INVESTIGATIONS ON POPULATION VARIABILITY AND EXPERIMENTAL STUDIES IN SOME SPECIES OF HETEROSPOROUS FERN *MARSILEA* L.

A
THESIS

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By

Priyanka Sharma

Under supervision of

Dr. (Mrs.) Nilima Bhardwaj (Sr.Lecturer)

P.G.Department of Botany

Government College, Kota

UNIVERSITY OF KOTA, KOTA (RAJASTHAN)

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CERTIFICATE

It is certified that the

a) Thesis entitled **"INVESTIGATIONS ON POPULATION VARIABILITY AND EXPERIMENTAL STUDIES IN SOME SPECIES OF HETEROSPOROUS FERN *MARSILEA* L."** submitted by Miss. Priyanka Sharma is an original piece of research work carried out by the candidate under my supervision.

b) Literary presentation is satisfactory and the thesis is in a form of suitable presentation.

c) Work evinces the capacity of the candidate for critical examination and independent judgment.

d) Candidate has put in at least 200 days of attendance every year.

Date: -

Dr. Nilima Bhardwaj
Lecturer in Botany
P.G. Department of Botany
Government College, Kota
(Rajasthan)

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CHAPTER I

INTRODUCTION & REVIEW

Marsileaceae, a perplexing family of rooted amphibious ferns has received unabated attention occupying a central and pivotal position in botanical compendium. There are three genera in this family- *Marsilea, Regnellidium* and *Pilularia*. *Regnellidium* is monotypic genus known only from three localities in Southern Brazil and adjacent Argentina. *Pilularia* is a genus of six species of wide but disjunctive distribution. In contrast *Marsilea* is a cosmopolitan genus of approximately 50-80 species. Genus *Marsilea* commonly known as water clove is named after the Italian count Luigi Ferdinando Marsigli by Linnaeus in 1754. These small plants are of unusual appearance and do not resemble common ferns.. *Marsilea* can be broadly classified into two categories- hydrophytic and xerophytic depending upon whether the life history of the species is passed mostly under aquatic or terrestrial environments. The ability to develop heterophylly is proposed as one of the adaptive traits enabling the group of amphibious ferns to survive in contrasting habitats. This habit of the plants not only seems to determine the shape and size of the vegetative and reproductive organs but also their morphological aspect. The range of morphological plasticity is so much pronounced that different populations of a species growing under diverse habitat conditions appear to be distinct species.

The present work was undertaken with the specific aim of studying various parameters of wild and cultivated populations of some selected *Marsilea* species of Kota and adjoining areas, including the screening of a rare, endangered and threatened endemic species population of *Marsilea coromandelina* complex and evaluating the main causes of threat and ways for the conservation of this species since pronounced morphological plasticity has led to a very wide degree of phenotypic variations causing an immense confusion in systematic treatment of this complex cosmopolitan genus with particular reference to species delimitation. An attempt has been made in the present work to identify eco-morphological, phenological, anatomical,

physiological, phytochemical, and reproductive biology parameters which may help to identify distinctive features for being employed towards specific delimitation in this highly plastic genus. Besides, analysis of the physiochemical property of soil has also been done, which might be helpful in detecting specific climatic conditions and nutritional preferences of *Marsilea* populations of hadauti region for a healthy grow in nature.

Ferns present an array of cytological complexities with allopolyploidy playing a pivotal role in speciation. It was this realization which prompted cytological study of the rare endemic population of *Marsilea cf coromandelina* found in a small patch enroute Borawas. Besides, spore germination experiments have also been taken up in *M.minuta* and *M.cf.coromandelina* populations of Kota. Though, such studies have been undertaken in the desert taxa of *Marsilea* eg. *M.aegyptiaca, M.rajasthanensis,* and *M.diffusa* by Bhardwaja and his associates (1997), such studies have now been carried out in *M.minuta* and *M.cf.coromandelina* for the first time in the hope that this will add to our knowledge of this complex genus and its distribution in the hadauti plateau.

Marsilea exhibits morphological variation within species and as such it becomes difficult to distinguish species depending on traditional morphology only. Recently, various molecular markers and PCR techniques have been used in several studies. In the present study genomic analysis through Random amplified polymorphic DNA (RAPD) method have been employed for the first time to study interpopulational differentiation reflecting various modes of reproductive biology.

Very few studies have been carried out on the phytochemical properties of pteridophytes and with this background an attempt has been made to evaluate the phytochemical properties of the genus.

Tissue culture studies in ferns have been utilized as a research instrument to study the development potentialities ever since early sixties. The present context deals with the study of the aspects of *In vitro* morphogenesis through all the stages beginning from inoculation, multiplication to rooting and acclimatization. The present study is a part of the current concern with the conservation of the highly endemic species of *Marsilea cf. coromandelina* complex population which seems to be declining at an alarming pace.

Present work has been arranged in seven chapters. It is hoped that these studies will result into a valuable reference for future researchers interested in this important and stimulating field of pteridology.

REVIEW

The phytogeographic dimensions of *Marsilea*, the widest genus of the family, have not been seriously pondered since Braun's contributions on the subject. The family Marsileaceae, customarily placed amongst the aquatic ferns in literature, is now recognized as an 'unfern like' group of three genera namely *Marsilea*, *Regnellidium* and *Pilularia.* The nature of amphibiousness covers the entire spectrum of aquatic to xeric habitat with paludal terrestrial zone lying in between.

