josekutty, Haneef & Augustine.*Taiwania.* 61(3): 218 – 220. 2016. **Fig. 16 and 17.**

Type: Kerala

Rhizomatous annual herbs; Rhizome vertical, covered by roots, 2 cm long and 5 mm wide. Leaves dimorphic; submerged leaves in rosettes and spatulate; petiole spongy, 2 cm; lamina ovate - rhomboid, 10 x 8 mm, round at apex. Fertile shoots arise from the axils of submerged leaves, 16 - 42 cm long; floating leaves 1.4 – 1.5 x 1.4 – 1.6 cm, ovate, deeply cordate at base; Petiole *ca.,* 3 mm long. Flowers 4 – 10, bisexual, pedicellate, bracteate; bracts membranous, 1.5 mm x 1.7 mm, light green; pedicel 2 mm; bracts triangular. Calyx gamosepalous, 4 or 3 lobed, green with pinkish apex , glabrous; lobes elliptic – lanceolate with hyaline margins, 2 mm x 1 mm. Corolla gamopetalousupto 3mm in diam., white with yellow throat, tube 0.8 mm; petals 3 or 4, ovate – lanceolate, centrally veined, margins involuted and minutely fimbriately toothed towards the apex, 3 mm x 1mm length. Stamens 4 or 3, epipetalous, bithecous; anthers cream coloured, 0.4 mm x 0.4 mm long; filaments 1 mm; Inter staminal glandular hairs absent. Pistil bottle shaped; style 0.3 mm; stigma bifid; Ovary 0.8 x 0.6 mm unilocular with parietal placentation; hypogynous glands not distinct. Capsule green, oblong, 3mm x 2 mm; stalk 6mm long. Seeds are brownish black, 4 – 10 in capsule, discoid, 0.8 mm across, smooth.

Flowering and Fruiting: July – December

Ecology: Growing in the seasonal ponds of the lateritic hillocks associated with *Microcarpaea minima* (K. D. Koenig ex Retz.) *Ludwigia hyssopifolia* (G. Don) Exell., *Eriocaulon cuspidatum* Dalz. and *Wiesneria triandra* (Dalz.) Micheli.

Distribution

Kerala: Kasaragod

World: Southern Western Ghats (endemic)

Fig. 16: *Nymphoides palyi* Biju, Josekutty, Haneef & Augustine. **A.** Habit, **B.** Habit-Herbarium specimen, **C.** Rhizome, **D.** Flower, **E & F.** Sepal, **G.** Petal, **H.** Stamen, **I.** Pistil, **J.** Fruit, **K.** Seed, **L.** Vegetative propagative plant from existing rhizome.

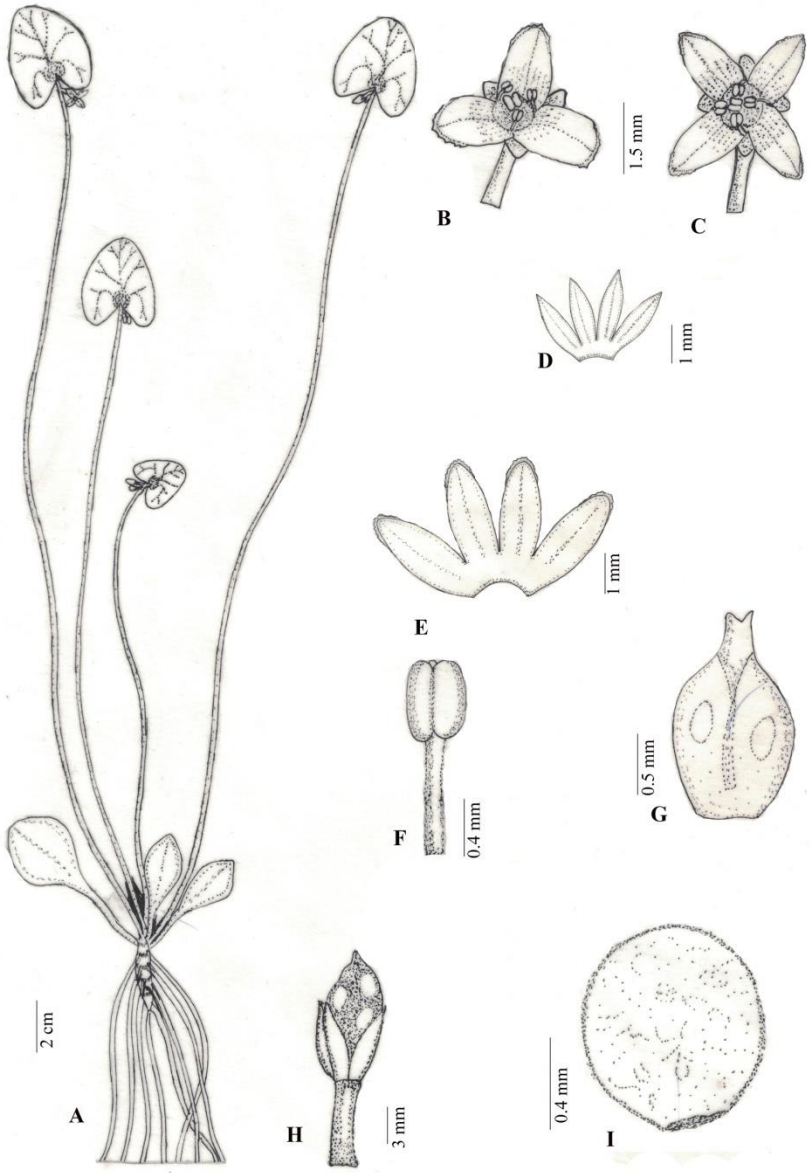

Fig 17: *Nymphoides palyi* Biju, Josekutty, Haneef & Augustine. **A**. Habit, **B & C.** Single flower, **D.** Sepal**, E.** Petal, **F**. Stamen, **G.** Pistil, **H.** Fruit, **I.** Seed.

Specimens examined: Kasaragod, Perla, 19. 09. 2016, Anoop K. P &Rajilesh V. K., 14595(MBGH); Kasaragod, Perla, 07.12.2018,Pavisha P, Hridhya P &Ajesh P. P., 15831(MBGH).

5.2. COMPARATIVE MORPHOLOGY

Species Name	***N. indica***	***N. macrosperma***	***N. krishnakesara***	***N. hydrophylla***	***N. parvifolia***	***N. balakrishnanii***	***N. palyi***
Habit	Herb	Herb	Herb	Herb	Herb	Herb	Herb
Rhizome	Stoloniferous, brown, 4 cm long and up to 1.3 cm wide.	Stoloniferous, pale Orange – brown, 3 – 7 cm long and 1 – 2 cm wide.	Non stoloniferous, pale orange – brown, 2 - 5 cm long and 1.2 cm wide.	Stoloniferous, 2 -3 cm long and 1 – 2 cm wide.	Non stoloniferous, short, obconical, 1 cm long and 3 mm wide.	Non stoloniferous, cylindrical, 2 cm long and 7 mm wide.	Non stoloniferous, vertical, 1 -2 cm long and 5 mm wide.
Shoot	Dimorphic, primary shoot arising from the rhizome, secondary shoot from the flowering region of primary shoot each bearing single leaf.	Dimorphic, primary shoot arising from the rhizome, secondary shoot from the flowering region of primary shoot each bearing single leaf.	Monomorphic, primary shoot arising from the rhizome. Secondary shoot absent	Dimorphic, primary shoot arising from the rhizome, secondary shoot from the flowering region of primary shoot each bearing single leaf.	Monomorphic, primary shoot arising from the rhizome. Secondary shoot absent.	Monomorphic, primary shoot arising from the rhizome. Secondary shoot absent.	Monomorphic, primary shoot arising from the rhizome. Secondary shoot absent.
Leaf	Monomorphic; floating, thick, entire, ovate – orbicular, rounded at apex, deeply cordate at base, glossy green above, pale- gland dotted below.	Monomorphic; floating, thick, entire, ovate – orbicular, rounded at apex, deeply cordate at base, green above, pale-gland dotted below.	Dimorphic; submerged leaves spatulate, rosulate, green, spongy petiolar part and ovate or oblong lamina; floating leaves ovate – orbicular, deeply cordate at base,	Monomorphic; floating, ovate – orbicular, obtuse at apex, deeply cordate at base, entire, green above with purplish bloaches or green with purplish margine,	Dimorphic; basal submerged leaves spatulate, rosulate, green, spongy petiolar part and ovate or oblong lamina;; floating leaves ovate – orbicular, rounded	Dimorphic; basal leaves submerged, spongy petiolar part and deltate – ovate lamina, green. Floating leaves orbicular – ovate, deeply cordate at base, rounded at apex,	Dimorphic; basal leaves submerged, spatulate, rosulate, green, spongy petiolar part and ovate – rhomboid lamina; floating leaves ovate, deeply cordate at base, obtuse

			obtuse at apex, entire, glossy green above.	purple coloured with pale- gland dotted below.	at apex, cordate at base, green or pink coloured.	glossy green above	at apex, dark green above.
Inflorescence	Umbel	Umbel	Umbel	Umbel	Umbel	Umbel	Umbel
Flower	Bisexual and heterostylous.	Unisexual and homostylous.	Unisexual and homostylous.	Bisexual and female flower in separate plants, homostylous.	Bisexual and homostylous.	Bisexual and homostylous.	Bisexual and homostylous.
Calyx	Gamosepalous, lobes 4 -8, oblong – acute, greenish brown with hyaline margins, 5 mm x 2 mm.	Gamosepalous; 5 lobed; lobes pink, oblong – obtuse, 2 mm x 1 mm long.	Gamosepalous; 5 lobed; lobes oblong, obtuse, glabrous, 4 mm x 1 mm.	Gamosepalous; 5 lobed; lobes linear – lanceolate, acute, *ca*. 5 x 1.5mm.	Gamosepalous; 3 or 4 lobed; lobes oblong – acute, pinkish at apex, hyaline at margine, up to 2mm x 1mm.	Gamosepalous; 4 lobed, green; lobes linear – lanceolate with hyaline margins, 4 x 1 mm long.	Gamosepalous; 4 or 3 lobed, green with pinkish apex, glabrous; lobes elliptic – lanceolate with hyaline margins, 2 mm x 1 mm.
Corolla	Gamopetalous; white with yellow throat; corolla tube ca. 2mm; lobes 4 -8, ovate-lanceolate, acute at apex, up to 16 mm x 4mm, covered with long white hairs.	Gamopetalous; white without yellow throat; corolla tube short; lobes 5, oblong or elliptic, acute, up to 5 mm x 3 mm, hairy at base and centre,fimbriately toothed wings on the margins.	Gamopetalous; white without yellow throat; tube short; 5 lobed; lobes obtuse, without hairs, up to 6mm x 2 mm, marginal and median wings fimbriate, median wings extend from the apex to the middle.	Gamopetalous; white with yellow throat; tube 2mm with glandular hairs; lobes 5, oblong, obtuse or retuse at apex, 8 x 5 mm, with flexuous membraneous wings on the margins and a	Gamopetalous; white with yellow throat and a ring of hyaline hairs at the throat, corolla tube 2mm long; lobes 3 or 4, oblong or elliptic, 4 mm x 2mm long, margin fimbriately	Gamopetalous; white with yellow throat; tube 2mm; lobes 4, ovate-lanceolate, hairy with fimbriately toothed wings on the margins, 5 x 4 mm	Gamopetalous; white with yellow throat, tube 0.8 mm; lobes 4 or 3, ovate – lanceolate, margins involuted and minutely fimbriately toothed

				median longitudinal crest	toothed towards the apex.		towards the apex, 3 mm x 1mm length.
Stamen and stamiode	Dimorphic, epipetalous, 4 – 8; stamen longer than the pistil in short styled flowers and shorter than the pistil in long styled flowers. Anther colour pale purple.	Monomorphic, epipetalous, 5 stamens in male flower; 5 staminode in female flower. Anther colour pale purple.	Monomorphic, epipetalous, 5 stamens in male flower; 5 staminode in female flower. Anther colour blue.	Monomorphic, epipetalous, 5 stamens in bisexual flower; 5 staminode in female flower. Anther colour yellow.	Monomorphic, epipetalous, 3 or 4 stamens. Anther colour yellow.	Monomorphic, epipetalous, 4 stamens. Anther colour black along the dorsal side and yellow along the ventral side	Monomorphic, epipetalous, 3 or 4 stamens. Anther colour cream.
Pistil and pistillode	Pistil bottle shaped, 4 mm x 2 mm in short style 7 mm x 2 mm in long style; style stout 1 mm in short styled, 3mm in long styled flowers. Stigma sinuately 2 – 4 lobed; hypogynous disc glands as many as stamens, hairy,	Pistil bottle shaped, 3 mm x 1 mm, style very short, stigma yellowish white, bifid and long minutely papillate stigmatic hairs. Hypogynous glands 5, glands hairy at the tip; pistillode bottle shaped, 3 mm long, stigmatic hairs short.	Pistil bottle – shaped, up to 3 mm; stigma sessile, 2 seriate, cream coloured, radiating, minutely papillose long hairs; hypogynous glands 5 without hairs; pistillode oblong, 3mm long; hypogynous glands 5 without hairs.	Pistil bottle – shaped, 3 x 2 mm, 5 hypogynous glands at the base, glands hairy on the upper side; style short; pistil 3mm x 1 mm in female flower.	Pistil bottle – shaped, 2mm x 1mm; hypogynous gland 3 or 4 without hairs; style short; stigma bifid.	Pistil bottle shaped; 1.2 mm x 1 mm ; style 0.4 – 0.6 mm; stigma bifid, hypogynous glands with hairs; hypogynous glands 4, hairy at the tip.	Pistil bottle shaped, 1.1 mm long; style 0.3 mm; stigma bifid; hypogynous glands absent.
Fruit	Capsule - ellipsoid, torulose when ripe.	Capsules - sub globose, 7 mm x 5 mm long.	Capsule - obovoid, *ca.* 9 mm long.	Capsule -oblong, up to 5 mm long. Pedicel 3.1 cm long.	Capsule - ellipsoid, up to 4mm x 2mm.	Capsule - oblong – obovoid, 4 x 2 mm, pedicel 2 cm long	Capsule - oblong, 3mm x 2 mm; pedicel 6mm long

Seed	Seeds 14- 28 per capsule, discoid, brown, 1- 1.5 mm across, smooth.	Seeds 2-4, light brown or creamy, obovate or ellipsoid, 3.5mm x 4.5 mm length, seed surface tuberculated.	Seeds 2 – 9, obovoid, rounded or obtuse at distal end, subacute at proximal end cream or brown in colour, surface tuberculated, 3.5 mm long and 2.5 mm wide.	Seeds 4 – 6, brown, discoid, up to 2mm across; surface tuberculated.	Seeds 8 – 15, light brown, 1mm across, discoid, surface tuberculated.	Seeds 5 – 13, discoid, brownish black, 1 mm across, tuberculate.	Seeds 4 – 10, discoid, brownish black, discoid, 0.8 mm across, smooth.
Pollen	Monad, triangular, heteropolar, radially symmetric, parasyncolpate and prolate speroidal, Pollen wall is spiny in nature	Monad, triangular, radially symmetric, tricolpate and prolate – speroidal to sub – prolate in shape. Pollen surface is spinulose.	Monad, triangular, heteropolar, radially symmetric, syncolpate and prolate – speroidal in shape	Monad, triangular, radially symmetric, tricolpate and prolate – spheroidal in shape	Monad, triangular, heteropolar, radially symmetric, parasyncolpate and oblate – speroidal in shape Pollen surface spinulose.	Monad, triangular, heteropolar, radially symmetric, syncolpate and prolate – speroidal in shape. The pollen surface spinulose.	Monad, triangular, heteropolar, radially symmetric, oblate – spheroidal. The pollen surface was spinulose.

Table.3. Comparative morphology of *Nymphoides* spp. in Kerala

5.3. PHENETICS

Phenetics (also known as taximetrics) is a method to classify organisms based on the overall morphological similarity notwithstanding the evolutionary relationships / characters. In the present study the results of multiple range tests of characters of the OTUs are given in the Table 5. For cluster analysis, all the twenty two characters were tabulated against the seven OTUs using the numerical codes given for character states and abbreviated codes of characters. This tabulated data (Table 6) were used to generate a Dendrogram using the statistical package STATISTICA version 7.0, loaded in a personal computer, adopting Unpaired Group Method with Arithmetic mean as algorithm (Sokal& Michener, 1958) as the statistical test.

5.3.1. Phylogenetic studies

The morphological and phylogenetic characters of the specimens were compared on the basis of visible characters. The phylogeny of seven species of *Nymphoides* in Kerala was done.

4.4.1. Cluster analysis

Seven *Nymphoides*species are considered in this study with their code (Table.4). The data for the analysis were collected from fresh material collected from different localities and also from herbarium specimens deposited in the Malabar Botanical Garden Herbarium (MBGH). In the present study, twenty two multi-state qualitative morphological characters of the *Nymphoides* spp. were considered. The characters (with abbreviated codes) used in the cluster analysis with their character states are given in Table 5.The characters with two states were coded as 1 and 2 and also given continuous numbers when the character states are with more than two character sates.

Code No.	Name of the species (OTU's)
1	*Nymphoides balakrishnanii* Biju, Josekutty, Augustine and Haneef
2	*Nymphoides hydrophylla* (Lour.) Kuntze
3	*Nymphoides indica* (L.) Kuntze
4	*Nymphoides krishnakesara*K.T.Joseph and Sivar.
5	*Nymphoides macrosperma*R.V.Nair
6	*Nymphoides palyi* Biju, Josekutty, Augustine and Haneef
7	*Nymphoides parvifolia* Kuntze

Table.4: *Nymphoides* spp. (OTU's) selected for the study

Sl. No.	Code	Characters	Characters States and their code numbers
1	Hab	Habitat	Clay soil (1) or lateritic soil (2), clay soil and lateritic soil (3)
2	Plt	Plant	Bisexual (1), Unisexual (2), Bisexual and female(3)
3	Rhi	Rhizome	Stoloniferous (1) or non stoloniferous (2)
4	Sht	Shoot	Dimorphic (1) or monomorphic (2)
5	Lvs	Leaves	Dimorphic (1) or monomorphic (2)
6	Flr	Flower	Bisexual (1), unisexual (2), bisexual or female(3)
7	ClxL	Calyx lobe	Oblong-acute (1), oblong–obtuse (2), linear–lanceolate (3), elliptic-lanceolate (4)
8	Co	Corolla	White with yellow throat (1) or white (2)
9	PetL	Petal lobe	Fimbriately toothed (1), shallowly fimbriate or undulate (2)
10	PetLS	Shape of petal lobe	Obtuse or retuse (1), ovate to lanceolate (2), oblong-obtuse (3), oblong or elliptic (4)
11	HrPL	Presence of hairs on petal lobe	Present (1) or absent (2)
12	Stm	Stamen	Dimorphic (1) or monomorphic (2)
13	ClrA	Colour of Anther	Yellow (1), pale purple (2), blue (3), black with yellow (4), Cream (5).
14	NoSt	No. of stamens	Three or four (1), four (2), five (3), four – eight (4)
15	ISG	Inter staminal gland	Present (1) or absent (2)
16	ArC	Stigmatic hair	Present (1) or absent (2)
17	Stl	Style	Heterostylous(1)or homostylous (2)
18	HgG	Hypogynous gland	Hairy (1), hairless (2)
19	Fr	Fruit	Ellipsoid (1), sub globose (2), obovoid (3), oblong (4), or oblong to obovoid (5)
20	Se	Seed	Tuberculate (1) or smooth (2)
21	SeS	Shape of seed	Discoid (1), obovate or elliptical (2), Obovoid (3)
22	PoS	Pollen shape	Prolate-spheroidal (1), prolate-spheroidal to subprolate (2) or oblate-spheroidal (3)

Table.5: Characters used in the cluster analysis

For cluster analysis, all the twenty two characters were tabulated against the seven OTU's using the numerical codes given for character states and abbreviated codes of characters. The character codes and OTU's codes were prepared based on the morphological data of seven *Nymphoides* species. This data were used to generate Dendrogram using the statistical package STATISTICA version 7.0 loaded in a personal computer, adopting Unpaired Group Method with Arithmetic mean as

algorithm (Sokal& Michener, 1958) and percent disagreement (Hill & Lewicki, 2006) as the statistical test.

Code for Characters	Characters	Grouping of Taxa (represented by code numbers) according to their character states				
		1	2	3	4	5
Hab	Habitat	Clay soil 2, 4	Laterite soil 3, 5, 6, 7	Caly soil and laterite soil 1		
Plt	Plant	Monoecious 1, 5, 6, 7	Dioecious 2, 3	Monoecious or gynodioecious 4		
Rhi	Rhizome	Stoloniferous 1,2,4	Nonstoloniferous 3,5,6,7			
Sht	Shoot	Dimorphic 1, 2, 4	Monomorphic 3, 5, 6, 7			
Lvs	Leaves	Dimorphic 3, 5, 6, 7	Monomorphic 1, 2, 4			
Flr	Flower	Bisexual 1, 5, 6, 7	Unisexual 2, 3	Bisexual and unisexual 4		
ClxL	Calyx lobe	oblong–acute 1, 5	Oblong-obtuse 2, 3	Linear-lanceolate 4, 6	Elliptic lanceola e 7	
Co	Corolla	White with yellow throat 1, 4, 5, 6, 7	white 2, 3			
PetL	Petal lobe	Fimbriately toothed 1, 2, 3, 5, 6, 7	Undulate 4			
PetLS	Shape of petal lobe	Obtuse or retuse 4	ovate to lanceolate 1,6, 7	Oblong or obtuse 3	oblong or elliptic 2, 5	
HrPL	Presence of hairs on petal lobe	Present 1, 2, 5, 6, 7	Absent 3, 4			
Stm	Stamen	Dimorphic 1	Monomorphic 2, 3, 4, 5, 6, 7			
ClrA	Colour of Anther	Yellow 4, 5	Pale purple 1, 2	Blue 3	Black with yellow 6	Cream 7
NoSt	No. of stamens	Three or four 5, 7	Four 6	Five 2, 3, 4	Four to eight 1	
ISG	Inter staminal gland	Present 1, 2, 3, 4, 5, 6	Absent 7			

ArC	Stigmatic hair	Prsent 2, 3	Absent 1, 4, 5, 6, 7			
Stl	Style	Heterostylous 1	Homostylous 2, 3, 4, 5, 6, 7			
HgG	Hypogynous gland	Hairy 1, 2, 4, 6	Hairless 3, 5	Absent 7		
Fr	Fruit	Ellipsoid 1, 5	Sub globose 2	Obovoid 3	Oblong 4, 7	Oblong to obovoid 6
SeO	Seed ornamentation	Tuberculate 2, 3, 4, 5, 6	Smooth 1, 7			
SeS	Seed shape	Discoid 1, 4, 5, 6, 7	Obovate or ellipsoid 2	Obovoid 3		
PoS	Pollen shape	Prolate-spheroidal 1, 3, 4, 6	Prolate-spheroidal to subprolate 2	Oblate-spheroidal 5, 7		

Table 6: Results of multiple range test of characters

Character codes	**Codes for OTUs**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Hab	3	1	2	1	2	2	2
Plt	1	2	2	3	1	1	1
Rhi	1	1	2	1	2	2	2
Sht	1	1	2	1	2	2	2
Lvs	2	2	1	2	1	1	1
Flr	1	2	2	4	1	1	1
ClxL	1	2	2	3	1	3	4
Co	1	2	2	1	1	1	1
PetL	1	1	1	2	1	1	1
PetLS	2	4	3	1	4	2	2
HrPL	1	1	2	2	1	1	1
Stm	1	2	2	2	2	2	2
ClrA	2	2	3	1	1	4	5
NoSt	4	3	3	3	1	2	1
ISG	1	1	1	1	1	1	2
ArC	2	1	1	2	2	2	2
Stl	1	2	2	2	2	2	2
HgG	1	1	2	1	2	1	3
Fr	1	2	3	4	1	5	4
Se	2	1	1	1	1	1	2
SeS	1	2	3	1	1	1	1
PoS	1	2	1	1	3	1	3

Table 7: Character states of the OTU's

OTU	OTU's arranged as per their numerical codes						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	0.00	0.64	0.86	0.59	0.55	0.50	0.59
2	0.64	0.00	0.50	0.55	0.68	0.68	0.82
3	0.86	0.50	0.00	0.68	0.55	0.55	0.68
4	0.59	0.55	0.68	0.00	0.64	0.55	0.73
5	0.55	0.68	0.55	0.64	0.00	0.32	0.32
6	0.50	0.68	0.55	0.55	0.32	0.00	0.36
7	0.59	0.82	0.68	0.73	0.32	0.36	0.00

Table 8: Percent disagreement between OTU's under study

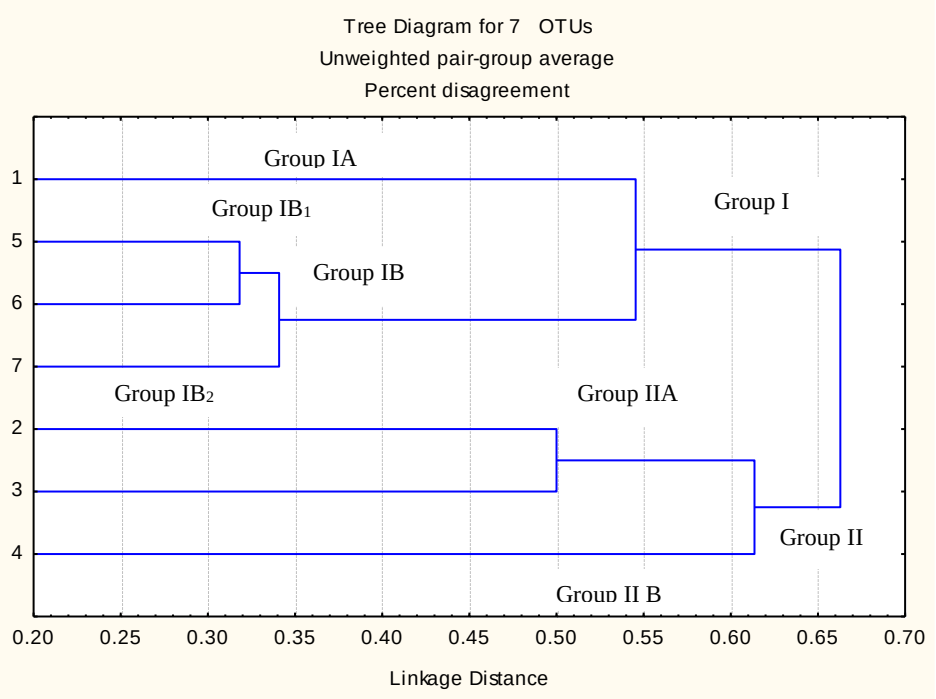

Fig. 18: Dendrogram of seven species of *Nymphoides* in Kerala

1.*N. indica*, 2. *N. macrosperma*, 3. *N. krishnakesara*, 4. *N. hydrophylla*, 5. *N. parvifolia*, 6. *N. balakrishnanii*, 7. *N. palyi*.

The Dendrogram of *Nymphoides* spp. in Kerala obtained through the cluster analysis. The results of dendrogram (fig.15) clearely revealed that the seven species of *Nymphoides* in Kerala come into two distinct groups.

Group 1: 1, 5, 6 & 7

Group II: 2, 3 &4

Group I and Group II were clustered together and show 66% dissimilarities. Group I is classified into Group IA and Group IB. In Group I, *N. indica*is clustered distantly from other species in this group and shows 55% dissimilarities from other species *viz., N. parvifolia, N. balakrishnanii* and *N. palyi.* Group IB is again classified into Group IB_1 and Group IB_2. *N. palyi i*s come under Group IB_2 and are separated from *N. balakrishnanii* and *N.* parvifolia (Group IB_1). *N. palyi* shows 34% dissimilarities with other two species. These two species come in Goup IB_1 are closely related, which shows highest affinities (68% similarities) between them.

Group II consists of two clusters Group IIA and Group IIB and represents three taxa *viz., N. hydrophylla, N. krishnakesara, N. macrosperma*. Group IIB is seperated from Group IIA which consists of two taxa *viz*., *N. krishnakesara* and *N. macrosperma.* Group IIB is 61% dissimilarities with Group IIA. In this study *N. krishnakesara* is clustered together with *N. macrosperma* instead of *N. hydrophylla. N. krishnakesara* shows more similarity (50%) with *N. macrosperma* than *N. hydrophylla.*

The phenetic analysis of seven species of *Nymphoides* spp. gives a picture on the affinities of *Nymphoides spp.* in Kerala. The results show that *N. parvifolia* and *N. balakrishnanii* are closely related species and shows highest affinities. *N. krishnakesara* and *N. macrosperma* in Group II also shows highest affinities among them. So the Kerala species of *Nymphoides* spp. shows two groups and these two groups show 66% dissimilarities.

5.4. REPRODUCTIVE BIOLOGY

5.4.1. *Nymphoides indica* (L.) Kuntze

Thefloral characteristics of *N. indica* are described in Table. 9.

Two types of flowers (1) with short style and (2) with long style in different plants of same population. Both the flowers are almost identical except for the length of the style.

Sl. No.	Floral Characters	Observations	
		Short Style Flower	**Long style Flower**
1	Blooming time	Throughout the year	Throughout the year
2	Flower type	Actinomorphic, complete, hypogynous and short style	Actinomorphic, complete, hypogynous and long style
3	Flower colour	White with yellow throat	White with yellow throat
4	Odour	Absent	Absent
5	Honey	Present	Present
6	Anthesis time	06.00 am – 08.00 am	06.00 am – 08.00 am
7	Anther dehiscence time	05.30 am – 6.00 am	05.30 am - 06.00 am
8	Anther dehiscence mode	Through longitudinal slit	Through longitudinal slit
9	No. of anthers per flower	4 – 8	4 – 8
10	Pollen type	Monad, heteropolar, radially symmetric trigonal and tricolpate	Monad, heteropolar, radially symmetric trigonal and tricolpate
11	Pollen size	41.72 ± 1.82 µm	36.58 ± 1.6 µm
12	Pollen shape	Prolate - Spheroidal	Prolate - Spheroidal
13	Stigma	Wet	Wet
14	Length of style	1.86±0.09	0.7 ± 0.08
14	Fruit	Capsule	Capsule
15	Flower- Fruit ratio	10 : 7	10 : 6
16	Ovule – Seed ratio	17 : 12	15 : 9
17	Flower closing time	12.30 pm – 1.30 pm	12.30 pm – 1.30 pm

Table.9: Floral characters of *N.indica*

5.4.2. Phenology

Leaf development and Flower – Bud development

The morphological changes of leaf and bud in their developmental stages are the following.

Leaf development(Figs. 19; Fig. 22)

Stage 1: Leaf initiation stage noted just visible to the naked eye.There is no distinction between the leaf, shoot and petiole. The mean length is 1mm.

Stage 2: The shoot and the leaves areclearly distinguished in this stage but the lamina is found to be in rolled conditionbelo the water level from initiation to

8th day. The colour of the lamina is brown and the shoot is white. The mean size of the lamina is 8mm x 2.2mm and the mean length of the shoots is 6.4mm.

Stage 3: Leaf petiole is clearly observed in this stage from 4th day to 8th day. Lamina isin the rolled condition under water. The mean length of the shoot is 392mm long and the lamina is 23mm x 3mm. Petiole length is 1 mm.

Stage 4: The leaf lamina is in the unrolled condition during this stage (Fig.). The lamina opened under water from 8th day to 12th day. The mean lengthof the shoot is 752mm.The mean length of the lamina is 56.8 x 47mm and the petiole length is 5.6mm.

Stage 5: Leaf lamina reachedabove the water surface from 12th - 14th / 15th day. The elongation of the shootdepends on the depth of water. It is glossy green in colour with pinkish colour. The meanlength of the shoot is 762mm and the lamina is 97 mm x 118 mm. The petiole length is 8mm.

Stage 6: The leaf lamina getsmatured; its glazing is disappeared and turned into dark green during 15th to 18th day. The mean length of the lamina is 280mm x 244 mm and the shoot is 96.4 mm with thickness 8 mm. Themean length of the petiole is 21.2mm.

Stage 7: During this stage green colour of the lamina changes to yellow during 34th –36th days and starts decaying.

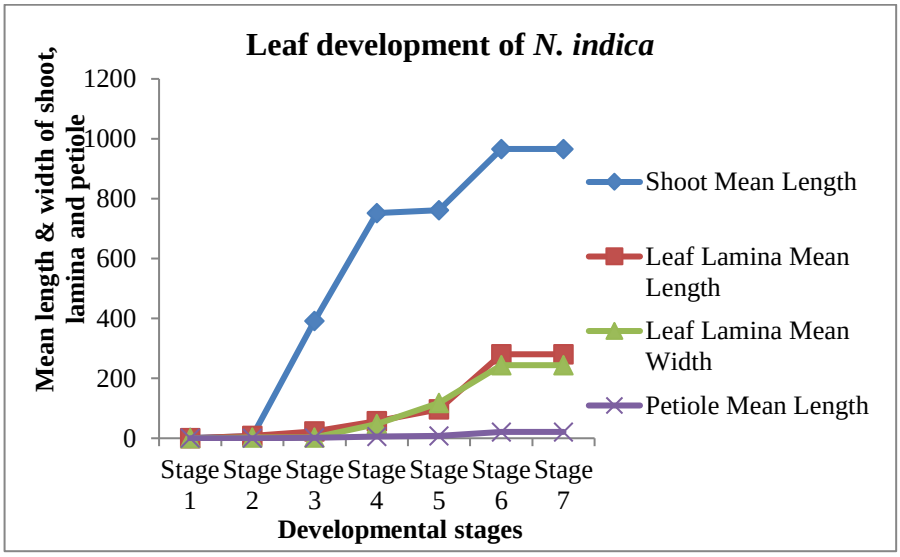

Fig. 19: Leaf Developmental stages of *N. indica*

Flower -Bud development(Fig. 20; Fig. 22)

The period of flower bud development in *N. indica*, from its initiation to the full bloom stage could be divided into seven different stages. They are:

Stage 1: In this stage flower buds are just visible with naked eye. Flower bud initiation started at the time of leaf initiation. It is cream coloured, found just below the leaf covered with brown bract (1st day). The size of the bud is 1mm.

Stage 2: From the day after initiation to 4th, day the flower bud is obovoid, cream and covered with bract. The bract is light brown. The mean size of the bud is 2mm x 1.2mm.

Stage 3: During 4th to 6th day, the bud emerges out from the bract and the sepal and pedicel observed during this stage.The mean size of the budis4.4mm x 3mm and the pedicel is 1.6mm.The bract islight green in colour.

Stage 4: Pedicel and sepals are clearly distinguished during 6th – 12th dayandthe plant also produced 5 buds in this stage. The mean size of the bud is 5.8mm x 4mm. The pedicellength is9mm.

Stage 5: Petals emerge out of the calyx. Pedicel grows very fast. Within 12 – 15 days of the pedicel and buds reach above the water surface. The mean size of the budis11mm x 5mm and the mean length of the pedicel is79.6mm.

Stage 6: This is the mature bud stage formed before2 hours of anthesis. The mean bud size is 20mm x 7.3mm (15th or 16th day). The mean length of the pedicel is124mm.

Stage 7: This is the full bloom stage in which the flower becomes open on the 15th or 16th day and exposing the various whorls and reproductive parts. The mean diameter of the flower is40mm. The mean length of the pedicel is 124mm.

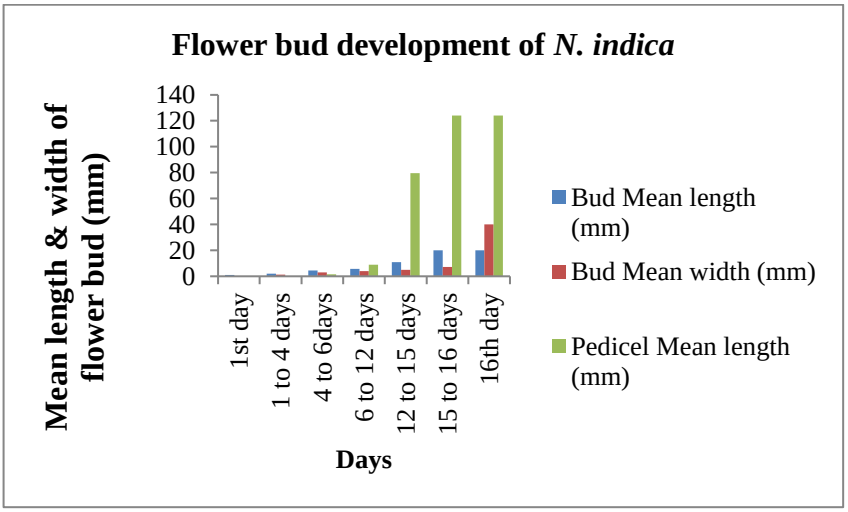

Fig.20: Flower Bud developmental stages of *N. indica*

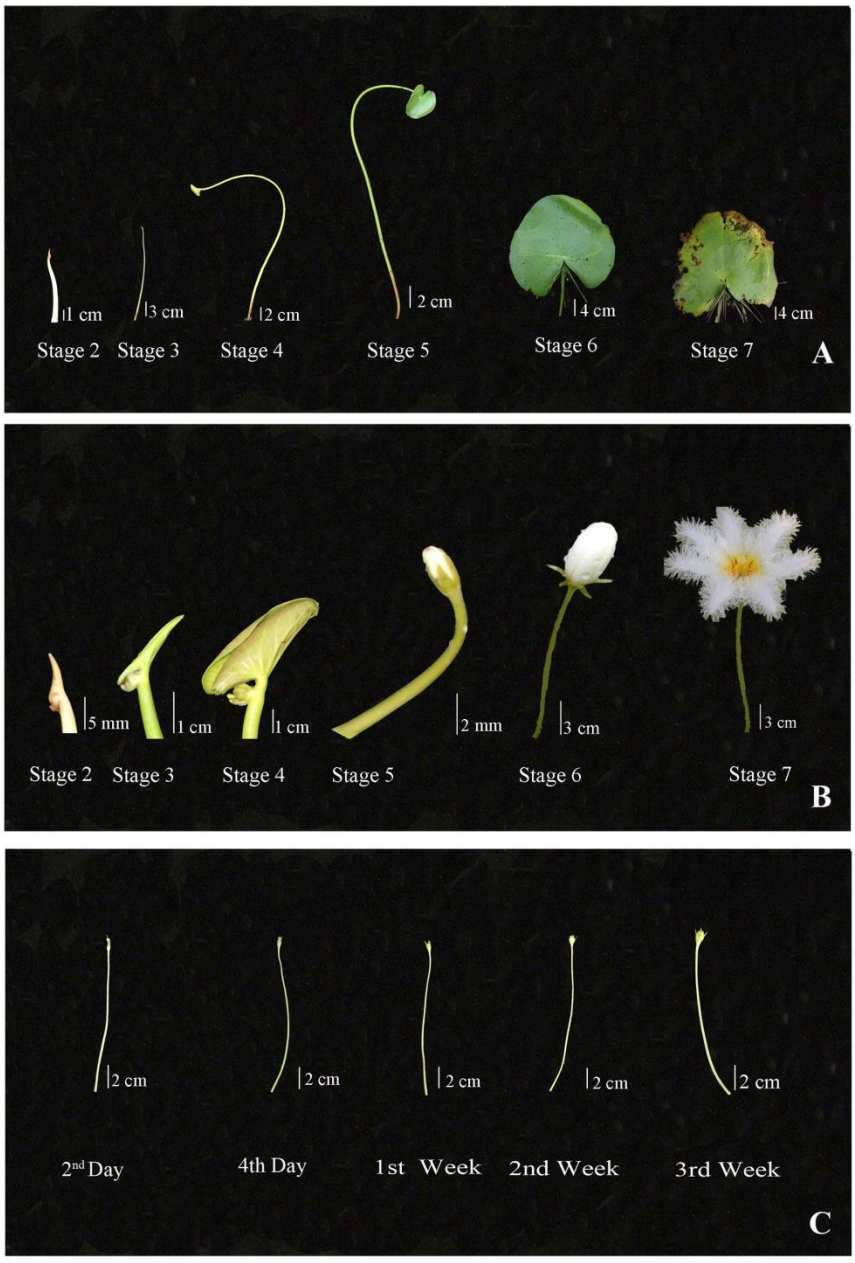

Fig. 22: Stages of development of leaf, Flower bud & Fruit of *N. indica*. **A**. Leaf developmental stages, **B.** Flower bud development, **C.** Fruit developmental stages.

Fruit development (Fig. 20; Fig.21)

Various stages of fruit development from pollination to the maturation and its dehiscence were recorded. The total time required for the starting of fruit development to the fruit dehiscence was noted as 26 – 30 days.

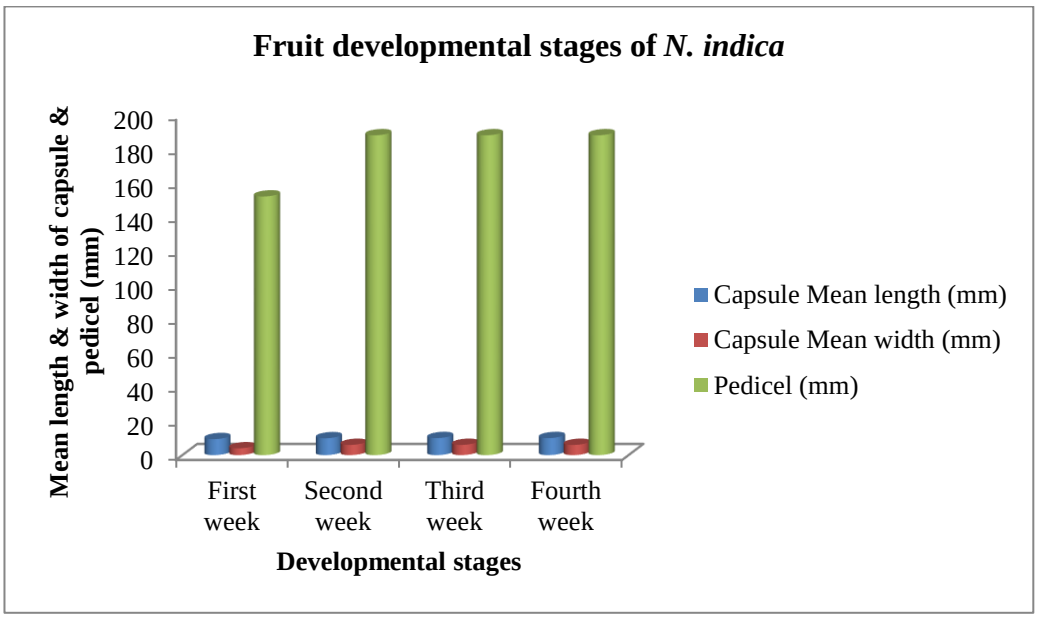

Fig. 21: Fruit developmental stages of *N. indica*

Flowering activity

The flowering time begins from the month of August. Fig. 23 showed that the peak time of flowering is November – January. New shoots are produced in the month of July from existing rhizome which later gets detached from the parent plant and grow as independent plants.

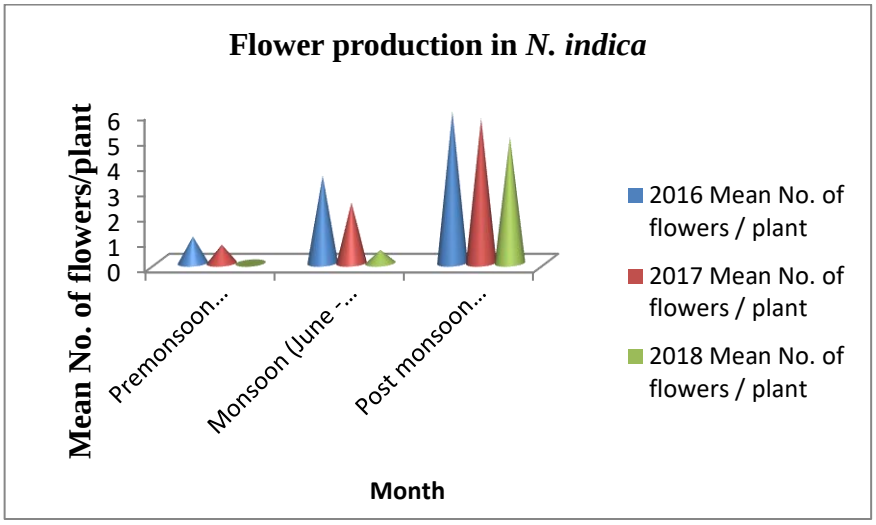

Fig. 23: Flowering seasonal variation in *N. indica*

Fig . 24: Anthesis and anther dehiscence in *N. indica*. **A - G.** Different stages of flower bud opening. **H.** Anther dehiscence.

Anthesis (Fig.24)

Anthesis of *N. indica* started during06.00 am – 08.00 am. The anther dehiscence starts before half an hour offlower opening. The anther dehisced through longitudinal slit.

Pollen morphology (Fig.25& 26)

The pollen grains are medium sized, monad, triangular - obtuse, heteropolar, radially symmetric, parasyncolpate and prolate – spheroidalin shape. The mean polar axis of pollen grains in short styled flowers is 41.72 ± 1.82 µm and the mean equatorial axis is 39.44 ± 2.4µm. P/ E ratio is 105.99 ± 4.89 µm (n =100). In long styled flowers the mean polar axis is 36.58 ± 1.6 µm and the mean equatorial axis is 34.40 ± 2.36µm. P/ E ratio is 106.80 ± 8.87 µm (n =100). Exine wall is spinulose in nature.

Pollen – Biochemical analysis (Fig. 25 & 26)

Pollen grains became brown due to the presence of newly formed starch when treated with I_2KI solution and become blue, when stained with Coomassie brilliant blue solution, which indicated the presence of protein on the stigmatic surface. The pollen grains become black when it stained with Sudan black due to the presence of lipids.

Pollen production (Fig.27)

N. indica produced 4 – 8 petals in flowers. The mean number of pollen per anther in short styled and long styled flower is recorded in Fig. 27. Pollen grains number increased gradually while increasing the number of stamens. The number of pollen in long styled flowers is always higher than in short styled flowers.

Stamen-pistil Length (Table 10)

Dimorphism of *Nymphoides indica* is observed in different plants of same population confirmed by calculating the stamen and pistil length of flowers. The mean length of the stamen of the short styled flower is 0.826± 0.03mm and in long styled flower is 0.686± 0.01mm. The pistil length of the two morphs is

different (Table 10). In short styled flower the mean length of the pistil is 0.516± 0.03mm and that of the long styled flower is 0.74± 0.01mm.

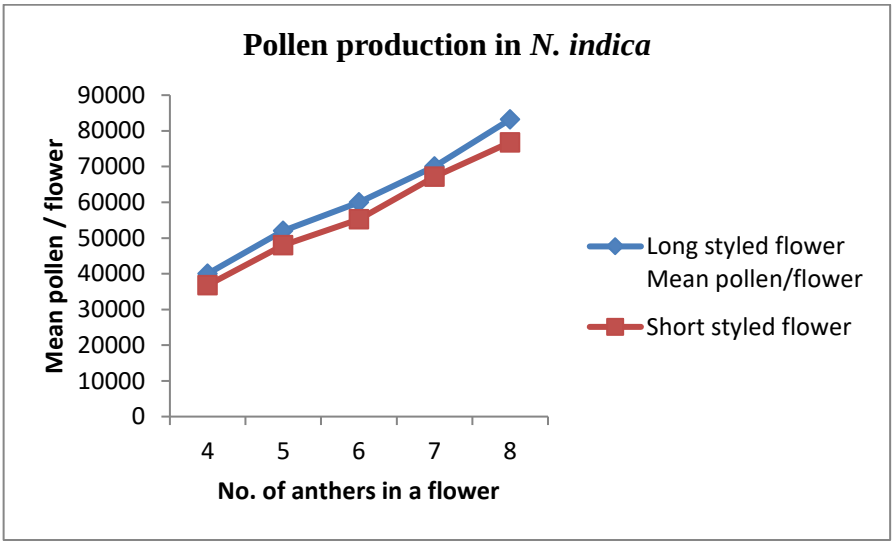

Fig.27: Pollen production in *N. indica*

Types of flower	Stamen length	Pistil length
Short style	0.826 ± 0.03	0.516 ± 0.03
Long style	0.686 ± 0.01	0.74 ± 0.01

Table.10: Stamen – pistil length in *N. indica*

Pollen – Ovule ratio

Table 11 below shows the pollen – ovule ratio of short styled and long styled flowers.