Braun's profound work comprising herbarium material examination supplemented with observations of the live *Marsilea* and *Pilularia* species from all over the world for nearly thirty five years (1839-1873) is the only transworld systematic account of Marsileaceae. The work on *Marsilea* has been extensively reviewed by Reed (1954, 1965). Sustained investigations by Braun provided a repository and reference point for ensuing regional systematic surveys initiated by Gupta & Bhardwaja on an international scale (1962).

Regional monographic studies have been earnestly taken up for the African species by Launert (1968) and Kornas (1985-1986) and the new world species by Johnson (1986). Recent advances in the knowledge of *Marsilea* from 1962 onwards have been reviewed by Bhardwaja (1980, 1997) which include various facets of study such as morphology, systematics, embryogeny, sporal aberrations, cytology, parthenogenesis, ecology, phytochemistry, morphogenesis, experimental and analytical studies.

Monographic presentations of the Indian and Asian species have been conducted by Bhardwaja (1958) and Gupta (1962). Bhardwaja (1977,1997) in the course of his researches

extending over almost half a century with the systematics and speciation aspects has concluded that speciation in this family reflects geographic divergences. Kornas (1983, 1985&1988) has coined out comprehensive and sustained field studies of *Marsilea* occurring in Northern Nigeria and Northern Cameron emphasizing the adaptive strategies of this genus to extreme environment. Heterospory, sporal aberrations, spore viability and spore germination, gametophytes, gametogenesis and embryogenesis and pteridophytic life-cycle phases manifested in Marsileaceae have been critically presented by Raghvan (1989) in an analytical work under the title 'Developmental Biology of Fern Gametophytes'. Members of this family have been widely utilized for physiological, morphogenetic and experimental studies.

Recent work in this family relates to phylogeny of the group with reference to the fossil record (Pryer, 1999) and structure and function of spores (Schneider & Pryer, 2002).

MORPHOLOGY

Genus *Marsilea* is well known for its morphological plasticity. Significant contributions in this field may be referred to morphological investigations by Russow (1872), Shattuck (1910), Bower (1928) and Tournay (1951) and the developmental studies conducted by Russow (1872), Shattauck (1910), Bower (1928), and Tournay (1951). The developmental studies conducted by Russow (1872) included morphological and anatomical aspects of the vegetative and reproductive organs of *M,drummondii*, *M.elata*, *M.quadrofolia*, and *M,aegyptica*. Pandeya (1953) published a short note on the shape and size variations in the leaves of *M.quadrifolia.* Water is an important ecological factor resulting into variations in size and shape of the vegetative organs and thus differentiating the genus into xerophytic and hydrophytic species.

Gupta (1961) published his monograph on *Marsilea* based on the doctoral studies of Bhardwaja (1959).

Johnson (1986) has stressed that internodal roots in *Marsilea* are of taxonomic significance. Soni (1988) has described morphological variations of *M.aegyptica* and *M.minuta* growing in Western Rajasthan. Bhardwaja (1981b, 1987, 1988, and 1989) and Bhardwaja & Gena (1989) have provided an exhaustive and up-to-date review on various aspects of biology of *Marsilea*.

Nagalingum et al (2007) has recently described molecular phylogenetic relationships and morphological evolution in the heterosporous fern *Marsilea.* Phylogenetic assessment of morphological evolution has suggested that the presence of an inferior sporocarp tooth and the place of sporocarp maturation are homoplastic characters, and are therefore of unreliable taxonomic use at an infrageneric level.

Tai-Chung Wu and Wen-Yuan Kao, (2011) investigated the adaptative traits of *M.crenata, M.quadrifolia, and M.schelpiana* distributed in various geographical regions comparing morphological features, optical properties and photosynthetic performance of these species. *M.quadrifolia* is distributed in temperate region where receiving low precipitation) has led to the highest trichome density on its leaflet surface and the highest water use efficiency.

ANATOMY

Anatomical studies of ferns and fern allies with special reference to stellar system have been extensively investigated during the present century. Pande (1923) reported the presence of a dictyostele in the tubers *of M.erosa* (*M.minuta*) and oil as a storage product in its cortex. Puri & Garg (1953) published a detailed description of the anatomy of the sporocarp of *M.minuta*

providing a new interpretation to the morphology of sporocarp stating it was equivalent to a single leaflet. Bhardwaja & D'souza (1987) have studied vascular tissue morphology of land and water forms *of M.aegyptica*, *M.diffusa* and *M.mutica* with a view to understand the effect of habitat variations on vascular tissue morphology of this plastic genus which is well known for its adaptive capabilities of growing on land as well as in aquatic habitats. A comparative analysis of the ultra structural peculiarities of *Marsilea quadrifolia* L. mesophyll cells of the leaves belonging to the plants grown in invitro system and in natural habitat has been recently described by Brezeanu Aurelia & C.Banchiu (2008)

CYTOLOGY

Cytology of the genus *Marsilea* was initiated by Mehra & Loyal (1959) who reported n=20 (2n=40) chromosome numbers of diploid *M.minuta* from North India along with a sterile triploid cytotype with 2n=60. Subsequently Mehra & Loyal (1959) recorded the occurrence of three different biotypes of *M.minuta* Patna biotype- which is perfectly normal producing typical mega and microsporangia; Jullundhar biotype- in which the megasporangia are normal but microsporangia have both typical and atypical microspores; and Saugar biotype- in which megasporangia are completely lacking but microsporangia with greater percentage of atypical microspores are produced as compared to the second bio-type. The triploid cytotype with 2n=60 was found to be completely abnormal, producing 16 sterile monads within a sporangium, all of which are of the same type alongwith numerous sporelets.

Cytological studies pertaining to chromosome numbers of Marsileaceae have been reviewed and summarized along with counts for additional species of *Marsilea* by Lesho (1944).

PHYTOCHEMISTRY

Phytochemistry is one of the more advanced and rapidly expanding areas of plant taxonomy (chemo-systematics) which utilizes chemical information to improve the classification of plants (Irudayaraj & Patrick Raja, 1998). Chemical taxonomy has grown rapidly and today it is applied to distinguish not only species but also individuals within a population. Like other areas of plant sciences, phytochemistry has been carried out comparatively on lesser number of non-flowering plants in contrast to flowering plants which have a wide range of chemicals in the form of pigments and aromatic compounds. Phytochemical analysis of pteridophytes with a view to solve taxonomical tangles has been taken up extensively with the works of Hegnauer (1962). Chatterjee et al. (1963) have studied the chemistry and pharmacology of marsilin, Berti & Botari (1968) and Swain & Cooper-Driver (1973). Phytochemical studies of *Marsilea* were initiated with a view of its economical exploitation by extraction of Marsilin from *M.minuta* and *M.rajasthanensis* by Chatterjee et al. (1963 a, b, 1964). Yadav (1995) has investigated the possible role and behavior of phytochemical compounds such as sugars, proteins, and amino acids during the biorhythmic movements of leaflets in the three species of *Marsilea*. Higher amount of amino acids has been observed during "sleeping" position.

However, Chakravarty & Debnath (1975) could not detect marsilin in the leaves of *M.minuta* investigated by them. Instead they reported an asymmetrical Hydroxy-ketone; 3, Hydrotriacon-II-one (first report of a C-30 ketone with a hydroxyl group in a plant) in the petroleum ether extract of the leaves. A mixture of secondary alcohols (Carbon content C_{27}-C_{31}) was also found to be present in the leaf extract. Methylamine, ß-sitosterol, a waxy material containing hydrocarbon and high molecular weight esters were also obtained by these authors from chloroform extract of the leaves. Alcoholic extract of the leaves yielded a saponin (m.p.

304-305 °C). Flavanoid compounds and some triterpenoid hydrocarbons have also been reported in *M.diffusa*, *M.polycarpa* and *M.quadrifolia* by these authors. Bhardwaja et al. (1977) have analysed sporocarps of *M.diffusa* and reported five steroidal compounds (Sitosterol, Cholesterol, Stigmasterol and two unknowns) and suggested their role in the pronounced viability possessed by the mega- and microspores of *Marsilea*.

Sharma (1978) carried out phytochemical studies of some Australian, European, African and Indian species of *Marsilea* with special reference to pigments, reducing sugars, ascorbic acid, steroids, amino acids, total soluble salts and circadian changes in the total soluble proteins in the leaf. Bhardwaja et al (1983) have drawn attention to the relevance of phytochemical investigations towards phylogeny and interspecific and intergeneric relationship in Marsileaceae. Wallace et al. (1984) investigated polyphenolics of the family Marsileaceae and their possible phylogenetic utility. Vyas & Sharma (1988) studied phytochemistry of 14 taxa of pteridophytes including *M.minuta* and *M.aegyptica*. Rathore & Sharma (1988) investigated proline contents during stress and non stress conditions in 11 species of ferns from Mt.Abu including *M.minuta*. It was confirmed that drought resistant ferns possess more amount of proline than the aquatic or moisture loving plants. Sharma & Bhardwaja (1989) recorded circadian changes in the total soluble leaf protein in water fern *Marsilea* L. Harsh et al (1989) detected the presence of steroids in the sporocarp and complete vegetative plants of *Marsilea* species collected from west Rajasthan.

Ecological investigations on some ferns from Rajasthan in relation to their drought tolerance and cell membrane permeability (Khan 1993) show that enhanced damage of chlorophylls and carotenoids is seen in *Christella dentata* when compared to *Hypodematium crenatum*. An overall higher content of various metabolites (soluble sugars, phenols, soluble

protein, amino acids, starch, proline and lipids) has been recorded in wild populations in comparison to the cultivated populations of three species of *Marsilea* as per Kumar, 1995 while a reverse trend has been observed in the carotenoids content (). Sharma et al. (1995) have studied phytochemistry in relation to ecology of pteridophytes of Rajasthan.