Types of flower	No. of anthers per flower	Mean No. of pollen per flower	Mean No. of ovules per flower	Pollen ovule ratio
Long styled	4	40000± 1414	29 ± 7	1379 : 1
	5	52000 ± 894	28 ± 9	1857 : 1
	6	60000 ± 1414	29 ± 9	2068 : 1
	7	70000 ± 1414	31 ± 9	2258 : 1
	8	83000 ± 1673	31 ± 8	2683 : 1
Short styled	4	36800 ± 1095	30 ± 8	1226 : 1
	5	48000 ± 894	29 ± 10	1655 : 1
	6	55200 ± 1095	30 ± 9	1840: 1
	7	67200 ± 894	31 ± 9	2167 : 1
	8	76800 ± 894	31 ± 8	2477 : 1

Table.11. Pollen – Ovule ratio

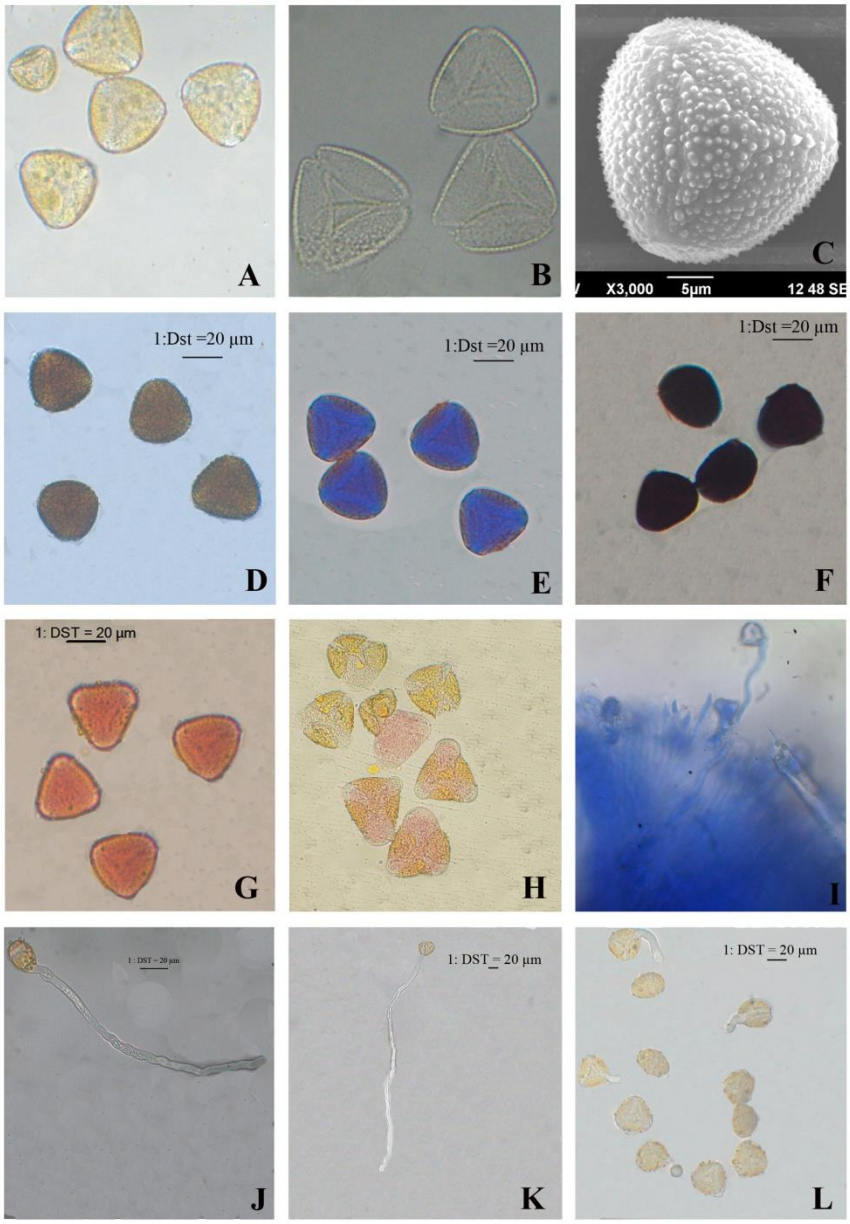

Fig. 25: Pollen biology of Short styled flowers of *N. indica*. **A.** Pollen normal, **B.** Pollen acetolysis, **C.** Pollen SEM photograph, **D - F.** Pollen test, **D.** starch test, **E.** Protein test, **F.** Lipid test, **G - H.** Pollen viability test, **G.** Acetocamine test, **H.** TTC Test, **I.** Pollen germination on stigma, **J.** Pollen germination in 8% sucrose solution, **K.** Pollen germination in 4% Brewbaker and Kwacks solution, **L.** Germination in 16% sucrose solution.

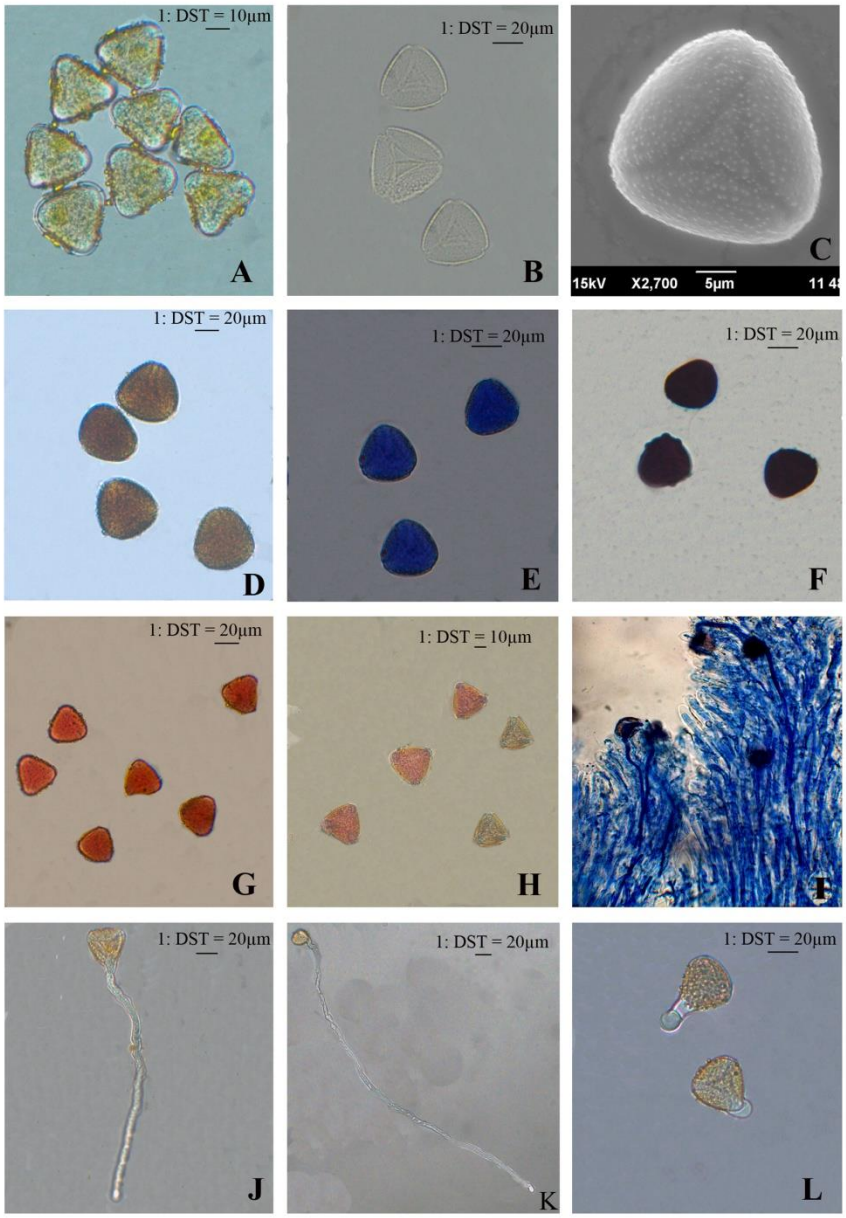

Fig. 26: Pollen biology of Long styled flowers of *N. indica*. **A.** Pollen normal, **B.** Pollen acetolysis, **C.** Pollen SEM photograph, **D - F**. Pollen test, **D.** starch test, **E.** Protein test, **F.** Lipid test, **G - H**. Pollen viability test, **G.** Acetocamine test, **H.** TTC Test, **I.** Pollen germination on stigma, **J.** Pollen germination in 8% sucrose solution, **K.** Pollen germination in 4% Brewbaker and Kwacks solution, **L.** Germination in 20% Brewbaker & Kwacks solution.

Pollen viability (Fig. 25, 26 &28)

The pollen viability was tested by 0.1% TTC at pH 7 and 1% acetocarmine. Fig.26below shows, the pollen grains have maximum viability at pre anthesis period (2 hours before anthesis). 83.64 % in TTC and 84.3 ± 1.7 % in acetocarmine (Fig). Pollen viability decreases after anthesis and lost its viability after 11 am.

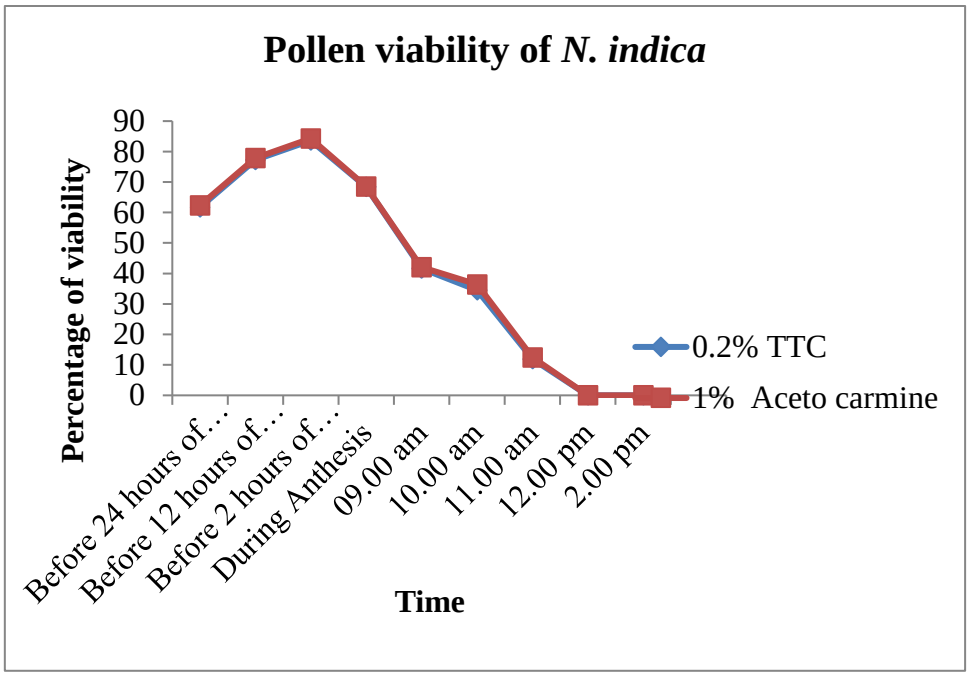

Fig. 28: Pollen viability of *N. indica*

Pollen germination method

***In vitro* method** (Fig. 25 & 26)

The germination of pollen grains is tested in Brewbaker and Kwack's medium and sucrose solutions. The pollen grains were maximum germinated in 8% solution of both the medium (83 %)with a mean pollen tube length521.89 ± 73.44µm(Brewbaker and Kwack's medium) and 476.8 ± 43.6 µm (sucrose solution) were observed after 3 hours.

Sl No.	Percentage of solutions	Brewbaker & Kwack's Solution		Sucrose solution	
		Percentage of germination	Mean pollen tube length (µm)	Percentage of germination	Mean pollen tube length (µm)
1	4	56	474.4 ± 42.3	80	372.97 ± 57.2
2	8	83	521.89 ± 73.44	83	476.8 ± 43.6
3	12	63	444.9 ± 88.82	65	141.4 ± 22.3
4	16	48	198.6 ± 50.49	13	20.37 ± 5.3
5	20	31	33.7 ± 9.74	0	0
6	25	0	0	0	0

Table 12: *In vitro* Pollen germination in *N. indica*

***In vivo* method** (Table 13, Fig. 25 & 26)

N. indica shows self incompatibility. Experiments for testing incompatibility were done by manually pollinating with pollen of same flower. It is found that these pollens do not germinate on the stigma indicating that these flowers show self incompatibility. Hence, pollen germination on stigma is studied by cross pollination method. *In vivo* pollen germination study shows that maximum pollen germination occurs in post anthesis period (Table 13). The percentage of pollen germination in post anthesis is 60.36 % with a mean of pollen tube length 205.6µm in short styled flowers and 70.72 % with a mean of 111.68µm long pollen tube in long styled flowers after 1 hour.

Types of flower	Flower opening time	Mean no. of total pollen on stigma	Mean no. of germinated pollen	% of mean no. of pollen germinated	Mean pollen tube length (µm)
Long Styled flower	Pre anthesis	17	0	0	0
	Anthesis	31	9	27.983	35.433
	Post anthesis	41	25	60.36	205.597
Short styled flower	Pre anthesis	33	0	0	0
	Anthesis	34	10	28.105	42.094
	Post anthesis	35	25	70.718	111.678

Table 13: *In vivo* pollen germination of *N. indica*

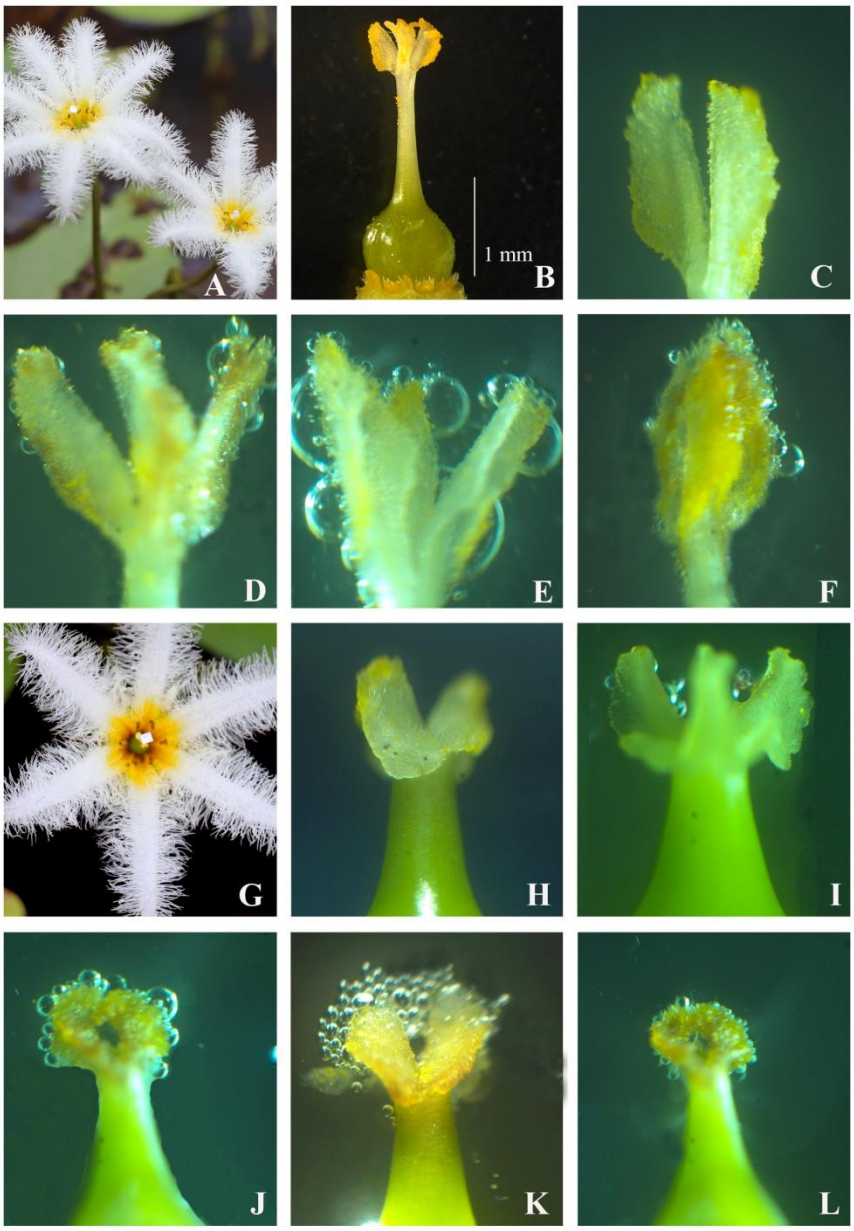

Fig. 29: Stigma receptivity of *N. indica*. **A - F.** Stigma receptivity of Longstyle flower. **A.** Observation method, **B - F.** H2O2 Method. **B.** Long styled pistil, **C.** During pre anthesis time, **D.** During anthesis time, **E.** At 1.00 pm, **F.** At 5.00 pm. **G - L.** Stigma receptivity of Short styled flower. **G.** Observation method, **H - L.** H2O2 Method. **H.** During Pre anthesis time, **I.** During athesis time, **J.** At 10.00 pm, **K**. At 1.00 pm, **L.** At 4 pm.

Stigma receptivity (Fig. 29)

Direct observation method: The stigma is transparent and wet, and also adheres to 1mm^2 piece of paper (considered as receptive).

Hydrogen peroxide test: The stigmatic surface produced oxygen bubbles at receptive time when it was treated with hydrogen peroxide. The stigma showed maximum bubble activity during the post anthesis period *ie.* 11.00 am – 2.00 pm both on long style and short style (Table 14). The peak time of stigma receptivity is at 1.00 pm. The receptivity decreases after 2.00 pm.

Sl. No.	Time	Short styled stigma			Long styled stigma		
		Mean no. of bubbles	H value	p value	Mean no. of bubbles	H value	p value
1.	08.00 am	2.4 ± 1.003	7.011	0.03003	2.733 ± 1.507	9.38	0.00919
2.	09.00 am	8.47 ± 1.07	7.4406	0.02423	8.533 ± 1.357	8.2994	0.01577
3.	10.00 am	29.9 ± 1.67	6.3529	0.04173	23.933 ± 1.72	6.4471	0.03981
4.	11.00 am	68.57 ± 1.14	8.3865	0.0151	76. 6 ± 1.19	8.4626	0.01453
5.	12.00 pm	71.67 ± 1.47	6.3181	0.04247	80.63 ± 1.52	6.5981	0.03692
6.	01.00 pm	85.53 ± 1.2	7.3826	0.02494	87.53 ± 1.72	10.4794	0.0053
7.	02.00 pm	81.7 ± 1.21	11.7335	0.00283	82.7 ± 2.09	10.6535	0.00486
8.	03.00 pm	50.2 ± 1.85	6.5981	0.03692	46.16 ± 1.84	6.469	0.03938
9.	04.00 pm	30.13 ± 1.11	9.4942	0.00868	27.47 ± 1.11	7.5335	0.2313
10.	05.00 pm	17.78 ± 5.73	10.8697	0.00436	17.27 ± 1.33	7.1813	0.02758
11.	06.00 pm	10.22 ± 3.53	7.34	0.2548	9.13 ± 1.81	6.1723	0.04568

Table 14: H_2O_2 Test of Short styled and long styled stigma of *N. indica*

Histochemical localization of esterases on stigmatic surface (Table 15; Fig. 30)

The stigma is dark brown - black at the time of more receptivity (11.00 am – 02. 00 pm) when it was treated with α-naphthyl acetate. The test proved that the stigma is

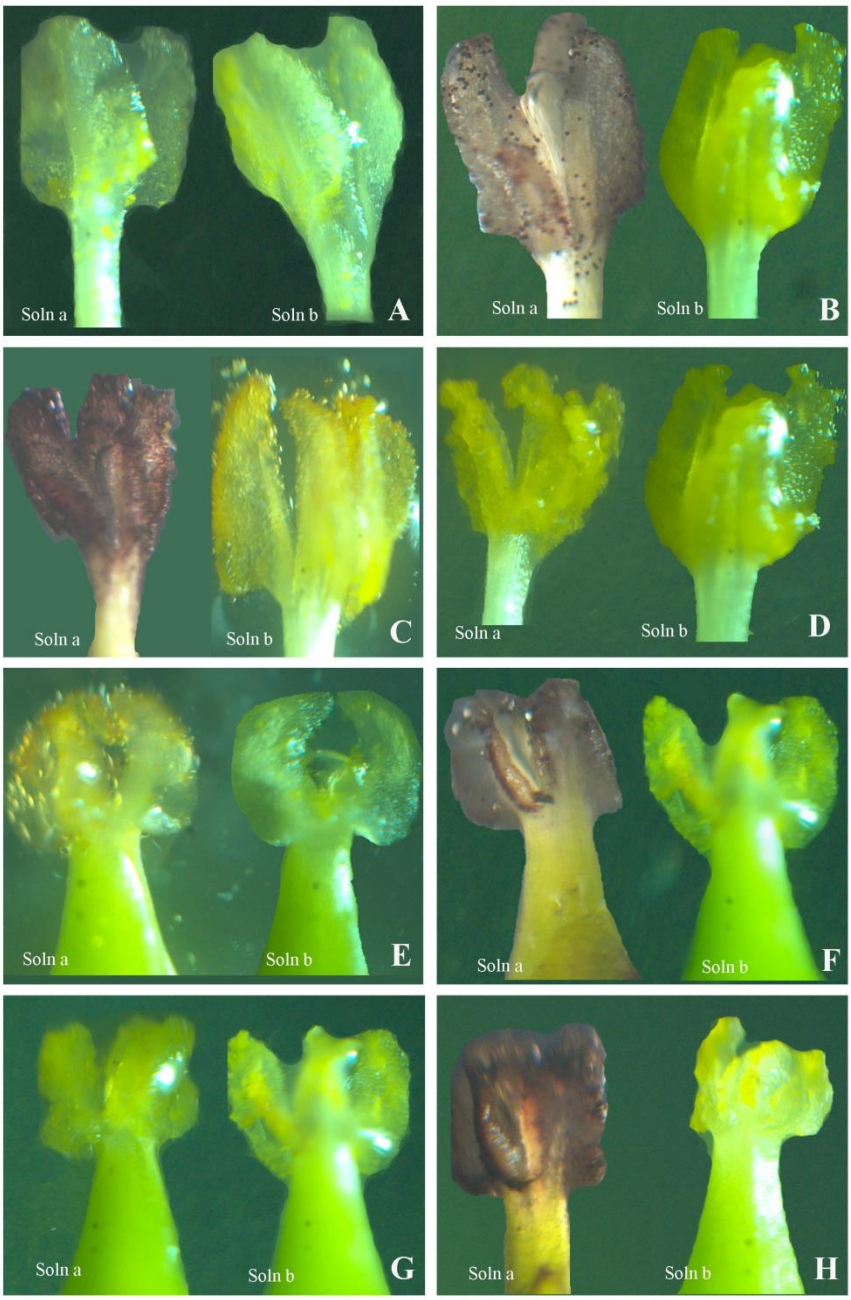

Fig. 30: Histotochemical localization of esterases on stigma of *N. indica.* **A - D.** α-Naphthyl test of Long styled stigma in solution a & Solution b, **A.** During Pre anthesis time, **B.** During anthesis time, **C.** During post anthesis time, **D**. At 6.00 pm, **E - H.** α-Naphthyl Acetate test of short styled stigma in Solution a & b, **E.** During pre anthesis time, **F.** During anthesis time, **G.** During Post anthesis time, **H.** At 6.00 pm.

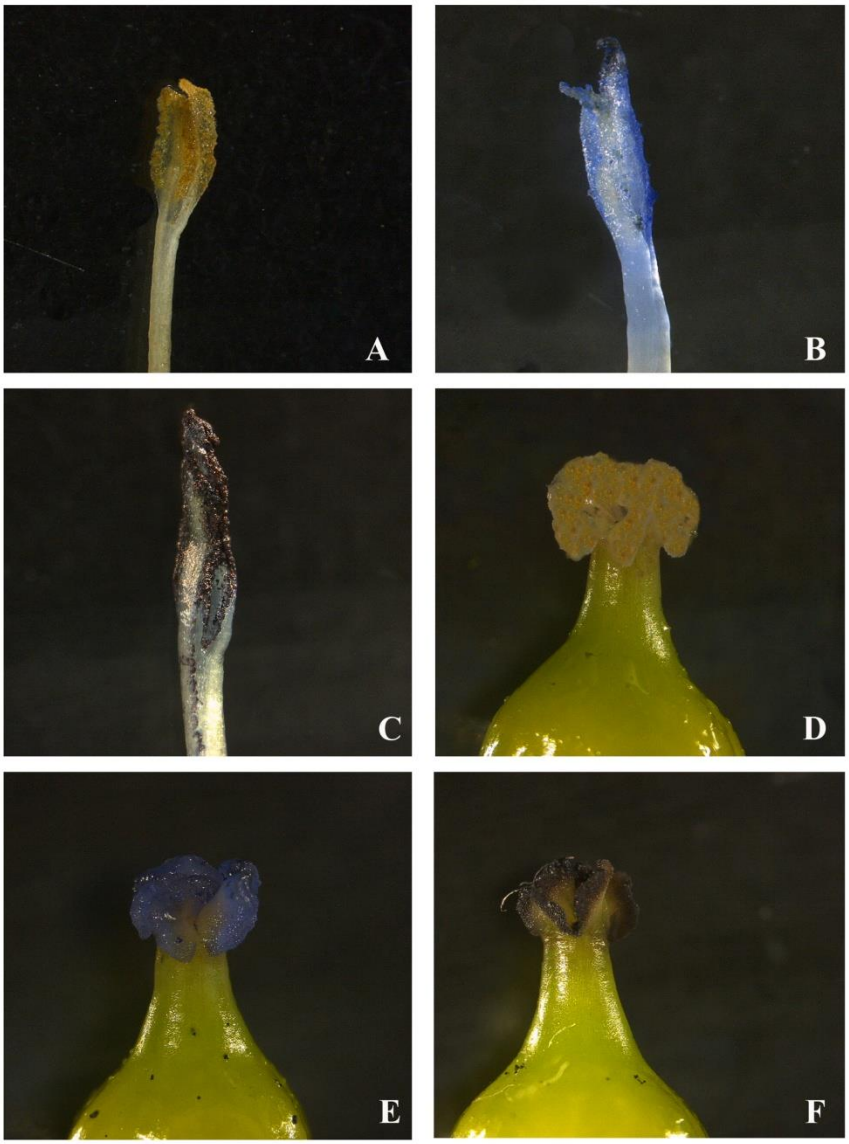

Fig. 31: Biochemical analysis of stigma of *N. indica*. **A - C.** Biochemical analysis of long styled stigma. **A.** Test for starch, **B.** Test for protein, **C.**Test for lipid; **D - F.** Biochemical analysis of short styled stigma. **D.** Test for starch, **E.** Test for protein, **F.** Test for lipid.

More receptive during 11.00 am – 02.00 pm. After that the receptivity gradually decreases.

Sl. No.	Time	Frequency of colour
1	06. 00 am - 08.00 am	Colourless
2	08.00 am - 09.00 am	Light brown
3	09. 00 am - 10.00 am	Brown
4	11.00 am - 12.00 pm	Black
5	01.00 pm - 02.00 pm	Black
6	02.00 pm - 03.00 pm	Brown
7	04.00 pm - 05.00 pm	Brown
8	06.00 pm – 07.00 pm	Colourless

Table.15. Histochemical localization of esterases on stigmatic surface

Stigma – Biochemical analysis (Fig. 31)

The stigma becomes blue black when it was stained with Sudan black due to the presence of lipids. The stigmatic colour changed into purple or brown colour when it stained with I_2KI indicated the presence of newly formed starch and when it become blue colour it stained with Coomassie brilliant blue indicated the presence of protein.

Pollination Biology:

Floral visitors and their behaviours (Table 16; Fig. 32 and 33)

Anthesis of *N. indica* is at 06.00 am – 08.00 am. Insect visits started during the time of anthesis just before flower opening. A little honey is present in the flower (less than 1 µl). During the study time several insect pollinators were observed foraging in *N. indica* flowers. The major visitors are *Apis mellifera, Apis florae* (Order hymenoptera), *Notiphila*spp. (Order Diptera), *Bagous* spp. (Family Curculionidae), *Lasioglossum curtis, Lasioglossum mutilum* (Family Halictidae), *Ceriagrion rubiae* and *Ceriagrion coromandelianum* (Family Coenagrionidae) (Table 16). Insects visit is not active at early morning. The peak time of visits are during 10.00 am – 12.00 pm. Some visitors enter into the bud through small

opening at the time of flower opening without damaging the flower (Eg. *Lasioglossum mutilum*). But some like *Bagous* spp. cause damage to the floral parts and feed the ovules and pollen. During visiting time, the body parts of the insects touch the stamen and the pollen grains adhere to the insect body. They deposit pollens on stigmatic surface of another flower, while foraging the next flower. The mouth of the *Lasioglossum mutilum* enters deep into the corolla tube and forage nectar from the flower. Durig the time of visit the body parts were touched to the dehisced anthers and the pollens get stuck on the insect's body. The flowers offered both the pollen and nectar to the visitors. During a single visit the *L. mutilum* and *Notiphila* spp. foraged on 4 – 6 flowers. The pollinators visited the flowers in the morning (7.00 am – 3.00 pm), while some pollinators were observed foraging in the evening time also.

Sl. No.	**Name of the visitors**	**Family**	**Foraging Nature**	**Foraging hours**	**Time spent in each flower**	**Stigma touch**	**Frequency of visit**
1	*Lasioglossum mutilum*	Halictidae	Nectar + pollen	7 am – 3 pm	4 – 8 minutes	+++	High
2	*Lasioglossum curtis*	Halictidae	Nectar + pollen	10 am – 2 pm	4 – 8 minutes	+++	Low
3	*Apis mellifera*	Apidae	Nectar + pollen	10 am – 12 pm	4 – 5 minutes	+++	Low
4	*Apis florae*	Apidae	Nectar + pollen	10 am – 12 pm	1 – 3 minutes	+++	Low
5	*Notiphila* spp.	Ephydridae	Nectar + pollen	6 am – 6 pm	5 – 15 minutes	+++	High
6	*Bagous* spp.	Curculionidae	Pollen	7 am – 6 pm	10 – 20 minutes	+	High
7	*Ceriagrion rubiae*	Coenagrionidae	No foraging Nature	10 am – 11 am	4 – 8 seconds	-	Low
8	*Ceriagrion coromandelianum*	Coenagrionidae	No foraging Nature	10 am – 11 am	4 – 8 seconds	-	Low

Table 16: *N. indica* floral visitors and their behaviours

Stigma touch +++: very good, ++: good, +: poor, -: no touch. Frequency of visit: high (10 – 40 visit/day), intermediate (4 – 9 visit/day), low (<3 visit/day).

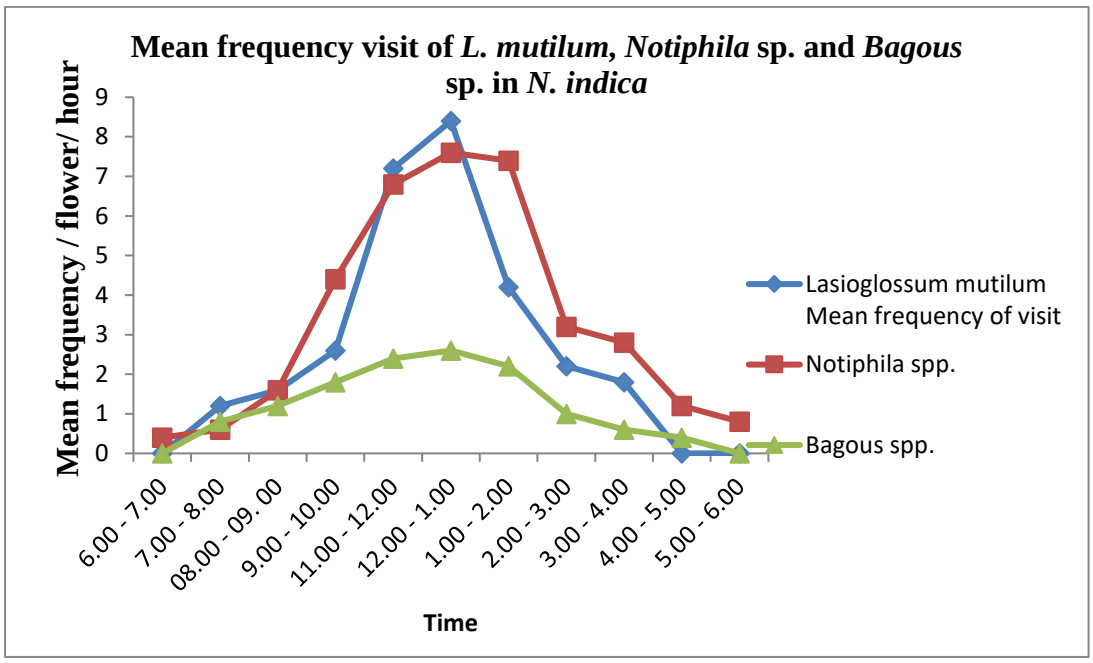

Fig. 33: Frequency of visit of *L. mutilum, Notiphila* spp. and *Bagous* spp. in *N. indica*

Pollination efficiency: Pollination efficiency is calculated using the method of Cruden *et al.* (1990). Pollination efficiency of short styled flower is 0.024 and that of the long styled flower is 0.023(Table 17).

Sl. No.	Type of Flowers	Mean No. of pollen on Stigma	Mean No. of pollen per flower	Pollination efficiency
1	Long styled	1336 ± 205.9	58400 ± 17276	0.023
2	Short styled	1408 ± 211.9	56760 ± 16212	0.024

Table.17. Pollination efficiency of *N. indica*

Breeding System (Fig.34)

N. indica shows self incompatibility. Different pollination techniques conducted and the result shown in Table 14. This experiment was done during the month of 2018.Self pollination test and cross pollination test also confirmed the self incompatibility of *N. indica.* The breeding system showed that *N. indica* could not produce fruits by selfing showing self incompatibility. The fruits produced by cross pollination only.

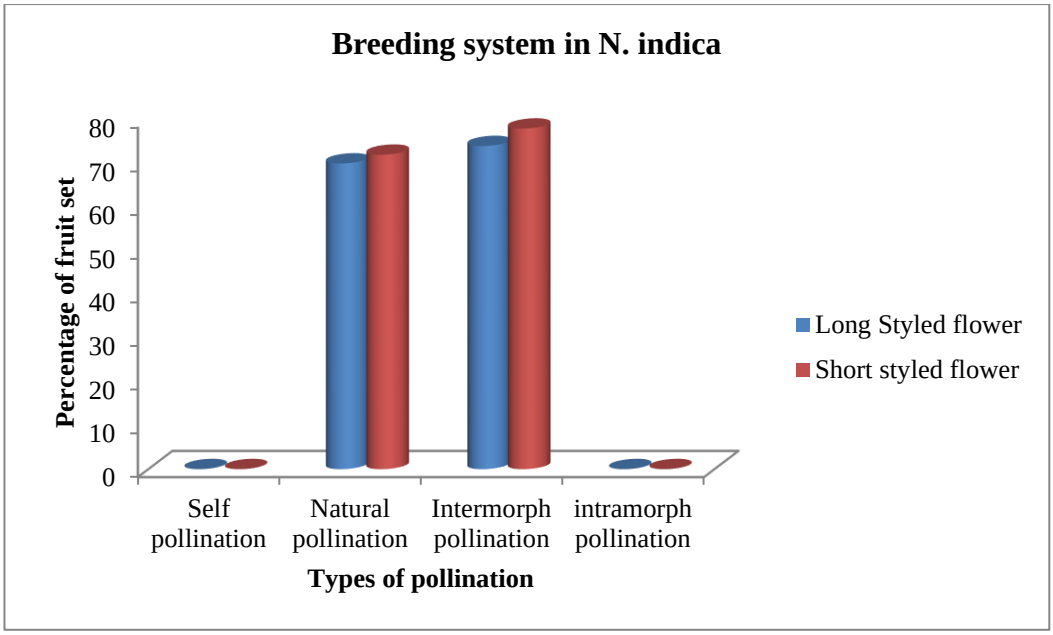

Fig. 34: Breeding system of *N. indica*

N. indica has two types of flowers, long styled flowers and short styled flowers in different plants in the same population. Fruits are produced only by intermorph cross pollination. In natural condition 72%mean number of fruit set was observed in short styled flowers and 70% in long styled flowers. But through manual pollination (Intermorph pollination)produced 78% of fruit set in short styled flowers and 74% fruit set were observed in short styled flowers.

Fruit and seed

Capsule ellipsoid, green and turned light yellow when it matured. The mean length and width of a capsule is8 mm x 5 mm and the mean pedicel length 5mm. Capsule consists of 14 – 32 seeds. Seeds are discoid and brown. Seed surface is smooth.

Flower fruit ratio: The flower fruit ratio of short styled plant is 10 : 7and that of long styled plant is 10 : 6. Short styled plants produced more fruits than long styled plant (Table 18).

Ovule – Seed ratio: The ovule - seed ratio of short styled plant is 17 : 12 and that of long styled plant is 15 : 9. Short styled plants produced more fruits than long styled plant (Table 18).

Types of flower	Parameter	Ratio
Short Styled	Flower : Fruit	10 : 7
	Ovule : seed	17 : 12
Long styled	Flower : Fruit	10 : 6
	Ovule : seed	15 : 9

Table.18. Seed set ratio of *N. indica*

Fruit and seed dispersal: Fruits matured at 26 – 30 days after pollination and dehisced three or four days after maturity. The fruits are grown under the water below the floating leaf and the dehiscence occurred in the water. The distal point of the fruit brakes first and the seeds are liberated out to the water. The seeds are hydrophobic in nature. It floats on the water surface. Later seeds sink to the bottom of the water.

Seed germination (Table.19; Fig.35)

Seed germination was not observed in the field. In controlled conditions, seeds germinated in dung-soil mixture and Gibberelline supplemented soil. Maximum germination observed in dung-soil mixture (60%) after 140 – 160 days. Germination not observed in control. Scarified seed germinated after 20 – 40 days.

Sl. No.	Days	Percentage of germination (%)				Scarified seed
		Seed				
		Soil	Soil + 50 ppm gibberellin	Soil + 100 ppm gibberellin	Dung soil	Soil
1	5 – 10	0	0	0	0	0
2	10 – 20	0	0	0	0	0
3	20 – 40	0	0	0	0	8.3
4	40 – 80	0	0	0	0	0
5	80 – 120	0	26.68	0	0	0
6	120 – 140	0	35	30	53.33	0
7	140 – 160	0	0	38	60	0
8	160 – 180	0	0	0	0	0

Table.19. Seed germination rate of *N. indica* in different condition

Fig. 35: Reproduction in *N. indica*. **A - F.** Vegetative reproduction. **A & B.** Stolon from rhizome and produced new plantlets, **C.** New plants germinate from existing rhizome, **D.** Roots from the base of the petiole, **E & F.** rooted region detached from mother plants and produced new plants, **G & H.** Seed germination. **G.** Germination in 0.05% Gibberellic acid, **H.** Germination in dung soil.

Vegetative propagation (Fig. 35)

Vegetative propagation of *N. indica* is mainly from the existing rhizomes, stolons and detached leaves with part of shoot attached. Many lateral roots and leaves were produced from the junction of flower producing parts of the shoot. After decaying of leaves and shoots these rooted region float on the water and produce new rhizome and leaves, then sunk into the bottom and get attached to the soil or it grows and produces new saplings floating on the water surface and gets harboured in the shores where water level is low.

By stolons, shoots were produced from the existing rhizome. Seed germination in natural condition has not been observed in *N. indica.* The propagation of *N. indica* is mainly through vegetative propagation.

5.4.2. *Nymphoide skrishnakesara* K.T.Joseph and Sivar.

The floral characteristics of *N. krishnakesara* are described in Table. 20

Sl.	Floral Characters	Observations
1	Blooming time	August - February
2	Flower type	Actinomorphic, Dioecious, hypogynous
3	Flower colour	White
4	Odour	Absent
5	Honey	Present
6	Anthesis time	6.00 am – 8.00 am
7	Anther dehiscence time	6.05 am – 8.05 am
8	Anther dehiscence mode	Through longitudinal slit
9	Number of anthers/ flower	5
10	Mean No. of pollen / anther	8600 ± 966
11	Mean No. of pollen / flower	43000 ± 4830
12	Mean No. of Ovules/ flower	8 ± 2.8
13	Pollen – Ovule ratio	6142 : 1
14	Pollen type	Monad, heteropolar, triagonal, symmetric and tricolpate
15	Pollen size	33.59 ± 1.4 µm
16	Pollen shape	prolate – speroidal
17	Stigma type	Wet
18	Fruit type	Capsule
19	Flower – fruit ratio	16 : 9
20	Ovule – seed ratio	4 : 3
21	Flower closinging time	2.00 pm - 2.30 pm

Table 20: Floral characters of *Nymphoides krishnakesara*

Phenology

Leaf development and Flower – Bud development

The development of leaf initiation to yellowing, bud to flower and flower to fruit development is divided into different stages. The developmental stages are the following:

Leaf development (Fig. 36& 38)

Stage 1: Leaf initiation stage is noted just visible to the naked eye. There is no distinction between the leaf, shoot and petiole. The mean length is 1mm.

Stage 2: During this stage, the shoot and leaf is clearly distinguished but the lamina is found to be in rolled condition below the water level from initiation to 3rd day. The mean length of the shoot is 16mm. The length and the width of the lamina is 13mm x 1mm.

Stage 3: The petiole is observed during 4th day. Here also the lamina is in the rolled condition under water. The mean length of the shoot is 37.1mmand the lamina is 20.2mm x 3.3mm. The mean length of the petiole is 1 mm long.

Stage 4: The lamina is opened during this stage. The lamina will be open when it is emerged at the water surface (Fig) and it takes 9 – 12 days depends on the depth of the water. The upper surface of the leaf is glossy and the lamina length is 32.9mm x 31.5mm.

Stage 5: The leaf lamina gets matured; its glazing is disappeared and turned into dark green during 18 to 20 day. The mean length of the shoot is 151.8mm and the lamina is 47.9 mm x 46.8 mm. The petiole length is 2mm.

Stage 6: During this stage green colour of the lamina changed to yellow during 32nd to 38thdays.

Stage 7: On the 40th to 48thday intervals, the leaf lamina become grey coloured and gets started for decaying.

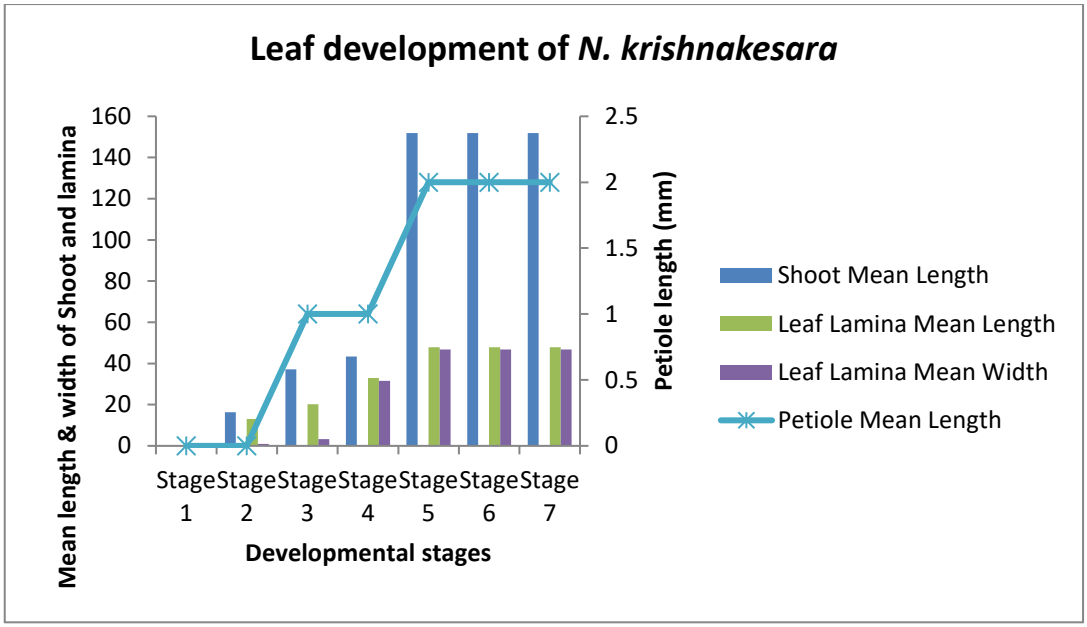

Fig. 36: Leaf developmental stages of *N. krishnakesara*

Flower -Bud development (Fig.37 & 38)

The period of flower bud development in *N. krishnakesara*, from its initiation to the full bloom stage was divided into different stages. They are:

Stage 1: In this stage flower bud is just visible to the naked eye. Flower bud initiation will be start at the time of leaf initiation. It is cream coloured, found just below the leaf on the petiole junction. There is no distinction between the bud and pedicel. The average size of the bud is 1mm (1st day).

Stage 2: From the day after initiation to 3rd day, the flower bud is slightly bigger, obovoid and covered with bract. The mean size of the bud is 1.9mm x 1mm.

Stage 3: During 4th – 6th day, the bud emerges out from the bract and its mean size is3mm x 1.9mm.

Stage 4: Pedicel and sepals are clearly distinguished during 6th – 7th day. The mean size of the bud is 3.7mm x 2.8mm and the length of the pedicel is 4.5mm.

Stage 5: During this stage the petals emerge out of the calyx. Within 7 – 12 days, the buds reach above the water surface. The mean size of the bud is 4.97mm x 3mm and the pedicel is 5mm.

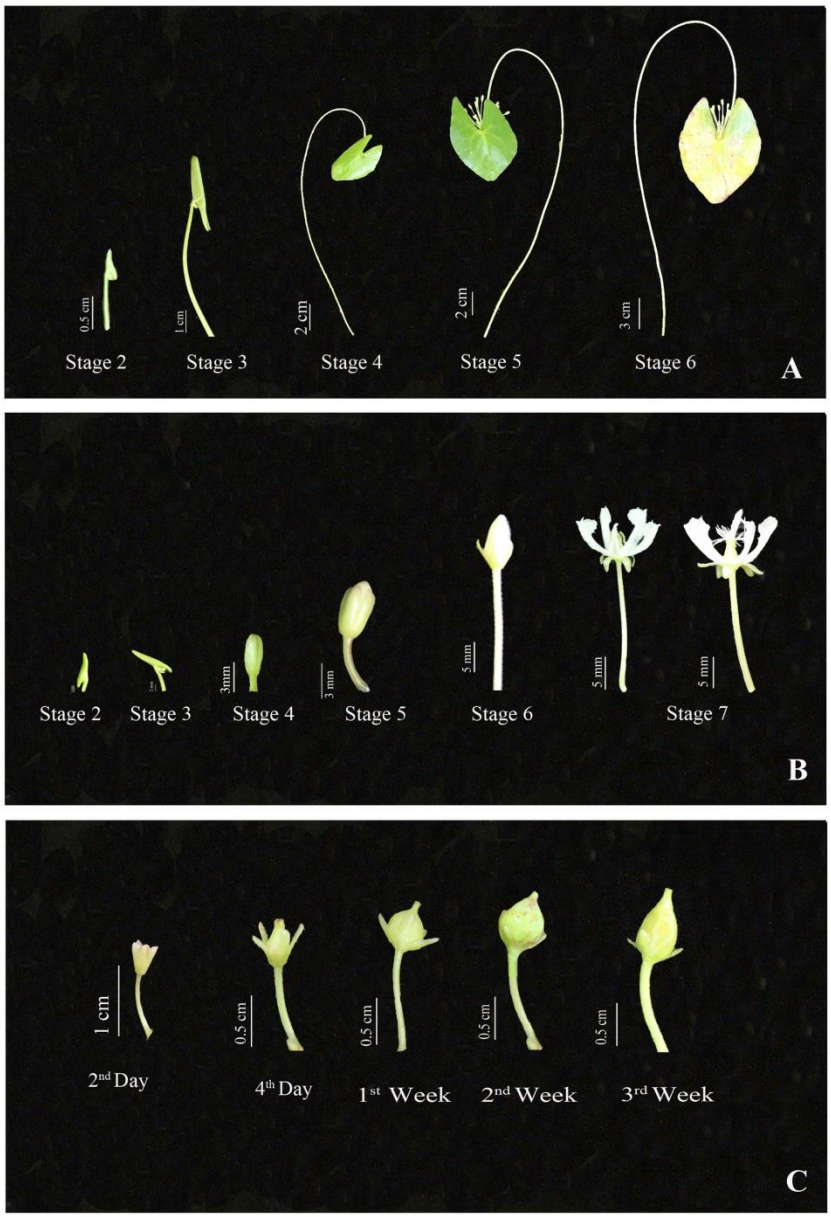

Fig. 38: Developmental stages of leaf, flower bud and fruit of *N. krishnakesara*. **A.** Stages of leaf development, **B.** Stages of flower bud development, **C.** Stages of fruit development.

Stage 6: This is the mature bud stage formed before 2 hours of anthesis. The mean bud size is 5.6mm x 3.4mm and the pedicel length is 15.3mm (13th or 14thdays).

Stage 7: This is the full bloom stage in which the flower becomes open on the 13th or 14th day and exposing the various whorls and reproductive parts. The flower size is10.44mm in diameter and the pedicel is 15.3mm long.

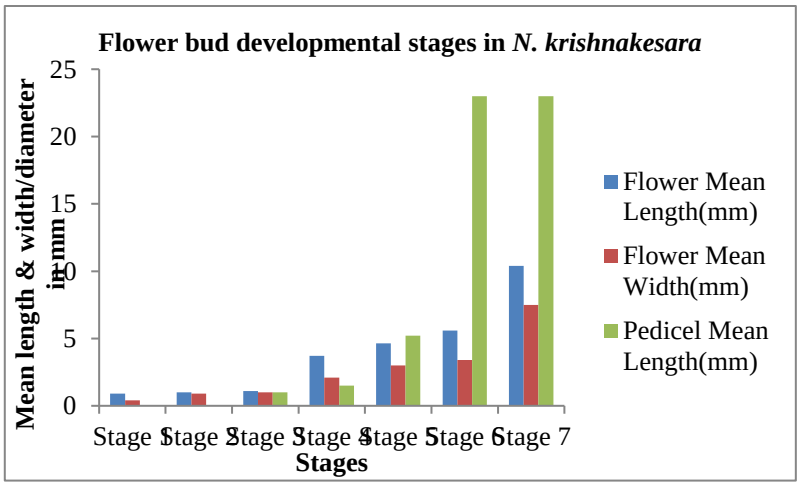

Fig.37. Flower bud developmental stages of *N. krishnakesara*

Fruit development (Fig. 38 & 39)

Various stages of fruit development from pollination to the maturation and its dehiscence were recorded (Fig. 39). The total time required during the first week the pistil is bulged and then slowly grows in to fruits. The total time required for the starting of fruit development to the fruit dehiscence was noted as 21 – 24 days.