Marsilea has been reported to possess sedative and anticonvulsant activities and has been found to be a non-toxic drug in the treatment of epilepsy, anxiety, depression and mental health problems in general and severe neurological disorders in particular which are widely prevalent in modern fast paced life with a multitude of stressful conditions. Regarding this view, recently Bhattamisra et al (2007) concluded that ethanolic extract of *M.minuta* possesses anxiolytic activity in rats. Other reported activities include ant fertility activity, hypocholestermic activity and as a cure of lactation difficulties and menorrhagia. The leaves of *M.minuta* are used as a vegetable in the different regions of India and some tribal areas. Recently Utkarsh Alok (2013) described various pharmacological properties and successfully demonstrated various pharmacological actions of *Marsilea minuta*. Recently A.John De Britto et al (2013) also detected the presence of phytochemicals in petroleum ether, benzene, chloroform, methanol, and aqueous extracts of *Marsilea minuta.*

Muraleedhara Nair et al. (2011) have after extensive investigation have conducted the phytochemical studies on *Azolla pinnata* R., *Marsilea minuta* L. and *Salvinia molesta* Mitch. and concluded that plant extracts of these three plants show the presence of many bioactive compounds.

John De Brito A, Gracelin and Kumar (2013) have recently described qualitative and quantitative analysis of phytochemical analysis of phytochemicals in *M.minuta* Linn. and reported the presence of photochemical compounds such as steroids, reducing sugars,

triterpinoids, sugars, alkaloids, phenolic compounds, flaonoids, saponins, tannins, anthroquinones and amino acids in five solvent extracts of petroleum ether, benzene, chloroform and methanol.

Recently much emphasis has been laid on the various pharmacological properties Utkarsh Alok et al. (2013) have studied the phytochemistry and pharmacological activities of *Marsilea minuta* Linn.

REPRODUCTIVE BIOLOGY

The phenomenon of parthenogenesis and apogamy in *Marsilea* is of wide occurrence and was first recorded as early as 1897 by Shaw in *M.drummondii*. Temperature certainly has some effect on the frequency of parthenogenesis. The sporocarp of *Marsilea* is a unique, unparalleled structure among pteridophytes. It is tougher than the sporocarps of *Regnellidium* and *Pilularia*. Allsopp (1952a). Bhardwaja & Sen (1961) observed that sporocarps of *M.rajasthanensis* could withstand sustained heating at 65° C for at least 54 hours. Malone & Proctor (1965) observed dispersal of sporocarps of *M.mucronata* by water birds and suggested that tough sporocarp wall provides ample protection to spores against avian digestive processes allowing sporocarps of this species to remain in the intestinal tracts of the water birds resulting into dispersal of spores by a flying bird.

The dehiscence mechanism of sporocarp wall was first described by Braun (1839). Subsequently, Cunningham and Reed (1933) studied dehiscence of sporocarp and germination of spores in *M.polycarpa*. Bhardwaja & Mohammad (1967) observed that extrusion of sorophore from scarified sporocarps of *M.quadrifolia* occurred only in complete darkness, while in *M.aegyptica*, *M.brownii*, *M.minuta* and *M.rajasthanensis* sorophore extrusion occurred even in

direct sunlight. Loyal & Kumar ((1977) have reported destruction of inner contents of *M.minuta* sporocarps by larvae of the weevil *Echinocnemus* Schonherr. These larvae could bore holes and puncture the sporocarp wall eating up food laden megaspores in this species.

Spore viability happens to be an important aspect of the biology of *Marsilea*. Allsop (1952) reported successful germination of spores and embryo formation from 68 year old sporocarp of *M.minuta* and 61 year old sporocarp of *M.fourneri*. Later, Bloom (1955) reported embryo formation from 80 year old sporocarps of *M.quadrifolia*. Machlis and Rawitsher kumel (1967) studied the hydrated megaspore of *M.vestita* and found that the gelatinous structure associated with hydrated megaspore is far more elaborate consisting of papillar and basal envelopes and a highly defined bell and basal layer. According to Bloom (1953) sporocarps of all ages show some loss of spore viability due to some retarded development and maturation. Moreover, microspores are comparatively more prone to ageing than megaspores.

Bilderback (1978) made a detailed study of the development of the sporocarp of *Marsilea vestita*. She investigated the ultra structure of developing sorophore and reported three phases of polysaccharide accumulation during the differentiation of sorophore cells.

Bhardwaja (1980) obtaining sporelings from 130 years *M.burchellii* sporocarp, this being the longest recorded time period of spore viability. Aspects of heterospory, especially microsporal aberrations have been studied in detail by Bhardwaja & Wadhwani (1984) and Bhardwaja (1986).

Sharma (1978) studied the effect of physiologically active substances on the development of sporelings in *M.diffusa* and *M.rajasthanensis*. He further showed that GA3 induced sporelings formation in isolated megaspores of *M.minuta* which otherwise would not produce

parthenogenetic sporelings. Bhardwaja (1981) suggested that the development of sporelings from sporelings from isolated megaspores is from a diploid egg and is expressed as apogamous parthenogenesis.