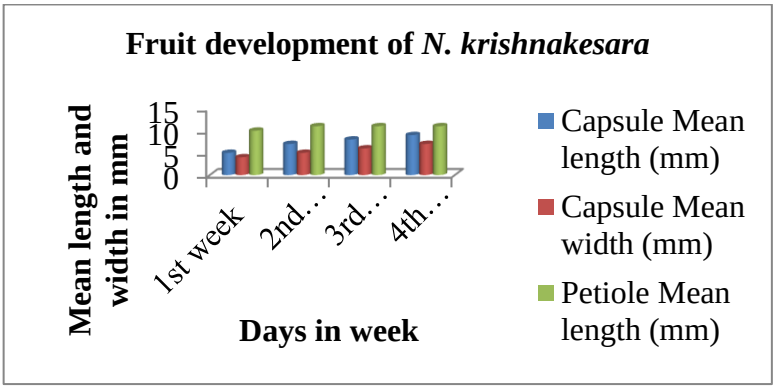

Fig.39. Fruit developmental stages of *N. krishnakesara*

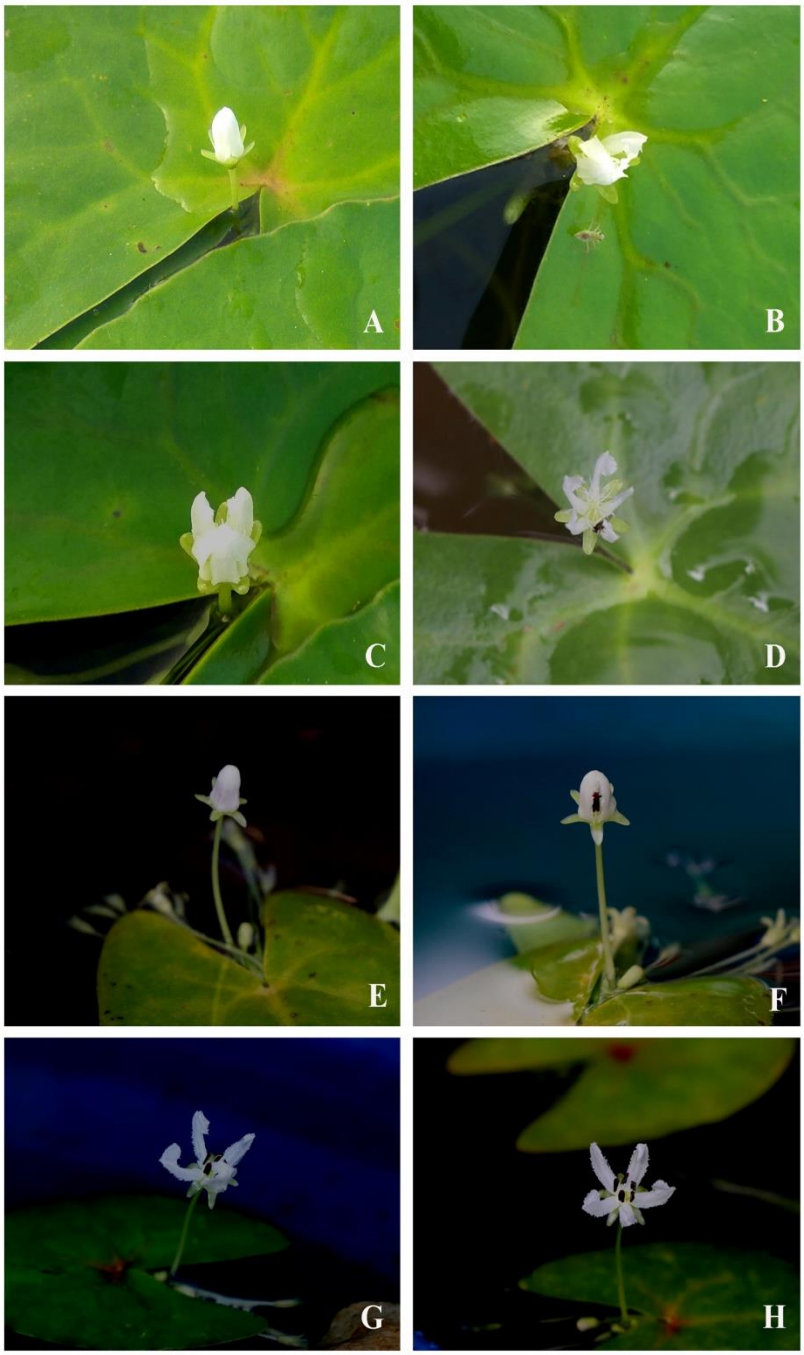

Fig. 40: Anthesis of *N. krishnakesara*. **A - D.** Anthesis of female flower, **E - H** Anthesis of male flower.

Flowering activity (Fig. 40)

N. krishnakesara is aseasonal perennial plant. The aerial shoots die off during the beginning of summer season and the rhizomes remain dormant under the soil till the end of summer and sprout soon after the next rainy season. The flowers are born on the junction between the shoot and the petiole. The flowering activity was studied in three different season viz., pre- monsoon (March - May), mosoon (June - November) and post-monsoon season (December - February). The flowering time start at the month of August. The peak time of flowering was observed during November - January. Pre monsoon season is the dormancy time of the rhizome *ie*, March – May is the dormant stage of rhizome (Table 21).

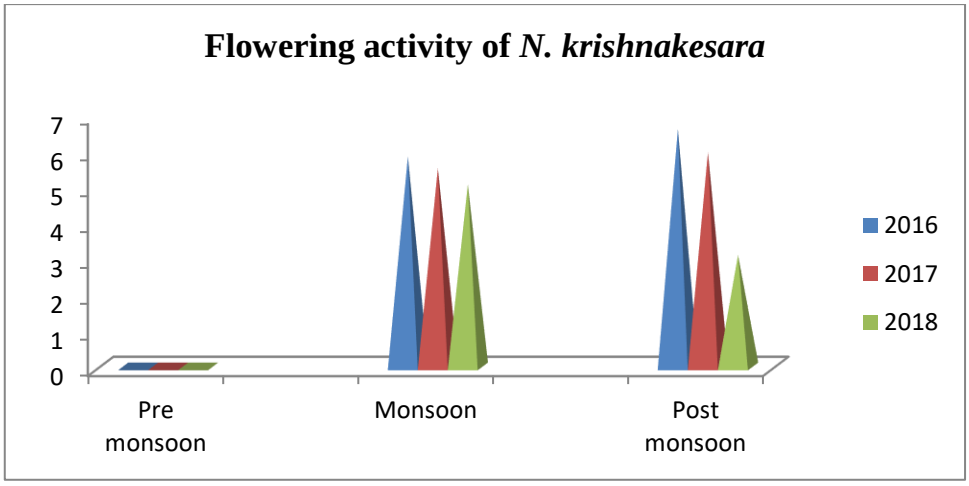

Figure 40: Flowering seasonal variation in *N. krishnakesara*

Anthesis (Fig. 41)

During the time of anthesis the flower opening started early in the morning 06.00 am – 08.00 am. The petals open one by one taking 5 – 10 minutes intervels and fully opened the flower. Anther dehiscence starts just after anthesis. The anthers dehisced through longitudinal slit and disburse the pollen grains.

Pollen morphology (Fig. 42)

The pollen grains are medium sized, monad, triangular, tricolpate, heteropolar, radially symmetric, trilete and prolate – spheroidal. Mean polar axis of pollen grains are 33.59 ± 1.4µm and the mean equatorial axis are30.38 ± 2.02µm. P/ E ratio is 110.99 ± 7.8µm(n =100).

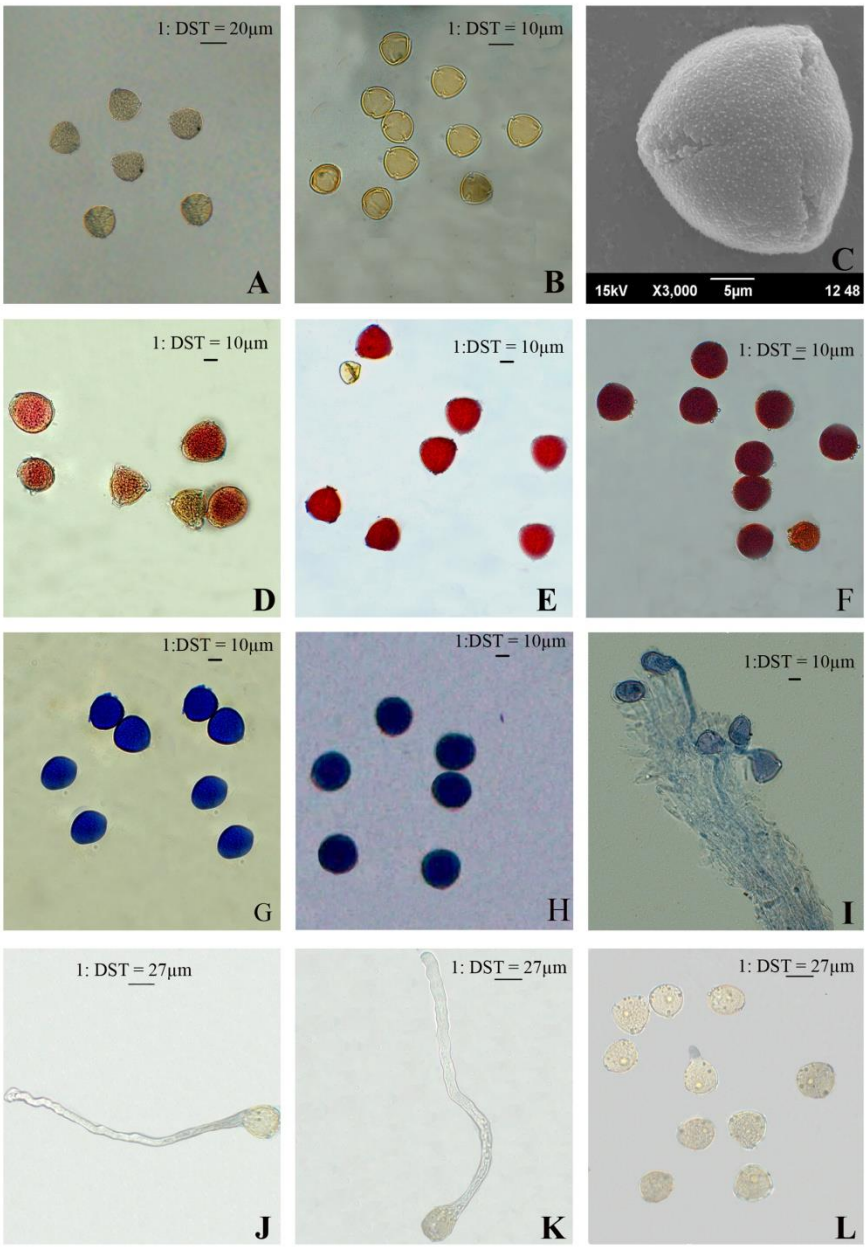

Fig. 41: Pollen biology of *N. krishnakesara*. **A.** Normal pollen, **B.** Pollen-Acetolysis, **C.** Pollen SEM photography, **E & F.** Pollen viability, **E.** 0.1 % TTC method, **F.** 1 % Acetocarmine Test, **F - H.** Pollen biochemical test. **F.** Starch test, G. Protein test, **H.** Lipid test, **I.** Pollen germination on stigma (*In vivo*), **J - L.** Pollen germination. **J.** Germination in 4% sucrose, **K.** Germination in 4% Brewbaker & Kwack's solution, **L.** Germination in 16% Brewbaker & Kwack,s solution

Pollen – Biochemical analysis (Fig. 42)

Brown colour pollen grains indicate the presence of starch, when it is treated with I_2KI. It is treated with Sudan black and obtained black colour, indicates the pollen contains lipids. The pollen grains stained with Coomassie brilliant blue R results blue colour indicate the presence of protein.

Pollen production

The mean number of pollen per flower is 43000 ± 4830 (n=10). The mean number of pollen per anther is 8600 ± 966 (n=10).

Pollen – Ovule ratio

The mean number of pollen per flower is43000 ± 4830 and the mean numbers of ovules are7 ± 1. Hence the P / O ratio is 6142: 1.

Pollen viability(Fig. 42 & 43)

The pollen viability was tested by 0.1% TTC at pH 7 and Acetocarmine (1%). In TTC (0.1%) test, the viable pollen grains shows red colour due to the accumulation of formazan, but the non-viable pollen grains remain colourless. Figure 42 below shows, the pollen viability of *N. krishnakesara*. The pollen grains have the maximum viability at two hours before anthesis (96.99 ± 3.9 % in TTC and 97.82 ± 4.9 % in Acetocarmine). During anthesis time the viability will be70.04 ± 2.8% in TTC and 70.32 ± 2.1% in Acetocarmine. Viability will become declined after anthesis.

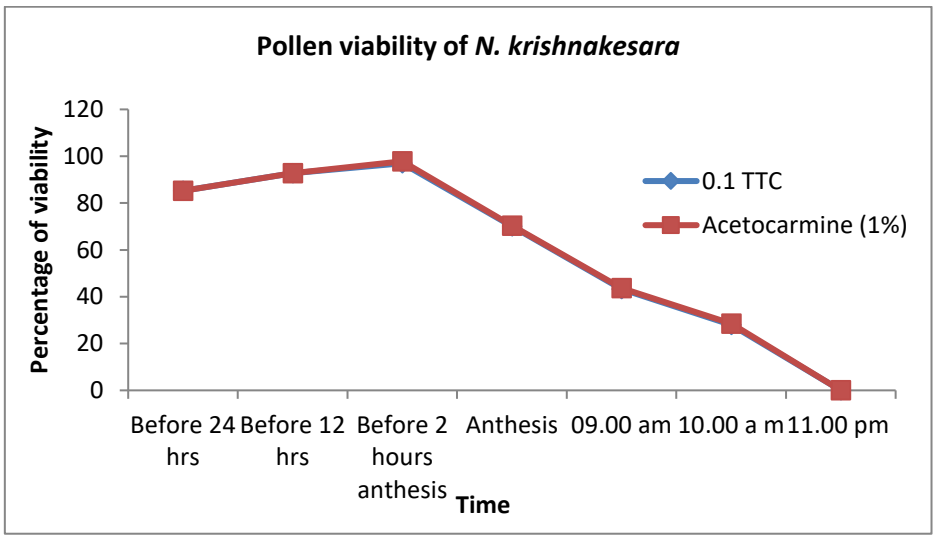

Fig. 43: Pollen viability of *N. krishnakesara*

Pollen germination method

***In vitro* method** (Table 21; Fig. 42)

In Brewbaker& Kwack's medium, the pollen grains germinated maximum in 4% solution (81%) with mean pollen tube length is310.32 ± 118.87 µm after 1 hour. In sucrose solution, 56% of pollen germinated in 4% of sucrose solution and the mean pollen tube length is 81.79µm ± 23.2µm after 1 hour (Table. 20).

Sl No.	Percentage of solutions	Brewbaker & Kwack's Solution		Sucrose solution	
		Percentage of germination	Mean pollen tube length (µm)	Percentage of germination	Mean pollen tube length (µm)
1	4	81	310.32 ± 118.87	56	81. 79 ± 23.2
2	8	80	209.96 ± 73.41	33	33.99 ± 5.58
3	12	61	120.36 ± 63.25	24	27.5 ± 11.78
4	16	47	21.68 ± 10.15	18	24.6 ± 4.76
5	20	0	0	5	12.23 ± 6.62
6	25	0	0	0	0

Table.21: *In vitro* pollen germination in *N. krishnakesara*

***In vivo* method** (Table 22; Fig. 42)

In vivo pollen germination study shows that maximum pollen germination occurs after anthesis. The percentage of pollen germination in post anthesis period is 46.15 with mean pollen tube length is81.36 ± 6.9.

Sl. No.	Parameter	Total pollen on stigma	Germinated pollen grain	% pollen germinated	Mean pollen tube length (µm)
1	Pre anthesis	58	0	0	0
2	Anthesis	31	11	35.48	68.47 ± 10.2
3	Post anthesis	26	12	46.15	81.36 ± 6.9

Table 22. *In vivo* pollen germination in *N. krishnakesara*

Stigma receptivity (Fig. 44)

Direct observation method: The stigma is transparent, shining and wet and also adheres to 1mm^2 piece of paper is considered as receptive

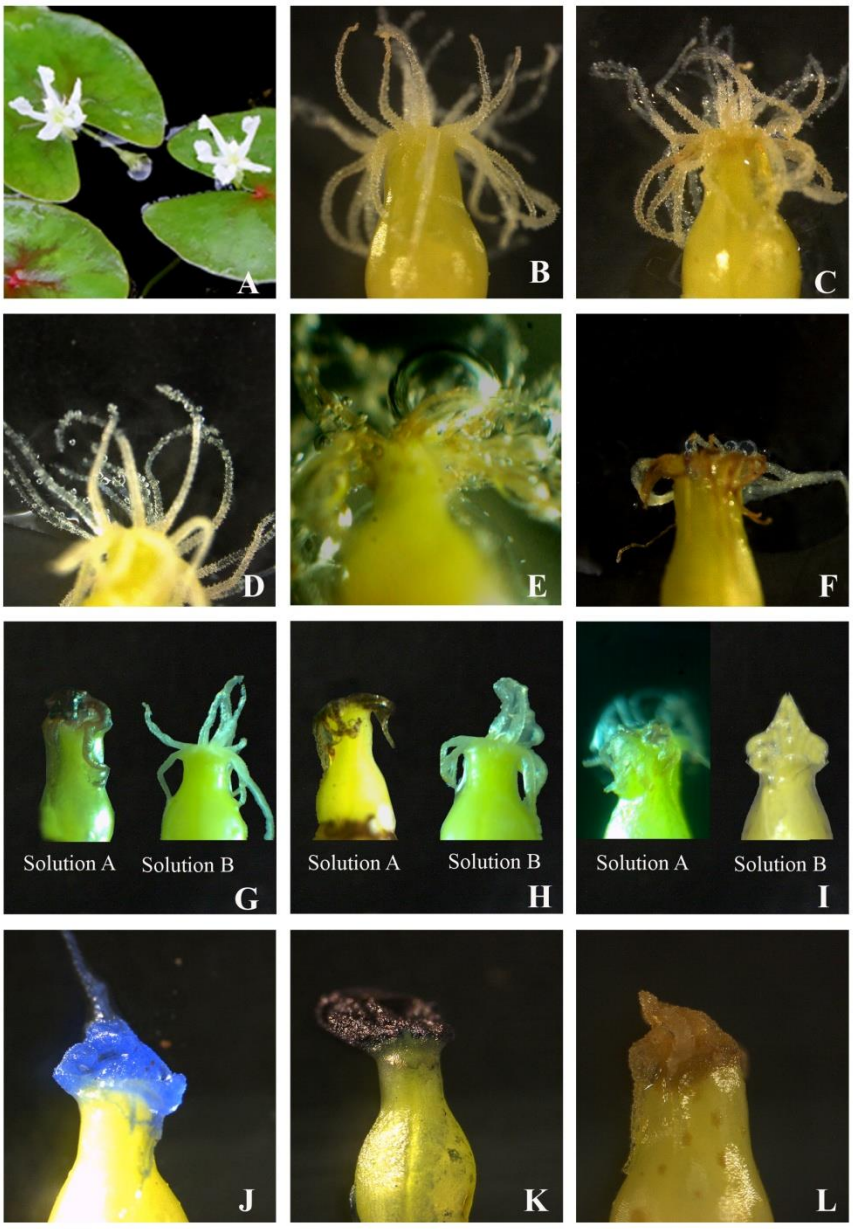

Fig. 44: Stigma receptivity & Stigma biolchemical analysis of *N. krishnakesara*. **A- F.** Stigma receptivity Tests. **A.** Observation method, **B.** Stigma of *N. krishnakesara*, **C - F.** H2O2 Method. **C.**During anthesis, **D.** At 11.00 am, **E.** At 1.00 pm, At 4.00 pm, **G - I.** Histochemical localization of esterases - α -Naphthyl test. **G.** During anthesis, **H.** During post anthesis, **I.** At 6.00 pm. **J - L.** Stigma biochemical test. **J.** Protein test, **K.** Lipid test, **L.** Starch test.

Hydrogen peroxide test (Table 23, Fig. 44)

The receptive stigma produced many enzymes at the time of receptive period. The enzyme peroxidase present on the stigmatic surface, the Oxygen bubbles are evolved from the receptive time when it was treated with hydrogen peroxide. The stigma produces maximum bubble at 1.00 pm and shows the maximum receptivity (62.07 ± 1.34, H value = 0.01336, p value = 0.00001) at that time. The receptivity gradually declined after 1.00 pm.

Sl. No.	Time	Mean no. of bubbles	H value	p value
1.	08.00 am	5.27 ± 1.80	7.1768	0.02825
2.	09.00 am	9.67 ± 2.21	6.9471	0.03110
3.	10.00 am	21.97 ± 1.71	6.5574	0.03768
4.	11.00 am	57.43 ± 1.73	7.2394	0.02679
5.	12.00 pm	59.9 ± 1.69	7.3471	0.02539
6.	01.00 pm	62.07 ± 1.34	0.01336	0.00001
7.	02.00 pm	53.87 ± 1.28	6.5968	0.03694
8.	03.00 pm	36.5 ± 1.55	9.4942	0.00868
9.	04.00 pm	21.5 ± 1.55	8.6632	0.01315
10.	05.00 pm	11.77 ± 1.83	8.2865	0.01587
11.	06.00 pm	5.47 ± 1.36	6.6703	0.03561

Table 23: H_2O_2 Test of stigma receptivity in *N. krishnakesara*

Histochemical localization of esterases on stigmatic surface (Table. 24;Fig. 44)

The stigma becomes black colour at the time of more receptivity when it was treated with α-Naphthyl acetate solution. In *N. krishnakesara* the stigma was more receptive at 11.00 am – 2.00 pm, after that the receptivity gradually decrease.

Stigma – Biochemical analysis (Fig. 44)

The stigma becomes blue black when stained with Sudan black indicated the presence of lipids on the stigmatic surface and change to brown colour indicates the presence of newly formed starch, when it stained with I_2KI. The stigma was

stained with Coomassie brilliant blue R. the colour of the stigma changed to blue colour, indicating the presence of protein.

Sl. No.	Time	Frequency of colour
1	05.00am - 06.00 am	colourless
2	07.00 am - 08.00 am	Colourless
3	09.00 am - 10.00 am	Brown colour
4	11.00 am - 12.00 pm	Black colour
5	01.00 pm -02.00 pm	Black colour
6	02.00 pm - 04.00 pm	Brown colour
7	04.00 pm -06.00 pm	Light brown

Table.24: Histochemical localization esterases on stigma of *N. krishnakesara*

Pollination Biology

Floral visitors and their behaviours (Table 25; Fig. 45 & 46)

Mode of pollination is entemophily. Anthesis of *N. krishnakesara* is at06.00 am – 07.00 am. Insect's visits started during the time of anthesis just after flower opening. A little honey is present in the flower (less than i µl). During the study time small flies under the order Diptera (*Notiphila*sp.) visited to the flowers. *Notiphila* species are the main pollinators of *N. krishnakesara*. A single fly visited 2 - 3 flowers in a single visit. This insects carries pollen from mal flowers and deposited it on the stigma of female flowers. After visiting male flower it carries pollen and deposited on the stigma of female flower. The bees spent 5 – 15 minutes per flower. Insects visit is more active at 10.00 am – 11.00 pm. The pollinators visited to the flowers from morning to evening (06.00 am – 06.00 pm).

Sl. No.	Name of the visitors	Family	Foraging Nature	Foraging hours	Time spent in each flower	Stigma touch	Frequency of visit
1	*Notiphila* spp.	Ephydridae	Nectar + pollen	6 am – 6 pm	5 – 15 minutes	+++	High
2	*Ceriagrion* spp.	Coenagrionidae	No foraging nature	10 am – 11 am	4 – 8 seconds	-	Low

Table 25: *N. krishnakesara* floral visitors and their behaviours

Stigma touch +++: very good, ++: good, +: poor, -: no touch. Frequency of visit: high (10 – 40 visit/day), intermediate (4 – 9 visit/day), low (<3 visit/day).

Fig. 44: Floral visitors of *N. krishnakesara*. **A - C**. *Notiphila* spp. on Male flower, **D & E**. *Notiphila* spp. on Female flower, **F**. *Ceriagrion* spp.

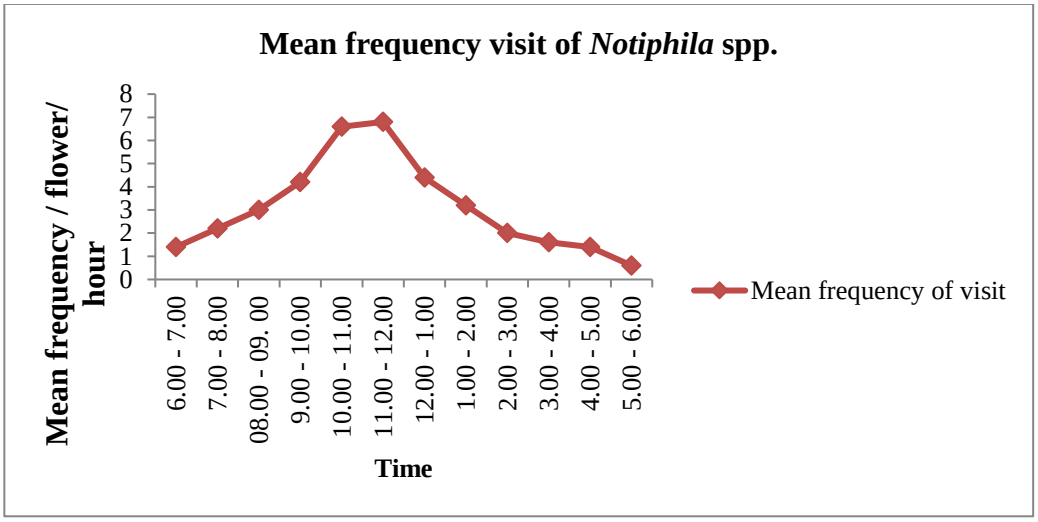

Fig. 46: Frequency visit of *Notiphila* spp. in *N. krishnakesara*

Pollination efficiency:

The total pollen per flower is 43000 ± 4830 and total number of pollen on stigma after pollination is 1004 ± 237.3. Pollination efficiency of *N. krishnakesara* is 0.023.

Breeding System (Fig. 47)

The fruit set and seed set obtained from different pollination treatment were observed and recorded. In natural pollination 54% of fruit set were observed but in manual cross pollination produce 100% of fruits set.

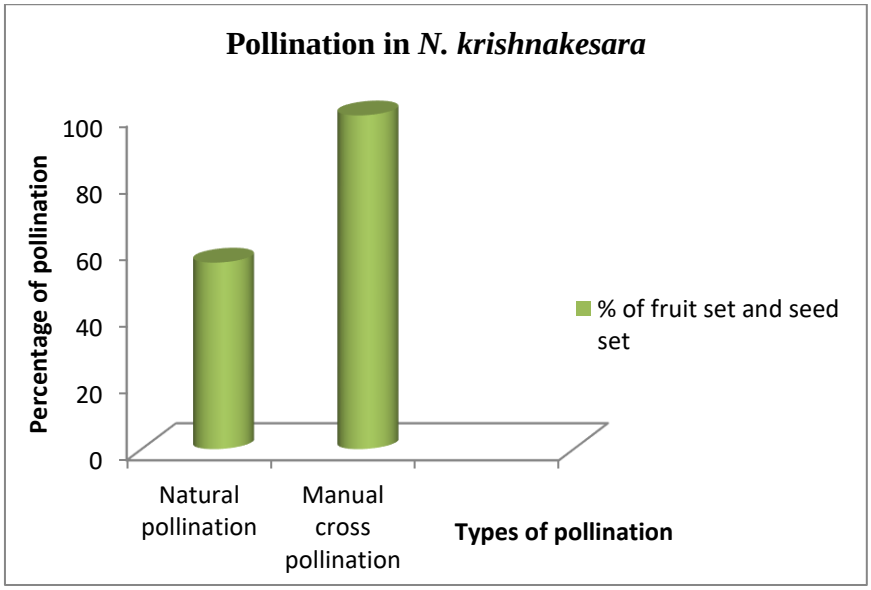

Fig. 47: Breeding in *N. krishnakesara*.

Fruit and seed

Capsule ellipsoid, light green and turned light yellow when it matured. The mean length and width of a capsule is9 mm x 7 mm and the mean pedicel length 1.06 cm. Capsule consists of 4 - 9 seeds. Seeds are discoid and brown. Seed surface is tuberculated.

Flower - fruit ratio and Ovule - seed ratio (Table 26)

Flower fruit ratio in *N. krishnakesara* is 16: 9 and ovule – seed ratio is 4 : 3.

Sl. No.	Parameter	Ratio
1	Flower:Fruit	16 : 9
2	Ovule : Seed	4 : 3

Table.26. Seed set of *N. krishnakesara*

Fruit and seed dispersal mechanisms

Fruits matured at 21 – 24 days after pollination and dehisced two or three days after maturity. The fruits are grown under the water below the floating leaf and the dehiscence occurred in the water. The distal point or the proximal point of the fruit breakes first and the seeds are liberated out to the water. The seeds are hydrophobic in nature. It floats on the water surface. Later seeds sink to the bottom of the water.

Seed germination (Table. 27; Fig.48)

Seed germination was not observed in the field. In controlled conditions, seeds germinated in dung-soil mixture and Gibberelline supplemented soil. Maximum germination observed in dung-soil mixture (61.67%) after 140 – 160 days. Germination not observed in control. Scarified seed germinated after 20 – 40 days in control.

Vegetative propagation (Fig. 48)

N. krishnakesara is a rhizomatous plant. Vegetative reproduction occurs from the rhizome. During the summer season, the aerial shoots wilted and the rhizomes became dormant state under the soil. In the beginning of next rainy season, the dormant rhizome sprouted and produced new suckers. One rhizome produces 3 – 5 suckers. These suckers grow as separate plants.

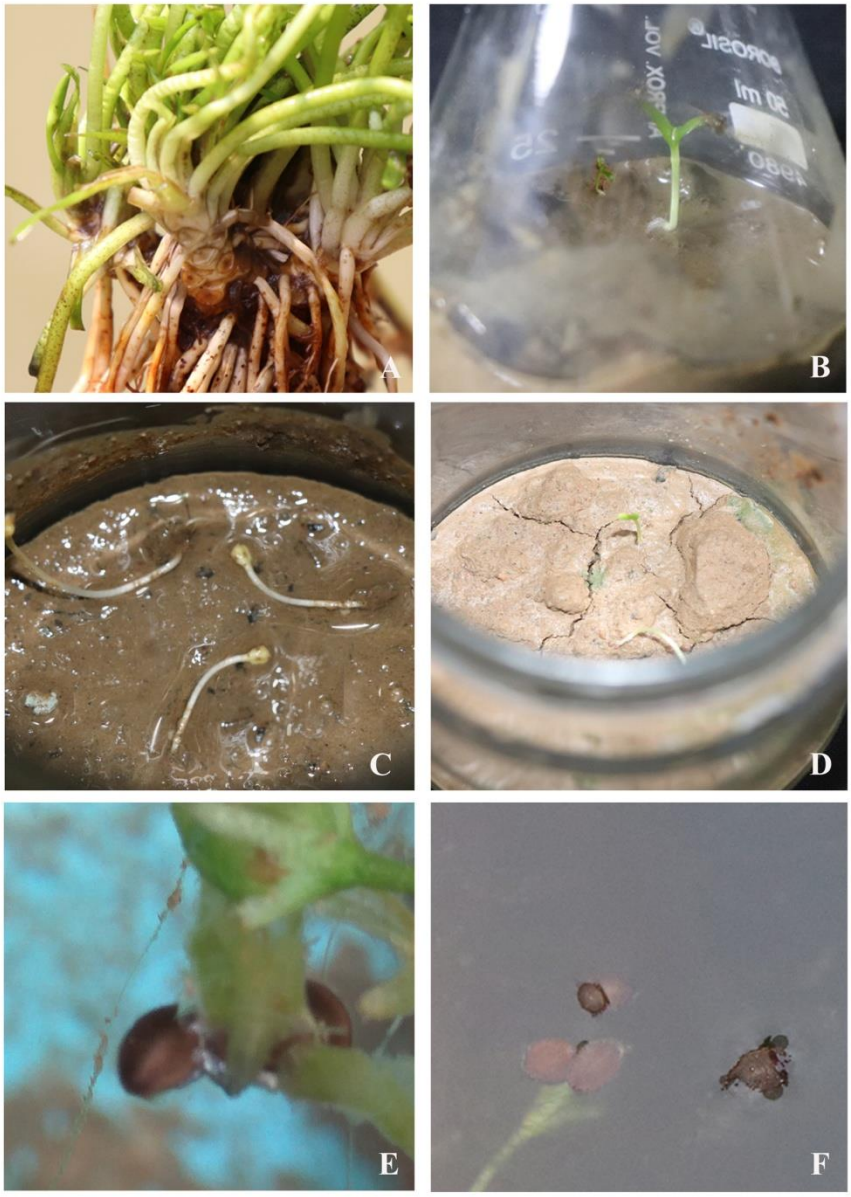

Fig. 47: Reproduction and fruit dehiscence of *N. krishnakesara.* **A.** Vegetative reproduction, **B .** Seed germination in 500 ppm gibberellic acid, **C.** Seed germination in dung soil, **D.** Seed germination in 1000 ppm gibberellic acid, **E.** Fruit dehiscence, **F.** Seed dispersal.

Sl. No.	Days	Percentage of germination (%)				Scarified seed
		Seed				
		Soil	Soil + 50 ppm gibberellin	Soil + 100 ppm gibberellin	Dung soil	Soil (Control)
2	5 – 10	0	0	0	0	0
3	10 – 20	0	0	0	0	0
4	20 – 40	0	0	0	0	6.67
5	40 – 80	0	36.67	0	0	0
6	80 – 120	0	50	40	0	0
7	120 – 140	0	0	50	50	0
8	140 – 160	0	0	0	61.67	0
9	160 – 180	0	0	0	0	0

Table.27: Seed germination rate of *N. krishnakesara* in different condition

5.4.3. *Nymphoides hydrophylla* (Lour.) Kuntze

The floral characteristics of *N. hydrophylla* are described in the Table.28.

Sl. No.	**Floral Characters**	**Observations**	
		Bisexual plant	**Female plant**
1	Blooming time	July – March	July – March
2	Flower type	Actinomorphic, complete, hypogynous, petals joined at the base and free above.	Actinomorphic, hypogynous, petals joined at the base and free above.
3	Flower colour	White with yellow throat.	White with yellow throat.
4	Odour	Absent	Absent
5	Honey	Present	Present
6	Anthesis time	08.00 am – 09.00 am	08.00 am – 09.00 am
7	Anther dehiscence time	08.00 am – 8.30 am	-
8	Anther dehiscence mode	Through longitudinal slit	
9	Number of stamen/ flower	5	-
10	Number of staminode/ flower	-	5
11	Mean No. of pollen / anther	5400 ± 966	-
12	Mean No. of pollen / flower	27000 ± 4830	-
13	Mean No. of Ovules/ flower	7	4
14	Pollen – Ovule ratio	3857	-
15	Pollen type	Monad, triagonal and tricolpate	-
16	Pollen size	29.09 ± 1.93 µm	-
17	Pollen shape	prolate – speroidal	-

18	Stigma type	Bifid, Wet	Bifid, Wet
19	Fruit type	Capsule	Capsule
20	Flower – fruit ratio	3 : 2	9 : 1
21	Ovule – seed ratio	7 : 5	2 : 1
22	Flower closing time	3.30 PM	3.30 pm

Table.28. Floral characters of *Nymphoides hydrophylla*

Phenology

Leaf development and Flower – Bud development

The development of leaf and bud to decaying of leaf and full bloom flower stage was divided into different stages. The morphological changes of leaf and bud in their developmental stages are:

Leaf development (Fig. 49& 51)

Stage 1: Leaf initiation stage is noted just visible to the naked eye. There is no distinction between the leaf, shoot and petiole. The mean length is 1mm (1^{st} day).

Stage 2: During this stage, the shoot and leaf is clearly distinguished on the 2^{nd} day but the lamina is found to be in rolled condition. The mean length of the shoot is 10mm and the length of the lamina is2.8mm x 0.5mm.

Stage 3: The petiole is observed during this stage. Here also the lamina is in the rolled condition. The mean length of the shoot is 15.2mmand the lamina is 5.4mm x 1mm. The mean length of the petiole is 1 mm long (3^{rd}–4^{th}day).

Stage 4: The leaf lamina is opened during this stage. The lamina will be open inside the water (Fig) and it takes 7^{th} - 8^{th}days depending on the depth of the water. The mean length of the lamina is 30mm x 31.8mm and the shoot is 231.7mm.The petiole length is 5.8mm.

Stage 5: Lamina will be reach above the water surface from 8^{th} to 11 or 12^{th} day due to the elongation of the shoot. Lamina is yellowish glossy green. The mean length of the shoot is 336.9mm and the mean length of the lamina is 49.3mm x 48.3mm. The petiole is 8.1mm.

Stage 6: This is the mature stage of leaf, the main veins are cleared and the leaves become matured and dark green during 18th to 20th days. During this stage mean length of the petiole is 12.3mm and the shoot is 471.8mm. The mean length of the lamina is 86.6mm x 84.6mm.

Stage 7: The mature leaf becomes yellow colouredduring 40 to 42ndday's intervals. The green colour changes to yellow colour and start to stop its life span. The mean length of the shoot is 471.8mm and the lamina is 86.6mm x 84.6mm.

Stage 8: The lamina is fully decayed during the 50thto 56thday intervals.

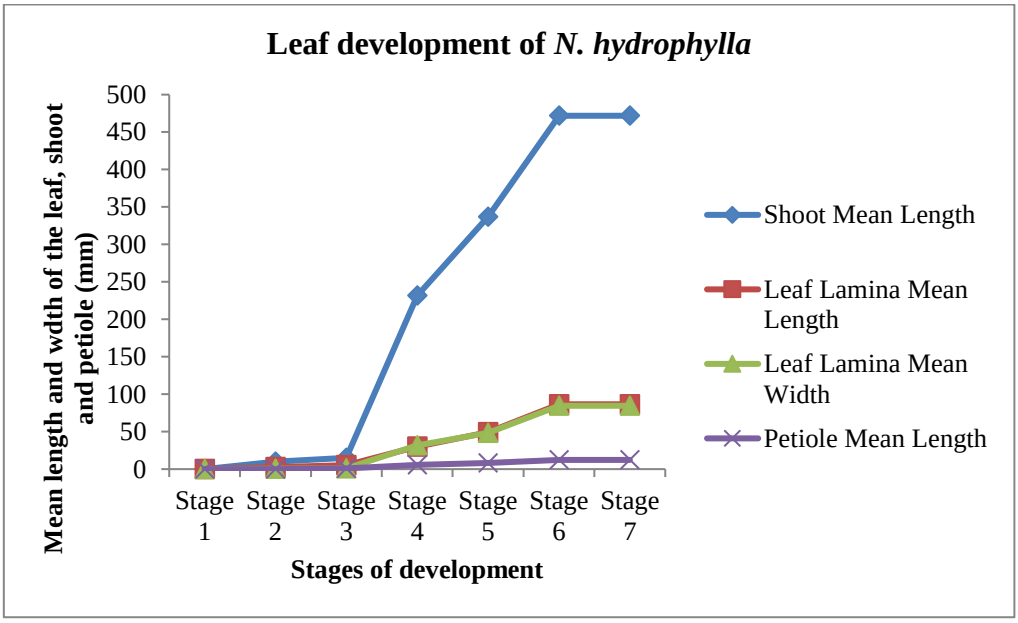

Fig. 49: Leaf developmental stages of *N. hydrophylla*

Flower -Bud development (Fig. 50& 51)

The period of flower bud development in *N. hydrophylla*, from its initiation to the full bloom stage was divided into different stages. They are:

Stage 1: In this stage flower buds are just visible with naked eye. Flower bud initiation started at the time of leaf initiation. It is cream coloured, found just below the leaf covered with bract (1st day).

Stage 2: From the day after initiation to 4th day, the flower bud is obovoid, cream coloured and covered with bract. The mean size of the buds is 1mm.

Fig. 51: Stages of development of leaf, Flower bud & Fruit of *N. hydrophylla*. **A**. Leaf developmental stages, **B.** Flower bud development, **C.** Fruit developmental stages.

Stage 3: During 4th day, the bud emerges out from the bract and its mean size is1.6 x 1mm. The bract is light green in colour. Two buds are formed in this stage.

Stage 4: Pedicel and sepals are clearly distinguished during 6th – 12th day of intervels. The mean size of the bud is 2mm x 1.4mm and the pedicel is1mm.

Stage 5: Petals emerges out of the calyx. Pedicel grows very fastly and attains 22.1mm length. Within 12th to 13thdas of interval the flower bud reached above the water surface. The mean size of the bud is 5.1mm x 2mm.

Stage 6: This is the mature bud formed before2 hours of anthesis. The mean bud size is 6.7mm x 3.6mm (15th or 16th day). The mean length of the pedicel is 32.5mm.

Stage 7: This is the full bloom stage in which the flower becomes open on the 15th or 16th day and exposing the various whorls and reproductive parts. The mean flower size is 21.9mm. The length of the pedicel is 32.5 mm.

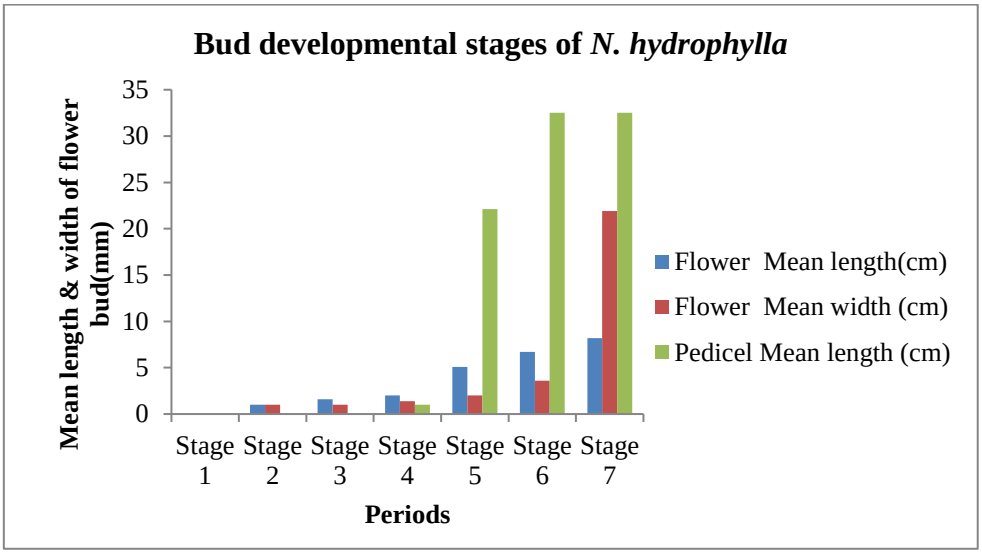

Fig. 50: Flower bud developmental stages in *N. hydrophylla*

Fruit development (Fig. 51 & 52)

Various stages of fruit development from pollination to the maturation and its dehiscence were recorded. The total time required for the starting of fruit development to the fruit dehiscence was noted as 21 - 22 days.

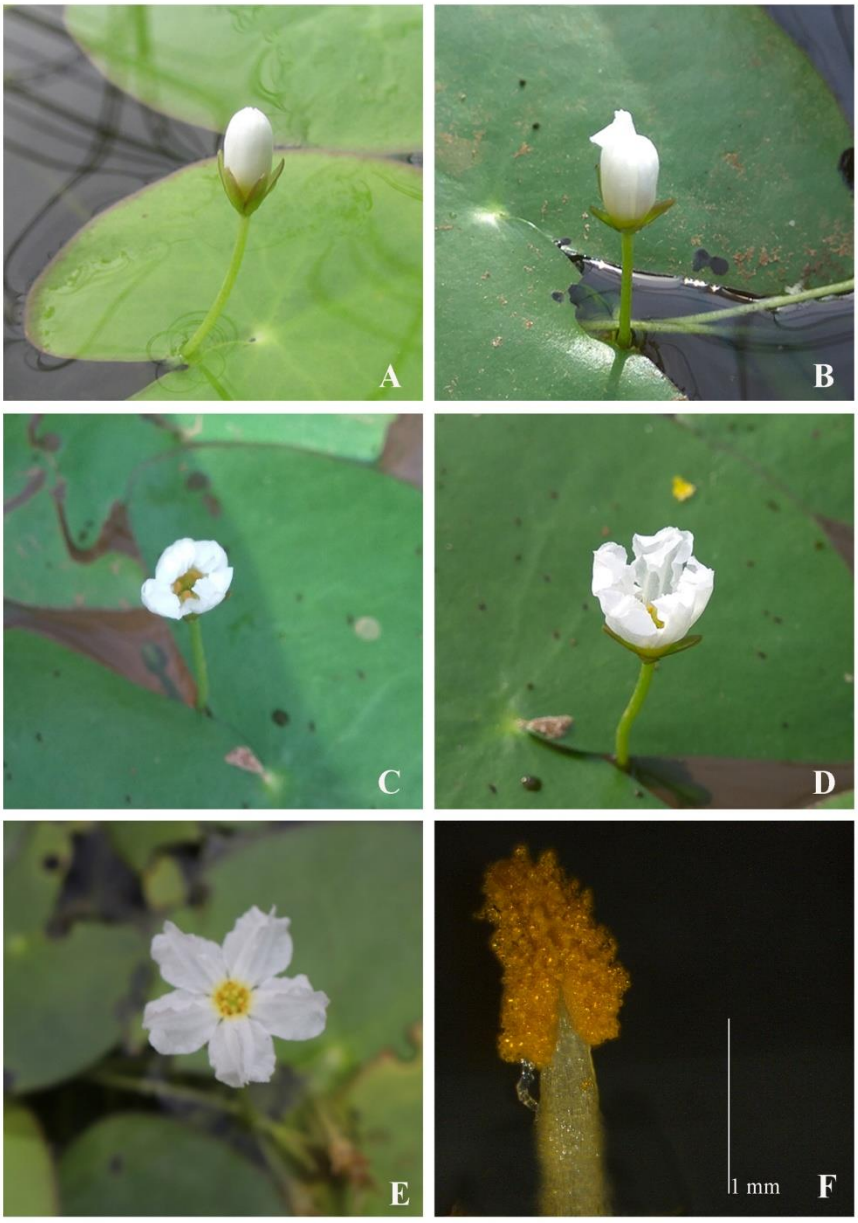

Fig. 53: Anthesis & Anther dehiscence of *N. hydrophylla.* **A - E.** Different stages of anthesis of flower, **F.** Anther dehiscence.

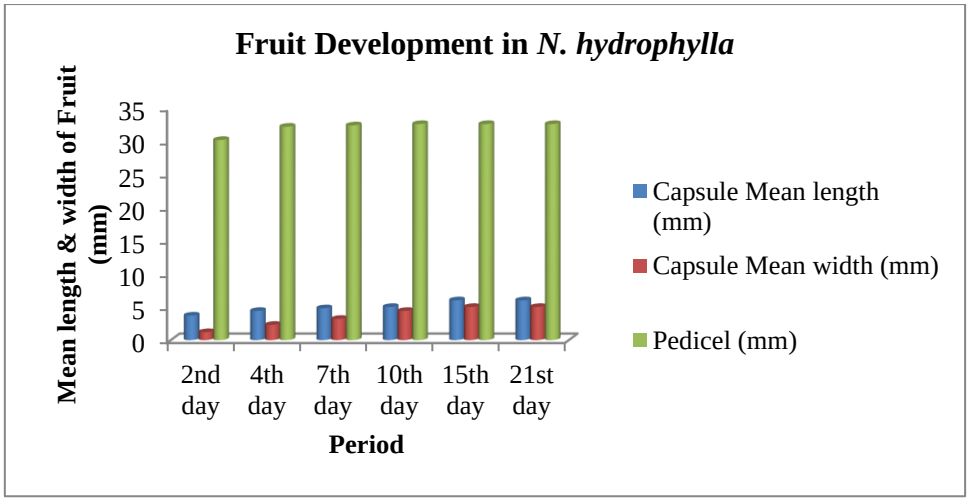

Fig. 52: Fruit developmental stages of *N. hydrophylla*

Flowering activity (Fig. 53)

Nymphoides hydrophylla (Lour.) Kuntze is a seasonal aquatic plant widely distributed throughout in India. The aerial shoots die off during the beginning of summer season. The rhizomes remains dormant under the soil till the end of summer and germinate soon as the next rainy season. New seedlings were also produced from the fertile shoots. The flowers were born on the junction between the shoot and petiole. The flowering time started at the month of July. The peak time of flowering is November – December. The month of June – July was its seedlings to maturity time. Flowering started at the middle July. Mid March – May showed the dormant stage of rhizome.

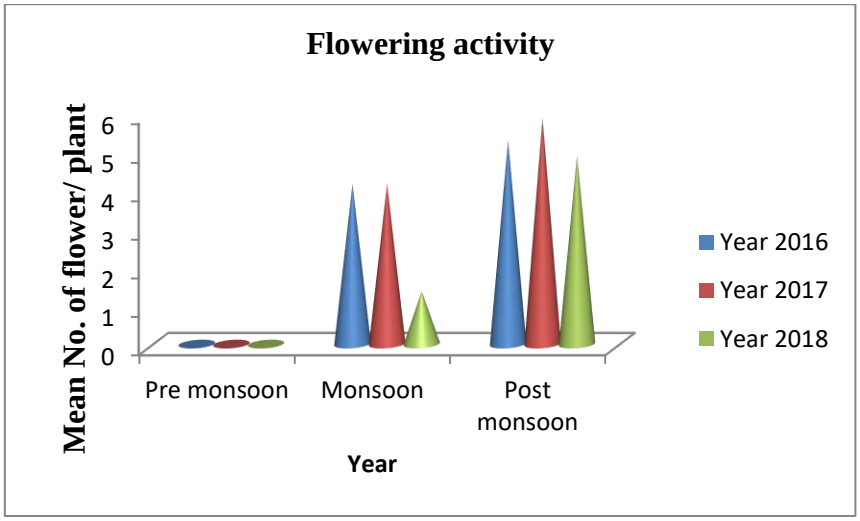

Fig. 53: Flowering activity of *hydrophylla*

Anthesis (Fig. 54)

Anthesis of *N. hydrophylla* is started during 08.00 am – 09.00 am. The anther dehiscence starts at the time of anthesis. The anther dehisced through longitudinal slit.

Pollen morphology (Fig. 55)

The pollen grains aremedium sized, heteropolar, monad, triangular, radially symmetric, tricolpate and oblate – spheroidal with spiny surface. Mean polar axis is 29.09 ± 1.93 µm and the mean equatorial axis is 27.93 ±1.88 µm. Mean P/ E ratio is 104.14 ± 3.17 µm (n =100).

Pollen – Biochemical analysis (Fig. 55)

The pollen grains became red or pinkish colour due to the presence of newly formed starch when treated withI_2KI solution and become blue, when stained with Coomassie brilliant blue solution, which indicated the presence of protein on the stigma. The pollen grains become black colour due to the presence of lipids.

Pollen production

The number of anthers in *N. hydrophylla* is five. The mean number of pollen per anther is 5400 ± 966 (n=10). The mean number of pollen per flower is 27000 ± 4830 (n=10).

Pollen – Ovule ratio

The mean number of pollen per flower was 27000 ± 4830 and the mean number of ovules was 7 ± 1. Hence the P / O ratio is 3857:1.

Pollen viability (Fig. 55 & 56)

The pollen viability was tested by 0.1% TTC at pH 7 and 1% Aceto carmine. Fig.56below shows, the pollen grains have maximum viability at pre anthesis period (2 hours before anthesis). 57.05 ± 3.2 % in TTC (0.1%) and 57.4 ± 2% in Acetocarmine (1%). Pollen viability decreases after anthesis period and lost its viability after 11 am.

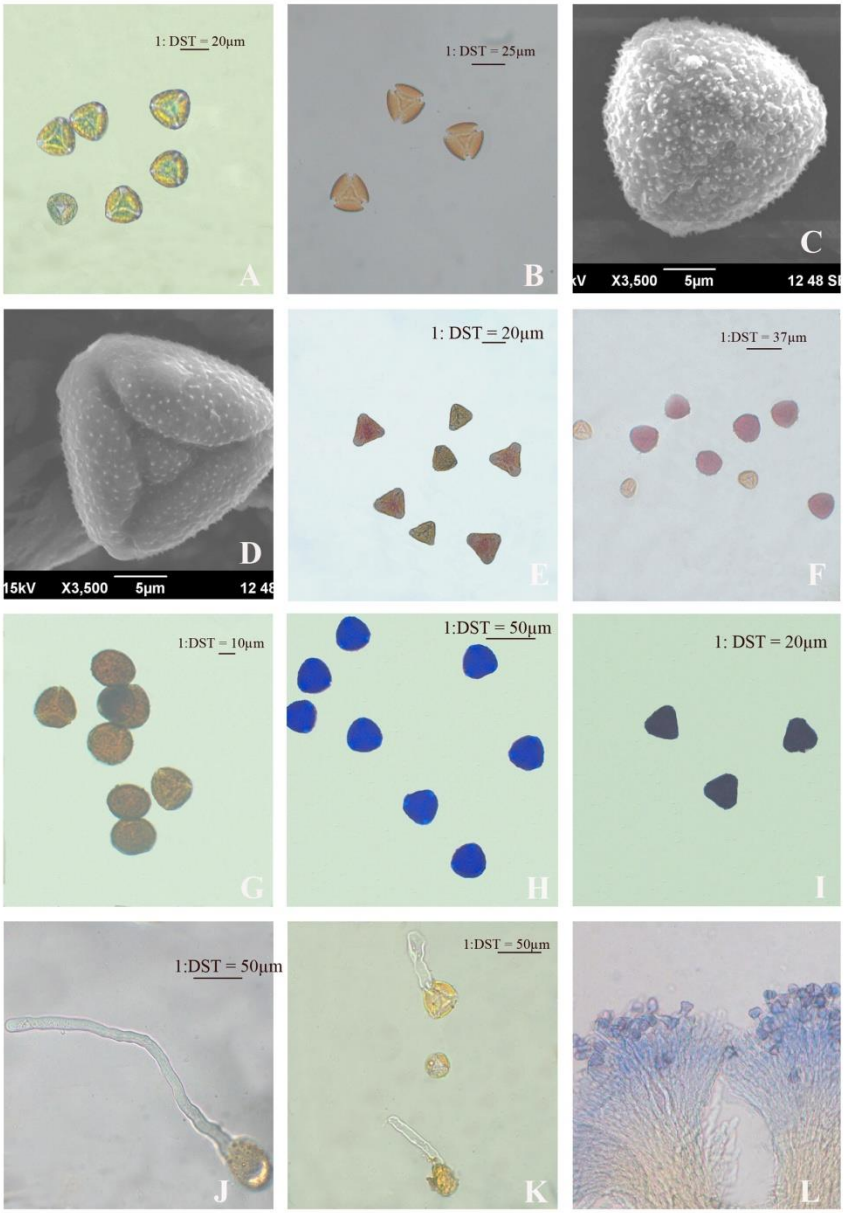

Fig. 54: Pollen biology of *N. hydrophylla*. A. Normal pollen, **B.** Pollen-Acetolysis, **C. & D.** Pollen SEM photography, **E & F.** Pollen viability, **E.** TTC method, **F.** Acetocarmine Test, **G - I.** Pollen biochemical test. **G.** Starch test, **H.** Protein test, **I.** Lipid test, J - L. Pollen germination. J. Germination in 4% Brewbaker & Kwack's solution, , **K.** Germination in 4% sucrose solution, **L.** Pollen germination on stigma (In vivo pollen germination).

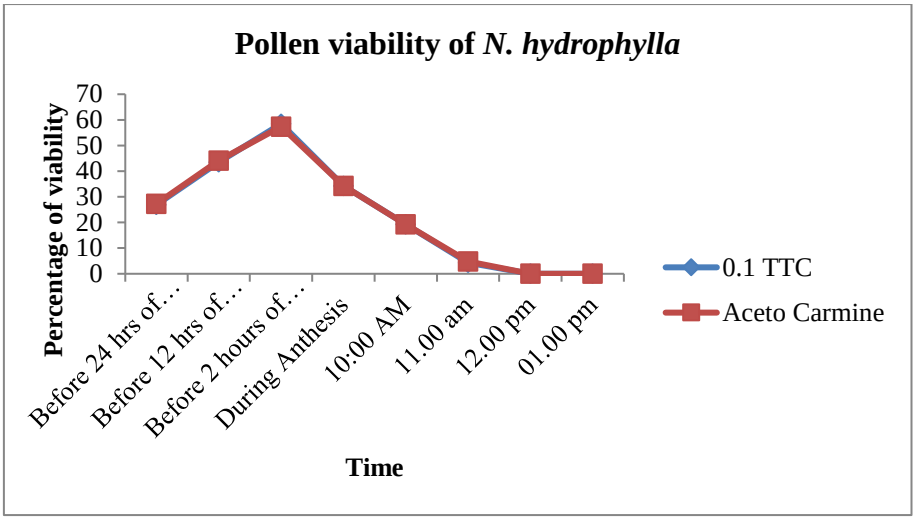

Fig. 56: Pollen viability of *N. hydrophylla*

Pollen germination method

***In vitro* method** (Table 29; Fig.55)

In Brewbaker medium the pollen grains germinated maximum in 4% solution (100%) with the mean pollen tube length is 565.63 ± 83.17 µm after 3 hours. In sucrose solution, 83% of pollen germinated in 4% of sucrose solution, and the mean pollen tube length is328.21 ± 33.03 µm after 3 hours. The growth of pollen tube length is maximum in 4% of both the solutions and minimum in 16 % of both the solution.

Sl. No.	Percentage of solutions	Brew baker &Kwack's Solution		Sucose solution	
		Percentage of germination	Mean pollen tube length (µm)	Percentage of germination	Mean pollen tube length (µm)
1	4	100	565.63 ± 83.17	83	328.21 ± 33.03
2	8	80	245.2 ± 28.77	50	202.73 ± 61.07
3	12	61	102.85 ± 41.75	28	32.59 ± 4.02
4	16	47	22.1 ± 10.17	13	21.87 ± 3.7
5	20	0	0	0	0
6	25	0	0	0	0

Table.29: *In vitro* pollen germination in *N. hydrophylla*

***In vivo* method** (Table 30; Fig. 55)

In vivo pollen germination study shows that maximum pollen germination occurs in post anthesis period. The percentage of pollen germination in post anthesis is 95% with a mean of pollen tube length 196.82 ± 32.87µmafter 1 hour.

Fig. 57: Stigma receptivity & Stigma biochemical analysis of *N. hydrophylla.* **A - F.** Stigma receptivity Tests. **A.** Observation method, **B.** Stigma, **C - F.** H2O2 Method. **C.** During Pre anthesis **D.** During Anthesis, **E.** At 1.00 pm, **F.** At 5.00 pm, **G -** I α-Naphthyl Test (Histochemical localization of esterases). **G.** Anthesis time, **H.** At 1.00 pm, **I.** At 5.00 pm. **J - L.** Stigma biochemical test. **J.** Protein test, **K.** Lipid test, **L.** Starch test.

Sl No.	Parameter	Total pollen on stigma	Germinated pollen grain	% pollen germinated	Mean pollen tube length (µm)
1	Pre anthesis	0	0	0	0
2	Anthesis	17	8	47.06	26.22 ± 8.57
3	Post anthesis	22	21	95	196.82 ± 32.87

Table.30: *In vivo* pollen germination in *N. hydrophylla*

Stigma receptivity

Direct observation method (Fig. 57)

The receptive stigma is transparent, shining, wet and adhere to $1mm^2$ piece of paper (considered as receptive).

Hydrogen peroxide test (Table 31; Fig. 57)

The stigmatic surface produced oxygen bubbles when it is treated with hydrogen peroxide. The stigma showed more bubbles during the time of post anthesis period (11.00 am – 3.00 pm) indicated that the stigma became more receptive at that time. The peak time of receptivity is at 1.00 pm (63.7 ± 1.99 bubbles/min, H= 6.54, p value = 0.03801).The stigmatic receptivity gradually decreases after 1.00 pm.

Sl. No.	Time	Mean no. of bubbles	H value	p value
1.	08.00 am	0	0	0
2.	09.00 am	14.7 ± 1.21	9.6026	0.00822
3.	10.00 am	37.80 ± 1.28	6.5832	0.03719
4.	11.00 am	59.5 ± 1.61	6.7606	0.03404
5.	12.00 pm	62.47 ± 1.50	9.0252	0.01097
6.	01.00 pm	63.7 ± 1.99	6.54	0.03801
7.	02.00 pm	55.57 ± 1.91	8.78	0.01242
8.	03.00 pm	50.03 ± 1.16	6.9116	0.03156
9.	04.00 pm	38.53 ± 1.34	8.4626	0.01453
10.	05.00 pm	21.43 ± 1.75	7.6342	0.02199
11.	06.00 pm	9.85 ± 1.16	11.4348	0.00329

Table.31: H2O2 Test of *N. hydrophylla*

Histochemical localization of esterases on stigmatic surface (Table 32, Fig. 57)

Table showed that the stigma becomes dark brown at 11.00 am – 2.00 pm indicated that the stigma was more receptive. At pre anthesis period (6.00 am – 8.00 am) stigma was not receptive. The stigmatic receptivity decreases after 3.00 pm.

Stigma – Biochemical analysis (Fig. 57)

The stigma become black when it is stained with Sudan black indicated the presence of lipids on the stigmatic surface and itbecomes purple or brown when it stained with I_2KI indicated the presence of newly formed starch. The stigma becomes blue indicate the presence of proteinwhen it stained with Coomassie brilliant blue R. The experiment resulted that the stigmatic surface contains lipid, starch and protein.

Sl. No.	Time	Frequency of colour
1	06.00 am – 07.00 am	No colour
2	08.00 am – 09.00 am	Light brown
3	09.00 am – 10.00 am	Brown colour
4	11.00 am – 12.00 pm	Dark Brown – Black colour
5	01.00 pm – 02.00 pm	Dark Brown – Black colour
6	02.00 pm – 03.00 pm	Brown
5	04.00 pm – 05.00 pm	Light brown colour
6	06.00 pm – 07.00 pm	colourless

Table.32: Histochemical localization of esterases on Stigma surface of *N. hydrophylla*

Pollination Biology

Floral visitors and their behaviours (Table 33; Fig. 58 and 59)

Entemophilic pollination was noticed in *N. hydrophylla.* Anthesis occurred in 07.00 am – 09.00 am. Bisexual flowers and female flowers are visited by same type of insect species. Floral scent is absent. Insects visited on the flower soon after anthesis. *Notiphila* sp., *Lasioglossum* sp., *Telicota bambusae* (Butterfly) and *Componatus* sp. are the flower visitors. *Notiphila* spp. is the effective pollinator. The flies visited 2 –4 flowers by a single visit. These insects visit in the bisexual flowers of different plants and carry pollen load. The pollen deposited on the stigma of the flowers of the other plant or on the same plant. Time spent by this bee in each flower was 5 – 10 minutes.

Fig. 58: Visitors of *N. hydrophylla.* **A.** *Lasioglosum mutilum*, *B. Telicota bambusae,* **C.** *Notiphila* spp., **B.** *Componatus* spp.

Sl. No.	Name of the visitors	Family	Foraging Nature	Foraging hours	Time spent in each flower	Stigma touch	Frequency of visit
1	*Notiphila* sp.	Ephydridae	Nectar + pollen	9 am – 6 pm	5 – 10 minutes	+++	High
2	*Lasioglossum* spp.	Coenagrionidae	Nectar + pollen	10 am – 11 am	4 – 8 seconds	+	Low
3	*Telicota bambusae*	Hesperiidae	Nectar	10 – 11 am	2 – 3 seconds	+	Low
4	*Componatus* sp.	Formicidae	Pollen	10 – 11 am	1 – 2 minutes	+	Low

Table 33: *N. indica* floral visitors and their behaviours

Stigma touch +++: very good, ++: good, +: poor, -: no touch.
Frequency of visit: high (10 – 40 visit/day), intermediate (4 – 9 visit/day), low (<3 visit/day).

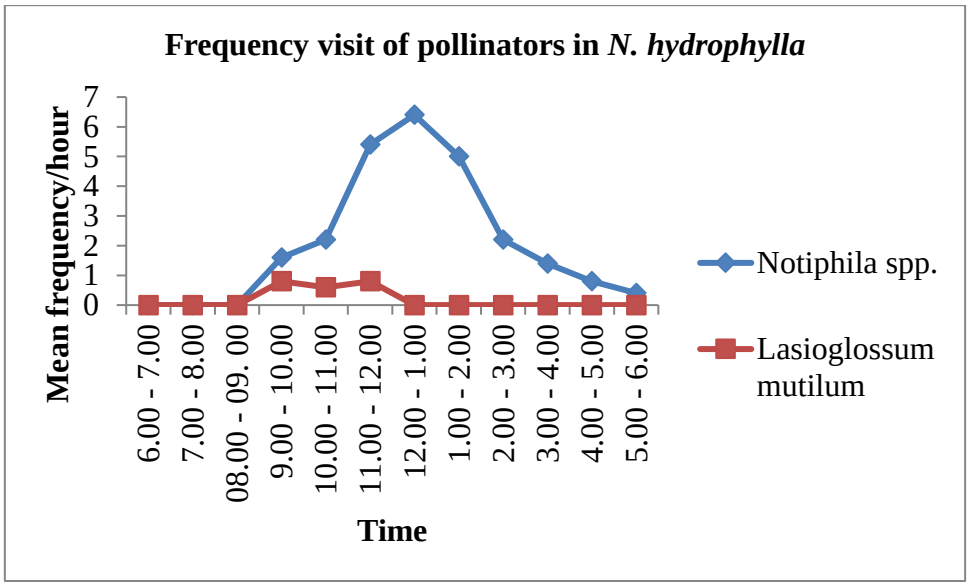

Fig. 59: Frequency of major floral visitors in *N. hydrophylla*

Pollination efficiency:

The total pollen per flower is 27000 ± 4830. In bisexual flower, mean number of pollen on stigma after pollination is 588 ± 237.02. Pollination efficiency of the bisexual plant of *N. hydrophylla* is 0.022. But the pollination efficiency of the female plant is zero in natural condition because pollen grains are absent in famale plant and both the plants grown in different population. In this case vegetative reproduction is dominant in female plant.

Breeding System (Fig. 60)

Fruit set was noticed only in the bisexual flowers under natural condition. The results of different pollination indicated that 60% of fruits are produced in bisexual plants under natural condition, but in female plant no fruits are produced in natural condition because both plants are not grow in same population. By hand pollination 80% fruits were produced in bisexual flowers and 28% in female flower. Self pollination in bisexual plant produced 20% of fruits. The fruit set and seed set obtained from different pollination treatment were observed and recorded.

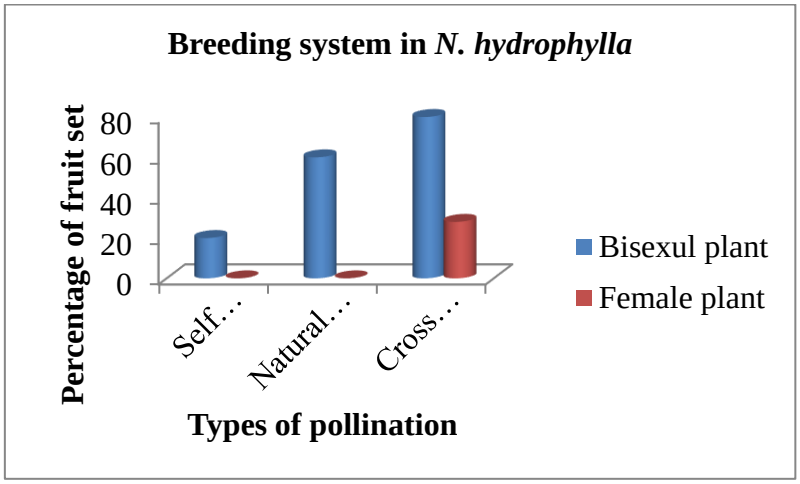

Fig. 60: Breeding system in *N. hydrophylla*

Fruit and seed

Capsule is green or brownish green with persistent calyx, obovoid, *ca.* 7 mm long, 3 - 6 seeds in each capsule of bisexual plant. The mean length and width of a mature capsule is 7 mm x 5 mm and the mean pedicel length is 3.46 cm. The fruits ripen under water below the floating leaves. Seeds are obovoid, rounded or obtuse at distal end, subacute at proximal end, brown in colour, 2 mm long and 1.9 mm wide. Seed surface is tuberculated..

Flower - fruit ratio and ovule - seed raio (Table 34)

Flower fruit ratio of bisexual plant in natural condition is 3: 2. But in female plant flower-fruit ratio was zero under natural condition. But in hand pollination treatment the flower-fruit ratio of female plant is 9: 1. Ovule seed ratio in bisexual plant is 7: 5 and that of female plant is 5: 4.

Types of flower	Parameter	Ratio
Bisexual plant (In Natural)	Flower : Fruit	3 :2
	Ovule : seed	7 : 5
Female plant (Manual cross pollination)	Flower : Fruit	9 : 1
	Ovule : seed	5 : 4

Table 34. Flower - fruit ratio and ovule - seed raio in *N. hydrophylla*

Seed dispersal

The matured fruits were monitored and studied throughout the fruiting period and observed the fruit dispersal mechanism. Fruit matured 21st – 23rdday after the pollination and dehisced two or three days after maturity. The capsule breaks under the water either at the proximal end or at the distal end of the capsule and the seeds is liberated out to the water or break down the region of capsule attached to the pedicel and float on the water, and then liberated the seeds. Seeds are light weighted, so the seeds floating on the water surface. Later seeds sink to the bottom of the water.

Seed germination(Table 35; Fig. 61)

Seed germination was not observed in the field. In controlled conditions, seeds germinated in dung-soil mixture and Gibberelline supplemented soil. Maximum germination observed in dung-soil mixture (40%) after 140 – 160 days. Germination not observed in control. Scarified seed seed germinated after 20 – 40 days.

Sl. No.	Days	Percentage of germination (%)				Scarified seed
		Seed				
		Soil	Soil + 50 ppm gibberellin	Soil + 100 ppm gibberellin	Dung soil	Soil
2	5 – 10	0	0	0	0	0
3	10 – 20	0	0	0	0	0
4	20 – 40	0	0	0	0	4.67
5	40 – 80	0	3.33	0	0	0
6	80 – 120	0	35	6.67	0	0
7	120 – 140	0	0	20	23.33	0
8	140 – 160	0	0	23.33	40	0
9	160 – 180	0	0	0	0	0

Table.35: Seed germination rate of *N. hydophylla* in different condition

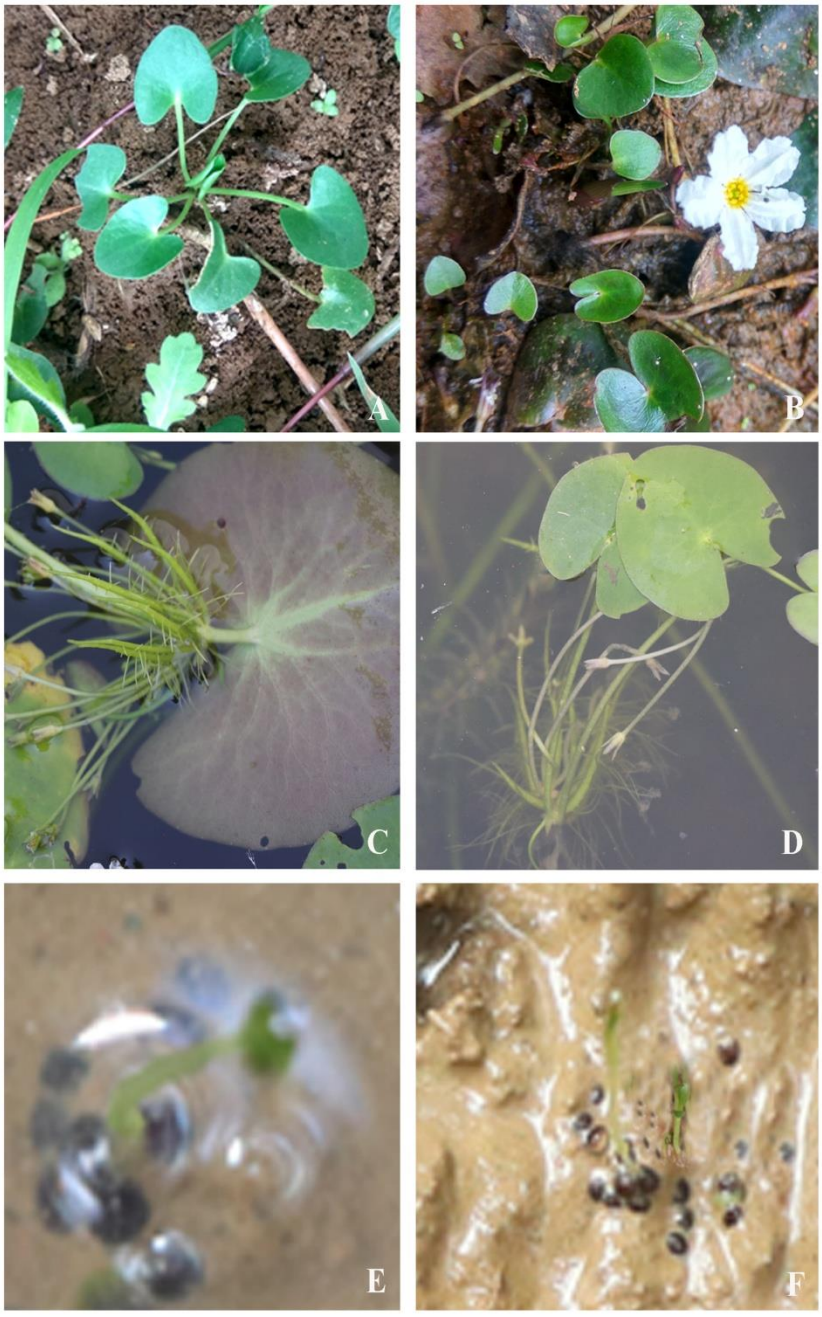

Fig. 63: Reproduction of *N. hydrophylla*. **A - D.** Vegetative reproduction. **A.** New plants germinate from existing rhizome **B.** Stolon from rhizome and produced new plantlets., **C.** Roots produced from cuttings of leaf, **D**. Portion of rooted region detached from mother plants, **E .** Seed germination in 500ppm Gibberellic acid, **F**. Seed germination in Dung soil.

Vegetative propagation (Fig. 61)

Vegetative reproduction of *Nymphoides hydrophylla* occurs from the rhizome, stolon and detached leaves with part of shoot attached. New plants produced from the rhizome in rainy season. Many lateral roots and leaves were produced from the junction of flower producing parts of the shoot. After decaying of leaves and shoots these rooted region float on the water and produce new rhizome and leaves, then sunk into the bottom and get attached to the soil or it grows and produces new saplings floating on the water surface and gets harboured in the shores where water level is low. Stolons were produced from the rhizome and produced new plants from it.

5.4.4. *Nymphoides parvifolia* Kuntze

The floral characteristics of *N. parvifolia* are described in Table.36

Sl. No.	Floral Characters	Observations
1	Blooming time	August - January
2	Flower type	Actinomorphic, complete, hypogynous, petals joined at the base and free above.
3	Flower colour	White with yellow throat.
4	Odour	Absent
5	Honey	Present
5	Anthesis time	08.00 am – 09.00 am
7	Anther dehiscence time	08.00 am – 08.30 am
8	Anther dehiscence mode	Through longitudinal slit
9	Number of anthers/ flower	3 or 4
10	Mean No. of pollen / anther	342 ± 5 (3 anther), 340 ± 8 (4 anther)
11	Mean No. of pollen / flower	1207 ± 18 (3 anther), 1360 ± 33 (4 anther)
12	Mean No. of Ovules/ flower	9.2 ± 2.15 (3 anther) or 10.6 ± 2.7 (4 anther)
13	Pollen – Ovule ratio	111: 1 (3 anther flower), 123 : 1 (4 anther flower)
14	Pollen type	Monad, triagonal and tricolpate
15	Pollen size	25.13 ± 1.09
16	Pollen shape	Oblate – speroidal to prolate - spheroidal
17	Stigma type	Bifid, Wet
18	Fruit type	Capsule
19	Flower – fruit ratio	1 : 1 (in both petal flower)
20	Ovule – seed ratio	5 : 4 (in both petal flower)
21	Flower drooping time	2.00 pm – 2.30 pm

Table 36. Floral characters of *N. parvifolia*

Phenology

Leaf development and Flower – Bud development

The morphological changes of leaf and bud in their developmental stages are the following.

Leaf development (Fig 62& 64)

Stage 1: Leaf initiation stage noted just visible to the naked eye.

Stage 2: On the second day the shoot and leaf is clearly distinguished from this stage but the lamina is found to be in the rolled condition (Plate...). The mean length of the leaf lamina is measured as 1.1 mm and the mean length of the shoots are 1mm.

Stage 3: Leaf petiole is clearly observed by naked eyes on the third day of intervals. Lamina is in the unrolled condition inside the water. The mean length of the shoot is 17.7 mm long and the mean length and width of the lamina is measured as 3.3 x 1 mm. Petiole length was measured as 1 mm.

Stage 4: During 7^{th} or 8^{th} day, the leaf lamina opened under water and the colour of the lamina turned to pink or brown. The mean length of the shoot is 120 mm and the lamina is 10.9 mm.

Stage 5: Lamina reached on the water surface on the 8^{th} – 9^{th} day. It is brown in colour and glazing. The length and width of the lamina is 14.3mm x 12.6 mm.

Stage 6: The colour of the leaf lamina changed to green, disappeared its glazing and brown colour. Brown and green patches seen on the leaf lamina after 15^{th} – 16^{th} day. The length and width of the lamina was 18.3 x 15.9 mm.

Stage 7: On the 28– 32 day intervals the lamina changed its colour and turned to yellow and ready for decay. The length and width of the lamina was 18.3 x 15.9 mm.

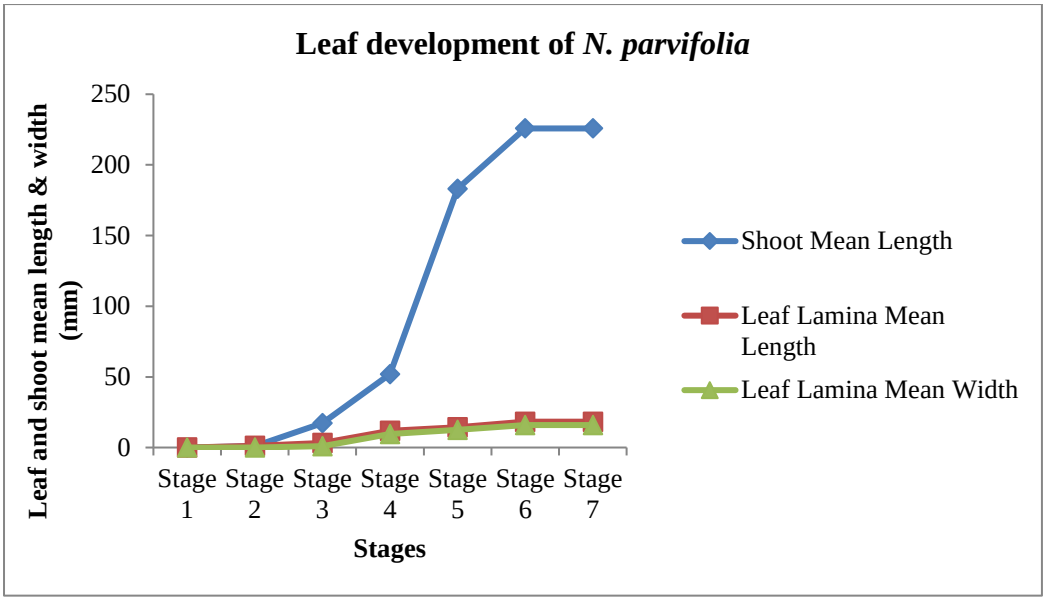

Fig. 62: Leaf developmental stages of *N. parvifolia*

Flower -Bud development (Fig 63& 64)

The period of flower bud development in *Nymphoides parvifolia*, from its initiation to the full bloom stage is divided into different stages. They are:

Stage 1: During this stage the flower bud is just visible to the naked eye. Flower bud initiation started at the time of leaf initiation. It is cream coloured just below the leaf on the shoot.

Stage 2: From the day after initiation to 4th day, the flower bud is obovoid, cream coloured and covered with bract. The mean size of the buds is 1mm.

Stage 3: From 5thto 7th/9th day, the bud emerges out from the bract and its mean size is1.15 mm x1 mm. The pedicel is just observed.

Stage 4: Pedicel and sepals are clearly distinguished during 8th – 9th day of intervals. The mean size of the bud is 2.5mm x 1.1mm and the pedicel is1mm.

Stage 5: Petals are seen on the tip of the flower bud on the 12th - 13th day. Pedicel slightly increased and its size is 1.5mm.

Stage 6: This stage is the mature bud formed before2 hours of anthesis. The mean bud size is 3mm x 2mm (13th or 14th day). The mean length of the pedicel is 4mm.

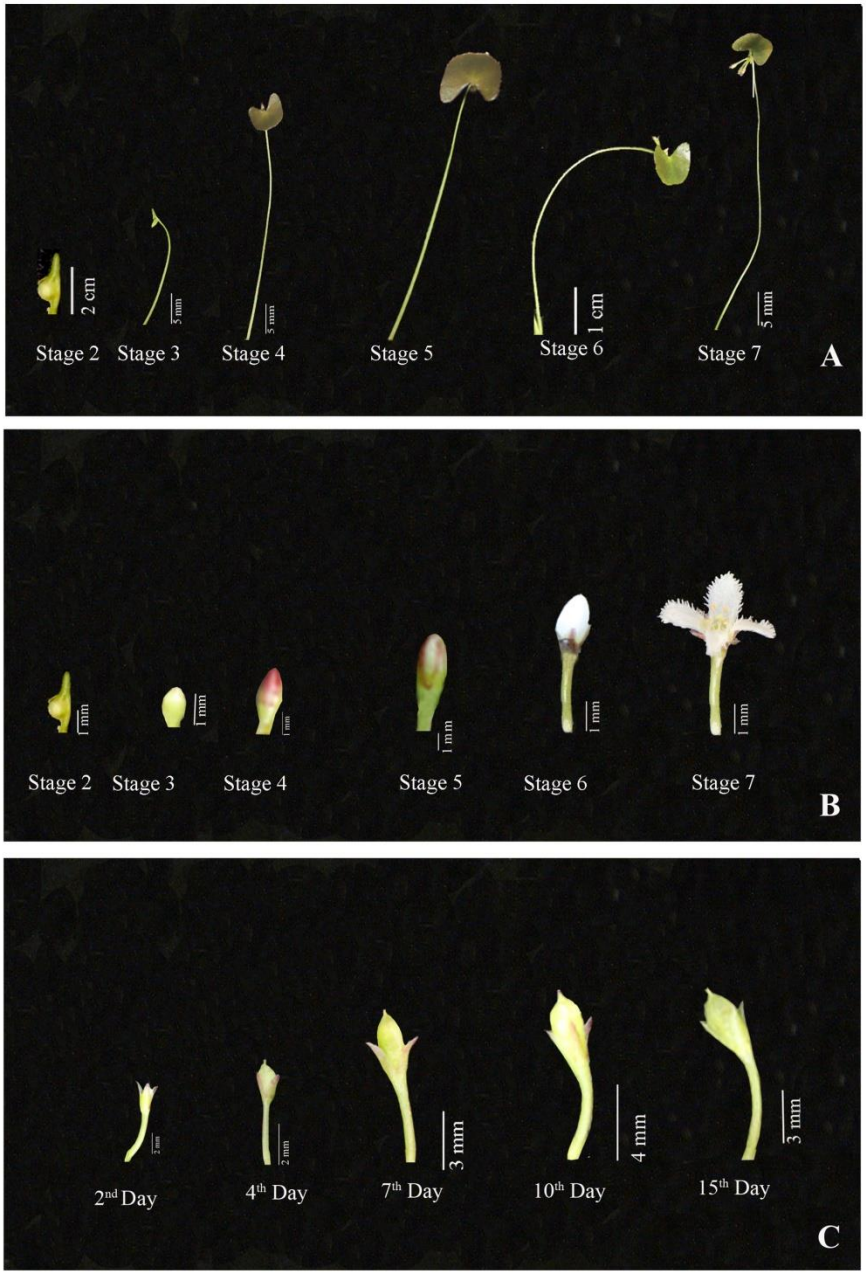

Fig. 64: Development of leaf, Flower bud & Fruit of *N. parvifolia*. **A.** Leaf developmental stages, **B**. Flower bud developmental stages, **C**. Fruit developmental stages.

Stage 7: This is the full bloom stage in which the flower becomes open on the 13th or 14th day and exposing the various whorls and reproductive parts. The mean flower size is3mm and the mean length of the pedicel is 4mm.

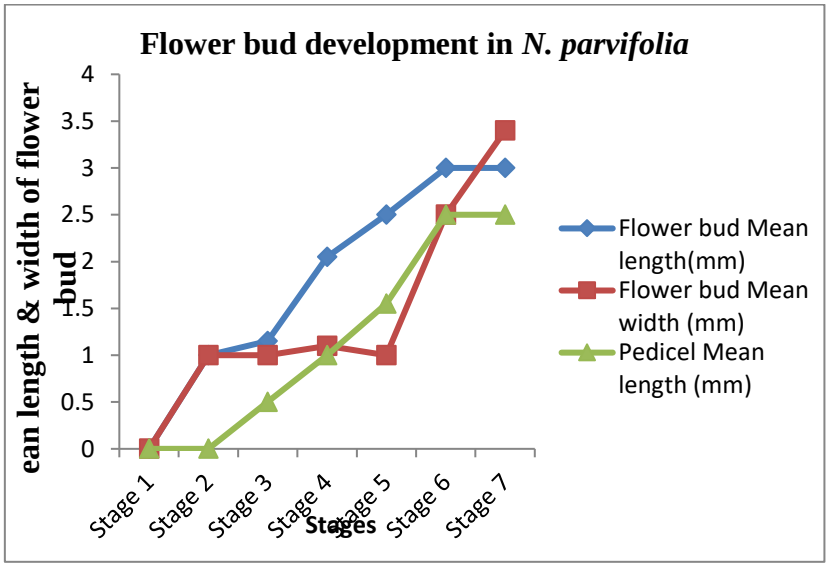

Fig. 63: Flower bud developmental stages in *N. parvifolia*

Fruit development (Fig. 64 & 65)

Fruit of *N. parvifolia* is a capsule. The fruits were produced after pollination. The pistil size is slowly increased after pollination and grows to become a fruit. Figure shows the changes of the fruit development from pollination to the maturation. The total time required for the development of fruit to its formation to maturation is 15th or 16th days and it dehisced 4 or 5 days after maturation.

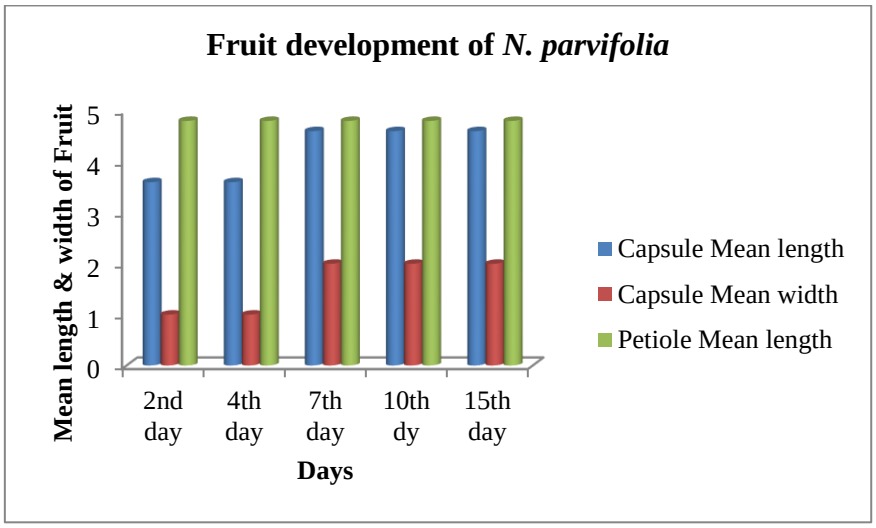

Fig. 65: Fruit developmental stages in *N. parvifolia*

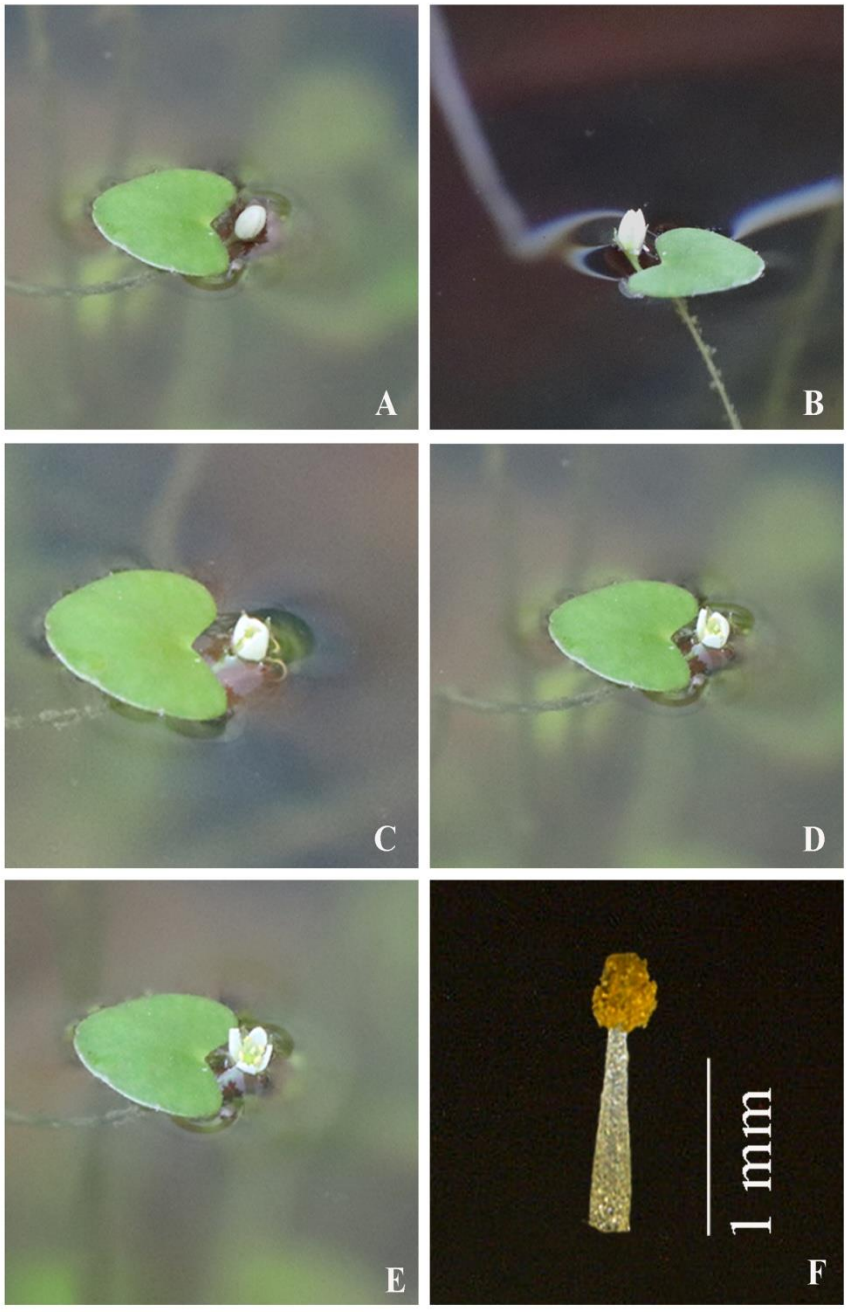

Fig. 66: Anthesis & Anther dehiscence of *N. parvifolia.* **A - E**. Different stages of anthesis of flower, **F.** Anther dehiscence.

Quantitative assessment of flowering(Fig. 66)

The rhizome of *N. parvifolia* is dormant in the summer and germinates at the time of rainy season. The flowering time started at the month of July last week. Figure shows the peak time of flowering (November–January). The month of June – middle of July is its seedlings to maturity time. February – May is the dormant stage.

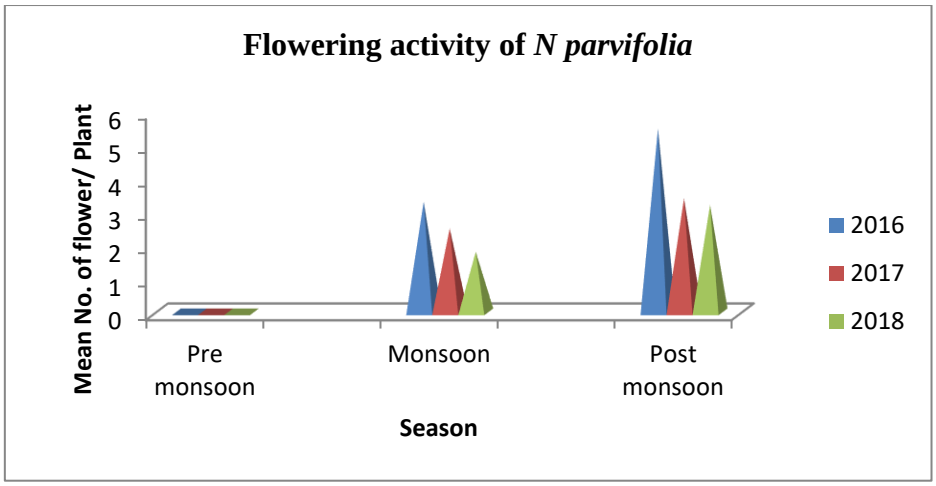

Fig. 66: Flowering activity of *N. parvifolia*

Anthesis (Fig. 67)

Flower opening time of *N. parvifolia* is extends from08.00 am to 09.00 am. The anther dehiscence starts at the time of anthesis. The anther dehisced through a longitudinal slit.

Pollen morphology (Fig.68)

The pollen grains are small sized or medium sized (23.63 – 27.75 µm), monad, triangular, heteropolar, radially symmetric, parasyncolpate and oblate – spheroidal to prolate spheroidal. Mean polar axis is 25.13 ± 1.09 µm and the mean equatorial is 24.4 ± 1.05 µm. P/ E ratio is 103.1 ± 4.37 µm (n =100).

Pollen – Biochemical analysis(Fig. 68)

The pollen grains became brownish due to the presence of starch when it treated with I_2KI solution. The pollen grains becomes black colour due to the presence of lipids when it stained with Sudan black and becomes blue coloured when it stained with Coomassie brilliant blue R showing the presence of protein.

Pollen production

N. parvifolia produces flower with three petals and four petals. These two types of flowers are born on different plants. Stamens of these flowers correspond to the petals. The mean number of pollen per anther in three-petal flower is 342 ± 5 (n=10) and the mean number of pollen per flower is 1027 ± 38 (n=10). The mean number of pollen per anther in four-petal flower is 340 ± 8 (n=10) and the mean number of pollen per flower is1360 ± 33 (n=10).

Pollen – Ovule ratio (Table 37)

The mean number of ovule in three petal flower is 9.2 ±2.15 and that of the four petal flower is 10.6 ± 2.7. Pollen ovule ratio in three petal flower is111:1 and that of the four petals flower is123:1.

Sl. No.	No. of anther in a flower	Pollen / flower	Mean No. of ovules	Pollen-Ovule ratio
1	Three anther	1027 ± 38	9.2 ± 2.1	111:1
2	Four anther	1360 ± 33	10.6 ± 2.7	123:1

Table.37. Pollen – Ovule ratio of *N. parvifolia*

Pollen viability (Fig. 68 & 69)

Fig.68 below shows, the pollen viability of *N. parvifolia.* The pollen grains have maximum viability at 2 hours before anthesis [4.11±4.8% in TTC (0.1%) and 54.36 ± 2.2 in Acetocarmine (1%)]. Pollen viability decreases after anthesis period and lost its viability after 11.00 am.

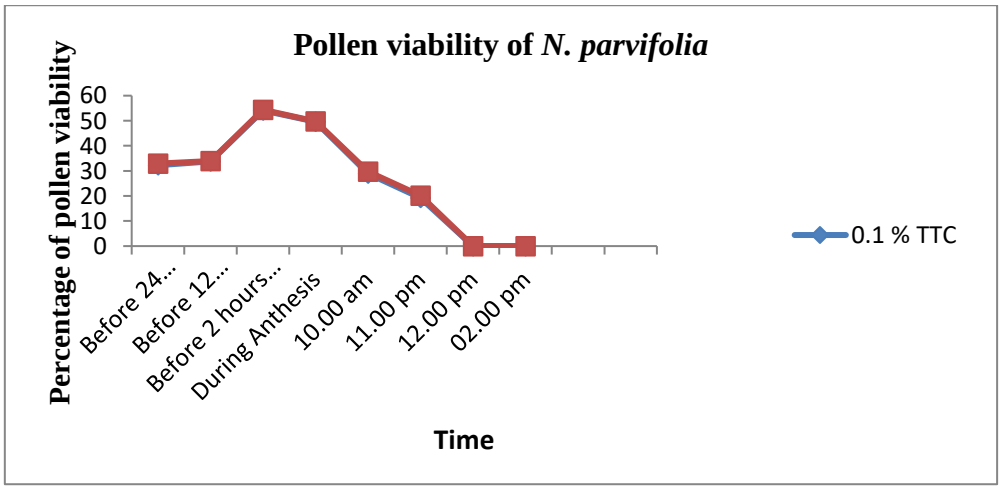

Fig. 69: Pollen viability of *N. parvifolia*

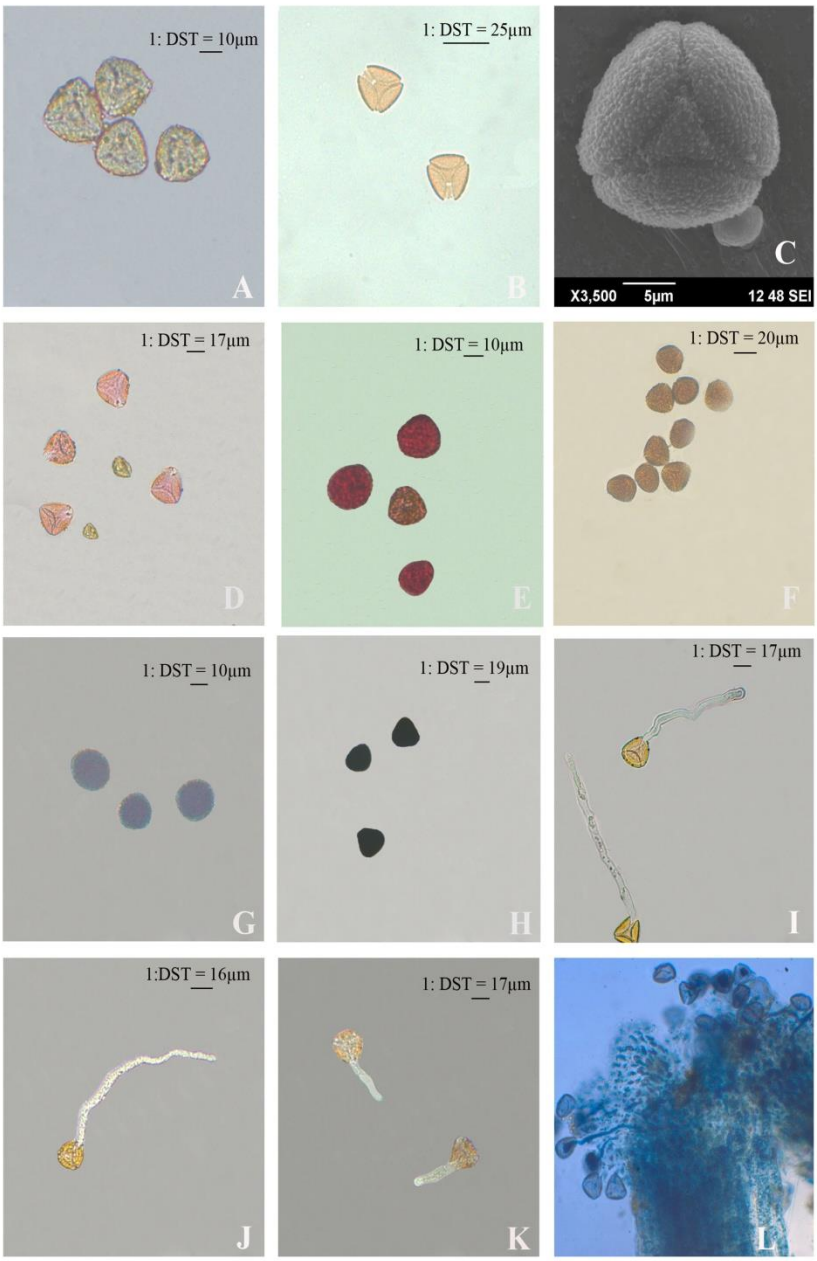

Fig. 67: Pollen biology of *N. parvifolia*. **A.** Normal pollen, **B.** Pollen-Acetolysis, **C.** Pollen SEM photography, **D & E.** Pollen viability, **D.** 0.1 % TTC method, **E.** Acetocarmine Test, **F - H.** Pollen biochemical test. **F.** Starch test, **G.** Protein test, **H.** Lipid test, **I - K.** *In vitro* pollen germination. **I.** Germination in 12% Brewbaker & Kwack's solution, **J.** Germination in 8% sucrose, **K.** Germination in 20% sucrose, **L.** Pollen germination on stigma.