Gupta (1957) studied epidermal and soral characters of some American species of *Marsilea*. Bhardwaja (1966) extensively investigated the phenomenon of microsporal aberrations in many taxa of *Marsilea*, belonging to widely separated geographical regions and established a definite relationship between occurrence of microsporal aberrations and leaf morphology of 32 taxa cultivated under identical conditions. He concluded that xeric habitat and ecological conditions play a vital role in sporogenesis leading to aberrant microspores and once this phenomenon is initiated in a population. It seems to acquire a genetic basis and such taxa even under humid conditions of cultivation in the garden continue to exhibit microsporal aberrations. Schneider & Pryer (2002) studied the structure and function of spores of the three living Marsileaceous fern genera (*Marsilea*, *Pilularia*, and *Regnellidium)* particularly with regard to the perine (outer spore wall) and acrolamella. According to Pryer, the gelatinous nature of the perine layer is possibly the result of acidic polysaccharide components in the spore wall that have hydrogel (swelling and shrinking) properties. Megaspores floating at the water/air interface form a concave meniscus, at the center of which is the gelatinous acrolamella that encloses a "sperm lake". This meniscus creates a vortex-like effect that serves as a trap for free swimming sperm cells propelling them into the sperm lake.

TISSUE CULTURE

Marsilea has been extensively employed for experimental studies. Allsop (1952) studied the effect of various physiologically active substances on the development of *Marsilea* in sterile

culture, effect of various sugars on the development and morphology, comparison of effect on the development of sporelings under varied cultural conditions and morphogenesis with special reference to origin of land and water forms. Rathore et al. (1989) have studied the effects of growth regulators and antibiotics (Cephalaxine and Cepharidine) on the germination, gametogenesis and sporophyte development in *Marsilea aegyptica*. Srivastava et al. (2008) conducted in vitro studies on development of gametophyte, sex ontogeny and reproductive biology of the threatened fern *Microsorium punctatum*. Aurelia Bwzeanu & C. Banciu (2009) presented a comparative analysis of the ultra structural peculiarities of *Marsilea quadrifolia* L. mesophyll cells of the leaves belonging to the plants grown *in vitro* system and in natural habitat and provided a protocol that could be used as a successful experimental system for ex situ conservation of this threatened species. Rolli E. et al. (2013) with a view to conserve the aquatic fern *Marsilea quadrifolia* L. in a long-term *in vitro* procedure, the effects of different cytokinins, varying their concentration and period of supplementation was investigated by

GENOMIC ANALYSIS

The water clovers bear few dependable morphological characters on which to base traditional identification. Morphological plasticity and molecular evolution among species of pteridophytes are remarkably prominent when compared with angiosperms. (Koall and Kenrick, 2004). Das et al, (2012) have recently described molecular marker based phylogenetic studies (RAPD) in complementing and supplementing taxonomy of *Selginella* species. DNA sequencing of several plastid regions to "fingerprint" *Marsilea* specimens from the southeastern U.S. to provide more accurate identifications was recently done by W. Mark Whitten et al. (2013)

The complete chloroplast genome sequences of *Lygodium japonicum*, a member of schizoid ferns and *Marsilea crenata* (Marsileaceae) have been determined by Lei Wang.et al. (2013). Comparative genomic analysis of all sequenced fern plastomes revealed that the gene order of *L.japonicum* plastome occupies an intermediate position between that of basal ferns and core leptosporangiates. Recently E.Rolli et al. (2013) assessed the genetic stability of *M.quadrifolia* by Random amplified polymorphic DNA (RAPD) by comparing eight randomly selected micro propagated plants derived from repeated subcultures with donor plant.

CHAPTER II

AREA OF INVESTIGATION

TOPOGRAPHY AND PHYSIOGRAPHY

Rajasthan, lying between 23°3'and 30°12'North latitude and 69°30'and 78°17' East longitudes is the second largest state of India and covers about 11% of the total area of the country. It is surrounded by Pakistan in the West while Punjab, Haryana, Uttar Pradesh and Gujarat lie in the North, East and South respectively. The presence of the Great Indian Thar Desert in its western province makes Rajasthan a unique state of India. One of the world's oldest mountain ranges, the Aravalli Range, cradles the only hill station of Rajasthan (**Figure 1,a**)

Pteridophytic flora of Rajasthan is mostly confined to Mt. Abu and Hadauti plateau which face interesting diversified status due to their variable climatic conditions. Extremity of climate is a characteristic feature of Rajasthan. Due to heavy rainfall, Mt. Abu possesses the richest vegetation of pteridophytes (approx 36 species, 15 genera) in entire Rajasthan. It is only during the rainy months that the ferns mainly flourish and are plentiful in number. In addition to Aravalli ranges, these vascular cryptogams are frequently observed in Hadauti plateau where thick and dense forests, wet and shady habitats, streams, springs and other water reservoirs exist and are known as favorite shelter places for pteridophytic species. The North and North-East portions of the state are poor in pteridophytic vegetation as these areas are full of sand dunes. However, along the banks of ponds, a few species of *Marsilea* e.g. *M.aegyptica* at Jodhpur, *M,minuta* and *M.rajasthanensis* at Kolyat (Bikaner) grow during rainy season. Therefore, pteridophytes of Hadauti plateau hold a significant position with respect to their occurrence and distribution. During rainy season a number of pteridophytes grow and survive in the valleys and ravines of River Chambal.

Present study mainly concerns with the survey of *Marsilea* populations of South Eastern Rajasthan constituting the Hadauti plateau.