Pollen germination method

***In vitro* method** (Table 38, Fig. 68)

The germination of pollen grains was tested in Brewbaker and Kwack's medium and sucrose solution in different concentration. In sucrose solution, 78% of pollen germinated in 8% of sucrose solution, and the mean pollen tube length is125.56 ± 21.52 after 3 hours. But in Brewbaker and Kwack's medium 78 % of pollen germinated in 12% solution with mean pollen tube length is176.25 ± 37.59 after 3 hours.

Sl No.	Percentage of solutions	Brew bakers &Kwack Solution		Sucose solution	
		Percentage of germination	Mean pollen tube length (µm)	Percentage of germination	Mean pollen tube length (µm)
1	4	30	67.16 ±12.4	69	65.4 ± 11.7
2	8	60	120.43 ± 17.52	78	125.56 ± 21.52
3	12	78	176.25 ± 37.59	57	42.89 ± 10.72
5	16	45	73.87 ± 18.1	38	51.11 ± 12.59
6	20	39	22.26 ± 2.9	18.33 ± 4.3	22.68 ± 6.8
7	24	0	0	0	0

Table.38. *In vitro* pollen germination of *N. parvifolia*

***In vivo* method** (Table 39, Fig. 68)

In vivo pollen germination study shows that maximum pollen germination occurs in post anthesis period. The percentage of pollen germination in post anthesis is 52.92 ± 3.99 % with a mean of pollen tube length 149.03 ± 55.37 µm after 1 hour.

Sl. No	Parameter	Mean no. of pollen on stigma	Mean no. of germinated pollen	Percentage of pollen germinated	Mean no. of pollen tube length
1	Pre anthesis	0	0	0	0
2	Anthesis	23.5 ± 4.9	6.8 ± 0.8	27.56 ± 4.6	33.81±9.7
3	Post anthesis	29.2 ± 5.6	15.4 ± 2.79	52.92±3.99	149.03±55.37

Table.39. *In vivo* pollen germination of *N. parvifolia*

Stigma receptivity

Direct observation method (Fig. 70)

The receptive stigma is transparent and wet, and also adheres to 1mm^2 piece of paper (considered as receptive).

Hydrogen peroxide test (Table 40; Fig. 70)

The stigmatic surface produced oxygen bubbles at receptive time when it is treated with hydrogen peroxide. The stigma showed more bubbles production during 12.00 pm (**53 ± 4** bubbles / min) – 2.00 pm **56 ± 2** bubbles / min). The peak time of receptivity of stigma is at 1.00 pm (*ie.* stigma produced 60.47 ± 1.54 bubbles/ min. H= 8.6884, p value = 0.01298).The receptivity gradually decreases after 1.00 pm.

Sl. No.	Time	Mean no. of bubbles	H value	p value
1.	08.00 am	0	0	0
2.	09.00 am	7.93 ± 1.41	14.82	0.0061
3.	10.00 am	24.73 ± 1.21	8.9232	0.01154
4.	11.00 am	49.57 ± 1.93	11.781	0.00277
5.	12.00 pm	52.57 ± 1.93	7.8548	0.01298
6.	01.00 pm	60.47 ± 1.54	8.6884	0.01298
7.	02.00 pm	51.1 ± 1.43	12.4652	0.00196
8.	03.00 pm	43.13 ± 1.31	12.4284	0.002
9.	04.00 pm	31.7 ± 1.469	7.8219	0.02002
10.	05.00 pm	24.1 ± 1.35	7.3181	0.02576
11.	06.00 pm	13.37 ± 1.74	10.2445	0.00596

Fig. 40: H_2O_2 Test of stigma receptivity in *N. parvifolia*

Histochemical localization of esterases on stigmatic surface (Table 41; Fig. 70)

The stigma becomes dark brown during more receptive time when it was treated with α-naphthyl acetate solution. The test proved that the stigma was more receptive during 12.00 am - 2.00 pm. After 2.00 pm the stigmatic colour changes from Black to brown when it treated with α-naphthyl acetate solution, indicated the stigma receptivity gradually decreases.

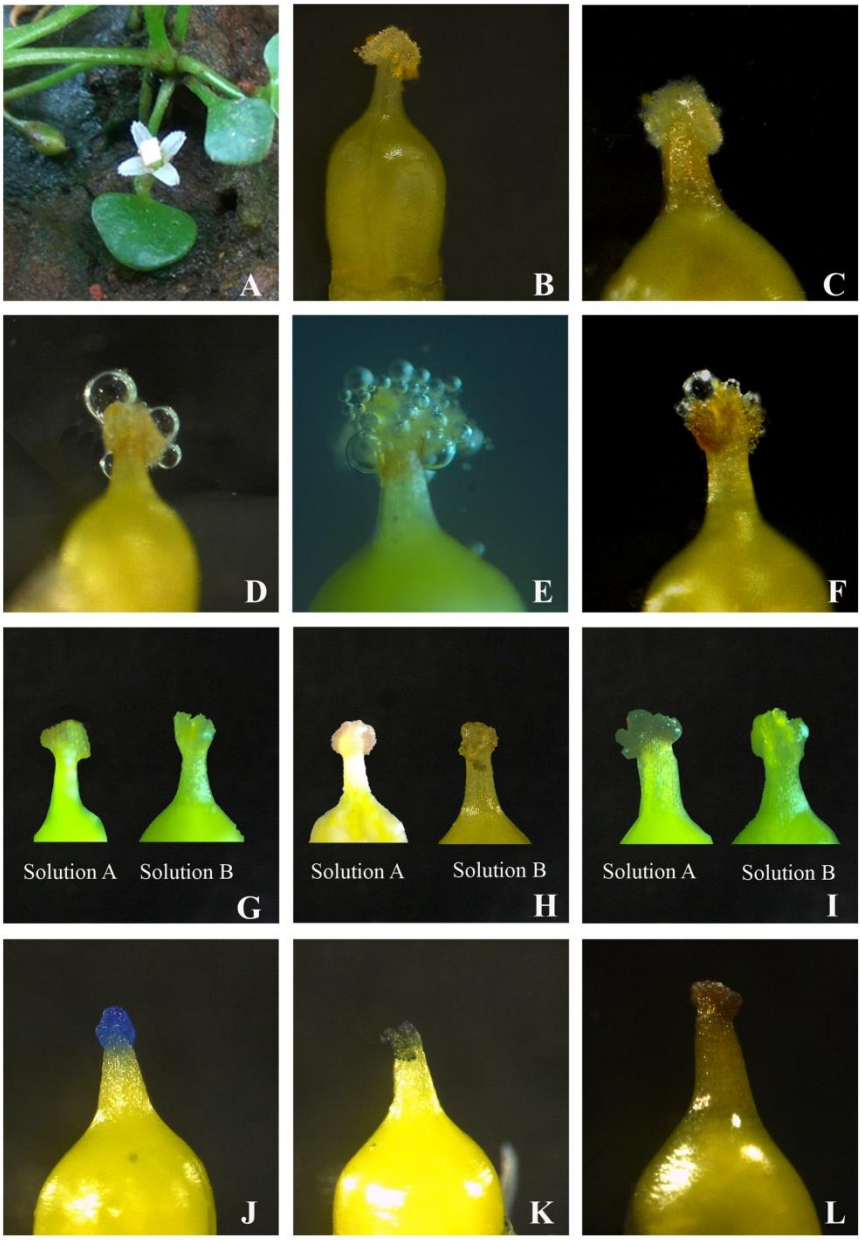

Fig. 69: Stigma receptivity & Stigma biology of *N. parvifolia*. **A - F.** Stigma receptivity Tests. **A.** Observation method, **B.** Stigma , **C - F.** H2O2 Method. **C.** During Pre anthesis, D. During Anthesis , **E.** At 1.00 pm, **F.** At 5.00 pm, **G - I.** α-Naphthyl Test (Histochemical localization of esterases). **G.** During pre anthesis, **H.** During anthesis, **I.** At 1.00 pm, **J - L.** Stigma biochemical test. **J.** Protein test, **K.** Lipid test, **L.** Starch test.

Sl. No.	Time	Frequency of colour
1	07.00 am	Colourless
2	08.00 am	Colourless
3	09.00 am	Colourless
3	10.00 am	Light brown colour
4	11.00 am	Brown Colour
5	12.00 pm	Dark Brown – Black colour
6	02.00 pm	Dark Brown – Black colour
5	03.00 pm	Bown
6	06.00 pm	Light brown

Table. 41. Histochemical localization of esterases on stigma of *N. parvifolia*

Stigma – Biochemical analysis (Fig. 70)

The stigma becomes blue black colour when it was stained with Sudan black, which indicating the presence of lipids. The stigmatic colour changed to black indicating the presence of starch when stained with I_2KI and it becomes blue colour when stained with Coomassie brilliant blue R indicating the presence of protein.

Pollination Biology:

Floral visitors and their behaviour (Fig. 71 and 72)

Anthesis of *N. parvifolia* is at 08.00 – 09.00 am. Insect's visits started soon after anthesis. Honey is present in the flower in very little amount. During the study time small flies under the order Dipteraare visited to the flowers. *Notiphila* spp. is the main pollinator. Insects visited 2 - 3 flowers by a single visit. At the time of visiting the body parts of the insects touched to the stamen and the pollen grains adhere on the insect body. They spent 5 – 10 minutes in to the flower and deposited pollen on stigma of the same flower or another flower.

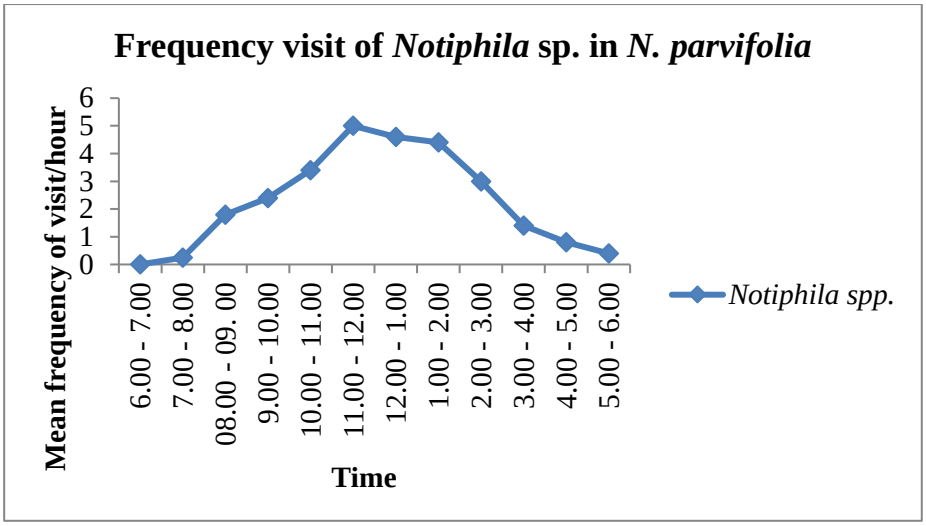

Fig. 71: Frequency of pollinator visit in *N. parvifolia*

Pollination efficiency (Table 42)

The Mean number of pollen per flower in 3 petal flower is 1027 ± 17.56 and mean number of pollen on stigma after pollination is 83.3 ± 8.08. Pollination efficiency is0.08. The Mean number of pollen per flower in 4 petal flower is 1360 ± 33 and total number of pollen on stigma after pollination is 90.4 ± 6.4. Pollination efficiency is 0.07.

Sl. No.	Flower type	Mean pollen per flower	Mean pollen on stigma	Pollination efficiency
1	3 anther flower	1027 ± 7.56	83.3 ± 8.08	0.08
2	4 anther flower	1360 ± 33	90.4 ± 6.4	0.07

Table.42: Pollination efficiency of *N. parvifolia*

Breeding System (Fig. 73)

The fruit set and seed set obtained from different pollination treatment were observed and shows in Fig.73.*N.parvifolia* produced fruits by self pollination and insect pollination in natural condition. In natural pollination 96% of fruit set was observed in both type of plant (3 petal flower and 4 petal flower). 90% of fruits were produced by self pollination and 100 % fruits are produced by manual cross pollination. The fruit set obtained through different pollination treatments were observed in Fig. 76.

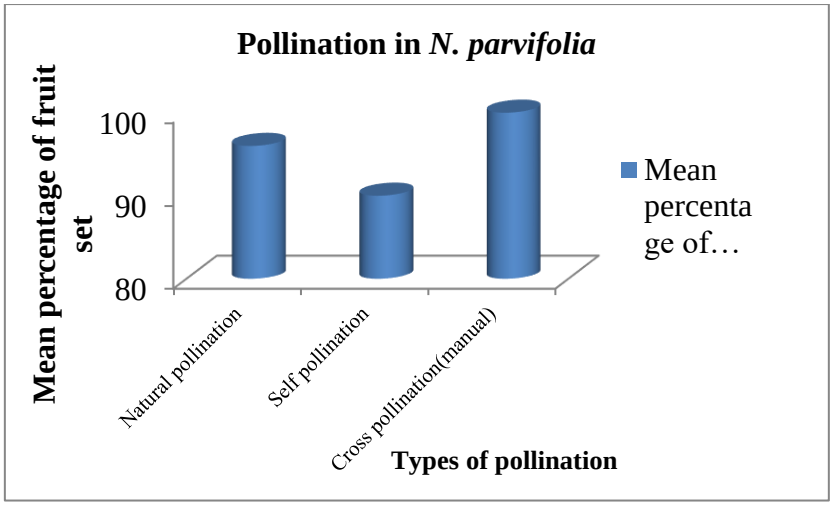

Fig. 73: Breeding in *N. parvifolia*

Fruit and seed

Capsule obovoid, ca. 8 mm long, 8 – 12 seeds in each capsule. The mean length and width of a capsule is 8 mm x 5 mm and the mean petiole length is 5mm. The fruits get ripened under water below the floating leaves. Seeds are obovoid, rounded or obtuse at distal end, subacute at proximal end, brown, 0.79mm long and 0.7 mm wide. Seed surface is tuberculated. The fruit set was observed in the natural habitat.

Flower fruit ratio and ovule -seed ratio (Table. 43)

The flower fruit ratio of *N. parvifolia* is 1 : 1 in both the flower (3 petal and 4 petal flower) and ovule – seed ratio of both flower is 5 :4.

Types of flower	Parameter	Ratio
3 petal flower	Flower : Fruit	1 : 1
	Ovule : seed	5 : 4
4 petal flower	Flower : Fruit	1 : 1
	Ovule : seed	5 :4

Table.43. Flower fruit ratio and ovule -seed ratio of *N. parvifolia*

Fruit and seed dispersal mechanisms: Fruits were matured during15th – 16th day after pollination and dehisced one or two days after maturity. The fruits detached from the mother plants. The distal point of the capsule breaks inside the

Fig. 71: Pollinator and reproduction in *N. parvifolia.***A & B.** *Diptera* spp., **C - F.** Vegetative reproduction. **C.** Vegetative reproduction in Natural place, **D - F.** New plantlets arrising from existing rhizome, **G - H.** Seed germination. G. Seed germination in 500 ppm GA, **H.** Seed germination in dung soil.

water and liberated seeds. Seeds were light weighted and float on the water surface. Later seeds sink to the bottom of the water.

Seed germination (Table 44; Fig. 72)

Seed germination was not observed in the field. In controlled conditions, seeds germinated in dung-soil mixture and Gibberelline supplemented soil. Maximum germination observed in dung-soil mixture (36.67%) after 160 - 180 days. Germination not observed in control. Scarified seed germinated after 40 - 80 days.

Sl. No.	Days	Percentage of germination (%)				Scarified seed
		Seed				
		Soil	Soil + 50 ppm gibberellin	Soil + 100 ppm gibberellin	Dung soil	Soil
2	5 – 10	0	0	0	0	0
3	10 – 20	0	0	0	0	0
4	20 – 40	0	0	0	0	1.67
5	40 – 80	0	0	0	0	0
6	80 – 120	0		0	0	0
7	120 – 140	0	5	0	0	0
8	140 – 160	0	16.67	0	0	0
9	160 – 180	0	0	13.33	23.33	0
10	180 - 200	0	0	20	36.67	0
11	200 - 220	0	0	0	0	0

Table.44. Seed germination rate of *N. parvifolia* in different condition

Vegetative propagation (Fig. 72)

Vegetative propagation of *Nymphoides parvifolia* from rhizome. The aerial shoots wilted, in summer season, but the rhizomes are in the dormant state under the soil. The dormant rhizome germinated and produced new suckers in the next rainy season. One rhizome produces 3 – 5 suckers Plate. These suckers growing into separate individual plants.

5. *Nymphoides balakrishnanii* Biju, Josekutty, Haneef & Augustine

The floral characteristics of *N. balakrishnanii* are described in Table 45.

Sl. No.	Floral Characters	Observations
1	Blooming time	August - February
2	Flower type	Actinomorphic, complete, hypogynous, petals joined at the base and free above.
3	Flower colour	White with yellow throat.
4	Odour	Absent
5	Anthesis time	08.00 am – 09.00 am
6	Anther dehiscence time	08.00 am – 08.45 am
7	Anther dehiscence mode	Through longitudinal slit
8	Number of anthers/ flower	4
9	Mean No. of pollen / anther	460 ± 93
10	Mean No. of pollen / flower	1840 ± 372
11	Mean No. of Ovules/ flower	10.8 ± 2.1
12	Pollen – Ovule ratio	157 : 1
13	Pollen type	Monad, heteropolar, radially symmetric trigonal and tricolpate
14	Pollen size	28.91 ± 2.36, medium sized grain
15	Pollen shape	Speroidal – prolate spheroidal
16	Stigma type	Bifid, Wet
17	Fruit type	Capsule
18	Flower – fruit ratio	1 : 1
19	Ovule – seed ratio	5 : 4
20	Flower closing time	03.00 -03.30 pm

Table.45. Floral characters of *Nymphoides balakrishnanii*

Phenology

Leaf development and Flower – Bud development

The morphological changes of leaf and bud in their developmental stages are the following.

Leaf development (Fig. 74and 77)

Stage 1: Leaf initiation stage noted just visible to the naked eye.

Stage 2: The shoot and the leaf are clearly distinguished in this stage but the lamina is found to be in the rolled condition below the water level from initiation to 3rd day. The mean size of the leaf lamina is 1.1 mm and the mean length of the shoot is 1.75 mm.

Stage 3: Leaf petiole is clearly observed in this stage from 5th - 6th day. Lamina is in the rolled condition below the water level. The mean length of the shoot is 11.2 mm and the mean size of the lamina is2.05 mm x 1 mm. The petiole length is 1 mm.

Stage 4: The lamina opened under water from 6th – 8th day and it is light green in colour. The mean length of the shoot is 53.1 mm and the lamina is5 mm x 2.7 mm. The mean petiole length is 1 mm.

Stage 5: Lamina reached above the water surface from 8th to 11th/12th day. It is light green in colour and glazing. The mean length of the shoot is 86.4 mm and the lamina is8.6 mm x 7.8 mm. The mean petiole length is 1 mm.

Stage 6: Lamina gets matured; disappeared its glazing and turned into dark green from 12th to 17th/18th day. The mean length of the shoot is 167.8 mm and the lamina is11.1 mm x 12.9 mm. The mean petiole length is 1 mm.

Stage 7: During this stage lamina changed its colour and turned to yellow with pink patches or yellow during 26th or 28th days. The mean length of the shoot is 167.8 mm and the lamina is 11.1 mm x 12.9 mm. The mean petiole length is 1 mm.

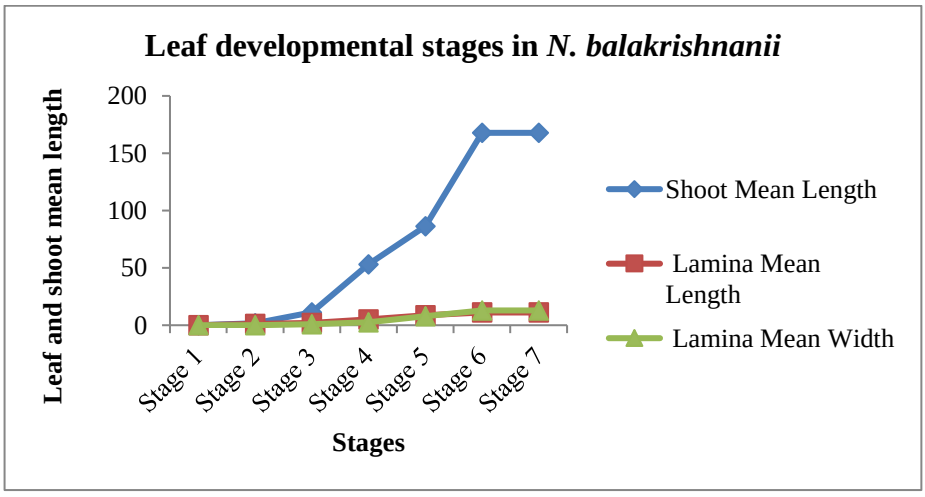

Fig. 74: Leaf developmental stages of *N.balakrishnanii*

Flower -Bud development (Fig. 75and 77)

The period of flower bud development in *N. balakrishnanii*, from its initiation to the full bloom stage could be divided into seven different stages. They are:

Stage 1: In this stage flower buds are just visible with naked eye. Flower bud initiation started at the time of leaf initiation. It is cream coloured found just below the leaf on the shoot (1st day). The mean size is 1 mm.

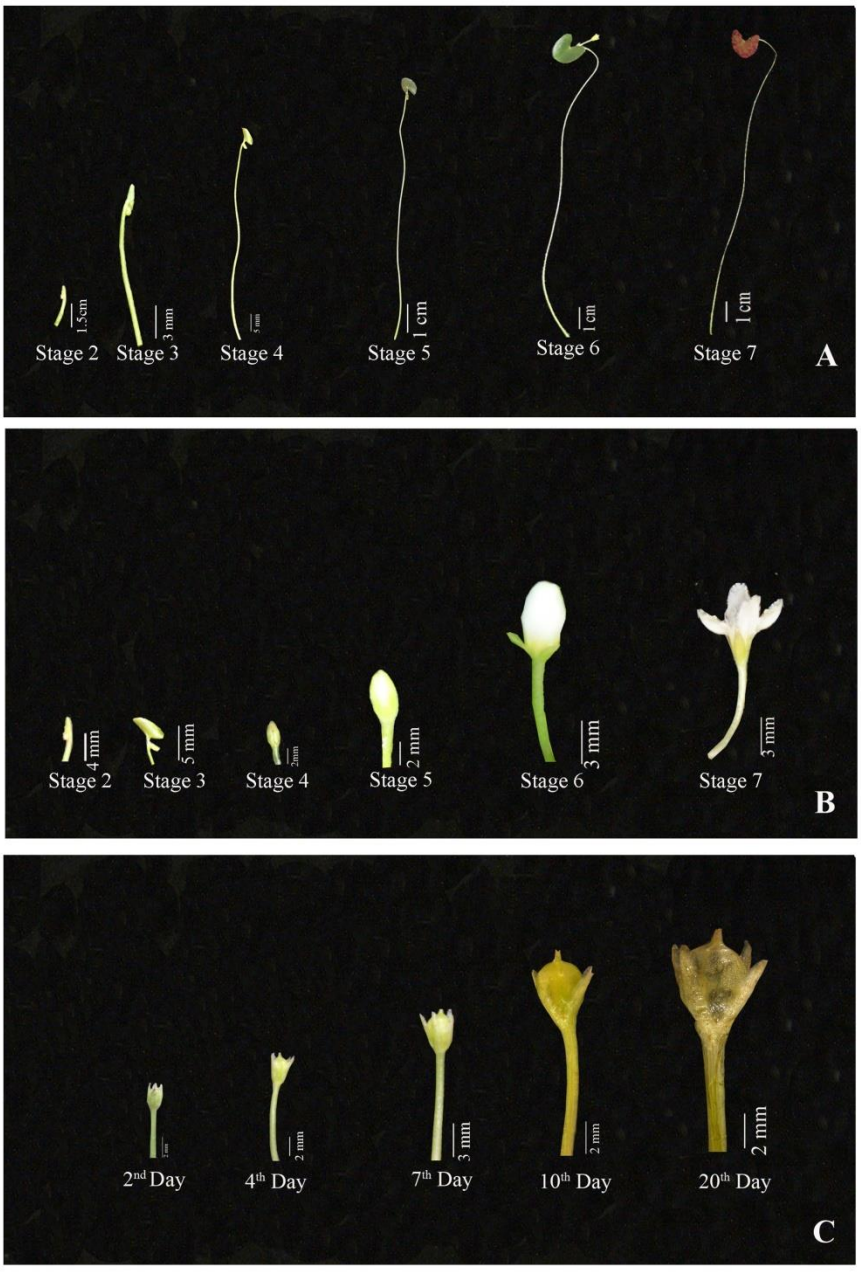

Fig. 75: Development of leaf, Flower bud & Fruit of *N. balakrishnanii*. **A.** Leaf developmental stages, **B.** Flower bud developmental stages, **C.** Fruit developmental stages.

Stage 2: From the day after initiation to 3rdday, the flower bud is obovoid, cream and covered with bract. The mean size of the bud is 1 mm.

Stage 3: During5th – 6th day, the bud emerges out to the bract. The mean size of the flower buds is 1.05 mm x 0.8 mm and the pedicel length is 0.65 mm.

Stage 4: Pedicel and sepals are clearly distinguished during 8th - 9th day. The mean size of the bud is 2.2 mm x 1.2 mm and the pedicel length is 2 mm.

Stage 5: Petals emerges out of the calyx. Pedicel slightly increased. Within 11 – 12 days the buds reach above the water surface. The mean size of the bud is 3.7 mm x 1.55 mm and the pedicel length is 4.2 mm.

Stage 6: This is the mature bud stage formed before 2 hours of anthesis (13th – 14th day). The mean bud size is 5.05 mm x 3 mm and the pedicel length is 7 mm.

Stage 6: This is the full bloom stage in which the flower becomes opened on the 14th/15th day and exposing the various whorls and reproductive parts. The mean diameter of the flower is 8.15 mm.

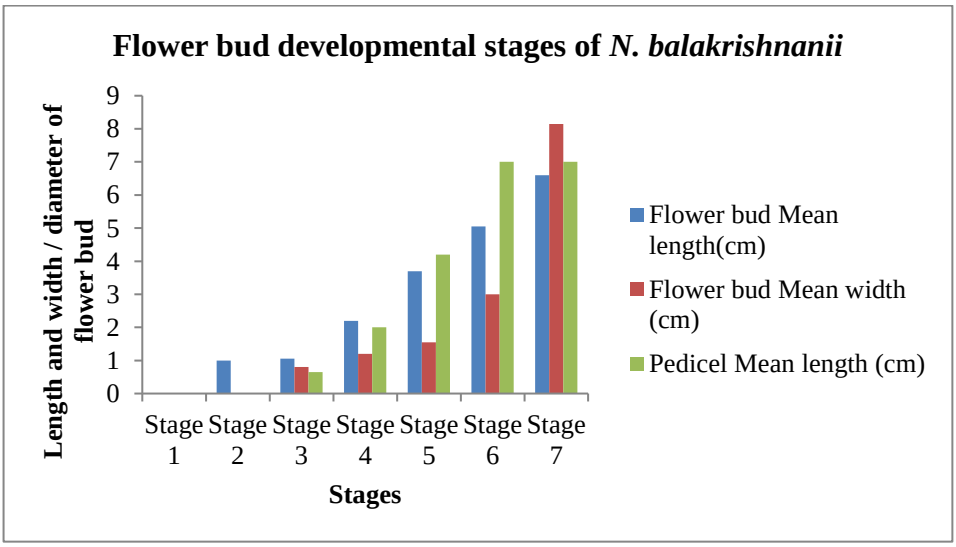

Fig. 75: Flower bud developmental stages in *N. balakrishnanii*

Fruit development (Fig. 76and 77)

Various stages of fruit development from pollination to the maturation and its dehiscence were recorded. The time required for the starting of fruit development to the fruit dehiscence was noted as 25th – 26th day.

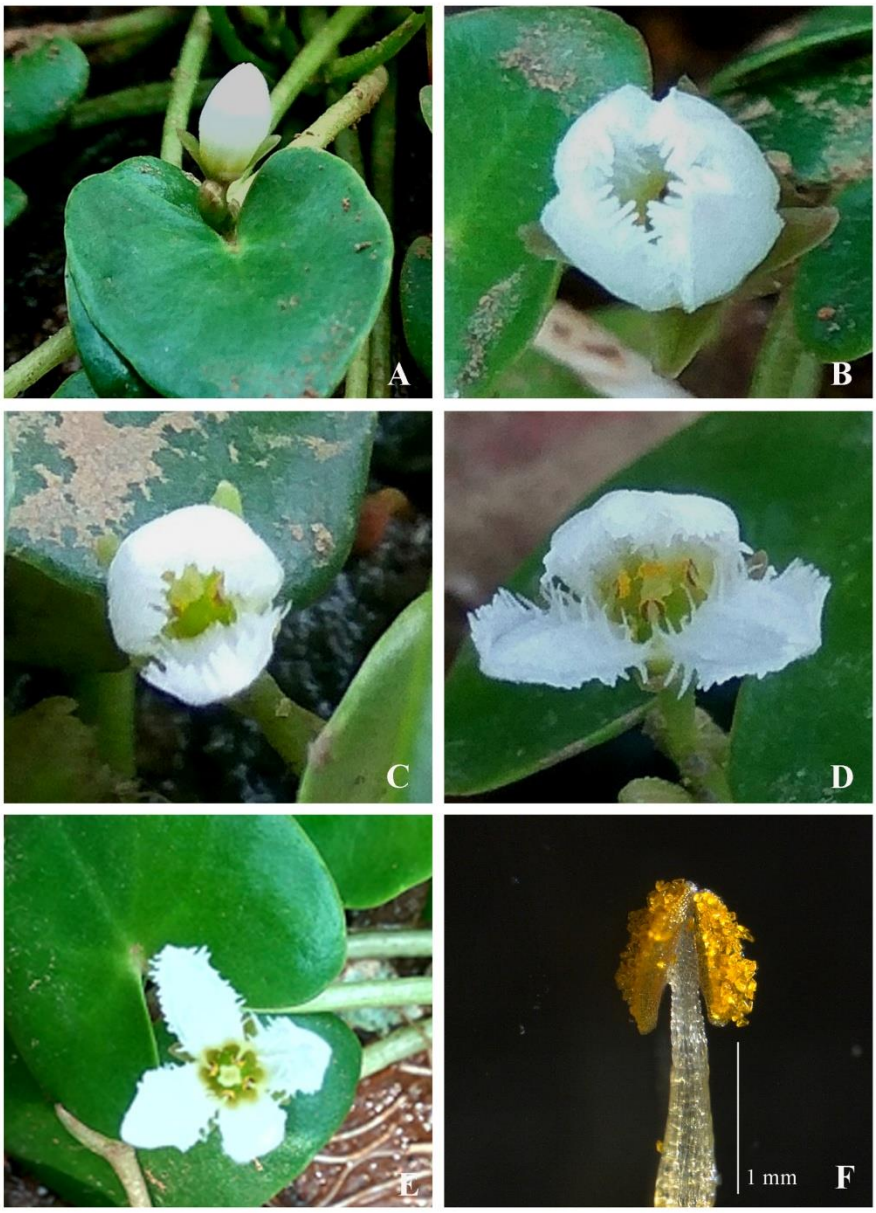

Fig. 77: Anthesis & Anther dehiscence of *N. balakrishnanii.* **A - E.** Different stages of anthesis of flower**, F.** Anther dehiscence.

Flowering activity in *N. balakrishnanii* (Fig. 78)

The flowering time begins from the month of August. Post monsoon season is the peak time of flowering. The month of June –July is the maturity time. February – May showed the dormant stage of rhizome.

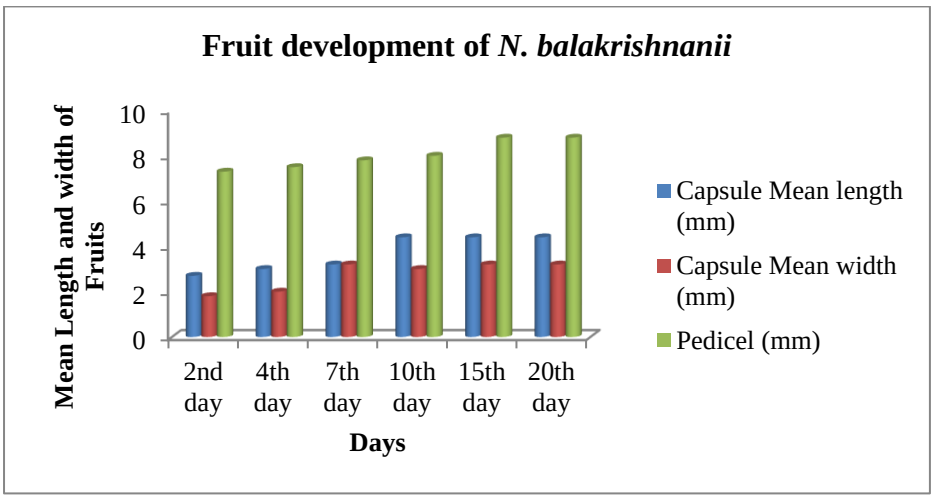

Fig. 76:Fruit developmental stages in *N. balakrishnanii*

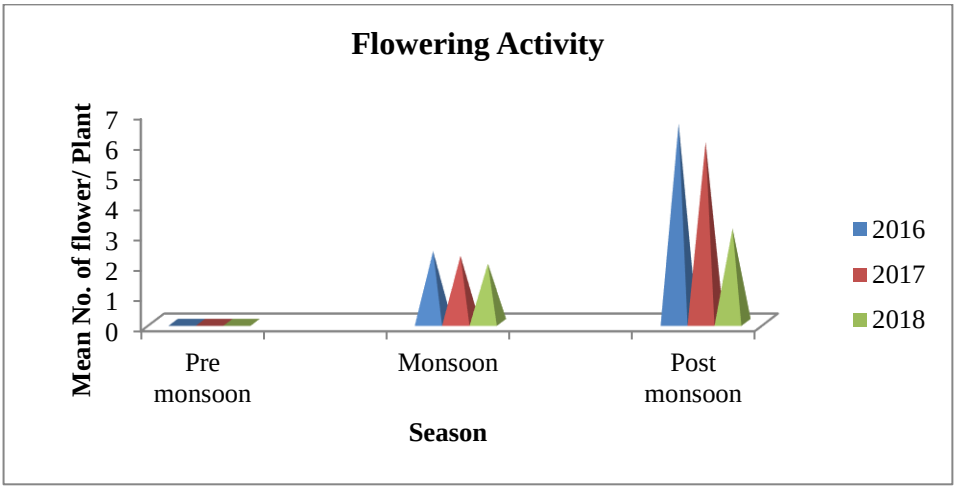

Fig. 78: Flowering activity of *N. balakrishnanii*

Anthesis (Fig. 79)

Anthesis of *N. balakrishnanii* started during 08.00 am – 09.00 am. The anther dehiscence starts at the time of anthesis. The anther dehisced through a longitudinal slit.

Pollen morphology (Fig. 80)

The pollen grains are medium sized, monad, triangular, heteropolar, radially symmetrical, parasyncolpate, spheroidalto prolate – spheroidal in shape. Mean

polar axis is 28.91 ± 2.36 µm and the mean equatorial axis is 28.02 ± 2.06 µm. P/ E ratio is 103.16 ± 3.4 µm (n =100). The pollen surface is spinulose.

Pollen – Biochemical analysis (Fig. 80)

Pollen grains became purple or brown due to the presence of newly formed starch when treated with I_2KI solution and become blue, when stained with Coomassie brilliant blue solution, which indicated the presence of protein on the stigmatic surface. The pollen grains become black when it stained with Sudan black due to the presence of lipids.

Pollen production

N. balakrishnanii has four stamens in a flower. The mean number of pollen per anther is 460 ± 93 (n=30) and the mean number of pollen per flower is 1840 ± 372 (n=30).

Pollen – Ovule ratio: Mean number of pollen / flower is1840 ± 372 and the mean number of ovule is 10.8 ± 2.1. Pollen ovule ratio is 170:1

Pollen viability (Fig. 80& 81)

The pollen grains starts its viability before 24 hours of anthesis and shows maximum viability before 2 hrs anthesis time (50.63 ± 3.6) and TTC (48.53 ± 3.3). Pollen viability decreases after anthesis and gradually lost.

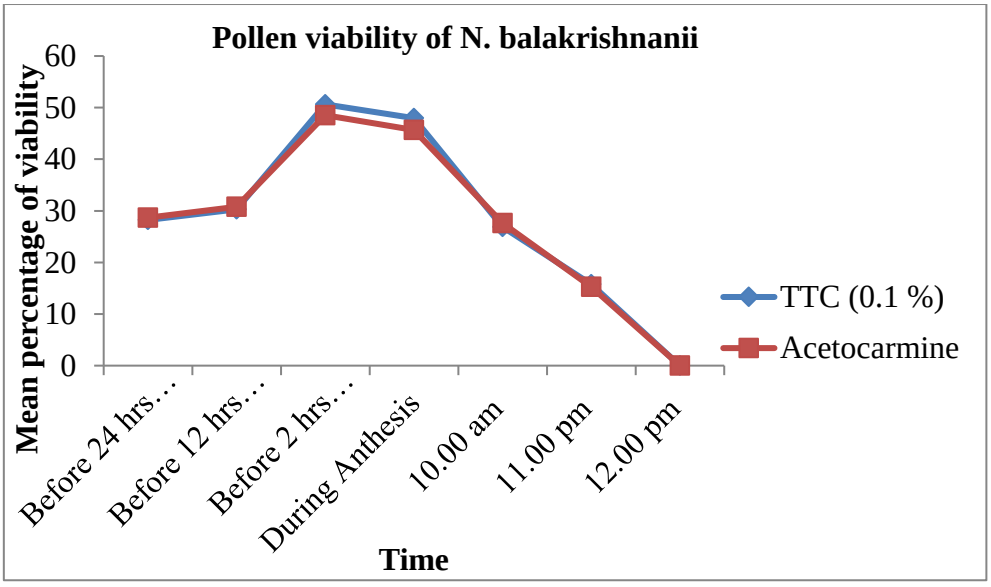

Fig. 81: Pollen viability of *N. balakrishnanii*

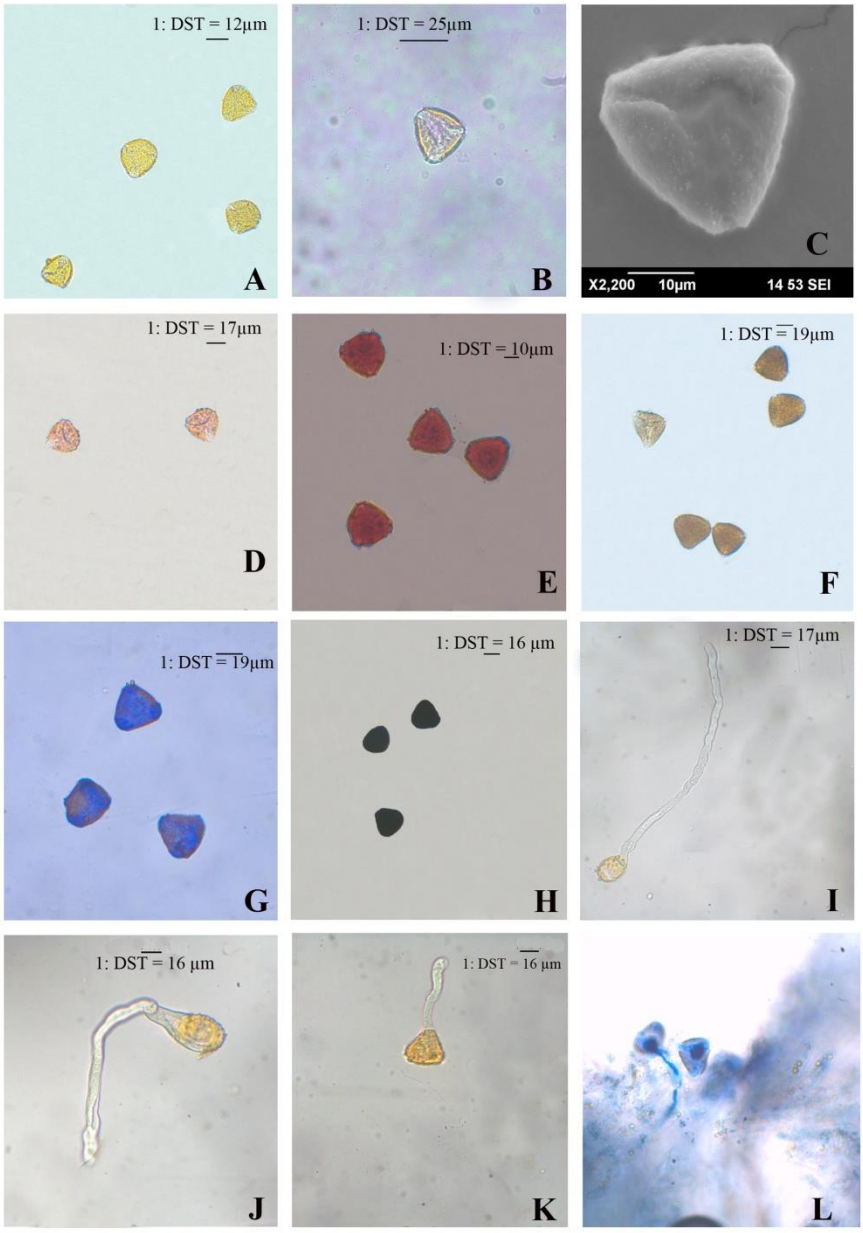

Fig. 78: Pollen biology of *N. balakrishnanii*. **A.** Normal pollen, **B.** Pollen showing exine ornamentation, **C.** Pollen SEM photography, **D & E.** Pollen viability, **D.** 0.1% TTC method, **E.** Acetocarmine Test, **F - H.** Pollen biochemical test. **F.** Starch test, **G.** Protein test, **H.** Lipid test, **I - K.** *In vitro* pollen germination. **I.** Germination in 16% Brewbaker & Kwacks solution, **J.** Germination in 12% sucrose, **K.** Germination in 20% sucrose, **L.** Pollen germination on stigma.

Pollen germination method

***In vitro* method** (Table 46, Fig. 80)

In Brewbaker and Kwack's medium the pollen grains germinated maximum in 12% solution (62 %)and the mean pollen tube length is632.03 ± 96.49.44 µm after 3 hours and77 % of pollen germinated in 12% of sucrose solution and the mean pollen tube length is 118.01 ± 28.05µm. The growth of pollen tube length is maximum in 12% Brewbaker and Kwack's solution and minimum in 20% solution.

Sl No.	Percentage of solutions	Brewbaker & Kwack's Solution		Sucrose solution	
		Percentage of germination	Mean pollen tube length (µm)	Percentage of germination	Mean pollen tube length (µm)
1	4	14	108.77 ± 13.82	37	65.85 ± 9.8
2	8	23	148.7 ± 40.59	53	99.32 ± 22.06
3	12	62	632.03 ± 96.49	77	118.01 ± 28.05
4	16	40	100.7 ± 26.03	47	59.87 ± 12.85
5	20	12	0	0	0
6	25	0	0	0	0

Table. 46. *In vitro* Germination of pollen in *N. balakrishnanii*

***In vivo* method** (Table 47, Fig. 80)

In vivo pollen germination study shows that maximum pollen germination recorded in post anthesis period. The percentage of pollen germination in post anthesis is 52.98 % with a mean of 129.33µm long pollen tube.

Sl. No	Parameter	Mean no. of pollen on stigma	Mean no. of germinated pollen	Percentage of pollen germinated	Mean no. of pollen tube length(µm)
1	Pre anthesis	0	0	0	0
2	Anthesis	18.4	2.4	12.76	8.23 ± 3
3	Post anthesis	29.2	15.4	52.98	129.33 ± 3

Table.47:*In vivo p*ollen germination in *N. balakrishnanii*

Stigma receptivity

Direct observation method (Fig. 82)

The receptive stigma is transparent, wet and also adhere to 1mm^2 piece of paper (considered as receptive).

Hydrogen peroxide test (Table 48; Fig. 82)

The stigma produced oxygen bubbles at receptive time when it was treated with hydrogen peroxide. The table shows the oxygen bubbles produced by stigma at different time intervals. Stigma produce maximum bubbles at 12.00 pm – 2.00 pm, indicatethat the stigma becomes more receptive at 12.00 pm – 2.00 pm. The peak time of receptivity is at 1.00 pm (59.93 ± 1.26 bubbles/min, H = 9.1413, p value = 0.01035). The stigmatic receptivity gradually decreases after 2.00 pm.

Sl. No.	Time	Mean no. of bubbles	H value	p value
1.	08.00 am	0	0	0
2.	09.00 am	4.6 ± 1.19	10.7439	0.00465
3.	10.00 am	21.23 ± 1.63	6.3877	0.04101
4.	11.00 am	36.57 ± 1.94	7.8374	0.01987
5.	12.00 pm	58.6 ± 1.83	9.7052	0.00781
6.	01.00 pm	59.93 ± 1.26	9.1413	0.01035
7.	02.00 pm	58 ± 1.29	9.7374	0.00768
8.	03.00 pm	39.83 ± 1.34	6.72	0.03474
9.	04.00 pm	33.87 ± 1.28	6.5968	0.03694
10.	05.00 pm	22.5 ± 1.95	12.2658	0.00217
11.	06.00 pm	18.1 ± 1.24	13.5348	0.00115

Table. 48 : Hydrogen peroxide test of *N. balakrishnanii*

Histochemical localization of esterases on stigmatic surface (Table 49;Fig. 82)

The dark brown coloured stigma between 1.00 pm – 2.00 pm showed maximum receptivity when treated with α – Naphthyl acetate solution. After 2.00 pm the stigmatic colour decreases in α – Naphthyl acetate solution (Soiution A) due to the decreases of stigma receptivity.

Sl. No.	**Time**	**Frequency of colour**
1	06.00 am – 08.00 am	Colourless
3	09.00 am – 10.00 am	Light brown
3	11.00 am – 12.00 pm	Dark Brown - Black
5	01.00 pm - 02.00 pm	Dark Brown – Black
6	02.00 pm – 03.00 pm	Brown
5	4.00 pm – 5.00 pm	Light brown colour
6	06.00 pm – 7.00 pm	Colourless

Table. 49. Histochemical localization of esterases on stigma of *N. balakrishnanii*

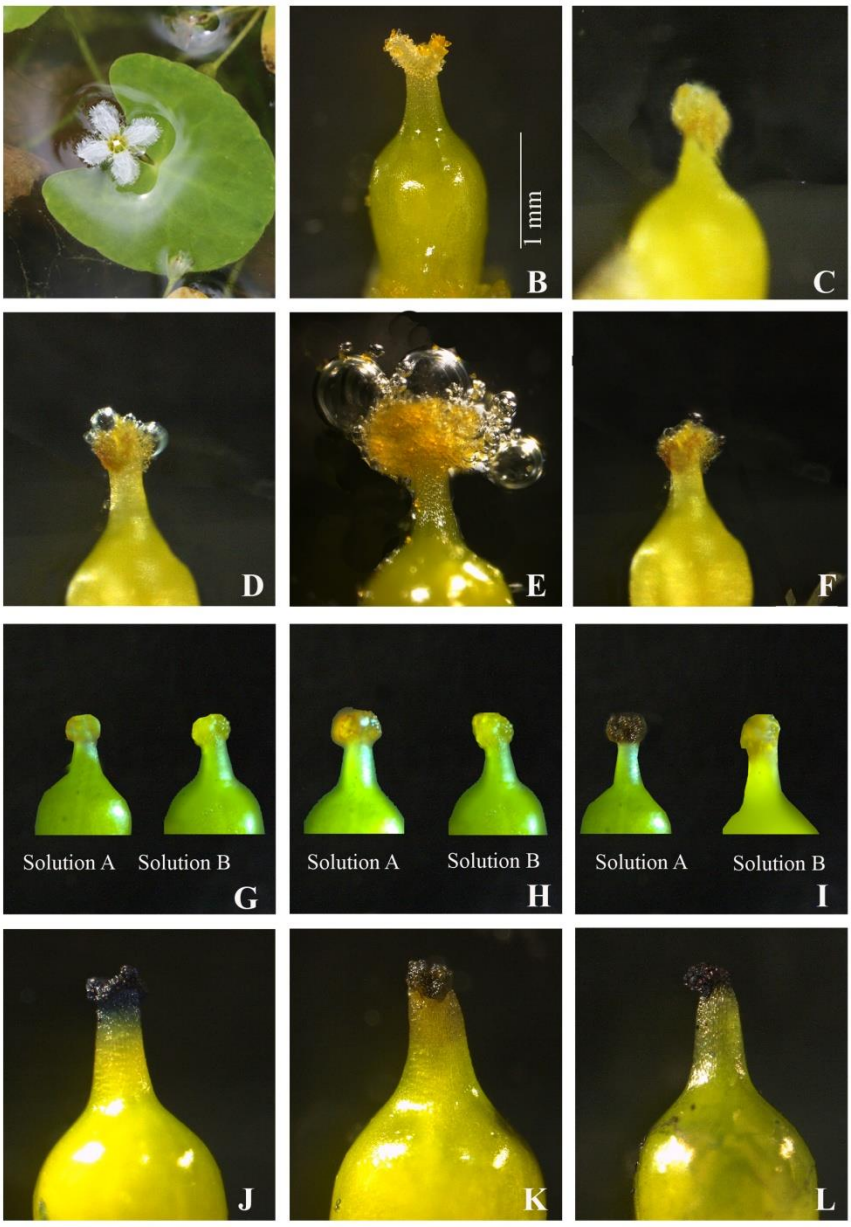

Fig. 82: Stigma receptivity & Stigma biology of *N. balakrishnanii*. **A - F.** Stigma receptivity Tests. **A.** Observation method, **B - F.** H_2O_2 Method. **B.** Stigma , **C.** During Pre anthesis, D. During Anthesis ,**E.** At 1.00 pm, **F.**At 5.00 pm, **G - I.** α-Naphthyl Test (Histochemical localization of esterases). **G.** During pre anthesis, **H.** During anthesis, **I.** During post anthesis, **J - L.** Stigma biochemical test. **J.** Protein test, **K.** Starch test, **L.** Lipid test.

Stigma – Biochemical analysis (Fig. 82)

The stigma becomes blue black when it was stained with Sudan black, which indicated the presence of lipids. The stigmatic colour changed in to brown indicated the presence of starchit stained withI_2KI solution and when it becomes blue colour stained with Coomassie brilliant blue indicate the presence of protein. The stigmatic surface contains lipids, proteins and starch.

Pollination Biology:

Floral visitors and their behaviours (Table 50; Fig. 83 and 84)

Insects are visited at the time of anthesis. Honey is present in very little amount(less than 1µl). During the study time ants, *Notiphila* sp. and the insects under the family Tridactylidaeareobserverved as the floral visitors in the flower. *Camponotus sericeus* (ant), *Componatus* sp., *Notiphila* sp. and *Xya* sp. are the main floral visitors of *N. balakrishnanii. Notiphila* sp. visit 2 – 3 flowers by a single visit and *C. sericeus*visit 3 - 5 flowers by a single visit. At the time of visit the body parts of the insects touched to the stamen and the pollen grains adhered to the insect body. They spent 6 – 8 minutes on flowers and deposited pollen on stigmatic surface. *Xya* spp. visited 1 – 2 flowers at a time.

Pollination efficiency:

The Mean number of pollen per flower 1840 ± 372.9 and total number of pollen on stigma after pollination is 71.3 ± 8.08. Pollination efficiency is 0.06.

Sl. No.	Name of the visitors	Family	Foraging Nature	Foraging hours	Time spent in each flower	Stigma touch	Frequency of visit
1	*Camponotussericeus*	Formicidae	Nectar + pollen	7am – 6 pm	5 – 8 minutes	+++	High
2	*Componatus*sp.	Formicidae	Nectar + pollen	8.00 am – 4 pm	3- 5 minutes	++	intermediate
3	*Notiphila sp.*	Ephhydridae	Nectar + pollen	8.00 am – 6 pm	4 – 5 minutes	+++	High
2	*Xya*spp.	Tridactylidae	pollen	9 am – 6 pm	4 – 8 seconds	++	intermediate

Table. 50. *N. balakrishnanii* floral visitors and their behaviours

Stigma touch +++: very good, ++: good, +: poor, -: no touch.
Frequency of visit: high (10 – 40 visit/day), intermediate (4 – 9 visit/day), low (<3 visit/day).

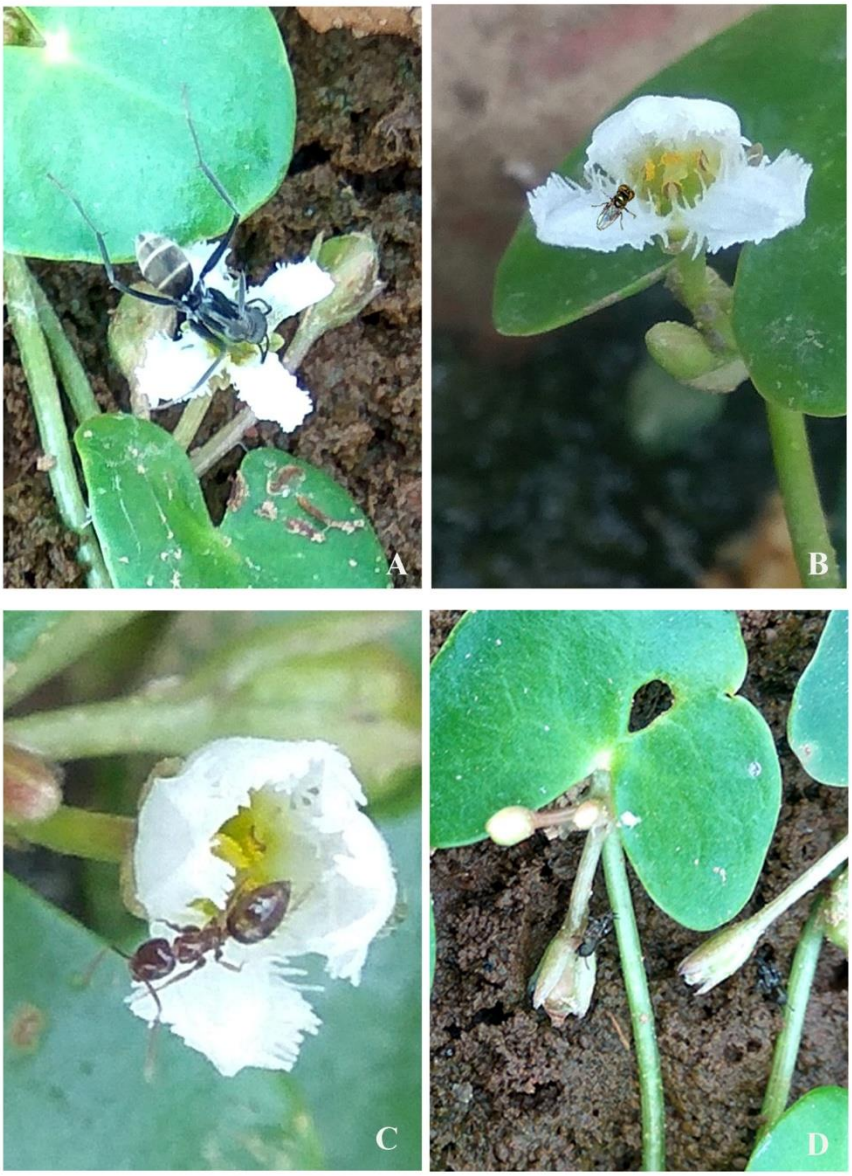

Fig. 81: Floral visitors of *N. balakrishnanii*. **A .** *Componatus sericeus,* **B.** *Notiphila* spp., **C.** *Componatus* spp., **D.** *Xya* spp.

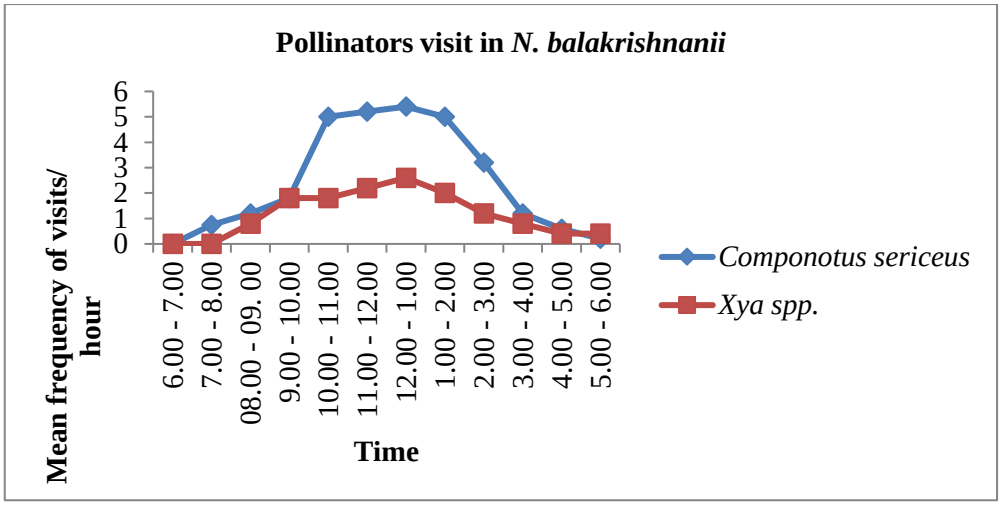

Fig. 84: Frequency visit of Pollinators in *N. balakrishnanii*

Breeding System (Fig. 85)

The fig. 85 shows the fruit set obtained from different pollination treatments. In natural pollination 98% fruits are produced. But in self pollination 94 % fruits were produced. Through manual cross pollination plant produced 100% fruits.

Fruit and seed

Capsule oblong – obovoid, 4 mm x 2 mm length, pedicel 2 cm and turned light yellow when it matured. Calyx is persistant and exeeds or equal to the fruits. The fruit consists of 5 – 13 seeds in each capsule. Seeds are obovoid, rounded, brownish black in colour, 1 mm long and 1 mm wide. Seed surface is tuberculate. Tubercles are clustered.

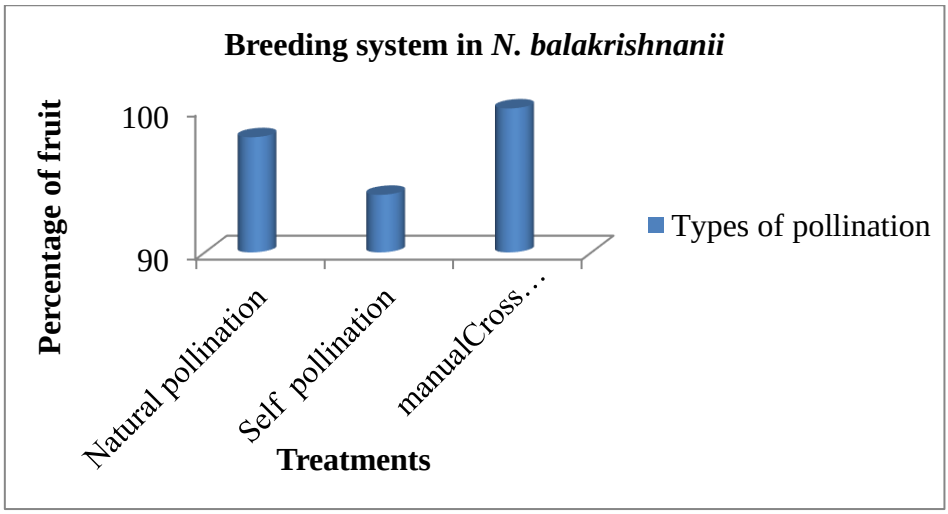

Fig.85: Breeding System in *N. balakrishnanii*

Flower fruit ratio and ovule -Seed ratio (Table 51)

The flower fruit ratio of *N. balakrishnanii* is 1 : 1 and ovule – seed ratio 5 :4.

Sl. No.	Parameter	ratio
1	Flower : Fruit	1 : 1
2	Ovule : Seed	5 : 4

Table.51. Flower fruit ratio and ovule -Seed ratio in*N. balakrishnanii*

Fruit and seed dispersal mechanisms

Fruits matured at 25th – 26thdays after pollination and dehisced one or two days after maturity. The fruit breaks and liberated seeds out to the water. Seeds were light weighted and float on the water surface. Later seeds sink to the bottom of the water.

Seed germination (Table 52; Fig. 86)

Seed germination was not observed in the field. In controlled conditions, seeds germinated in dung-soil mixture and Gibberelline supplemented soil. Maximum germination observed in dung-soil mixture (43.33%) after 180 - 200 days. Germination not observed in control. Scarified seedsgerminated after 20 – 40 days.

Sl. No.	Days	Percentage of germination (%)				Scarified seed
		Seed				
		Soil	Soil + 50 ppm gibberellin	Soil + 100 ppm gibberellin	Dung soil	Soil
2	5 – 10	0	0	0	0	0
3	10 – 20	0	0	0	0	0
4	20 – 40	0	0	0	0	5
5	40 – 80	0	0	0	0	0
6	80 – 120	0	35	0	0	0
7	120 – 140	0	6.67	3.33	0	0
8	140 – 160	0	23.33	16.67	13.33	0
9	160 – 180	0	0	0	33.33	0
10	180 - 200	0	0	0	43.33	0
11	200 - 220	0	0	0	0	0

Table.52. Seed germination rate of *N. balakrishnanii* in different condition

Vegetative propagation (Fig. 86)

The plant reproduces vegetatively through rhizome and detached leaves with part of shoot attached. The dormant rhizome germinated and produced new suckers in the next rainy season. One rhizome produces 3 – 5 suckers. These suckers growing into separate individual plants.

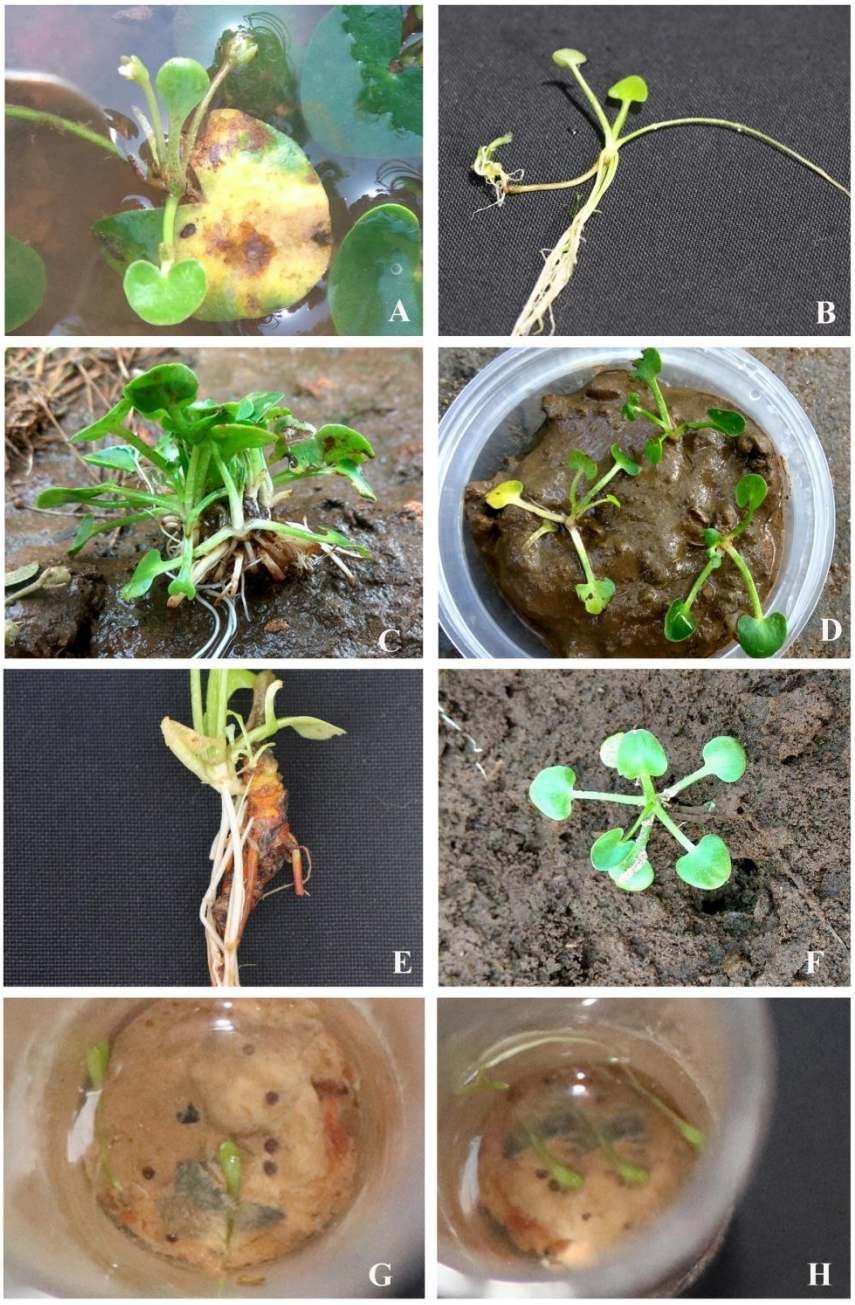

Fig. 84: Reproduction in *N. balakrishnanii*. **A - E.** Vegetative reproduction. **A & B.** New plants produce from shoots, **C - E.** New plants produce from rhizome, **F.** New plants in natural habitat, **G - H.** Seed germination. G. Seed germination in 500 ppm GA, **H.** Seed germination in dung soil

7. *Nymphoides macrosperma* R.V.Nair

The floral characteristics of *N. macrosperma are described in the* Table. 53

Sl. No.	Floral Characters	Observations
1	Blooming time	October - December
2	Flower type	Actinomorphic, unisexual, hypogynous, petals joined at the base and free above.
3	Flower colour	White
4	Odour	Absent
5	Anthesis time	08.00 am – 09.00 am
6	Anther dehiscence time	08.00 am - 08.30 am
7	Anther dehiscence mode	Through longitudinal slit
8	Number of anthers/ flower	5
9	Mean No. of pollen / anther	620 ± 160.6
10	Mean No. of pollen / flower	3100 ± 803
11	Mean No. of Ovules/ flower	3.6 ± 0.81
12	Pollen – Ovule ratio	861.11 : 1
13	Pollen type	Monad, triagonal and tricolpate
14	Pollen size	28.81 ± 0.82 µm
15	Pollen shape	prolate – speroidal – sub-prolate
16	Stigma type	Bifid, Wet and hairy
17	Fruit type	Capsule
20	Flower closing time	4.00 pm – 4.30 pm

Table.53. Floral characters of *N. macrosperma*

Phenology

Leaf development and Flower – Bud development

The morphological changes of leaf and bud in their developmental stages are the following.

Leaf development (Fig. 86 and 87)

Stage 1: Leaf initiation stage noted just visible to the naked eye.

Stage 2: The shoot and leaf was clearly distinguished from this stage but the lamina was found to be in the rolled condition. The mean length of the shoot was 2.2 mm and that of the leaf lamina was recorded as 2 mm.

Stage 3: Leaf petiole was clearly observed in this stage. Lamina was in the unrolled condition inside the water. The mean length of the shoot was 60.4 mm x 4 mm long and the mean length and width of the lamina were recorded as 4.2 x 2.2 mm. Petiole length was measured as 3.8 mm x 1.9 mm.

Stage 4: The lamina was opened during this stage (Plate). The lamina opened under water and pink colour appears on the leaf. The mean length of the shoot was 121.6mm x 5 mm and lamina was recorded as 33.4 mm x 11.4 mm.

Stage 5: Leaf lamina reached on the water surface. Lamina become glazing and green with brown patches on the lamina. Mean length of the shoot is 154.2 mm. The length and width of the lamina was recorded as 41.6 mm x 49.8 mm.

Stage 6: The colour of the leaf lamina changed to green, disappeared its glazing and brown colour. Mean length of the shoot is 210 mm. The length and width of the lamina was 68.4 mm x 66.8 mm.

Stage 7: During this stage lamina changed its colour and turned to yellow. The length and width of the lamina was 68.4 mm x 66.8 mm.

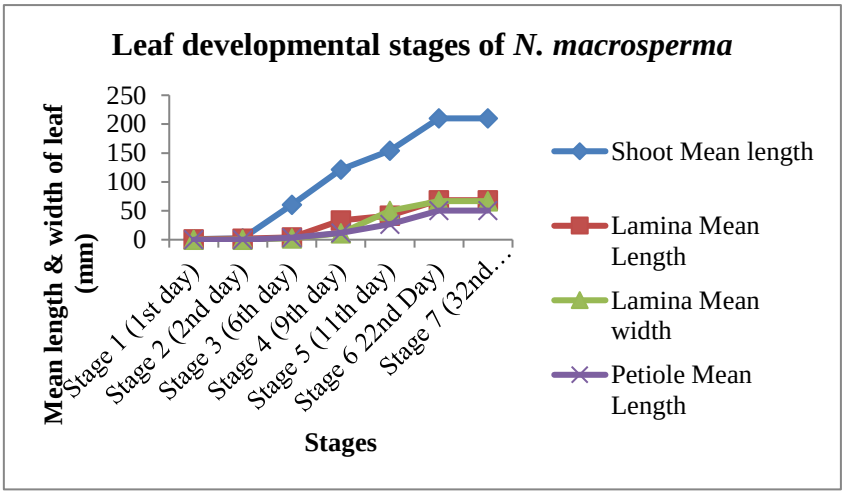

Fig. 87: Leaf developmental stages of *N. macrosperma*

Flower -Bud development (Fig. 87 and 88)

The period of flower bud development in *N. macrosperma* from its initiation to the full bloom stage could be divided into seven different stages. They are:

Stage 1: In this stage flower buds are just visible with naked eye. Flower bud initiation started at the time of leaf initiation. It is cream coloured, found just below the leaf (1st day).

Stage 2: From the day after initiation to 3rdday flower bud is obovoid and covered with bract. The size of the buds was recorded a 1.15 mm x 1.7 mm.

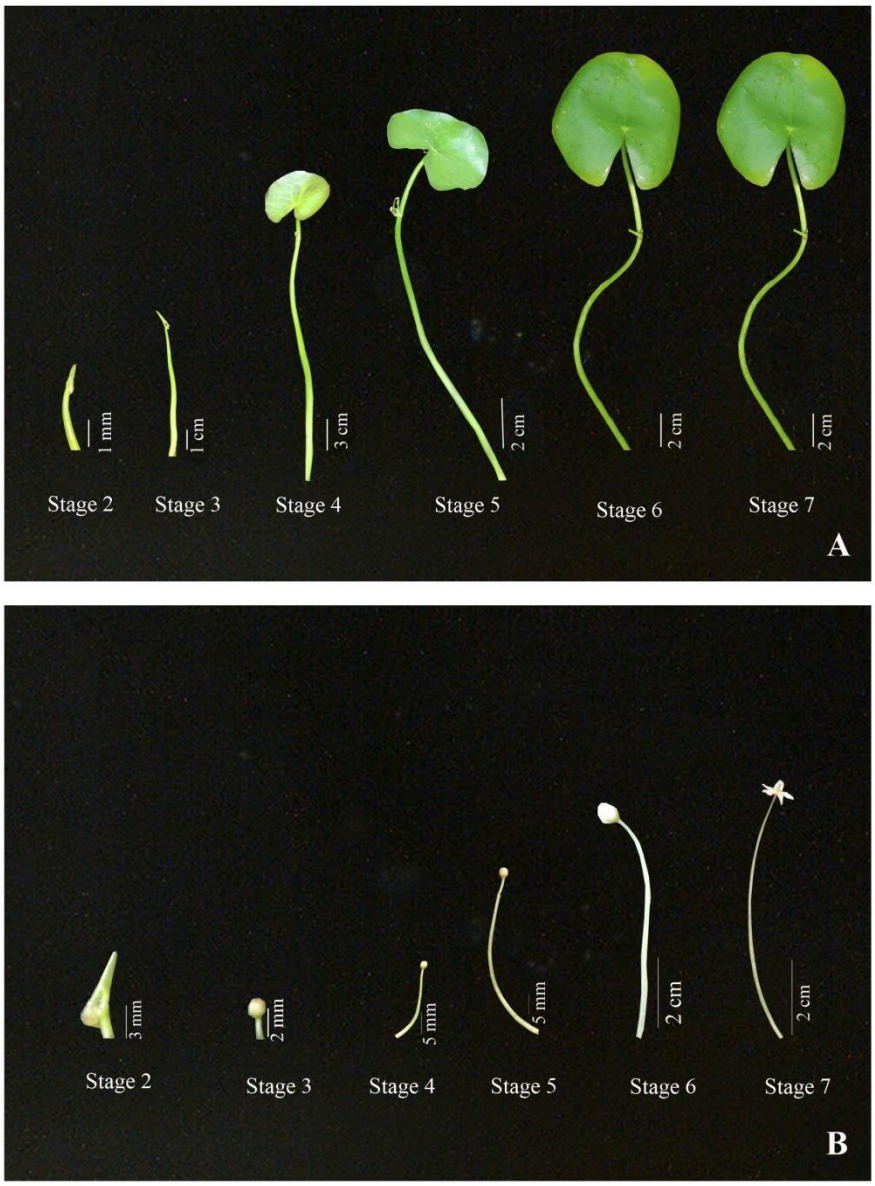

Fig. 87: Leaf and flower bud stages of *N. macrosperma.* **A.** Leaf developmental stages, **B.** Flower bud developmental stages.

Stage 3: during 6th day, the bud emerges out from the bract and its pedicel and sepal clearly distinguished. The mean size of the bud is 1.9mm x 1.85 mm. The pedicel length is 1.9 mm.

Stage 4: during 9th day the bud grows and appears pink colour on sepals. The pedicel grows very fast. The mean size of the bud is 2.04 mm length and 2mm. The pedicel length was 16.45mm.

Stage 5: Petal emerges out of the calyx. Within 12th - 13th days, the flower bud reached above the water surface. The mean size of the bud is 2.95 mm x 2 mm and the mean length of pedicel is 38.7 mm.

Stage 6: This is the mature bud form before 2 hours of anthesis. The mean size of the bud is 3.85 mm x 3 mm and the mean length of pedicel is 63.5 mm.

Stage 7: This is the full bloom stage in which the flower becomes open on the 15th or 16th day and exposing the various whorls and reproductive parts. The mean diameter of the flower is 7.5 mm and the mean length of the pedicel is 63.5 mm.

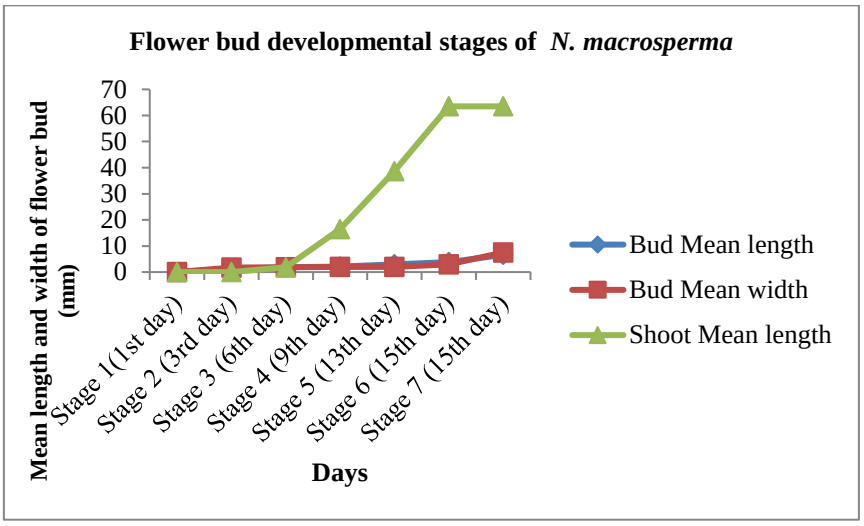

Fig. 89: Flower bud developmental stages of *N. macrosperma*

Flowering activity

We could locate only four female plants during the year 2017 from Kozhiode. None of the plants could be observed there in the next two years. Hence, flowering activity cannot be assed. Male plants were collected from Aakode, Malappuram in 2018. However, 2018 flood totally wiped out the entire population from the locality.

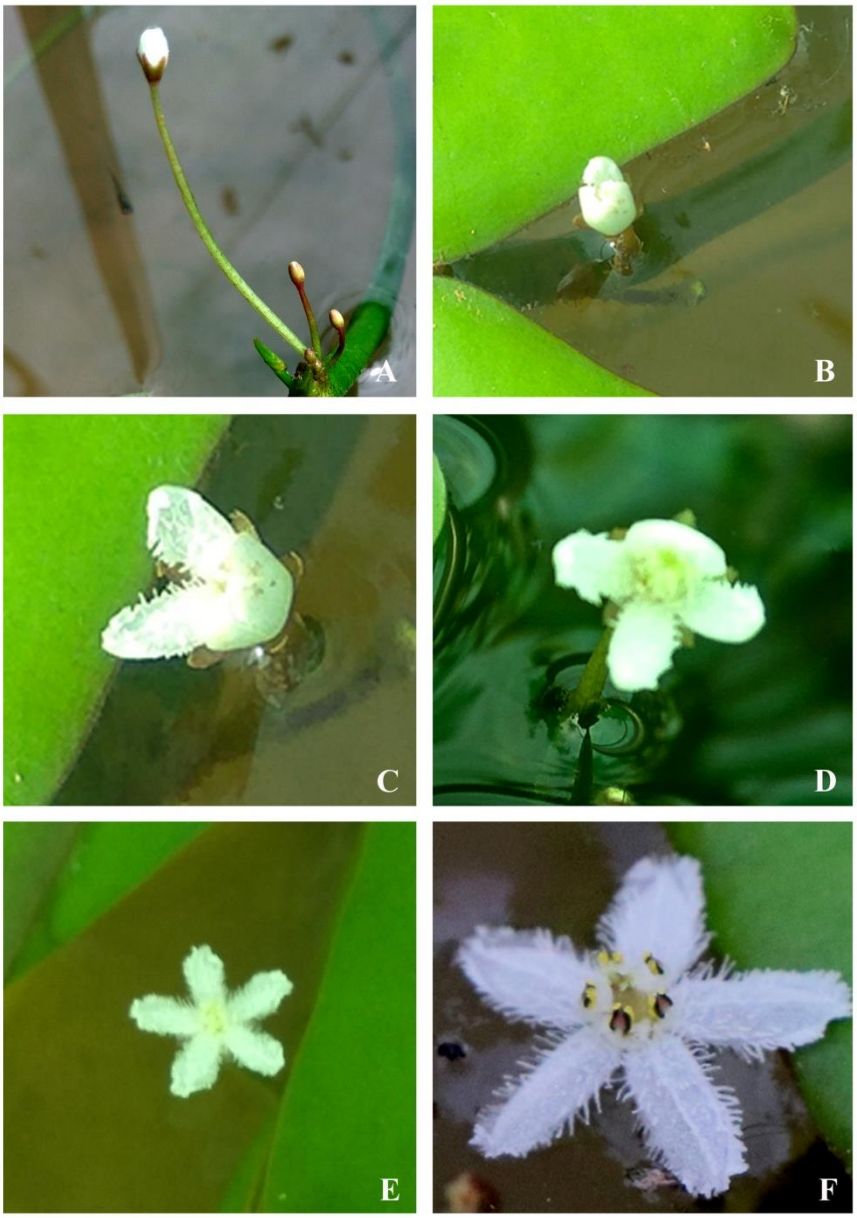

fig. 88: Anthesis and anther dehiscence in *N. macrosperma.* **A- E.** Anthesis of female flower, **F.** Anther dehiscence in male flower.

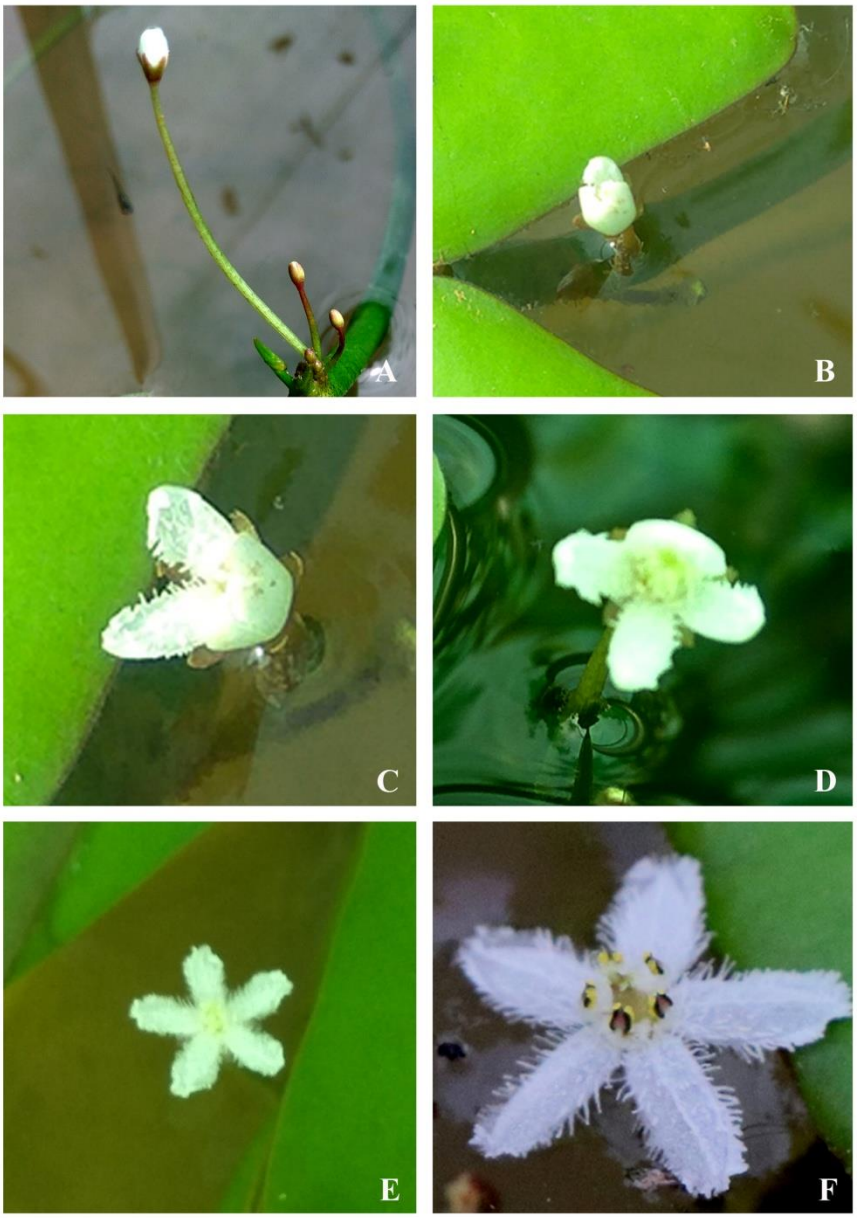

fig. 88: Anthesis and anther dehiscence in *N. macrosperma.* **A- E.** Anthesis of female flower, **F.** Anther dehiscence in male flower.

Anthesis (Fig. 90)

Anthesis of *N. macrosperma* started during 8.00 am to 9.00 am. The anther dehiscence was observed at the time of anthesis. The anther dehisced through longitudinal slit.

Pollen morphology (Fig. 91)

The pollen grains are medium sized, monad, triangular, radially symmetric, medium sized, tricolpate and prolate – speroidal to sub-prolate in shape. Mean polar axis is 28.81 ± 0.82 µm and the mean equatorial axis is 26.56 ± 0.46 µm. P/ E ratio is 108.44 ± 1.9 µm (n =100). Pollen surface is spinulose.

Pollen production

*N.macrosperma*has five stamens in a flower. The mean number of pollen per anther is 620 ± 160.6 (n=30) and the mean number of pollen per flower is 3100 ± 803 (n=30).

Pollen – Ovule ratio

Mean number of pollen / flower is 3100 ± 803 and the mean number of ovule is 3.6 ± 0.81. Pollen ovule ratio is 861:1

Stigma receptivity

Direct observation method (Fig. 92)

The stigma is transparent, wet and also adheres to $1mm^2$ piece of paper considered as receptive.

Hydrogen peroxide test (Table.54; Fig. 92**)**

The stigma produces oxygen bubbles at receptive time when it was treated with hydrogen peroxide. The receptivity starts at 9.00 am. Based on the number of bubbles evolved from stigma surface the stigma showed maximum receptivity at 1.00 pm (H = 11.12, p value = 0.00385). The receptivity gradually declined after 1.00 pm.

Histochemical localization esterases on stigmatic surface (Table. 55, Fig. 91**)**

The stigma is black at the time of more receptivity when it was stained with α-naphthyl acetate solution. In *N. macrosperma* the stigma was more receptive at 11.00 am – 2.00 pm, after that the receptivity was gradually decreases.

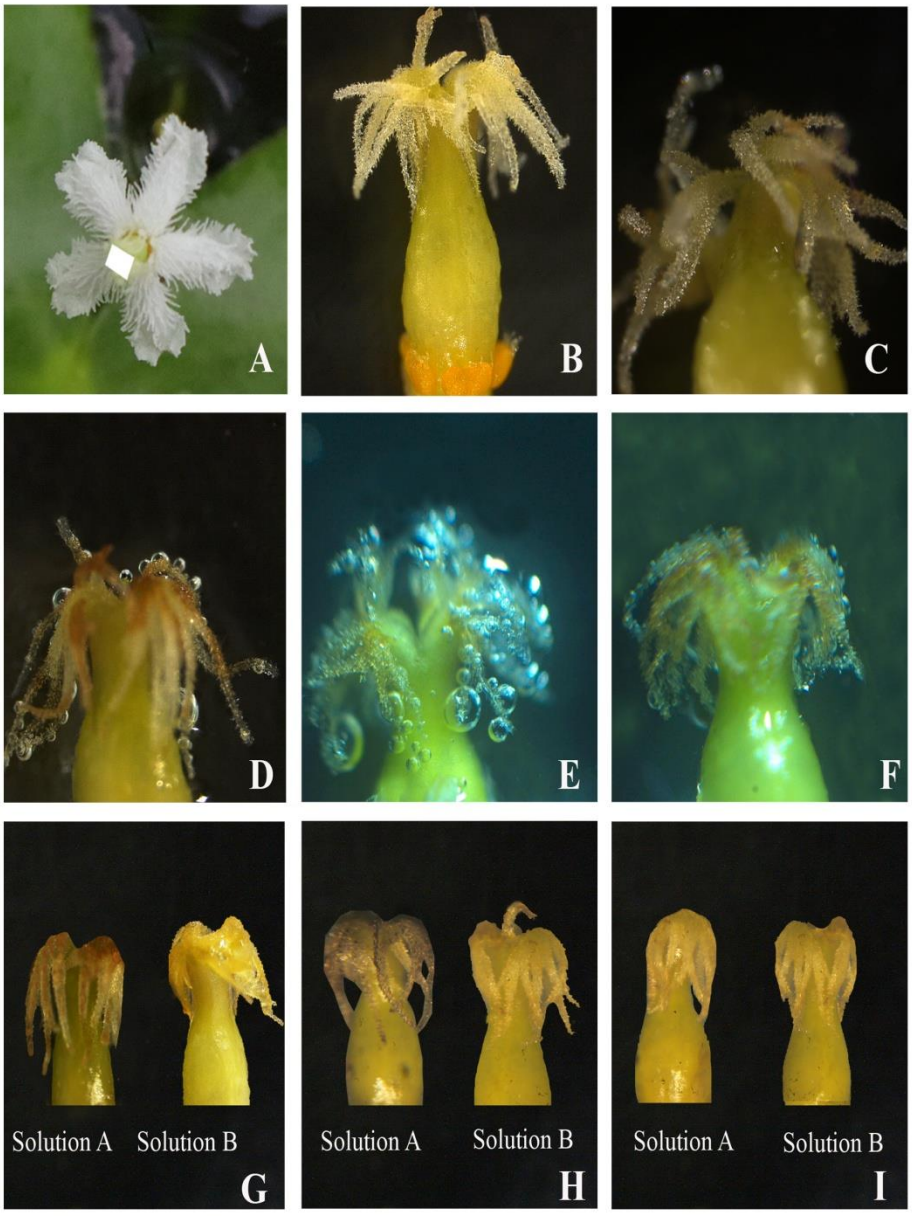

Fig. 92: Stigma receptivity & Stigma biology of *N. macrosperma*. **A - F.** Stigma receptivity Tests. **A.** Observation method, **B.** Stigma, **C - F.** H_2O_2 Method. **C.** During pre-anthesis, **D**. During anthesis, **E.** At 1.00 pm, **F.** At 5.00 pm, **G - I.** α-Naphthyl Test (Histochemical localization of esterases). **G.** During anthesis, **H.** At 1.00 pm **I.** At 6.00 pm,

Sl. No.	Time	Mean no. of bubbles	H value	p value
1.	08.00 am	0	0	0
2.	09.00 am	11.833 ± 1.88	8.3845	0.01511
3.	10.00 am	27.63 ± 1.11	9.76	0.00761
4.	11.00 am	51.61 ± 1.94	15.7026	0.00039
5.	12.00 pm	53.67 ± 1.44	9.7961	0.00746
6.	01.00 pm	58.03 ± 1.27	11.12	0.00385
7.	02.00 pm	54.07 ± 1.46	7.9103	0.01916
8.	03.00 pm	41.81 ± 1.98	9.0316	0.01093
9.	04.00 pm	30.27 ± 1.67	11.331	0.00346
10.	05.00 pm	29.92 ± 1.55	6.72	0.03474
11.	06.00 pm	21.04 ± 1.62	15.5542	0.00042

Table.54. H_2O_2 Test of stigma receptivity in *N. macrosperma*

Sl. No.	Time	Frequency of colour
1	07.00 am	Colourless
2	08.00 am	Colourless
3	09.00 am	Light brown
3	10.00 am	Brown
4	11.00 am	Black
5	12.00 pm	Black
6	02.00 pm	Black
5	3.00 pm	Brown
6	06.00 pm	colourless

Table.55. Histochemical localization of esterases on stigma of *N. macrosperma*

Pollination Biology

Floral visitors and their behaviours (Fig. 94)

Anthesis of *N. macrosperma* is at 8.00 – 9.00 am. The mode of pollination is entemophily. *N. macrosperma* is a dioecious plant. Insect's visits started soon after anthesis. Honey is little in the flower (less than 1 µl). During the study time small flies *Notiphila* spp. under the order Diptera are visited to the male flowers. Only this fly is observed on the male plant of *N. macrosperma.* But male and female plants grow in different localities. Female plant collected from the locality, Pantheerankavu, Kozhikode and female plant from Aakode, Malappuram. So pollination and fruit setting is very difficult. A single fly visited 1- 2 male flowers in a single visit. Time spent by this bee in each flower is 8 – 10 minutes. But female flower visitors were not found its natural locality.

Because the population of the plant is very less (*ie.* only 4 female plant was observed in natural locality).

Fig. 93: Pollintors of *N. macrosperma*

Fig. 94: Vegetative reproduction

Vegetative propagation (Fig. 94)

Vegetative propagation is more frequent in *N. marosperma.* Lateral roots and leaves are produced from the junction of flower producing region of shoot and detached with part of shoot attached. After decaying the mature leaf the rooted portion with two or three young leaves float on the water and sunk to the bottom where it get attached to the soil and grows as new plant.

The male plant of *N. macrosperma* was collected from Aakode, Malappuram Dist. and the female plant from Pantheerankavu, Kozhikode Dist. The experimental part of the pollen biology, viz., pollen number / flower, pollen morphology study, pollen biochemical test; stigma receptivity and histochemical localization of esterase enzyme on stigma etc. was carried out in the laboratory and phenological part viz., leaf development, flower bud development and observation of pollinator etc. These two plants were fully destroyed along with its habitat during the flood, 2018 and was not revived till date. The two plants were fully destroyed from its natural habitat after flood. So its reproductive study is restricted. Though both female plant of *N. macrosperma* was established successfully in the aquatic plant conservatory of MBGIPS, it's yet to flower. Hence, *In vitro* and *In vivo* pollen germination, pollen viability, pollination, seed germination was not carried out.

6

DISCUSSION

6. Discussion

Kerala State is blessed with vast marshy areas, extensive wetlands and aquatic systems. All of these systems are very important and are rich in plant diversity. The present work deals with the taxonomy and reproductive biology of *Nymphoides*, an aquatic plant genus in Kerala. The main objective of this study is to find out the distribution of *Nymphoides* spp. in Kerala and to study its taxonomic and reproductive aspects. For this purpose, extensive field explorations throughout the Kerala state were conducted and collected the specimens. Collected live specimens were introduced to the Aquatic plant conservatory of MBGIPS Garden (Aquagene) and conserved *ex situ*. Two or three specimens of each species have been processed into herbarium and deposited in MBGH, Kozhikode. Fresh specimens were studied in the laboratory and upon which detailed taxonomic descriptions were prepared. Updated nomenclature, phenological details, distribution in Kerala, India and World, conservation status and details of voucher specimens examined are provided for each taxon. Herbaria of Botanical Survey of India at Kolkata (CAL) and Coimbatore (MH), Calicut University Herbaria (CALI) and Herbarium of Malabar Botanical Garden and Institute for Plant Sciences, Kozhikode (MBGH) were consulted for this purpose. The nomenclature of each taxon was updated using authentic websites, viz., The Plant List (http://www.theplantlist.org), Tropicos (http://www.tropicos.org), IPNI (http://www.ipni.org), etc. The reproductive characters *viz*. pollen morphology, pollen biology, pollen viability, pollen germination, stigma receptivity and stigma biology were studied in the laboratory condition and phenology, pollinators study, pollination, breeding analysis, flowering and fruiting activity in field. Shenzhen Code (Turland *et al*., 2018) was used for the clarification of nomenclature.

During the present study out of nine species reported, seven species were collected from Kerala and conserved in the Aquagene conservatory of KSCSTE – MBGIPS. Six species could be successfully established (*ex situ*) in the garden. Taxonomic study was carried out in seven species, however reproductive studies were restricted to five species, because *N. macrosperma*

which was fully destroyed from its natural habitat after flood. *N. palyi* which could not established in MBGIPS conservatory. *N. aurantiacum* reported earlier from Aleppy (without mentioning the exact locality) could not be relocated from anywhere else. Sunil and Sivadasan (2009), though reported its occurancce in Thalavadi, Aleppy, we could not get the same from the said locality. Sunil (2018), also intimated the destruction of thenative flora of the above locality consequent to the extension of agriculture in this area (pers. Communication)

All species of *Nymphoides* are true aquatic. *N. indica* and *N. hydrophylla* grow in shallow ponds, pools, paddy fields and flooded lowlands. *N. macrosperma* grow inundated fallow paddy fields. *N. parvifolia, N. krishnakesara, N. balakrishnanii* and *N. palyi* grow in the seasonal ponds of lateritic hillocks. In the revision work of *Nymphoides* spp. in India, Sivarajan & Joseph (1993), reported that *N. aurantiacum* was growing in shallow ponds, pools, flooded lowlands and canals associated with *Eleocharis* sp., *Eriocaulon* sp. and *Najas* sp. Even after thorough survey, we could not locate the herbarium specimens of *N. aurantiacum* from Kerala state said to be deposited in the Central National Herbarium, Kolkata (CAL).

N. indica are found associated with *Nymphaea nouchali, Nymphoides hydrophylla* are found associated with *Cabomba caroliniana* and *Hydrilla verticillata. N. macrosperma* is found in the abandoned paddy fields associated with *N. indica, Nymphaea nouchali, Eichhornia crassipes* and *Cabomba caroliniana. N. krishnakesara* grows in association with *Blyxa octandra, Wiesneria triandra, Eriocaulon truncatum* and *Rotala malampuzhensis. N. parvifolia* is found associated with *Dopatrium junceum, Rotalamala mpuzhensis* and *N. indica. N. balakrishnanii* grows with *Ludwigia hyssopifolia* and *Eriocaulon* spp. *N. palyi* grows in association with *Microcarpaea minima, Ludwigia hyssopifolia, Eriocaulon cuspidatum and Wiesneria triandra*, as a seasonal transient community.

Comparative morphology of *Nymphoides* spp.

Rhizome:

All the species are annual or perennial rhizomatic aquatic herbs. The size and shape of the rhizome varied from species to species. Rhizome of *N. indica, N. macrosperma and N. hydrophylla* are stoloniferous and orange –brown but 2 – 4 cm long in *N. indica*; 3–7cm long and 1–2cm thick in *N. macrosperma* and 2-3 cm long in *N. hydrophylla. N. krishnakesara*, *N. parvifolia*, *N. balakrishnanii* and *N. palyi* have non-stoloniferous rhizome; 2-5cm long and 1.2 cm thick in *N. krishnakesara,*, 1cm long and 3mm thick in *N. parvifolia,* 2cm long and 7 mm thick in *N. balakrishnanii* and 1-2cm long and 5 mm thick in *N. palyi*. Sivarajan and Joseph (1993), reported rhizome of *N. hydrophylla* has 1-2cm thick, 1-5cm thick in *N. indica*, 5cm thick in *N. krishnakesara,* 1–2cm thick in *N. macrosperma* and rhizome short in *N. parvifolia.* Rhizomes of *N. balakrishnanii* is 2–4cm long and *N. palyi* is 2–2.5cm long (Biju *et al.* 2016).

Shoots and Leaves

Shoots and leaves of *Nymphoides* spp. are monomorphic or dimorphic. The plant with dimorphic leaves have basal submerged leaves and floating leaves. Leaves are monomorphic in *N. indica, N. macrosperma* and *N. hydrophylla* but dimorphic in *N. krishnakesara, N. parvifolia* (Sivarajan and Joseph, 1993) *N. balakrishnanii* and *N. palyi* (Biju *et al*., 2016). The shoots are petiole like, bearing flowers, monomorphic or dimorphic. Shoots branched in *N. indica, N. macrosperma* and *N. hydrophylla* but not branched in *N. krishnakesara, N. parvifolia* (Sivarajan and Joseph, 1993) *N. balakrishnanii* and *N. palyi* (Biju *et al*., 2016).

Flower, Fruit and Seed

Flowers are snow white in colour except in *N. aurantiacum*, which has orange yellow flowers. Flowers are heterostylous in *N. indica* (Ornduff 1966; Reddy and Bahadur 1976; Hamashima 1979; Barret 1980; Shibayama & Kadono 2003). Other species *viz*., *N. macrosperma, N. hydrophylla, N. krishnakesara, N. parvifolia, N. balakrishnanii* and *N. palyi* are homostylous. In the present study *N. indica* is found as heterostylous and other species in Kerala are homostylous.

Sepals gamosepalous; petals gamopetalous. Flowers are umbellate clusters of 4 – many, born on the junction of the shoot and the petiole. Flowers 15 – 45 in *N. indica,* 15 – 22 in *N. macrosperma,* 20–40 in *N. krishnakesara,* 10 – 20 in *N. hydrophylla,* 4–8 in *N. parvifolia,* 4–10 in *N. balakrishnanii* and 4–8 in *N. palyi. N. indica* produced more flowers than other species. *N. parvifolia* and *N. palyi* produced less number of flowers. Calyx gamosepalous in all *Nymphoides* spp. 4 – 8 lobed in *N. indica,* 5 lobed in *N. macrosperma, N. krishnakesara* and *N. hydrophylla;* 3 or 4 lobed in *N. parvifolia* and *N. palyi;* 4 lobed in *N. balakrishnanii.* Corolla gamopetalous; white with yellow throat in *N. indica, N. hydrophylla, N. parvifolia, N. balakrishnanii* and *N. palyi* .Corolla without yellow throat in *N. krishnakesara* and *N. macrosperma.*

Fruit

Fruit is a capsule producing many seeds. Fruit of *N. indica* is ellipsoid, 6mm x 5 mm in long; fruit of in *N. macrosperma* is sub-globose, 7 mm x 5 mm long obovoid; *ca.* 9 mm and oblong in *N. krishnakesara,* up to 5 mm and ellipsoid in *N. hydrophylla,* up to 4mm x 2mm and oblong – obovoid in *N. parvifolia*, 4 x 2 mm and oblong in *N.balakrishnanii* and 3mm x 2 mm in *N. palyi. N. macrosperma* has large fruit and *N. palyi* has small fruits.