Hadauti plateau

It is situated at the edge of Malwa Plateau at 23°45' to 25°53'N latitude and 75°9' to 77°26' E longitude in south eastern corner of the state. The total area of the plateau is 24,156.6 sq. km. and from administrative point of view, it covers Kota division of Rajasthan including Kota, Bundi, Jhalawar and Baran districts respectively [Figure1]. The plateau is quite unique due to its perennial and seasonal rivers and other water reservoirs, thick and dense forest supporting the growth and development of different species of various plant groups. Hadauti is predominantly an agricultural region with an agrarian economy. The Hadauti plateau comprises many kinds of habitats viz. crevices of rocks and ravines, shady and moistureful valleys, waterfalls, marshy land and ditches, aquatic and semi – aquatic as also xerophytic and lithophytic substrata where several genera of pteridophytes are found growing in rainy season and round the year.

Topography, geographical features and description of various *Marsilea* localities observed in Kota and sub-urban areas during the survey period are being described.

KOTA [FIGURE 1(b)]

Kota, formerly known as *Kotah*, is the third biggest city in the Northern Indian state of Rajasthan after Jaipur and Jodhpur. Kota region is situated on the banks of Chambal river and has been identified as a counter-magnet city for the National Capital Region to attract migrants and develop as an alternative centre of growth to Delhi. The city is the trade centre for an area in which millet, wheat, rice, pulses, coriander and oilseeds are grown; Industries include cotton and oilseed milling, textile weaving, distilling, dairying, manufacture of metal handcrafts, fertilizers, chemicals and engineering equipment. It is one of the principal cities of Rajasthan state.

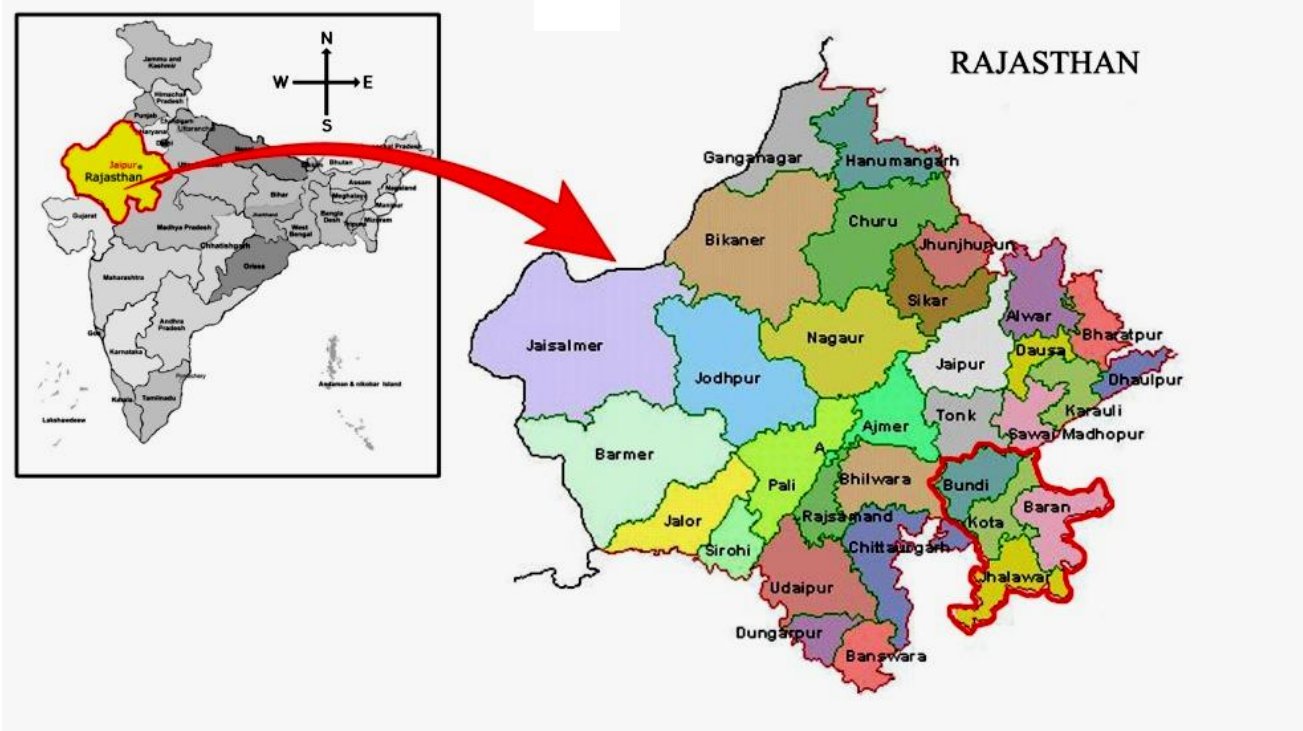
Figure- 1

(a) Location map of study area

(b) Hadauti Plateau highlighting surveyed localities.