Seeds

Seeds vary in number. *N. indica* produces 14 – 32 seeds per capsule, which is discoid, brown, 1 – 1.5 mm across, smooth. *N. macrosperma* produces 2 – 4 per capsule, which is obovate or ellipsoid, light brown or creamy, 3.5mm x 4.5 mm long. *N. krishnakesara* produces 4 – 9 seeds per capsule, which is obovoid, rounded or obtuse at distal end, sub-acute at proximal end, surface spiny. *N. hydrophylla*produces4 – 6 seeds per capsule, which is brown, discoid, surface tuberculated. *N. parvifolia* produces8 – 12 seeds per capsule, which is discoid, tuberculated, light brown in colour. *N. balakrishnanii* produces 5 - 13 seeds per capsule, which is 1 mm across, brown and tuberculated. *N. palyi* produces 4 – 10 seeds per capsule, which is discoid, brownish black, 0.8 mm across, smooth. Sivarajan and Joseph (1993), reported 18–25 seeds per capsule.

Seed are more or less smooth in *N. indica* (Aston, 2003). Sivarajan *et al*. (1989), reported the seeds of *N. indica* are light brown to brown, concave at funiculus scars, surface with clusters of tuberculate funicles. *N. macrosperma* possess the large fruit and *N. palyi* has the smallest fruit. The largest seed is produced by *N. macrosperma* and the smallest seed produced by *N. palyi.* Maximum number of seeds produced by *N. indica* (14 - 42) but less number of seeds produced by *N. macrosperma* (2 -4). A comparative morphological analysis of *Nymphoides* is done in Results; Page No.58.

Endemism and Rarity

Four species of *Nymphoides* are endemic to Kerala *viz. N. sivarajanii, N. krishnakesara, N. balakrishnanii* and *N. palyi.* Among them, *N. sivarajanii* is Critically Endangered (IUCN, 2019), so far reported only from the type locality, *ie.* Chettipadi, Malappuram (Joseph, 1991). However, we failed to collect the species despite extensive field surveys and purposeful search in and around the type locality and are feared as become extinct. One specimen from Ochira, Kollam kept at MH identified as *N. indica* looked like *N. sivarajanii*, but this specimen has no flowers (Sivarajan & Joseph, 1993). However, we could not get this species from the said locality in spite of concerted efforts. *N. krishnakesara, N. balakrishnanii* and *N. palyi* are distributed in a restricted area of Northern Kerala (Kannur and Kasaragod). These species are restricted to small seasonal ponds / indentations in lateritic hillocks in the above mentioned districts. The discontinuous distribution of these seasonal ponds leads to the fragmented distribution of these highly habitat specific threatened species. The other reasons for the rarity were, local people used these seasonal water bodies for domestic purposes especially for bathing, cloth washing, etc. which ultimately leads to the detergent polluted water that repels natural pollinators. The altered water pH due to detergents and soaps also adversely affect seed germination. Laterite brick mining, road and building constructions, land filling, cleaning out and deepening of natural ponds for various purposes, etc. also create impediments on the sustainability of *Nymphoides* populations threatening the survival of this highly sensitive taxon, in these areas.

Conservation

The collected seven *Nymphoides* spp. are conserved *ex situ* in the aquatic plant conservatory, 'Aquagene' of MBGIPS, Kozhikode. Six species could be successfully established in this garden. They are *N. indica, N. hydrophylla, N. krishnakesara, N. parvifolia, N.balakrishnanii* and *N. macrosperma.* Among them, *N. krishnakesara, N. parvifolia* and *N. balakrishnanii,* are lateritic specific and attempts to conserve them in *ex-situ* suceeded only after supplementing a solution of lateritic soil. This indicates the specific ecological niche required for the establishment of these species to lateritic soil which contains excess Iron (ferrous oxide) and probably this is essential and inevitable for the growth of all lateritic species of *Nymphoides* in Kerala (*N. krishnakesara, N. parvifolia, N. balakrishnanii* and *N. palyi*).

Reproductive Biology

Reproductive biology is the scientific study of the reproductive system which includes both asexual and sexual reproduction. Knowledge of reproductive biology is a prerequisite for both evolutionary and conservation studies (Fishbein, 2001; Silva and Silingardi, 2008). Study of reproductive mechanisms of plants can help to assess adaptive significance and homology of descriptive characters used in plant systematics (Simpson 2010). In recent years the studies on the reproductive biology of aquatic plants have greater value.The present study is the first study on the reproductive biology of *Nymphoides* spp. in Kerala. The work includes phenology, pollen biology, stigma biology, pollination biology, seed set, flower fruit ratio, ovule seed ratio, seed viability, fruit and seed dispersal and germination. The reproductive biology of angiosperm is important in determining barriers to seed and fruit set, understanding fruit set and breeding systems that regulate the genetic structure of a population (Tandon *et al.* 2003).

Phenology

Exstensive field survey done in Kerala revealed that, *Nymphoides indica* and *N. hydrophylla* are distributed in all districts. *N. macrosperma* is recorded from Kozhikode and Malappuram. Male plant and female plant were growing in different localities. *N. parvifolia, N. krishnakesara, N. balakrishnanii*

and N. palyi was growing extensively in Kannur and Kasaragod Districts. The leaf developmental study of all species indicated that the length of the shoot vary depending upon the depth of the water bodies. Development of leaf (initiation – Yellowing), buds (initiation – Full blooming) and fruits (after pollination – maturation) were passed through different stages. The average time required for the leaf, fruit maturation in *Nymphoides* spp. are different in various species. Flower production of all the species is found decreasing after flood. The maximum flowering was during November – January.

Heterostyly was shown in *N. indica.* Other species in Kerala ishomostylous. Shibayama (2003), reported that *N. indica* is a heterostylous species and studied the differences between the length of short style and long style, pollen size of two morphs and stamen – pistil length was studied in the field. The mean length of the stamen of the short styled and long styled flower was 0.826 mm in short styled flowers and 0.686 mm in long styled flowers.

Anthesis

The flowers of all *Nymphoides* survive for only one day. Anthesis in *N. indica* and *N. krishnakesara* was 06.00 am – 08.00 am. In *N. hydrophylla, N. macrosperma, N. parvifolia* and *N. balakrishnanii* anthesis occurred in 08.00 am to 09.00 am. Anther dehisced through longitudinal slits. In *N. indica* the anther dehiscence occurs half an hour before anthesis. But in all other species anther dehiscence occurred at the time of anthesis.

Sl. No.	Species	Flowering and fruiting	Anthesis time	Vegetative propagation	Seed dispersal
1.	*N. indica*	August – March	6 – 8 am	Stolon, Rhizome	Through water
2.	*N. Krishnakesara*	August – February	6 – 8 am	Rhizome	Through water
3.	*N. hydrophylla*	August – March	8 – 9 am	Stolon, Rhizome, shoot	Through water
4.	*N. parvifolia*	August – February	8 – 9 am	Rhizome	Through water
5.	*N. balakrishnanii*	August – January	8 – 9 am	Rhizome	Through water
6.	*N. macrosperma*	August – February	8 – 9 am	Rhizome, shoot	Through water

Table: 57. Phenological status of the different *Nymphoides* species in Kerala

Pollen Biology

The morphology, size and shape of the pollen grains vary from species to species. All *Nymphoides* species studied here have monad, triangular, heteropolar, radially symmetric, tricolpate and medium sized pollen grains. Pollen surface is spinulose. Perveen (1999), reported the pollen grains of *N. cristata* was heteropolar, oblate, tricolpate and triangular. Saadi and Mayah (2012), studied the pollen grains of *Nymphoides indica* and *N. peltata* and reported that the pollen grains were heteropolar, subprolate and suboblate. They also reported the pollen grains of *N. indica* was tricolpate spinuliferous and that of *N. peltata* was tricolpate reticulate. Gandhi *et al.*, (2014), reported that *N. hydophylla* has heteropolar and oblate pollen grains. Mourelle and Prieto (2016), reported the pollen of *N. indica* was monad, isopolar, tricolporate, perprolate and circular but in this study pollen grains are hetero polar, tricolpate, and prolate – spheroidal.

In the present investigation pollen grains of *N. indica* are medium sized, monad, triangular - obtuse, heteropolar, radially symmetric, parasyncolpate and prolate – spheroidal. The pollen grains of *N. krishnakesara* are medium sized, monad, triangular, tricolpate, heteropolar, radially symmetric, trilete and prolate – spheroidal. The pollen grains of *N. hydrophylla* are medium sized, heteropolar, monad, triangular, radially symmetric, parasyncolpate, prolate – spheroidal with spiny surface. In *N. parvifolia* the pollen grains are medium sized, monad, triangular, heteropolar, radially symmetric, parasyncolpate and oblate – spheroidal.In *N. macrosperma* the pollen grains are medium sized, monad, triangular, radially symmetric, medium sized, tricolpate and prolate – speroidal to sub prolate. In *N.balakrishnanii* the pollen grains are medium sized, monad, triangular, heteropolar, radially symmetrical, parasyncolpate, prolate – spheroidal. The pollen grains of *N. palyi* are medium sized, monad, triangular, radially symmetric, medium sized, tricolpate and oblate – speroidal. The exine surface of all the *Nymphoides* spp are spinulose.

Pollen number/ flower vary in different species. In *N. indica* number of anthers varies from 4 – 8 and the number ofpollen grains depending on the

number of anther present in the flower (Table. 53), in *N. macrosperma* the mean number of pollen per flower is 3100 ± 803, *N. krishnakesara* the mean number of pollen per flower is 43000 ± 4830, *N. hydrophylla* produces 27000 ± 4830 pollen/flower, *N. parvifolia* produces 1027 ± 38 in 3 petal flower and 1360 ± 33 pollen in 4 petal flower and *N. balakrishnanii*produces1840 ± 372 pollen/flower.

Gandhi *et al.* (2014), reported *N. hydrophylla* have 5133 pollen per anther. They reported that the pollen diameter was 27.018 ± 0.713 µm. In *N. indica* the P/E ratio in short styled flower is 105.99 ± 4.9 µm and in long styled flower is 106.80 ± 8.87 µm. Shibayama and Kadono (2003), reported the pollen grain diameter of long styled flower was 31.7 ± 1.5 µm and that of short styled flower was 39.6 ± 2.0 µm.In the present investigation the diameter of pollen grains in short styled flowers is 41.72 ± 1.82 µm and in long styled flowers is 36.58 ± 1.6 µm. In *N. krishnakesara* the pollen diameter is 33.59 ± 1.4µm. The pollen diameter of *N. macrosperma is* 28.81 ± 0.82 µm. In *N. hydrophylla* pollen diameter is 29.09 ± 1.93 µm.The diameter of pollen grains in *N. parvifolia* is 25.13 ± 1.01µm (in both the petal flower) and in *N. balakrishnanii* the pollen diameter is 28.91±2.36 µm.

P / E ratio of short styled flower of *N. indica* is 105.99 ± 4.89 µm and that of long styled flower is 106.80 ± 8.87 µm. P/ E ratio of *N. macrosperma* is 108.44 ± 1.9 µm, *N. krishnakesara* is 110.99 ± 78 µm, *N. hydrophylla* is 104.14± 3.17 µm, *N. parvifolia* is 103.1± 4.37 µm and *N. balakrishnanii* is 103.16± 3.4 µm.

Pollen biochemical analysis in *Nymphoides* spp. showed that pollen grains became purplish or brownish colour due to the presence of newly formed starch and become blue coloured which indicated the presence of protein when appropriate histochemical stains were used. The pollen grains become black colour due to the presence of lipids. Aswani and Sabu (2019), studied the biochemical analysis of pollen grains of some selected taxa of Zingiberaceae. They reported that the pollen contains starch, protein and lipids.

The pollen ovule ratio of *Cabomba aquatica* (hydrophyte) was 1055:1 (Silva and Leite, 2011).Pollen – ovule ratio of *N. indica* shows variation based on the number of petals. The mean ovule number and pollen ovule ratio of different *Nymphoides* are shown in the table below (Table. 53).

Sl. No.	Species Name	No. of anthers	Pollen/flower	Mean Ovule No.	Pollen – Ovule ratio
1	*N. indica* (No. of anthers varies from 4 – 8) Long styled flower	4	40000 ± 1414	29 ± 7	1379: 1
		5	52000 ± 894	28 ± 9	1857:1
		6	60000 ± 1414	29 ± 9	2068:1
		7	70000 ± 1414	31 ± 9	2258:1
		8	83000 ± 1673	31 ± 8	2683:1
	N. indica Short styled flower	4	36800 ± 1095	30 ± 8	1226:1
		5	48000 ± 894	29 ± 10	1655:1
		6	55200 ± 1095	30 ± 9	1840:1
		7	67200 ± 894	31 ± 9	2167:1
		8	76800 ± 894	31 ± 8	2477:1
2	*N. krishnakesara*	5	43000 ± 4830	7 ± 1	6142: 1
3	*N. hydrophylla*	5	27000 ± 4830	7 ± 1	3857: 1.
4	*N. parvifolia* (No. of anthers 3 – 4)	3	1027 ± 38	9.2 ± 1	111 :1
		4	1360 ± 33	10.6 ± 2.1	123 :1
6	*N. balakrishnanii*	4	1840 ± 372	10 ± 2.7	157: 1
7	*N. macrosperma*	5	3100 ± 803	3.6 ± 0.81	861.11:1

Table.58. Comparison of pollen production and pollen ovule ratio

Pollen viability starts at 24 hour before anthesis in all species. Maximum viability shown before 2 hours of anthesis and after anthesis the viability became reduced and lost in all the species of *Nymphoides*. Das *et al.* (2018), studied pollen viability of *N. indica* in 2% TTC and acetocarmine and reported 81.60% pollen viable in TTC and 79.45% viable in acetocarmine. In the present study pollen viability was tested in 1% acetocarmine and 0.1% TTC and showed that the pollen grains are viable till at 11.00 am. The maximum viability of pollen grains (before 2 hours of anthesis) in *N. indica* is 83.63 ± 1.92% in TTC and 84.3 ± 1.67% in acetocarmine, *N. krishnakesara* is 96.99 ±3.9% in TTC and 97.82 ± 4.9% in acetocarmine, *N. hydrophylla* is 57.05 ±3.2% in TTC and 57.4 ± 2% in acetocarmine, *N. parvifolia* is 54.11 ±4.8% in TTC and 54.36 ± 2.2% in acetocarmine, and *N. balakrishnanii* is 50.63 ±3.6% in TTC and 48.53 ± 3.3% in acetocarmine.

In vitro pollen germination of *Nymphoides* sp. were studied in Brewbaker and Kwak's medium and sucrose solution. In *N. indica*, maximum pollen grains germinated in 8% of both the medium and the mean pollen the length was 521.89 µm (in Brewbaker and Kwak's medium) and 476.8 µm (sucrose solution) in long styled flower. In short styled flower maximum germination observed in 8 % of both the solutions with mean pollen tube length of 509.5 µm (in Brewbaker and Kwak's medium) and 438.8 µm (sucrose solution) after 3 hours. Das *et al.* (2018), studied *in vitro* pollen germination in sucrose, Brewbakers solution, boric acid, calcium nitrate, Magnesium sulphate, potassium sulphate. 76% germinating pollen with a mean of 1105µm long pollen tube development was observed in 15% sucrose, solution, 100 ppm boric acid showed 51% pollen germination along with 533 µm long pollen tube, maximum 20% pollen germination along with 416 µm long pollen tube developed after 3 hours in 200 ppm calcium nitrate solution while 16% pollen germination along with 276 µm long pollen tube development was observed in 100 ppm magnesium sulphate solution and 13% pollen germination along with 221 µm long pollen tube developed in 100 ppm potassium nitrate solution. In *N. krishnakesara* maximum pollen germination is shown in 4% Brewbaker and Kwack's medium (81%) and sucrose solution (56%) with a mean pollen tube length 310.32 µm and 81. 79 µm respectively observed after 1 hour. *N. hydrophylla* shows maximum pollen germination in 4% in both the solution with a mean pollen tube length is 565.63 ± 83.17µm (Brewbaker and Kwack's medium) and 328.21 ± 33.03 µm (sucrose solution) after 3 hour. In *N. parvifolia*, maximum germination occurred in 12% Brewbaker and Kwack's medium (78%) with a mean pollen tube length is 176.25 µm and 8% sucrose solution with a mean pollen tube length is 125.56 µm after 3 hours. In *N. balakrishnanii* maximum germination observed in 12 % of both the solution with mean pollen tube length is 632.03 µm (Brewbaker and Kwack's medium) and 118.01 µm (sucrose solution) after 3 hours. Above and below these concentrations the germination and pollen tube development was less.

In vivo pollen germination tested in the field. Pollen – Pistil interaction was also studied in these experiments. Pollen- pistil interaction is a critical factor in postpollination phases. Pollen germinated maximum during post anthesis period in all the species.In *N. indica* the pollen tube length was 205.597 µm in long styled flower and 111.678 µm in short styled flower after 1 hour. In *N. krishnakesara* pollen tube has 81.36µm long after half hour and 196.82 µm long in *N. hydrophylla* after 1 hour. In *N. parvifolia*149.03 µm long after 1 hour and 129.33µm long pollen tube observed in *N. balakrishnanii* after 1 hour.

Stigma receptivity

Stigma receptivity is an important factor for pollination success. If the stigma had not been receptive at the time of pollination, the pollen grains cannot germinate on the stigma and resulted failure of fertilization and subsequent seed production. In the case of *Nymphoides* spp. the stigma receptivity starts just after anthesis. The stigma shows maximum receptivity during 11.00 am to 2.00 pm.After 2.00 pm receptivity gradually declined. Arshid and Wani (2012), studied the stigma receptivity of *Myriophyllum spicatum* L. and reported that the receptivity lasts for 3 - 4 days, the percentage germination of the pollen grains was highest on 4^{th} day after the spike comes out of the sheathing leaf and after that the receptivity of the stigma decreases gradually within one or two days as the stigma becomes brown and dry. But in *Nymphoides* spp. the stigma receptivity lasts for one day and the peak receptivity was observed at 1.00 pm.

The receptive stigma adheres to 1 mm^2 of paper during receptive time. Peroxidase enzyme and Esterase enzyme play an important role in stigma receptivity. Hydrogen peroxide test and α - naphthyl acetate test study also proved the stigmatic receptivity of *Nymphoides* spp. H_2O_2 test in *Nymphoides* spp. shows that the receptivity starts at the time of anthesis and the maximum receptivity at 11.00 am to 2.00 pm. After 2.00 pm the receptivity gradually decreases. By α – naphthyl acetate test, the stigma of all *Nymphoides* spp. was stained in dark brown – black shows the maximum receptive time. The biochemical study on the stigma showed that the stigmatic surface contains protein, lipids and starch.

Pollination Biology

Pollination is the most important event in sexual reproduction of angiosperms. Pollination biology is the transfer of pollen from male reproductive part to the stigma surface and the biological changes occur in post pollination phase. Majority of aquatic plants are not hydrophilous; The flowers of aquatic plants growing above the water surface and these flowers are pollinated by other biotic agents (Shivanna and Tandon, 2003). *Nymphoides* spp. are entemophilous. Nectar and pollen grains are the major rewards in insect pollinated plants (Faegri and Pijil, 1979). Shibayama & Kadono (2003), observed and reported *Parnara guttata, Notiphila* spp., *Mesembrius flaviceps, Lasioglossum mutilum, Apis mellifera, Bagous* spp. are the pollinating agents of *Nymphoides indica.* In the present investigation the main floral visitors in *N. indica* come under the Diptera, Hymenoptera and Coleoptera. The visitors are *Apis florae*, *Notiphila* sp., *Bagou s*sp. and *Lasioglossum mutilum. In N. krishnakesara, Notiphila* spp. is the main pollinating agents. *N. hydrophila, N. parvifolia* and *N. macrosperma* are also pollinated by *Notiphila* spp. under the order Diptera. In *N. balakrishnanii* the pollinators are *Componotus sericeus* under order Hymenoptera and *Xya* sp. under the order Orthoptera.

Pollination efficiency in *Nymphoides* spp. was tested by observing a particular number of stigmas after the visit of the pollinators under the microscope for the presence of pollen grains on the stigmatic surface. In *N. indica* pollination efficiency of short styled flower was 0.024 and that of the long styled flower was 0.023. Pollination efficiency of *N. krishnakesara* was 0.023. Bisexual flower of *N. hydrophylla* was 0.022. In *N. parvifolia,* pollination efficeiency of 3 petal flower was 0.08 and in 4 petal flower was 0.07 and in *N. balakrishnanii* the pollination efficiency was 0.06.

Pollination Treatment

Pollination treatments were carried out in different *Nymphoides* spp. In *N. indica* fruits were produced only by intermorph cross pollination not by self-pollination. In natural condition 72% mean number of fruit set was observed in short styled flowers and 70% in long styled flowers. Through manual

pollination (Intermorph pollination)78% of fruit set observed in short styled flowers and 74% fruit set were observed in short styled flowers. Short styled flowers produced more fruits than long styled flowers. Shibayama and Kadono (2003), also reported the fruit and seed set of *N. indica* was occurred only in intermorph pollination. They reported that 74.3% fruit and seed set rate in long styled flower and 76% in short styled flower. In *N. krishnakesara* 54% of fruit set was observed in natural but 100% of fruits were produced by hand pollination. In *N. hydrophylla* 60% of fruits were produced in bisexual plants under natural condition, but in female plants no fruits were produced because these two types of flowers were growing in different population. By hand pollination 80 % fruits produced in bisexual flower and 28% in female flower. *N. parvifolia* produced fruits by self-pollination and insect pollination. In natural pollination 96 % of fruit set was observed in naturally. But in cross pollination 100% of fruits were produced. In *N. balakrishnanii*98% fruits were produced in natural, 94% fruits produced in self-pollination and 100% fruits produced in manual cross pollination.

Fruit dehiscence and Seed dispersal

The fruit of all seven species of *Nymphoides* is a capsule. The capsule is ellipsoid in *N. indica* and *N. parvifolia,* subglobose in *N. macrosperma*, obovoid in *N. krishnakesara,* oblong in *N. hydrophylla* and *N. palyi,* oblong to obovoid in *N. balakrishnanii.* Calyx lobe is persistant with fruit. The flower buds are growing inside the water and are emerged on the water surface one day before anthesis. The flowers opened above the water level. After pollination the flowers sink in to the water and develop fruits. The fruits dehisced in water. Velde and Heijden (1981), reported that the fruit development of *N. peltata* takes place just below the water surface and each flower produces one beaked capsule about 2.5 cm long, which splits along one side to disperse many smooth seeds with winged margins.

Seeds are dispersed through water. The dehisced seeds float on the water for one or two days and later sink to the bottom of the water. Harris (2014), observed that *Nymphoides* seeds are released from the fruits and form floating

chains. The seeds float on the water surface due to a coating of a weak hydrophobic substance and by the marginal hairs (Cook 1990).

Natural seed germination of *Nymphoides* was not observed in the field. In controlled condition seeds germinated in dung –soil mixture and Gibberellin supplemented soil. Maximum seed germination observed in dung-soil mixture. Bhimachar (1955) reported that 25 seeds of *N. hydrophylla* sown in the soil. Out of the 25 seeds, one seed germinated after the 92 days.

In natural population vegetative propagation was observed. The vegetative propagation mainly through rhizome, brocken of leaves with part of shoot attached or stolon. It is observed that the thick floating vegetation associated with *N. indica* (*Eichornia crassipes, Salvinia molesta* etc.) prevents the vegetative propagation by blocking the newly formed vegetative buds on the floral axis from sinking and subsequent sinking at the bottom leading to the diminishing of the population.

The vegetative propagation is usually performed by:

1. Suckers produced on the rhizome (as in *N. krishnakesara, N. balakrishnanii, N. parvifolia, N. indica,)*
2. By runners produced from the rhizome (as in *N. hydrophylla, N. indica).*
3. Through vegetative buds produced adjacent to the floral axis. Usually both sides of the floral axis decay after senescence and the axis with the developing vegetative bud and roots sink to the bottom. This may also be carried by water currents to distribute to other parts of the water bodies. The thick floating vegetation, sometimes prevent the sinking of these vegetative buds leading to the prevention of proliferation.

Natural enemies (Fig.95)

Pond snails and the caterpillar of some aquatic moths damage the floating leaves of some *Nymphoides* spp. The leaves of *N. indica* is consumed and damaged by pond snails viz., *Succine raoi, Glessula* sp., *Ditropis* sp. and the aquatic caterpillar *Paraponyx* spp. in the hydrophobous stage.

The need for conservation of *Nymphoides* spp.

Earlier the distribution of *Nymphoides* spp. in Kerala was very frequent occupying almost all shallow ponds, canals and paddy fields. But during the last few decades a tremendous decrease in the population of *Nymphoides* spp. is observed. The snow white flowers with heart shaped glabrous leaves were a beautiful scene in the serene beauty of Kerala villages. Probably, the prolonged summer season with hike in day temperature (consequent to the global warming and subsequent climate change) might have adversely affected the *Nymphoides* populations like any other vegetation. The unusual unpredicted flash floods and the landslides have changed the geographic stability of Kerala in recent years. Flash floods hitherto unknown to Kerala have become a regular incidence for the last few years such drastic climate change leading to soil erosion and devastation of vegetation from certain areas have become a common phenomenon in the state. These climatic changes and their consequences have mainly affected aquatic plants in Kerala. The floods carry alluvial soils as sediments but the same time uproot the aquatic vegetation and displace them to unfavourable habitats, eventually leading the destruction of such aquatic species. Moreover, this phenomenon spreads enormous exotic aquatic weeds which were contained in same local ponds such as water hyacinth, *Salvinia molesta*, etc. and distributed in almost all water bodies including inundated paddy fields. This also augment the destruction of local flora. The physiological factors of the existing water bodies such as pH, salinity, eutrophication, etc. were changed drastically making the habitat inimical to the already established aquatic vegetation.

There are mainly two ways of conservation of (aquatic) plants. The *ex situ* conservation and *in situ* conservation. Since the ecology of *in situ* environment is changed owing to global warming and climate change either the plants should acquire adaptive features suitable for the altered environmental conditions or it should move to other congenial environments for its maintenance and proliferation. Since plants cannot move by itself human interaction is necessary for the conservation of these species for creating a suitable niche existed earlier and transport plants to such conditions available elsewhere. In botanical gardens

such situations could be created with proper understanding of the requirements of plants which are in danger, facing habitat loss and subsequent destruction from its natural habitat. The biotechnological methods, such as *in vitro* multiplication could be tried in order to break the reproduction barriers and can reintroduce them with proper modification such as developing tolerance and adaptability and save from the danger of extinction.

Of the *Nymphoides* spp., species like *N. krishnakesara, N. balakrishnanii* and *N. palyi* are endemic to the transient ditches of laterite hills of North Kerala. They show specific survival strategy by perennating through dormant rhizome during summer when the habitat becomes dry and hot. They come up as soon as the first rain of next monsoon is onset and live only for 4 – 6 months. Unfortunately these areas are under serious threat of destruction due to severe anthropogenic activities.

Species like *N. indica, N. macrosperma, N. hydrophylla and N. parvifolia* are purely aquatic and they do not survive in severe droughts. The prolonged summer season which has become characteristic to recent climatic change could be determined to population of these species and unless urgent remedial actions are not taken the extinction of these species may happen in the immediate future.

In the aquatic plant conservatory of Malabar Botanical garden and Institute for Plant Sciences where the theme of conservatory is Aquatic plant and Primitive plants, Six species of *Nymphoides* viz. *N. indica, N. macrosperma, N. krishnakesara, N. hydrophylla, N. parvifolia and N. balakrishnanii* are conserved as live plants. These plants are grown in cement troughs half filled with the original soil collected along with the plant species. In the case of lateritic species viz., *N. krishnakesara, N. balakrishnanii and N. palyi of Northern* Kerala it is found that the soil mixed with powder of laterite is highly beneficial for better growth of these plants.

Moreover, *in vitro propagation* of four species of *Nymphoides* has been successfully done and introduced to the field after hardening are growing up well. The protocol for successful *in vitro* multiplication of these species have been standardised.

7

SUMMARY & CONCLUSION

7. Summary & Conclusion

Nymphoides Seg. is an emergent aquatic plant resembling to *Nymphaea* spp. come under the family Menyanthaceae. The genus can be identified by its rounded or cordate, floating leaves, that hold the small flowers above the water surface on petiole like shoots. *Nymphoides* spp. are both dioecious and monoecious. Nine species and one variety are found in Kerala. Two species are dioecious and the remaining are monoecious. Among the nine species three spp. are endemic to Northern Kerala and growing on lateritic hillocks. Rhizome of the three species *viz*., *N. indica*, *N. hydrophylla* and *N. macrosperma* are medicinally important. Taxonomy and reproductive biology of *Nymphoides* spp. are done with detailed phenological observation and the conservation strategies of these species are discussed.

During the present study seven *Nymphoides* spp. were collected from different areas in Kerala. The species identification and morphological study was done using conventional taxonomic methods. At the time of field survey and collection, it was noted that some species are common, some are endemic and some are endangered. *N. indica* and *N. hydrophylla* are found to be common in Kerala while species like *N. balakrishnanii*, *N. krishnakesara*, *N. macrosperma*, *N. palyii,* etc. are highly endemic to certain seasonal ponds and streams in the state. All the collected specimens of *Nymphoides* spp. are preserved and mounted on herbarium sheets and deposited at MBGH. Six species could be successfully established in the aquatic plant conservatory (Aquagene) of Malabar Botanical Garden and Institute of Plant Sciences. A taxonomic key based on the morphological characters was also prepared to facilitate the easy identification of the species in the field. A comparative morphological study will be very useful for cluster analysis and tree making (Dendrogram).

This is the first attempt to study the reproductive biology of *Nymphoides* spp. in Kerala. It covers phenology, pollen biology, stigma biology, pollination biology, seed set, flower fruit ratio, ovule seed ratio, seed viability,

fruit and seed dispersal and seed germination. These studies were carried out during the period 2016 – 2018 and the results are summarized below.

N. indica, commonly known as *Chinnambel, Panchi, Thakaram, Elayambel, Thettambaral, etc.* The flowers are heterostylous, white with yellow throat covered with hairs. Leaf development from initiation to yellowing is completed in 34th to 36th days. 15th to 16th days are taken for flower development and the fruit development is completed within 26 – 30 days. The peak time of flowering is November – January. Anthesis was in early morning between 06.00 am - 08.00 am. The anther dehiscence starts before half an hour of anthesis. The pollen grains are medium sized, monad, triangular - obtuse, heteropolar, radially symmetric, parasyncolpate and prolate – spheroidal. P/ E ratio of pollen grain of short styled flowers is 105.99 ± 4.89 µm (n =100) and that of long styled flowers is 106.80 ± 8.87 µm (n =100). Pollen surface is spinulose. The pollen grains have maximum viability at 2 hours before anthesis (83.64% in TTC and 84.3% in Acetocarmine). In vitro pollen germination was maximum in in 8% Brewbaker and Kwack's medium and in 8 % sucrose solution after 3 hour. The stigma was most receptive from 11.00 am – 2.00 pm. Insect visits started at the time of anthesis before flower opening. The flower visitors are *Apis mellifera, Apis florae, Notiphila* spp., *Bagous* spp., *Lasioglossum curtis, Lasioglossum mutilum, Ceriagrion rubiae* and *Ceriagrion coromandelianum*. *Lasioglossum mutilum, Bagous* spp. and *Notiphila* spp. Pollination efficiency of short styled flower is 0.024 and that of the long styled flower is 0.023. Fruits are produced only by intermorph pollination. The mean percentage of fruit set in short styled flowers in natural population is 72% and that of the long styled flowers is 70%. The mean ovule: seed ratio of short style flower 17 : 12 and that of long styled flower is 15 : 9.

N. krishnakesara commonly known as 'krishnambel. The flowers are monoecious, white. Leaf development is completed during 32nd – 38th day of intervals. The flower development is completed on 13 or 14th days and the fruit development is completed in 21– 24 days. The peak time of flowering is November - January. Anthesis was in the early morning between 06.00 am –

08.00 am. The pollen grains are 43000 ± 4830 per flower, medium sized, monad, triangular, heteropolar, radially symmetric, syncolpate and prolate – spheroidal with spinulose exine. The pollen grains have maximum viability at 2 hours before anthesis. The pollen germination is maximum in 4% solution of Brewbakers and Kwack's solution and sucrose solution. The stigma receptivity is maximum during 11.00 am – 2.00 pm. Insect visits started at the time of anthesis. The floral visitors are *Notiphila* species and *Ceriagrion* spp. 54% of fruit set were observed in natural. Vegetative propagation is prominent through rhizomes.

N. hydrophylla commonly known as 'Cheruthettambel, Neythelambel. The flowers are gynodioecious, white with yellow throat without hairs. Leaf development is completed in 40 to 42nd days, flower development is completed in 15th or 16th days and the fruit development is completed in 21 – 22 days. The peak time of flowering is November – January. Anthesis is 08.00 am – 09.00 am. The pollen grains 27000 ± 4830 per flower, medium sized, heteropolar, monad, triangular, radially symmetric, prolate – spheroidal, exine spinulose. The pollen grains have maximum viability at 2 hours before anthesis. Pollen germination is maximum in 4% Brewbaker and Kwack's solution and sucrose solution. The stigma receptivity is maximum during 11.00 am – 2.00 pm. Insect visits started at the time of anthesis. The floral visitors are *Notiphila* sp., *Lasioglossum* sp. *Telicota bambusae* (Butterfly) and *Componatus* sp. *Notiphila* spp. is the effective pollinators in *N. hydrophylla*. Pollination efficiency of bisexual plant is 0.022 and that of the female plant in natural condition is zero. Fruit set was noticed only in the bisexual flowers under natural condition. Through manual cross pollination 80% fruits were produced in bisexual flower and 28% in female flower. The fruits are developed under the water below the floating leaf, after 22nd day, the seeds are dispersed in water, floats on the water and later sunk in to the bottom of the water body. Vegetative propagation is prominent through stolons, and brocken leaf with part of stem attached.

N. parvifolia is commonly known as 'Kunjambel". The flowers are bisexual with 3 – 4 petals, small, white with yellow throat and fimbriate petal.

Leaf development is completed in 28 – 32nd days, flower development is completed in 13 – 14th days and the fruit development is completed in 15th or 16th days. The peak time of flowering is November - January. Anthesis is at 08.00 am – 09.00 am. The pollen grains are 1027 ± 38 in 3 petal petal, 1360 ± 33 in 4 petal per flower. The pollen grains are medium sized, monad, triangular, heteropolar, radially symmetric, syncolpate and prolate spheroidal, exine spinulose. The pollen grains have maximum viability at 2 hours before anthesis. The pollen germination is maximum in 12% Brewbaker and Kwack's medium (78%) and 8% of sucrose solution (78%). The stigma receptivity is maximum during 12.00 am – 2.00 pm. Insect visits started at the time of anthesis. The floral visitor is *Notiphila* sp. pollination efficeiency of 3 petal flower was 0.08 and in 4 petal flower was 0.07 Fruit set is 96% in natural condition, 94% of fruits are produced by self pollination and 100 % fruits are produced by manual cross pollination. The fruits are developed under water below the floating leaf, on the 18 – 20 day the fruit wall is broken and the seeds dispersed in the water, floats on the water and later sunk to the bottom. Vegetative propagation is prominent.

N. balakrishnanii is endemic to Kasaragod District. The flowers are small, white with yellow throat and fimbriate petal. Leaf development is completed in 26 – 28 days, flower bud development is completed in 14-15 days and the fruit development is completed in 25 - 26 days. The peak time of flowering is November – Janury. Anthesis is at 08.00 am – 09.00 am. The pollen grains are 1840 ± 372 per flower, medium sized, monad, triangular, heteropolar, radially symmetric, syncolpate and prolate – spheroidal, exine spinulose. Pollen-ovule ratio is 170 : 1. The pollen viability maximum at 2 hours before anthesis. The pollen germination is maximum in 12% Brewbaker and Kwack's medium and sucrose solution. The stigma receptivity is maximum during 12.00 am – 2.00 pm. Insect visits started at the time of anthesis. The flower visitors are *Componotus sericeous, Componotus* sp. *and Xya* spp. *Componotus sericeous* is the effective pollinators in *N. balakrishnanii.* Pollination efficiency is 0.06. Fruit set is 98% in natural condition, 94% of fruits are produced by self pollination and 100 % fruits are produced by manual cross pollination. Seed set percentage is

84%. The fruits are grown under the water below the floating leaf, on the 24 - 26days the fruit wall is broken and the seeds liberated in the water, floats on the water for sometimes and later sunk into the bottom of the water body. Vegetative propagation is prominent through tillering.

N. macrosperma is a critically endangered plant. The flowers are small, hairy, white with yellow throat and fimbriate petal. The flower development is completed in 31 -32 days. The flower development is completed in 15 -16 days. Anthesis is at 08.00 am – 09.00 am. The pollen grains are 3100 ± 803 per flower, monad, triangular, radially symmetric, medium sized, tricolpate and prolate – speroidal – sub-prolate, exine spinulose. The stigma receptivity is maximum during 11.00 am – 2.00 pm. Insect visits started at the time of anthesis itself. The flower visitor in male flower is *Notiphila* sp. Vegetative propagation is prominent through brocken leaf with part of rooted stem attached.

N. palyii is endemic to Kasaragod district restricted to shallow temporary pools of laterite hills. The flowers are bisexual, small, hairy, white with yellow throat and minute fimbriate petal. Stamens 3 or 4, epipetalous; pistil bottle shaped and bifid. Ovary hypogynous with parietal placentation. Capsule green, oblong with 4- 10 brownish black seeds. This is a feeble species and failed to persist when introduced to the conservatory of MBGIPS. The phenetic analysis of *Nymphoides* spp. in Kerala shows that species of *Nymphoides* in Kerala come into two distinct groups. Group I includes *N. indica*, *N. parvifolia, N. balakrishnanii* and *N. palyii. N. parvifolia* and *N. balakrishnanii* are closely related species and shows highest affinities in group I. Group II consist of three species viz., *N. hydrophylla, N. krishnakesara, N. macrosperma.* In group II *N. krishnakesara* and *N. macrosperma* show highest affinities among them. So the Kerala species of *Nymphoides* spp. show two groups and these two groups show 66% dissimilarities.

The *Nymphoides* spp. in Kerala are facing serious problems owing to environmental changes such as high temperature, destruction of habitat due to anthropogenetic activities, etc. The effect of global warming is very conspicuous

in Kerala climate with prolonged summer and high rise in temperature and flooding during the rainy season. The prolonged dry season cause drought in shallow warm ponds killing the perennating rhizome of *Nymphoides* spp. which due to the prime habitat or this genus. Even the most common species such as *N. indica* are also facing this problem. Unless and until imminent control measures such as *ex situ* conservation are being taken the very existence of this genus in Kerala is at risk.

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List of Publications

Pavisha P, Pradeep N S & Madhusoodanan P V. 2020. **"Comparative morphology and Phnetics of *Nymphoides* species in Kerala".** *Plant Science Today.*Vol. 7(2): 233 – 239.

Pavisha Pongott, P V Madhusoodanan & R Prakashkumar. 2020**. "Pollen Biology and Siggma receptivity of *Nymphoides indica* L. (O.) Kuntze (Menyanthaceae)"**. *Research & Reviews: A Journal of Life Science*. Vol. 10(1):72 – 80p.

Others

Pavisha P, P V Madhusoodanan & R Prakashkumar. 2015. **"*Fasciation in Crotalaria retusa* L."**. *Phytotaxonomy.* Vol.14: 161 – 163.

PLANT SCIENCE TODAY, 2020
Vol 7(2): 233–239
https://doi.org/10.14719/pst.2020.7.2.735
ISSN 2348-1900 (online)

HORIZON
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RESEARCH ARTICLE

Comparative morphology and phenetics of *Nymphoides* species in Kerala

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ARTICLE HISTORY
Received: 23 January 2020
Accepted: 31 March 2020
Published: 02 May 2020

KEYWORDS
Nymphoides
Menyanthaceae
phenetics
dendrogram

ABSTRACT

Aquatic plants play an important role in maintaining the ecosystem balance. *Nymphoides* Seguier (Menyanthaceae) is an emergent, rhizomatous or stoloniferous genus, commonly known as "floating hearts", identified by its rounded or cordate, floating leaves, petiole like branches and unique floral characters. The present study deals with the comparative morphology and the phenetics of *Nymphoides* spp. in Kerala. These species are classified into two groups with the help of the dendrogram, which showed 66% dissimilarities among the two groups. *N. parvifolia* and *N. balakrishnanii* are closely related species in one group and *N. krishnakesara* and *N. macrosperma* are closely related species in other group.

Introduction

Menyanthaceae Dumort. are the most diverse and widespread family of aquatic and wetland plants under the order Asteralses. The family has five genera with 60–70 species distributed all over the world. The five genera are *Menyanthes* L., *Liparophyllum* Hook. f., *Nephrophyllidium* Gilg., *Villarsia* Vent. and *Nymphoides* Seguier. Of these five genera, *Nymphoides* are cosmopolitan in distribution. *Menyanthes* and *Nephrophyllidium* are restricted to Northern Hemisphere, *Liparophyllum* and *Villarsia* are found in Southern Hemisphere (11). The genus *Nymphoides* can be identified by its rounded or cordate, floating leaves, petiole like branches and its floral characters. *Nymphoides* species are both dioecious and monoecious.

The genus *Nymphoides* has 40–50 species in world, which are found in tropical regions of Africa, Australia, the Americas, India, and southeastern Asia (1, 5–8, 11). Eight species are found in India, seven of which are growing in Kerala (8). Recently two new species and one variety are reported (2, 3). The present study deals with the comparative morphology and phenetics of seven *Nymphoides* species in Kerala.

Materials and Methods

Seven *Nymphoides* spp. were collected from different parts of Kerala and conserved in the aquatic plant conservatory (Aquagene) of the Malabar Botanical Garden and Institute for Plant Sciences. The morphological and phylogenetic characters of the specimens were compared on the basis of visible characters. The phylogeny of seven species of *Nymphoides* in Kerala was done (Fig. 1 & Fig. 2).

The data for the analysis were collected from fresh material collected from different localities and also from herbarium specimens deposited in the Central National Herbarium (CAL), Botanical Survey of India, Southern Regional Centre (MH), Calicut University Herbarium (CALI) and Malabar Botanical Garden Herbarium (MBGH). In the present study, twenty two multi-state qualitative morphological characters of the *Nymphoides* spp. were considered (Table 1) and gave code numbers to the seven species (Table 2). The characters (with abbreviated codes) used in the cluster analysis with their character states are given in Table 1. The characters with two states were coded as 1 and 2 and also given continuous numbers when the character states are more than two. The results of multiple

To cite this article: Pavisha P, Pradeep NS, Madhusoodanan PV. Comparative morphology and phenetics of *Nymphoides* species in Kerala. Plant Science Today. 2020;7(2):233–239. https://doi.org/10.14719/pst.2020.7.2.735

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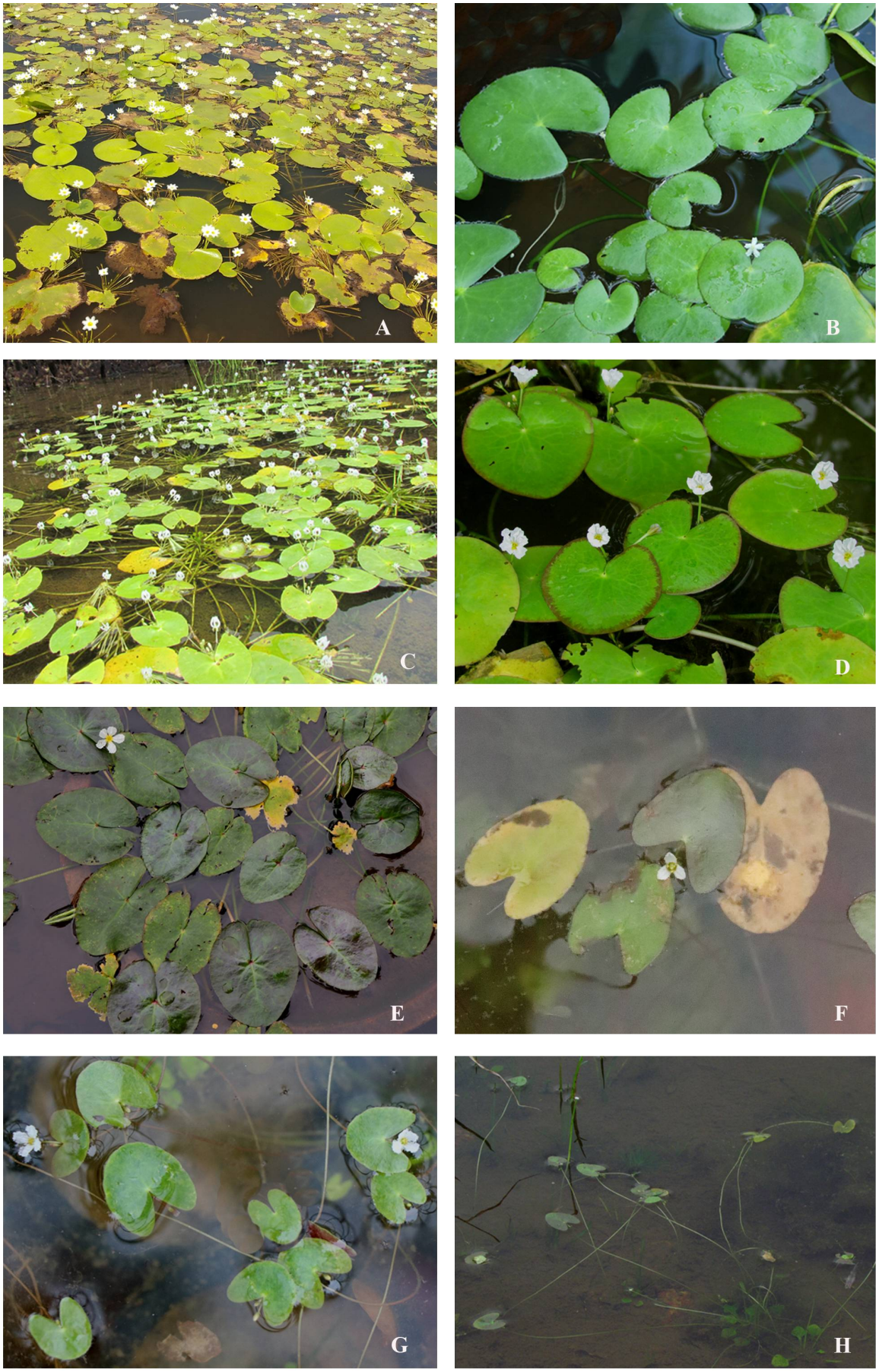

Fig.1. *Nymphoides* species in Kerala. **A.** *N. indica* (L) Kuntze, **B**. *N. macrospema* R.V.Nair, **C.** *N. krishnakesara* K.T. Joseph & Sivar., **D & E**. *N. hydrophylla* (Lour.) Kuntze, **F**. *N. parvifolia* Kuntze, **G**. *N. balakrishnanii* Biju, Josekutty, Haneef & Augustine., **H**. *N. palyii* Biju, Josekutty, Haneef & Augustine

range tests of characters of the OTUs are given in the Table 3. For cluster analysis, all the twenty two characters were tabulated against the seven OTUs using the numerical codes given for character

Fig. 2. Flowers of *Nymphoides* species in Kerala. **A & B.** Short styled and Long styled flowers of *N. indica*, **C & D**. Male and Female flowers of *N. macrosperma*, **E & F.** Male and Female flowers of *N. krishnakesara*, **G & H.** Bisexual & Female flowers of *N. hydrophylla*, **I & J.** Flowers of *N. parvifolia*, **K**. *N. balakrishnanii* flower, **L**. Flower of *N. palyii* (Herbarium)

states and abbreviated codes of characters (Table 4). These data were used to generate dendrogram using the statistical package STATISTICA version 7.0, adopting Unpaired Group Method with Arithmetic mean as algorithm (9) and percent disagreement (4) as the statistical test.

Table 1. Characters used in the cluster analysis

Sl. No.	Code	Characters	Characters states and their code numbers
1.	Hab	Habitat	Clay soil (1) or lateritic soil (2), clay soil and lateritic soil (3)
2.	Plt	Plant	Bisexual (1) or Unisexual (2), Bisexual and female (3)
3.	Rhi	Rhizome	Stoloniferous (1) or non stoloniferous (2)
4.	Sht	Shoot	Dimorphic (1) or monomorphic (2)
5.	Lvs	Leaves	Dimorphic (1) or Monomorphic (2)
6.	Flr	Flower	Bisexual (1), unisexual (2), bisexual or unisexual (3)
7.	ClxL	Calyx lobe	Oblong-acute (1), oblong–obtuse (2), linear– lanceolate (3), elliptic-lanceolate (4)
8.	Co	Corolla	White with yellow throat (1) or white (2)
9.	PetL	Petal lobe	Fimbriately toothed (1), shallowly fimbriate or undulate (2)
10.	PetLS	Shape of petal lobe	Obtuse or retuse (1), ovate to lanceolate (2), oblong-obtuse (3), oblong or elliptic (4)
11.	HrPL	Presence of hairs on petal lobe	Present (1) or absent (2)
12.	Stm	Stamen	Dimorphic (1) or monomorphic (2)
13.	ClrA	Colour of Anther	Yellow (1), pale purple (2), blue (3), black with yellow (4), Cream (5)
14.	NoSt	No. of stamens	Three or four (1), four (2), five (3), four to eight (4)
15.	ISG	Inter staminal gland	Present (1) or absent (2)
16.	ArC	Stigmatic hair	Present (1) or absent (2)
17.	Stl	Style	Heterostylous (1) or homostylous (2)
18.	HgG	Hypogynous gland	Hairy (1), hairless (2)
19.	Fr	Fruit	Ellipsoid (1), sub globose (2), obovoid (3), oblong (4), or oblong to obovoid (5)
20.	Se	Seed	Tuberculate (1) or smooth (2)
21.	SeS	Shape of seed	Discoid (1), obovate or elliptical (2), Obovoid (3)
22.	PoS	Pollen shape	Prolate-spheroidal (1), prolate-spheroidal to subprolate (2) or oblate-spheroidal (3)

Phenetics

Phenetics (also known as taximetrics) is a method to classify organisms based on the overall morphological similarity notwithstanding the evolutionary relationships/ characters. In the present study the results of multiple range tests of characters of the OTUs are given in the Supplementary Table 1. For cluster analysis, all the twenty two characters were tabulated against the seven OTUs using the numerical codes given for character states and abbreviated codes of twenty two characters.

Table 2. *Nymphoides* spp. (OTUs) selected for the study

Code No.	Name of the species (OTUs)
1.	*Nymphoides indica* (L.) Kuntze
2.	*Nymphoides macrosperma* R.V.Nair
3.	*Nymphoides krishnakesara* K.T. Joseph & Sivar.
4.	*Nymphoides hydrophylla* (Lour.) Kuntze
5.	*Nymphoides parvifolia* Kuntze
6.	*Nymphoides balakrishnanii* Biju, Josekutty, Haneef & Augustine
7.	*Nymphoides palyii* Biju, , Josekutty, Haneef & Augustine

Results and Discussion

The genus *Nymphoides* in Kerala state is represented by nine species and one variety, namely, *Nymphoides aurantiacum, N. balakrishnanii, N. hydrophylla, N. indica, N. krishnakesara, N. krishnakesara* var. *bispinosa*, *N. macrosperma*, *N. parvifolia*, *N. palyii* and *N. sivarajanii*. Eventhough after extensive explorations in the previous known localities, we could not relocate *N. aurantiacum, N. krishnakesara* var. *bispinosa* and *N. sivarajanii* and hence, they were not included in the present study. The worst flood occurred in Kerala state in 2018 may also adversely affected the fragmented population of these highly vulnerable species.

Key to the species of *Nymphoides* in Kerala

1a	Flowers corolla upto 40 mm in diam., distylous; corolla upper side totally covered with dense hairs.	*N. indica*
1b	Flowers corolla upto 22 mm in diam., small, homostylous; corolla partially or not covered with hairs	(2)
2a.	Plants dioecious; stigma bilobed with a whorl of radiating glandular hairs	(3)
2b.	Plants monoecious or gynodioecious; stigma bilobed without a whorl of radiating glandular hairs	(4)
3a.	Shoot dimorphic, many jointed each joint bearing single leaf; leaves monomorphic; corolla lobes without median wings; hypogynous glands hairy	*N. macrosperma*
3b.	Shoot monomorphic, single leaf arising from the axils of rhizome; leaves dimorphic; corolla lobes with median wings; hypogynous glands not hair	*N. krishnakesara*
4a.	Flowers unisexual (female) or bisexual; corolla lobes not fimbriate, undulate margine with upper median wing; seeds 2 mm across.	*N. hydrophylla*
4b.	Flowers bisexual; corolla lobes fimbriate without median wing; Seeds small, less than 2 mm across	(5)
5a.	Petals 4 in number; floating leaves obovate – orbicular; calyx exceeds the fruit	*N. balakrishnanii*
5b.	Petals 3 or 4 in number; floating leaves ovate – orbicular; calyx not exceeds the fruit	(6)
6a.	Floating leaves pale green – brownish green with pinkish tinge; hypogynous gland present; seed surface tuberculated	*N. parvifolia*
6b.	Floating leaves dark green; hypogynous gland not distinct; seed surface smooth	*N. palyii*

The result of comparative morphology of the seven *Nymphoides* spp. is given in Supplementary Table 1. The dendrogram of seven *Nymphoides* spp. in Kerala was obtained through the cluster analysis, using character states of the OTUs and resulted the percent disagreement between OTUs (Table 5 & Fig. 3. The results of dendrogram clearly revealed that the seven species come into two distinct groups Group I and Group II.

Group 1: 1, 5, 6 & 7

Group II: 2, 3 & 4

Group I and Group II were clustered together and show 66% dissimilarities. Group I is classified into Group IA and Group IB. In Group I *Nymphoides indica* is clustered distantly from other species and shows 55% dissimilarities from other species *viz*., *Nymphoides parvifolia, N. balakrishnanii* and *N. palyii.* Group IB is again classified into Group IB_1 and Group IB_2. *N. palyii* is come under Group IB_2 and are separated from *N. balakrishnanii* and *N. parvifolia* (Group IB_1). *N. palyii* shows 34% dissimilarities with other two species. These two species come in Goup IB_1 are closely related, which shows highest affinities (68% similarities).

Group II consists of two clusters Group IIA and Group IIB and represents three taxa *viz*., *N. hydrophylla, N. krishnakesara, N. macrosperma.* Group IIB is separated from Group IIA which consists of two taxa *viz*., *N. krishnakesara* and *N. macrosperma.* Group IIB is 60% dissimilar with Group IIA. In this study *N. krishnakesara* is clustered together with *N. macrospermum* instead of *N. hydrophylla. N. krishnakesara* shows more similarity (50%) with *N. macrosperma* than *N. hydrophylla.* On

Table 3. Results of multiple range test of characters

Code for Characters	Characters	Grouping of Taxa (represented by code numbers) according to their character states				
		1	2	3	4	5
Hab	Habitat	Clay soil 2, 4	Laterite soil 3, 5, 6, 7	Caly soil and laterite soil 1		
Plt	Plant	Bisexual 1, 5, 6, 7	Unisexual 2, 3	Bisexual and female 4		
Rhi	Rhizome	Stoloniferous 1,2,4	Nonstoloniferous 3, 5,6, 7			
Sht	Shoot	Dimorphic 1, 2, 4	Monomorphic 3, 5, 6, 7			
Lvs	Leaves	Dimorphic 3, 5, 6, 7	Monomorphic 1, 2, 4			
Flr	Flower	Bisexual 1, 5, 6, 7	Unisexual 2, 3	Bisexual and unisexual 4		
ClxL	Calyx lobe	oblong–acute 1, 5	Oblong-obtuse 2, 3	Linear- lanceolate 4, 6	Elliptic-lanceolate 7	
Co	Corolla	White with yellow throat 1, 4, 5, 6, 7	white 2, 3			
PetL	Petal lobe	Fimbriately toothed 1, 2, 3, 5, 6, 7	Undulate 4			
PetLS	Shape of petal lobe	Obtuse or retuse 4	ovate to lanceolate 1, 6, 7	Oblong or obtuse 3	oblong or elliptic 2, 5	
HrPL	Presence of hairs on petal lobe	Present 1, 2, 5, 6, 7	Absent 3, 4			
Stm	Stamen	Dimorphic 1	Monomorphic 2, 3, 4, 5, 6, 7			
ClrA	Colour of Anther	Yellow 4, 5	Pale purple 1, 2	Blue 3	Black with yellow 6	Cream 7
NoSt	No. of stamens	Three or four 5, 7	Four 6	Five 2, 3, 4	Four to eight 1	
ISG	Inter staminal gland	Present 1, 2, 3, 4, 5, 6	Absent 7			
ArC	Stigmatic hair	Prsent 2, 3	Absent 1, 4, 5, 6, 7			
Stl	Style	Heterostylous 1	Homostylous 2, 3, 4, 5, 6, 7			
HgG	Hypogynous gland	Hairy 1, 2, 4, 6	Hairless 3, 5	Absent 7		
Fr	Fruit	Ellipsoid 1, 5	Sub globose 2	Obovoid 3	Oblong 4, 7	Oblong to obovoid 6
SeO	Seed ornamentation	Tuberculate 2, 3, 4, 5, 6	Smooth 1, 7			
SeS	Seed shape	Discoid 1, 4, 5, 6, 7	Obovate or ellipsoid 2	Obovoid 3		
PoS	Pollen shape	Prolate-spheroidal 1, 3, 4, 6	Prolate-spheroidal to subprolate 2	Oblate-spheroidal 5, 7		

Table 4. Character states of the OTUs

Character codes	Codes for OTUs						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Hab	3	1	2	1	2	2	2
Plt	1	2	2	3	1	1	1
Rhi	1	1	2	1	2	2	2
Sht	1	1	2	1	2	2	2
Lvs	2	2	1	2	1	1	1
Flr	1	2	2	4	1	1	1
ClxL	1	2	2	3	1	3	4
Co	1	2	2	1	1	1	1
PetL	1	1	1	2	1	1	1
PetLS	2	4	3	1	4	2	2
HrPL	1	1	2	2	1	1	1
Stm	1	2	2	2	2	2	2
ClrA	2	2	3	1	1	4	5
NoSt	4	3	3	3	1	2	1
ISG	1	1	1	1	1	1	2
ArC	2	1	1	2	2	2	2
Stl	1	2	2	2	2	2	2
HgG	1	1	2	1	2	1	3
Fr	1	2	3	4	1	5	4
Se	2	1	1	1	1	1	2
SeS	1	2	3	1	1	1	1
PoS	1	2	1	1	3	1	3

the basis of morphological data (including pollen, seed and flavonoid data) and molecular data (10) the floating-leaved genus *Nymphoides* was reported as monophyletic, except for *N. exigua.* Based on the morphological and molecular study the Indian species of *Nymphoides* viz. *N. hydophylla, N. parvifolia* and *N. sivarajanii* were closely related to *N. krishnakesara* and *N. macosperma* (12). The phenetic analysis of seven species of *Nymphoides* spp. gives a picture on the affinities of *Nymphoides* spp. in Kerala. The results shows that *N. parvifolia* and *N. balakrishnanii* are closely related species and shows highest affinities. *N. krishnakesara* and *N. macrosperma* in Group II also shows high affinity. *Nymphoides* spp. of Kerala shows two groups showing 66% dissimilarities.

Table 5. Percent disagreement between OTU's under study

OTU	OTU's arranged as per their numerical codes						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	0.00	0.64	0.86	0.59	0.55	0.50	0.59
2	0.64	0.00	0.50	0.55	0.68	0.68	0.82
3	0.86	0.50	0.00	0.68	0.55	0.55	0.68
4	0.59	0.55	0.68	0.00	0.64	0.55	0.73
5	0.55	0.68	0.55	0.64	0.00	0.32	0.32
6	0.50	0.68	0.55	0.55	0.32	0.00	0.36
7	0.59	0.82	0.68	0.73	0.32	0.36	0.00

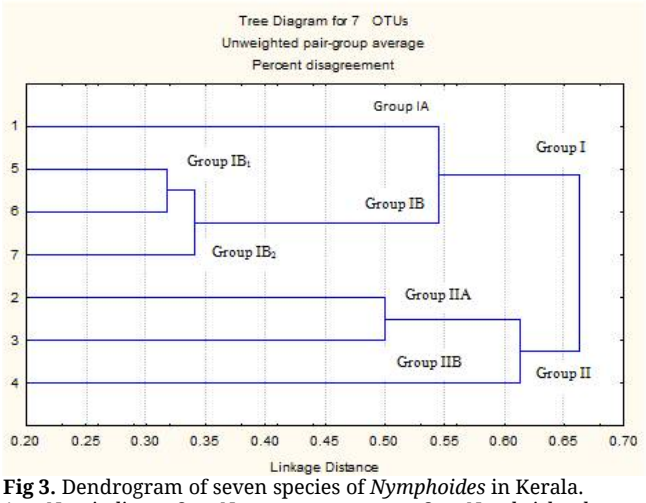

Fig 3. Dendrogram of seven species of *Nymphoides* in Kerala. 1. *N. indica,* 2. *N. macrosperma,* 3. *N. krishnakesara,* 4. *N. hydrophylla,* 5. *N. parvifolia,* 6. *N. balakrishnanii,* 7. *N. palyii.*

Specimens examined

a. *Nymphoides indica*: INDIA. Kerala: Kottayam District, Vaikom, 21 April 1988, *Swaminathan M. S. 88274* (CAL!). Trichur District, Paliappara, Chalakkudy, 22 Septmeber 1982, *Ramamurthy 74703*; *ibid*., *Ramamurthy 74863* (CAL!). Alappuzha District, Kanichukulangara junction, *s.die*, *Sunil C. N. 2210* (CALI!); *ibid*., Angadikal, *s.die*, *Sunil C. N. 2624* (CALI!); Kasaragod District, Peelikode, 16 February 2007, *Suresh K. K 03152* (MBGH!). Kozhikode District, Olavanna, 25 September 1999, *Krishnan P. N. 825* (MBGH!); *ibid*., 27 November 2015, *Pavisha P. 12370* (MBGH!); Oorkadavu, 27 June 2008, *Suresh K. K. 03994* (MBGH!); Pantheerankavu, 21 December 2016, *Pavisha P. 12375 (MBGH!)*. Kasaragod District, Peelikode, 16 February 2007, *Suresh K. K. 03317* (MBGH!); Kayyur, 05 February 2007, *Suresh K. K. & Jaris P. K. 04405* (MBGH!); *ibid*., *s. die*, *Suresh K. K. & Jaris P. K. 03181* (MBGH!).

b. *Nymphoides macrosperma*: INDIA. Kerala: Kozhikode District, Olavanna, 14 July 2006, *Krishnan P. N. 2064* (MBGH!); Pantheerankavu, 22 February 2016, *Pavisha P. 12372* (MBGH!).

c. *Nymphoides krishnakesara*: INDIA. Kerala: Kannur District, Madaippara, 25 October 1988, *Joseph K. T. 43001* (MH!); *ibid*., 20 September 2005, *Krishnan P. N. & Ansari R. 2563* (MBGH!); *ibid*., 04 September 2008, *Suresh K. K. 4004* (MBGH!); *ibid*., 12 December 2016, *Pavisha P, Rajilesh V. K. & Ajesh P. P. 12373* (MBGH!); Payyannur, 29 July 2009, *Suresh K. K. 4964* (MBGH!); *ibid*., 07 October 2008, *Suresh K. K. 4288* (MBGH!); *ibid*., 04 September 2008, *Suresh K. K. & Jaris P. K. 3317* (MBGH!); Korom, 15 January 2019, *Pavisha P, Jaseela V. T & Sinisha P. 17748* (MBGH!).

d. *Nymphoides hydrophylla*: British Burma, 04 January 1971, *S Kurz. 2264* (CAL – Holotype!). INDIA. Kerala: Palakkad District, Elavanchery, 02 November 1976, *Vajravelu M. S.* 48836 (CAL!). Kannur District, Ezhuvamalai, 17 December 1979, *Ramachandran 65266* (CAL!); *ibid*., 20 July 1981, *Ansari R. 70966* (CAL!). Thrissur District, Chalakudi, 15 September 1976, *Ramamurthy 48535* (CAL!). Thiruvananthapuram District, Pulimath, 02 December 1977, *Mohanan 52675* (CAL!). Kasaragod District, Peelikode, 06 February 2007, *Suresh K. K. & Jaris P. K. 03162* (MBGH!); Kayyur, 06 February 2007, *Suresh K. K. & Jaris P. K. 03188* (MBGH!). Kollam District, 16 February 2007, *Suresh K. K. 03229* (MBGH!). Idukki District, Moolamattam, 17 December 2008, *Suresh K. K. 04436* (MBGH!). Malappuram District, Koottumoochi, 18 November 2011, *Anoop K. P. & Hareesh K. T. 6537* (MBGH!); Kozhikode District, Palazhi, 22 February 2016, *Pavisha P. 12371* (MBGH!).

e. *Nymphoides parvifolia*: INDIA. Kerala: Kasaragod, Beemanadi, 27 September 1982, *Ansari R. 74328* (CAL!); *ibid*., 18 December 2008, *Suresh K. K. 4954* (MBGH!); Kayyur, 07 December 2018, *Pavisha P, Hridhya P & Ajesh P. P. 15813* (MBGH!).

f. *Nymphoides balakrishnanii*: INDIA. Kerala: Kasaragod, Koovappara, 24 January 2019, *Pavisha P. 17747* (MBGH!).

g. *Nymphoides palyii*: INDIA. Kerala: Kasaragod, Perla, 19 September 2016, *Anoop K. P. & Rajilesh V. K. 14595* (MBGH!); *ibid*., 07 December 2018, *Pavisha P, Hridhya P. & Ajesh P. P. 15831* (MBGH!).

Authors' contributions

PP carried out the field studies, identified the specimens and prepared the manuscript. MPV and PNS confirmed the species identity and helped in the manuscript preparation. All authors read and approved the final manuscript.

Acknowledgements

The authors are thankful to the Kerala State Council for Science Technology & Environment (KSCSTE) for financial support. The authors are thankful to the Director, KSCSTE Malabar Botanical Garden & Institute for Plant Science for providing the facilities. We also acknowledge the support and help provided by Dr. Mohanan K. V., Professor of Genetics, Calicut University in the preparation of the Dendrogram.

Competing Interest

The authors declare that they have no competing interests.

Supplementary file

Supplementary Table 1

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STM JOURNALS

Research & Reviews: A Journal of Life Sciences
ISSN: 2249-8656 (Online), ISSN: 2348-9545 (Print)
Volume 10, Issue 1
www.stmjournals.com

Pollen Biology and Stigma Receptivity in *Nymphoides indica* (L.) O. Kuntze (Menyanthaceae)

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Abstract

Nymphoides indica (L.) O. Kuntze *an emergent aquatic plant of Menyanthaceae, commonly called 'floating hearts" is bisexual, hypogynous and distylous (Two types of style – long style and short style) with 4 – 8 sepals and petals. Stamens correspond to the number of petals. The present study deals with phenology, pollen biology and stigma receptivity of N. indica. The pollen grains are yellow, medium, monad, triangular, heteropolar, radially symmetric, parasyncolpate and prolate – spheroidal in shape. The viable pollen grains becomes pink or red in 1% aceto carmine, blue coloured in aniline blue lactophenol stain and brown coloured in IKI solution. Stigma receptivity was high in the post pollination period.*

Keywords: *Nymphoides indica, floating hearts, phenology, stigma receptivity, pollen viability*

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INTRODUCTION

The genus *Nymphoides* Seguier is an aquatic herb either floating or creeping characterized by graceful and elegant snow-white flowers and heart shaped floating leaves. It usually occurs at the shallow periphery of streams, pools and as a weed in rice fields and aquaducts [1]. The *Nymphoides* species usually possess dioecious or monoecious, distylous or homostylous breeding systems [2].

Taxonomic revision of the genus *Nymphoides* Seguier was made in India based on the floral morphology and reported eight species [3]. Out of these species *Nymphoides peltata* (Gmel.) O. Kuntze is restricted in Kashmir while the other seven *Nymphoides* spp. are pantropical in distribution. Recently two new species and one variety have been reported from Kerala. At present ten species of *Nymphoides* seems to occur in India. Out of these nine species and one variety are found in Kerala viz. *Nymphoides aurantiaca* (Dalzell) Kuntze, *N. hydrophylla* (Lour.) Kuntze, *N. indica* (L.) Kuntze, *N. krishnakesara* K. T. Joseph & Sivar., *N. macrosperma* R. V. Nair, *N. parvifolia* Kuntze, *N. balakrishnanii* Biju, Josekutty, Haneef and Augustine, *N. palyii* Biju, Josekutty, Haneef and Augustine *and N. krishnakesara* var. bispinosa Biju, Josekutty, Haneef and Augustine is the most common and widely distributed [4,5]. The floral dimorphism is associated with self-incompatibility system of *N. indica* [5,6]. It is recorded in IUCN Red list under least concern category [7]. This species is distributed in all the districts of Kerala. The present study deals with the flowering phenology, pollen viability, pollen morphology, pollen germination and stigma receptivity of *N. indica* (L.) O. Kuntze.

MATERIALS AND METHODS

The materials for the present study were collected from different parts of the Kerala and maintained in the Aquagene conservatory of the Malabar Botanical Garden and Institute for Plant Sciences (MBGIPS), Kozhikode. The Flowering phenology and floral morphology were observed in the field as well in the laboratory with the help of a stereomicroscope

(Labomed CZM4). Anthesis was observed in the field at regular intervals from 6.00 AM – 8.00 AM. Insect pollination was studied by observing the kind and the number of floral visitors. Insect visitors were fixed in 70% alcohol and stored for identification.

Pollen production

The number of pollen per anther was counted by using haemocytometer and multiplied this figure by number of anthers per flower [8]. The mature anthers were crushed in lactophenol glycerine with aniline blue. A known dilution was placed on the grid and 10 replicate counts were made using haemocytometer [9].

Pollen – Ovule ratio

The number of ovules per flower was counted. The number of pollen grains divided by the number of ovules per flower gives the pollen – ovule ratio [8].

Pollen morphology

The pollen grains were collected from the mature anthers in a separate vial and studied under light microscope and scanning electron microscope. For light microscopic study, a sample of pollen grains was stained with 1% aceto carmine and observed the morphological features of pollen grains. For SEM study, the pollen grains were collected and stored in 100 % alcohol in a separate vial. One or two drops of pollen mixture were placed on a double-sided conductive tap on the material sputter coated with gold was observed under SEM at the Sophisticated Test and Instrumentation Centre (STIC), Ernakulam, Kerala. Pollen morphology was also studied by acetolysis method [10].

Pollen viability

Pollen viability was studied by different methods proposed by different authors.

a. Aniline blue – lactophenol Test [11]

Aniline blue – lactophenol (1%) was prepared first. Mature and un-dehisced anthers were collected and squashed in 1% aniline blue – lactophenol on a micro slide and observed after 15 minutes under stereomicroscope. The healthy and plumped stained pollen grains were recorded as viable. The pollen grains viability of pre - anthesis, anthesis and post anthesis period was checked.

b. 1% acetocarmine Test [12,13]

Pollengrains were checked at the time of pre - anthesis, anthesis and post anthesis using 1% aceto carmine on a micro slide under stereo microscope. Pollen grains turned red were considered as viable and the colourless grains were considered as nonviable.

c. IKI Solution Test [14]

One gram of KI and 0.5 gram of Iodine dissolved in 100 ml distilled water and kept it in a 100ml bottle. One drop of IKI solution was placed on a micro slide and pollen grains were sprinkled on it. Kept for 5 minutes and observed under Labomed Stereo microscope. Pink or red pollen grains are considered viable.

Stigma Receptivity

The Stigma receptivity was studied by different methods.

a. Hydrogen Peroxide Test [15]

Fresh, mature, unpollinated flower buds were collected and the pistils were carefully excised from the flower. The pistils were kept on a dry micro slide in a humid chamber. One drop of H_2O_2 was dropped on the stigmatic surface of the excised pistil kept on the micro slide. The stigma surface produced bubbles and counted the number of bubbles produced in 1 minute.

b. Aniline Blue – Lactophenol Method [16]

Stigma receptivity was recorded by fixing stigmas of different ages in Carnoy's fixatives. Carnoy's fixatives (6alcohol: 3 chloroform: 1 acetic acid) were prepared and stored in room temperature. The stigmas of *Nymphoides* spp. were fixed first in Carnoy's fixatives at different intervals (Pre - anthesis, Anthesis and Post anthesis) for 24 h and stored in 70 % ethanol. Fixed pistils were transferred to 4N NaOH for 12 hours to make the tissue soft. After washed thoroughly in distilled water, the pistils were stained with 1 % aniline blue lactophenol solution and mounted on a drop of glycerine. The preparation was observed under light microscope.

c. By Visual Observation [17]

According to Teryokin *et al*., stigma receptivity was determined by visual observation. Stigmas which were transparent,

shining and also adhere to $1mm^2$ piece of paper were considered as receptive.

d. Alpha – Naphthyl Acetate Test [18]

Fresh, mature, unpollinated flower buds were collected. The flowers were handled with forceps without injuring the stigma and style and carefully excised the pistil from the flower. The pistils were kept on a dry microslide in a humid chamber. Two types of solutions A and solution B were prepared for this experiment. A few drops of solution A took on a microslide and solution B on another microslide. The excised stigmas were dipped into solution A and B separately. The slides were then incubated at 25^0 C in a humidity chamber for 22 minutes. The stigmas were removed after incubation period and rinsed thoroughly with phosphate buffer, pH 6.8. The stigmas were mounted in 50 % glycerine and observed under light microscope and studied the stigmatic surface.

RESULTS

Nymphoides indica (L.) O. Kuntze is an aquatic heterostylous, rhizomatous herb and exhibits incompatibility mechanism. Flowers are dimorphic (long styled and short styled flowers) borne on separate plants. The flowers are tetramerous to octamerous borne in the same plant or in the separate plants. The stamens are corresponding to the petals number. The number of ovules varies in each flower. Pollinators were found to be attracted by pollen grains as well as honey. The floral visitors of *N. indica* are insects in the order Hymenoptera, Diptera and Curculionidae family. *Apis* spp. is one of the floral visitors of this species (Figure-3, Plate 2).

The mean length of the stamen of the short styled flower is 0.826 ± 0.03mm and in long styled flower is 0.686 ± 0.01mm. The pistil length of long styled flower is is 0.74 ± 0.01 mm and that of short styled flower is 0.516 ± 0.03 mm. Anthesis was in early morning between 06.00 am and 08.00 am. The anther dehisced before anthesis by longitudinal slits. Table–1 shows that stigma received the maximum pollen grains during the post anthesis period and maximum pollen germination and pollen tube growth in post anthesis time.

Figure 1 shows the number of pollen grains produced per flower in short styled and long styled flowers. Pollen grains number increased gradually while increasing the number of stamens.

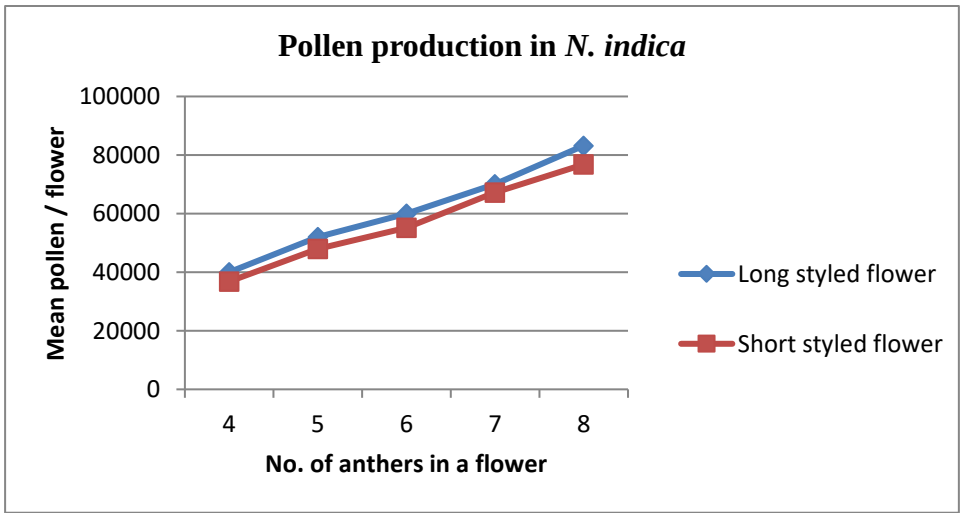

Fig. 1: *Pollen number in Long styled & Short styled flowers.*

Table 1: *In vivo pollen germination of N. indica.*

Types of flower	Flower opening time	Mean no. of total pollen on stigma	Mean no. of germinated pollen	% of mean no. of pollen germinated	Mean pollen tube length (µm)
Long Styled flower	Pre anthesis	17	0	0	0
	Anthesis	31	9	27.983	35.433
	Post anthesis	41	25	60.36	205.597
Short styled	Pre anthesis	33	0	0	0

flower	Anthesis	34	10	28.105	42.094
	Post anthesis	35	25	70.718	111.678

Table 2: *Pollen ovule ratio in N. indica.*

Types of flower	No. of anthers per flower	Mean No. of pollen per flower	Mean No. of ovules per flower	Pollen ovule ratio
Long styled	4	40000 ± 1414	29 ± 7	1379:1
	5	52000 ± 894	28 ± 9	1857:1
	6	60000 ± 1414	29 ± 9	2068:1
	7	70000 ± 1414	31 ± 9	2258:1
	8	83000 ± 1673	31 ± 8	2683:1
Short styled	4	36800 ± 1095	30 ± 8	1226:1
	5	48000 ± 894	29 ± 10	1655:1
	6	55200 ± 1095	30 ± 9	1840:1
	7	67200 ± 894	31 ± 9	2167:1
	8	76800 ± 894	31 ± 8	2477:1

Table 2 below shows the pollen – ovule ratio of *N. indica*. Both type of the flowers have different number of pollen grains and pollen ovule ratio.

Based on Scanning electron microscopic studies and acetolysis, the pollen grains are medium, monad, triangular, heteropolar, radially symmetric, parasyncolpate and prolate – spheroidal in shape. The mean polar axis of pollen grains in short styled flowers is 41.72 ± 1.82 µm and the mean equatorial axis is 39.44 ± 2.4µm. P/ E ratio is 105.99 ± 4.89 µm (n =100). In long styled flowers the mean polar axis is 36.58 ± 1.6 µm and the mean equatorial axis is 34.40 ± 2.36µm. P/ E ratio is 106.80 ± 8.87 µm (n =100). Exine wall is spinulose. The viable pollen grains becomes blue when it stained with aniline blue lactophenol, becomes pink or red coloured it stained with 1% aceto carmine and becomes brown or coloured when it stained with IKI solution Plate 1, Plate 2 (Figure 2, 3).The non-viable pollen grains are colourless in these solutions. To study the pollen germination *in vitro*, pollen grains were incubated in different concentrations of sucrose. The pollen germination is maximum in 8% sucrose solution (83%) and the mean pollen tube length is476.8 ± 43.6µm (Table 3).

S. No.	Percentage of solutions	Brewbaker & Kwack's Solution		Sucrose solution	
		Percentage of germination	Mean pollen tube length (µm)	Percentage of germination	Mean pollen tube length (µm)
1	4	56	850.5 ± 150.7	80	372.97 ± 57.2
2	8	83	521.8 9 ± 73.44	83	476.8 ± 43.6
3	12	63	444.9 ± 88.82	65	141.4 ± 22.3
4	16	48	198.6 ± 50.49	13	20.37 ± 5.3
5	20	31	33.7 ± 9.74	0	0
6	25	0	0	0	0

The stigma receptivity was studied by using different methods. By visual method, the receptive stigma was transparent, shining, cream coloured and wet and also adheres to 1mm^2 piece of paper. Table 4 shows H_2O_2 Method of stigma receptivity in *N. indica.* The stigma showed maximum bubble activity during the post anthesis period *i.e.* 11.00 am – 2.00 pm both on long style and short style. The receptivity decreases after 2.00 pm Plate 1, Plate 2 (Figure 2, 3).

Table 3: *In vitro Pollen germination in N. indica.*

Table 4: *H_2O_2 Method.*

Sl. No.	Time	Mean no. of bubbles / minutes	
		Short styled stigma	Long styled stigma
1.	08.00 am	2.4 ± 1.003	2.733 ± 1.507
2.	09.00 am	8.47 ± 1.07	8.533 ± 1.357
3.	10.00 am	29.9 ± 1.67	23.933 ± 1.72
4.	11.00 am	68.57 ± 1.14	76. 6 ± 1.19
5.	12.00 pm	71.67 ± 1.47	80.63 ± 1.52
6.	01.00 pm	85.53 ± 1.2	87.53 ± 1.72

7.	02.00 pm	81.7 ± 1.21	82.7 ± 2.09
8.	03.00 pm	50.2 ± 1.85	46.16 ± 1.84
9.	04.00 pm	30.13 ± 1.11	27.47 ± 1.11
10.	05.00 pm	17.78 ± 5.73	17.27 ± 1.33
11.	06.00 pm	10.22 ± 3.53	9.13 ± 1.81

Aniline blue – Lactophenol method shows the *in vivo* pollen germination on stigmatic head. The pollen germination on stigmatic head is maximum at post anthesis period in both morphs and the mean pollen tube length of long styled flower was 205.597 µm and that of short styled flower was 111.678 µm after 1 hour Plate 1, Plate 2 (Figure 2, 3).

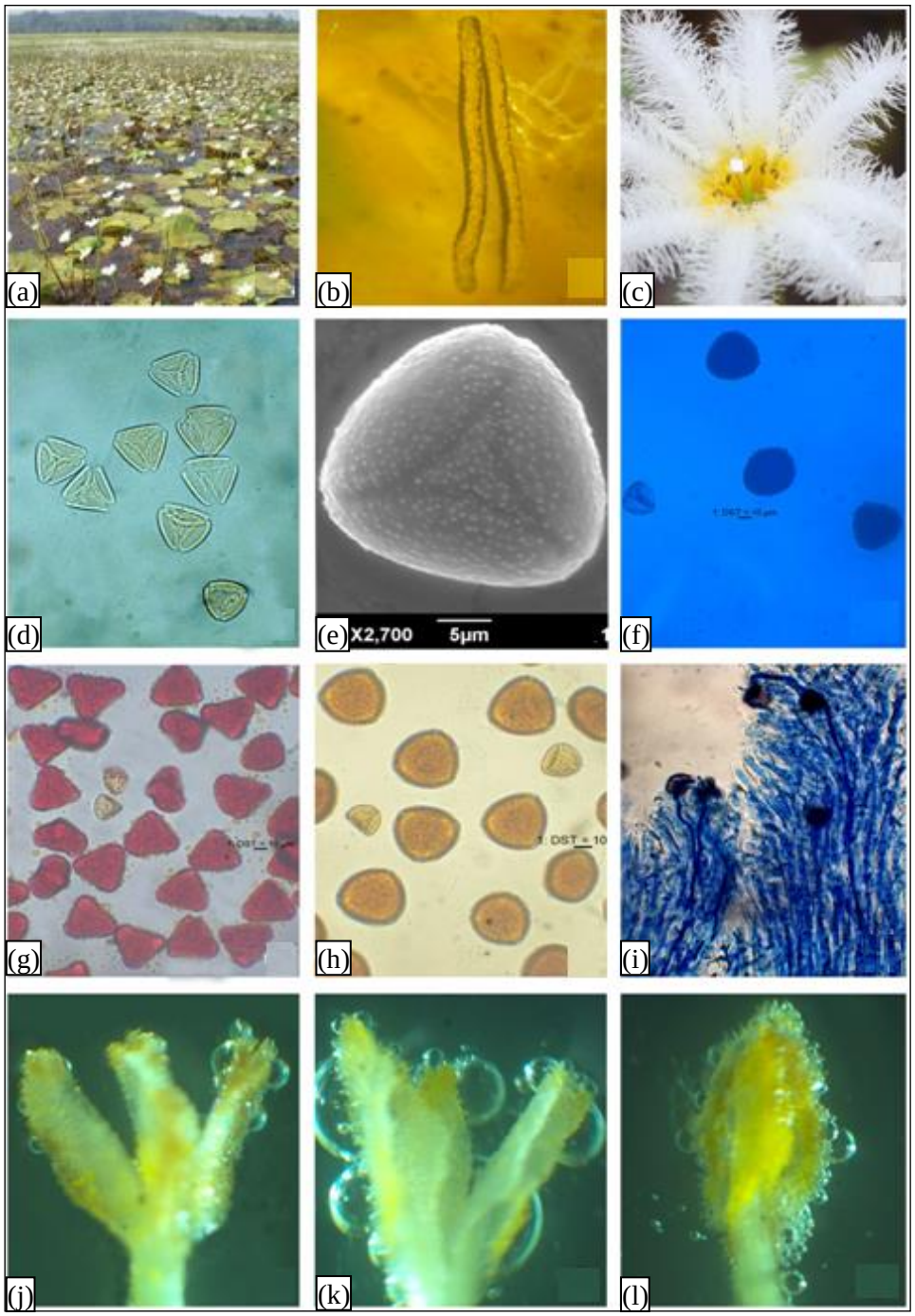

Fig. 2: (Plate 1) *Pollen morphology viability and stigma receptivity of long styled flower of Nymphoides indica (a.) Natural habitat, (b.) Anther dehiscence, (c.) Stigma receptivity by visual*

method, (d.) Acetolysis pollen, (e.) SEM photograph of pollen grains, (f.) 1% aniline blue lactophenol pollen visibility test, (g.) 1% aceto carmine test, (h.) IKI test, (i.) Stigma receptivity by aniline blue lactophenol method, (j-l) H_2O_2 method of stigma receptivity in pre-anthesis, anthesis and post-anthesis.

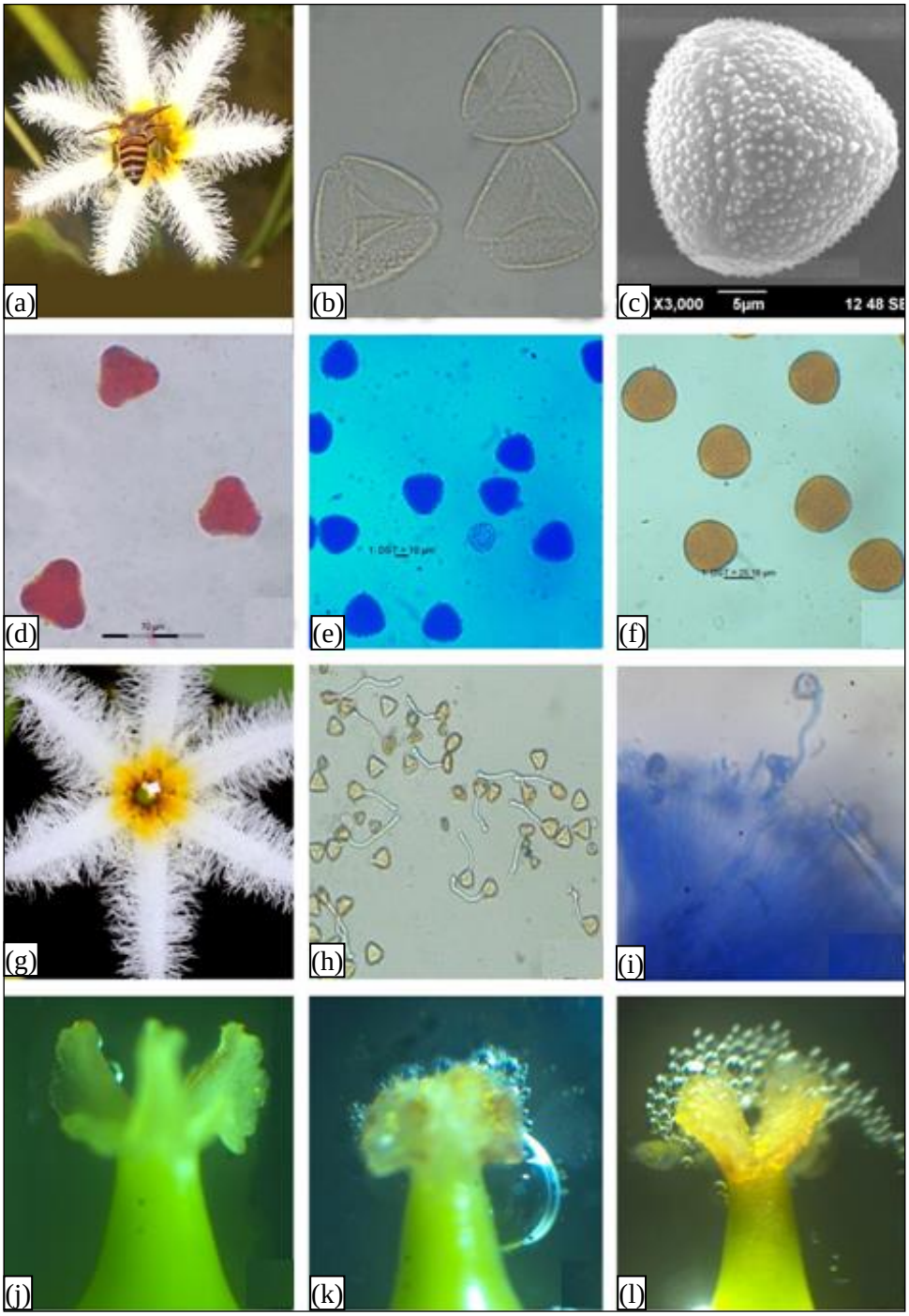

Fig. 3: (Plate 2) *Pollen morphology viability and stigma receptivity of short styled flower of Nymphoides indica (L.) O. Kuntze. (a.) Pollinator- Apisflorea, (b.) Acetolysis, (c.) SEM photograph of pollen, (d.) 1% aceto carmine test, (e.) 15 aniline blue lactophenol pollen visibility test, (f.) IKI test, (g.) stigma receptivity by visual method, (h.) Germination of pollen grain in sucrose solution, (i.) Stigma receptivity by aniline blue lactophenol method, (j-l) H_2O_2 method of stigma receptivity in pre-anthesis, anthesis and post-anthesis period.*

By alpha naphthyl acetate test, the stigma becomes black colour during maximum receptive time (11.00 am – 2.00 pm). After 2.00 pm receptivity gradually decreases Table 5, Plate 3 (Figure 4).

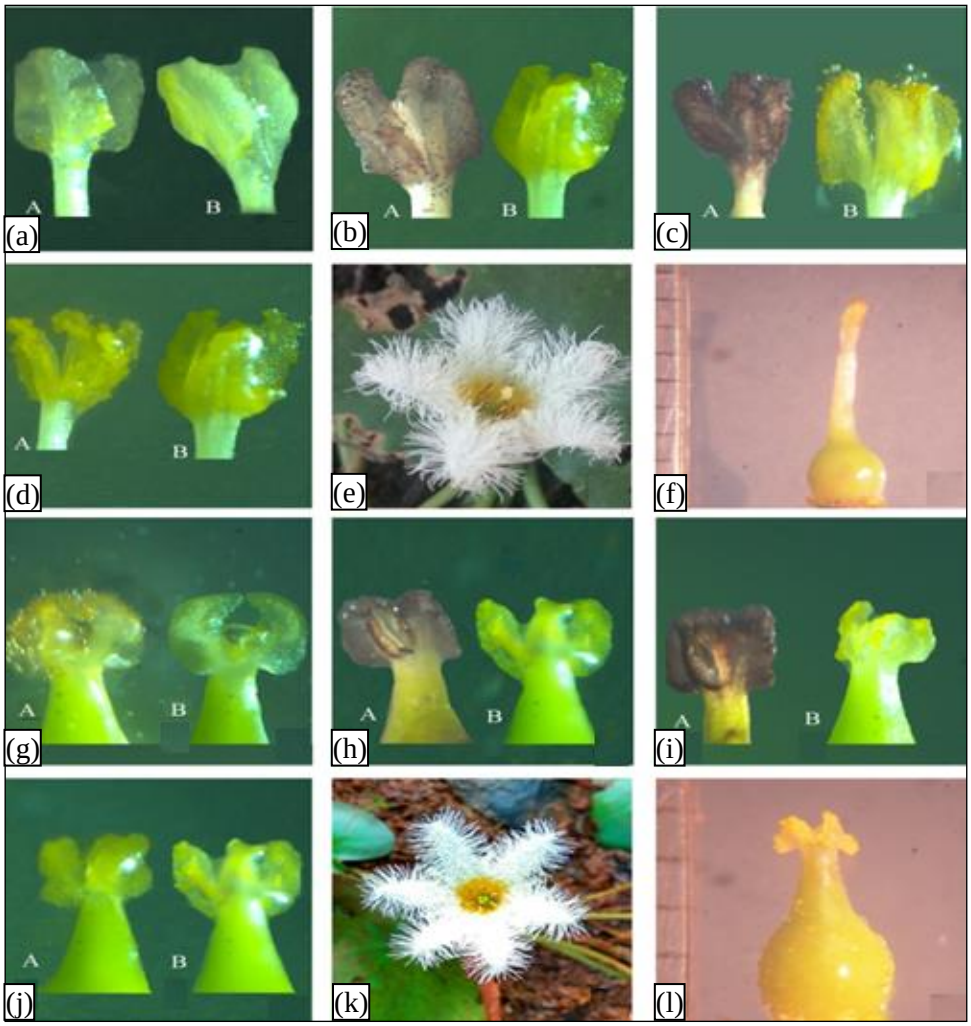

Fig. 4: (Plate 3) *Alpha naphthyl test on stigma receptivity of long & Short style flowers of Nymphoids indica (L.) O. Kuntze (a.) Pre-anthesis in solution A & B, (b.) Anthesis in Solution A&B, (c.) Post-anthesis in solution A&B, (d.) drooping stage in solution A&B, (e.) long styled flower, (f.) Long styled pistil, (g.) Pre-anthesis in solution A&B of short styled flower, (h.) Anthesis in solution A&B, (i.) Post-anthesis in solution A&B, (j.) Drooping stage in solution A&B, (k.) Short styled flower, (l.) short styled pistil.*

Table 5: *Alpha Naphthyl Acetate Test on stigma receptivity.*

S.N.	Time	Frequency of colour
1	06. 00 am - 08.00 am	Colourless
2	08.00 am - 09.00 am	Light brown
3	09. 00 am - 10.00 am	Brown
4	11.00 am - 12.00 pm	Black
5	01.00 pm - 02.00 pm	Black
6	02.00 pm - 03.00 pm	Brown
7	04.00 pm - 05.00 pm	Brown
8	06.00 pm – 07.00 pm	Colourless

DISCUSSION

Studies of pollen biology and stigma receptivity are most important for breeding programme of plants. The probability of adequate amount of pollen deposition on stigmatic surface is directly proportional to the amount of pollen production by a flower [8]. In *N. indica* flowers are of two morphs and have different numbers of stamens (four to eight). Whenever the number of anthers increased, the pollen count per flower also increased. The life span of flowers is one day

so the stigmatic receptivity remains only for one day. The pollen grains were matured before pre anthesis period (24 hours before the beginning of anthesis). The important parameter of pollen quality is the pollen viability [19]. The measurement of pollen diameter is the quickest and simplest method of assessing the viability of pollen [13]. In the present study pollen viability was confirmed by 1% aceto carmine, IKI and 1% aniline blue – lactophenol test and confirmed that viable pollen grains becomes pink or red in 1% aceto carmine, blue coloured in aniline blue lactophenol stain and brown coloured in IKI solution. Non-viable pollen grains are colourless. The stigmatic receptivity is a critical factor for successful completion of the post pollination events [15]. The receptivity of *N. indica* is maximum during 11.00 am – 2.00 pm. Pollen grain germination on the stigmatic surface was maximum after anthesis i.e., post anthesis and maximum pollen tube length was observed at the post anthesis period.

CONCLUSION

Nymphoides indica is a heterostylous aquatic plant, which produces two types of plants *i.e*., plants with short styled flowers and long styled flowers. The number of sepals and petals vary from 4 to 8 and this corresponds to the number of stamens in each flower. The dehiscence of anther occurs within the bud half an hour before anthesis. The anthesis is from 06.00 am to 08.00 am and the pollen grain viability extends up to 11.00 am (even three hours after full anthesis). The stigma receptivity starts at8.00 am, maximum at 2.00 pm and pollination occurred during this period augmented fruit setting percentage indicating that the stigmatic receptivity is a critical factor for successful production of perfect fruits and seeds.

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Cite this Article
P. Pavisha, P. V. Madhusoodanan, R. Prakashkumar. Pollen Biology and Stigma Receptivity in Nymphoides Indica (L.) O. Kuntze (Menyanthaceae). *Research & Reviews: A Journal of Life Sciences*. 2020; 10(1): 72–80p.

PHYTOTAXONOMY
Vol. 14, 2014. pp. 161-163

Date of Publication : April - 2015

Fasciation in *Crotalaria retusa* L.

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Fasciation is reported in *Crotalaria retusa* L. growing in Malabar Botanical Garden, Calicut – 673014, Kerala, South India. In a normal plant, growth in the apical meristem occurs at a single point, producing essentially cylindrical growth (Fig. 2 a-b). But, fasciation causes an elongation of the apical meristem so that flattened, ribbon-like growth is produced instead. The phenomenon may occur in almost any plant part, but is most noticeable in the stems or inflorescence. The linear fasciation with a flat, ribbon-shaped appearance is most common. Geneve (1990) reported bifurcated fasciations (linear fasciations which split to produce a 'Y' shaped double ribbon), multiradiate (stellate) shaped fasciations (the stem splits into three or more short branches) and ring fasciations (the growing point fuses to form a funnel shape). Fasciation at stem apex is observed in *Crotalaria retusa* L. (Fabaceae) growing in a pot in the Malabar Botanical Garden, Calicut-14, India. Fasciation is found on the main stem, all the branches being normal. Fasciation in this plant begins as a flat (1 cm broad) ribbon-like stem (Fig.1b), then broadens up to 12 cm, originally naked (Fig.1c), then becomes closely covered with normal looking leaves but with reduced petiole (Fig.1d). The cause of this phenomenon is unknown. This is the first report of fasciation in *Crotalaria retusa* L. in Fabaceae. However, fasciation has been reported in *Pisum sativum* and *Glycine max.*

Fasciation is a wide spread phenomena reported in more than 100 vascular plant species (Tang & Knap, 1998) affecting dicots and monocots in 39 plant families and 86 genera (Goethals et al., 2001). This has been reported in some members of the genera, *viz. Acer, Aloe, Cannabis, Euphorbia, Celosia, Primula, Prunus, Salix, Pisum, Lycopersicum, Solanum* and many genera of the Cactaceae family. Some varieties of *Celosia* are raised especially for their dependably fasciated flower heads, which are called "Cockcomb".

Fasciation can probably be due to some viral infection causing random genetic mutation as reported in many plants earlier. Fasciation can also be caused by hormonal imbalances in the meristematic cells of plants and additional environmental factors, such as fungi, mite or insect attack and exposure to chemicals. The bacteria *Rhodococcus fascians* causes fasciation in *Pisum sativum* plants. Some plants, such as peas and *Celosia*, may inherit the trait. Fasciation is not contagious, but bacteria that cause fasciation can be spread from infected plants to others by contact with wounds on infected plants and from water that carries the bacteria to other plants.

Acknowledgement

Authors are thankful to Mr. Rajilesh V.K., Research Fellow, Malabar Botanical Garden for his help and photographs. Authors are also thankful to Mrs.T.K. Rajisha for typing the manuscript.

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Fig. 1: Fasciation in *Crtalaria retusa* L.

A. Normal Plant B. Fasciation begins as narrow flat stem
C. Broadens at the apical region (almost naked) D. Leaves cover the faciated flax stem.

Fig. 2. A. Normal Inflorescence of *Crotalaria restusa* L., B. Flower of C. *restusa* L. - close

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