Figure-1

a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Jaisalmer
Nagaur
Jaipur
Dhaulpur
Jodhpur
Tonk
Karauli
Ajmer
Sawai Madhopur
Barmer
Pali
Bhilwara
Bundi
Baran
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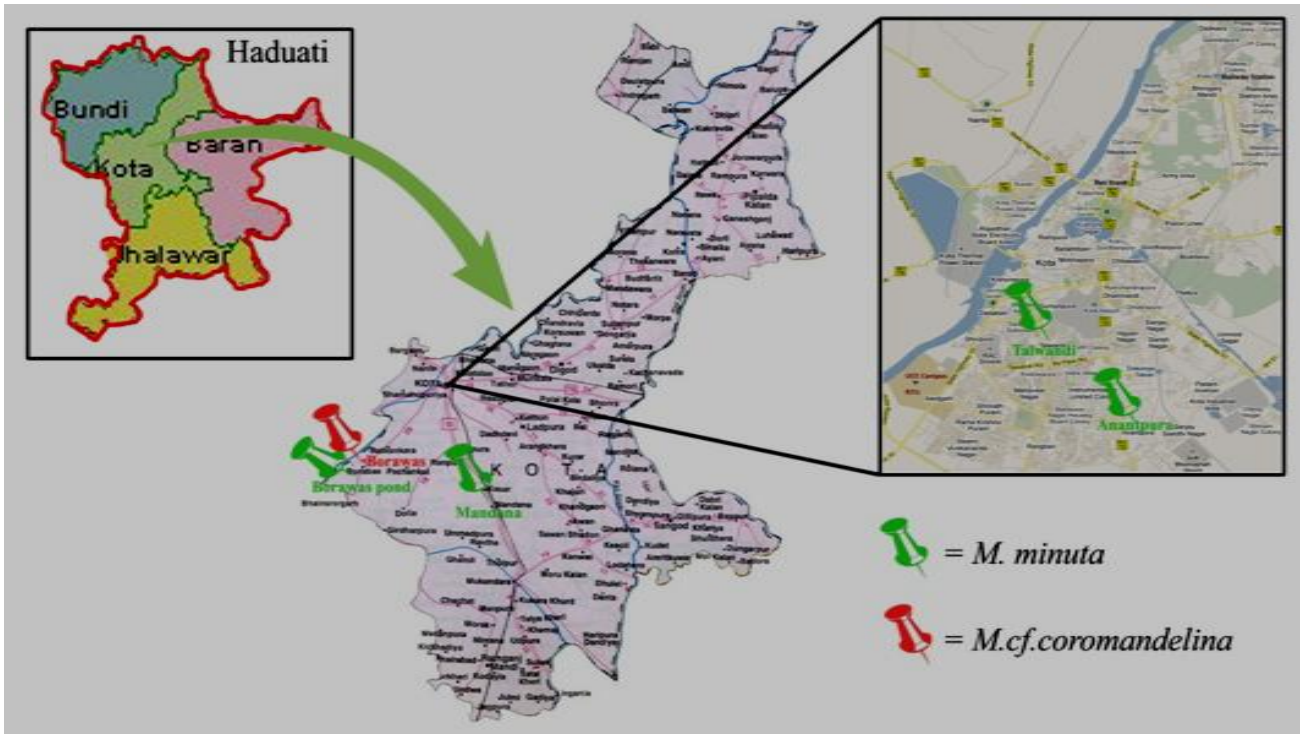
Chittaurgarh
Jhalawar
Udaipur
Dungarpur
Banswara

[B]


Haduati
Bundi
Baran
Kota
Jhalawar
KOTA
Borawas
Borawas pond
Mandana
Talwandi
Anantpura
= M. minuta
= M.cf.coromandelina

The city of Kota is situated at the center of the southeastern region of Rajasthan, and is widely known as Hadauti the land of the Hadas. Kota lies along the banks of the Chambal river and is the third largest city in Rajasthan. The cartographic coordinates are 25.18°N75.83°E It covers an area of 12,436 km^2 (3.63 per cent of the Rajasthan State). It has an average elevation of 271 metres (889 ft). The district is bound on the north and north west by Sawai Madhopur, Tonk and Bundi districts. The Chambal River separates these districts from Kota district, forming a natural boundary.

The historical places and temples are getting surrounded with signs of modern development. Kota is on a high sloping tableland forming a part of the Malwa Plateau. The Mukandarra hills run from southeast to northwest axis of the town. It is 36 km from Bundi. The town of Kota was once the part of the erstwhile Rajput kingdom of Bundi. It became a separate princely state in the 17th century. Apart from the several monuments that reflect the erstwhile glory of the town, Kota is also known for its palaces and gardens. It has fertile land and greenery with irrigation facilities through canals. Kota is one of the industrial hubs in northern India, with chemical, engineering and power plants based there

Kota has a semi arid climate (Köppen climate classification *BSh*) with high temperatures throughout the year. Summers are long, hot and dry, starting in late March and lasting till the end of June. The monsoon season follows with comparatively lower temperatures, but higher humidity and frequent, torrential downpours. The monsoons subside in October and temperatures rise again. The brief, mild winter starts in late November and lasts until the last week of February. Temperatures however between 26.7°C (max) to 12°C (min)

The average annual rainfall in the Kota district is 660.6 mm. Most of the rainfall can be attributed to the southwest monsoon which has its beginning around the last week of June and

may last till mid-September. Pre-monsoon showers begin towards the middle of June with post-monsoon rains occasionally occurring in October. The winter is largely dry, although some rainfall does occur as a result of the Western Disturbance passing over the region.

BORAWAS

This locality is situated approximately 25kms. from government college, Kota along Kota Rawatbhata route. It is one of the important locality of an endemic rare pteridophyte, *Marsilea cf. coromandelina* which is confined to a small patch in a deep depression. It has been observed that during rainy season the patch gets submerged with water and this species appears in mid July-August and completes its life cycle within a short span around mid October. This particular locality needs to be protected and is in danger of extinction due to anthropogenic and construction activities of the villagers.

ABHERA

This historical place is located about 7Km. from village Nanta and situated northwest from Kota city. In ancient times, Abhera was a dense forest having great importance. Prince Dhir Dev constructed a large pond in the year 1346. A beautiful palace and garden is also located at the eastern bank of Abhera pond having an approximate area of 100 hectares. *Marsilea minuta* was found growing contiguously with *Isoetes* in this locality.

MANDANA

It is a village of Ladpura Tehsil in Kota District of Rajasthan State. It is located 34 kms towards South from district head quarter Kota, 101 kms from Ladpura, 258 kms from State capital Jaipur.

It is situated about 33 kms away from Government College, Kota. A *Marsilea minuta* rich locality was found along the roadside of Mandana bus stand. Besides this *Actinopteris radiata* was also found growing as epiphyte on old buildings of this village.

TALWANDI

It is in the heart of Kota city. *Marsilea* populations are found growing abundantly along the road side throughout the year.

SURVEY AND COLLECTION

Following species of *Marsilea* populations growing at selected localities in Kota and adjoining areas were taken up for the present study.

1. *M.minuta* Linn.
2. *M.minuta* (hybrid)
3. *M.cf. coromandelina* complex.

The populations of these *Marsilea* species collected from the selected localities are represented in Table-1. Figure 2 to 6 represent the *Marsilea* populations of the selected localities growing in nature and under cultivated conditions.

Table 1

Surveyed localities of *Marsilea* population of Kota District

S.No.	POPULATION	LOCALITY	SITE
A	*M.minuta*	Talwandi (Kota city)	A massive patch along the road side situated in the centre of Kota, which remains flooded with water throughout the year.
B	*M.minuta*	Anantpura (Kota city)	A small patch on the City Mall road representing both hydrophytic and xerophytic condition.
C	*M.minuta* (hybrid)	Mandana (sub urban)	A small patch near Mandana bus stand
D	*M.cf.coromandelina*	Borawas (sub urban)	A small patch located ahead of Borawas, 27 Km away from Kota city.
E	*M.minuta*	Borawas pond (sub urban)	Dense patch along the edges of the Borawas pond. Flooded with water only during rainy season.

Figure – 2(a-e)

***Marsilea minuta* population at Talwandi locality**

(a) Road side view : *Marsilea minuta* population
(b) Under pot cultivation
(c) Herbarium specimen
(d) Horse grazing : All plants except *Marsilea*
(e) *M.minuta* population growing exclusively with the most problematic weed water hyacinth *Eichhornia crassipes* at Talwandi site.

Figure-2

Figure – 3(a-d)

***Marsilea minuta* (hybrid) population at Anantpura locality**

(a) Site view
(b) In nature
(c) Under pot cultivation
(d) Herbarium specimen

Figure-3

Figure – 4(a-e)

***Marsilea minuta* (hybrid) population at Mandana locality**

(a) Site view
(b) In nature
(c) Dried patch showing withered leaves
(d) Under pot cultivation
(e) Herbarium specimen

Figure-4

Figure – 5(a-e)

***M.cf.coromandelina* population at Borawas locality**

(a) A key mark (empty well like) for the easy location of endemic *M.cf.coromandelina* locality at Borawas

(b) Site view- Isolated patch of *M.cf.coromandelina* filled with water in rainy season

(c) In nature (vegetative phase)

(d) *M.cf.coromandelina* in association with grasses and angiospermic plants.

Figure- 05(a-h)

(e) In nature (reproductive phase- finger showing squarish sporocarp)

(f) Completely dried patch during summer season

(g) Under pot cultivation

(h) Herbarium specimen

(e)

(f)

(g)

(h)

Figure – 6(a-e)

***Marsilea minuta* population at Borawas pond locality**

(a) Site view
(b) In nature
(c) *M.minuta* leaflets floating in water
(d) Under pot cultivation
(e) Herbarium specimen

Figure-6

CHAPTER III

MATERIAL & METHODS

Various methods employed for the present study are cited below:-

PHYSICOCHEMICAL PROPERTIES OF SOIL

Soils of the area are important natural gift which are resultant of combined interaction of living organisms and climate on the barren rocks. Soils of any region may serve as the base or foundation of agriculture and economy of the area controlling the major activities of the region. The soils of this area are clay loam to clayey in texture. Soil sampling is perhaps the most vital step for any analysis. As a very small fraction of the huge soil mass is used for analysis, it becomes extremely important to get a truly representative soil sample of the field. Soil samples were invariably collected from each locality; soils from Talwandi, Anantpura, Mandana and Borawas were analyzed in detail. In present study physicochemical properties of soil such as soil pH, electrical conductivity(EC), organic carbon (OC), cation exchange capacity (CEC), exchangeable cations such as calcium, magnesium, ferrous, copper, manganese, zinc, nitrogen and phosphorous were analyzed at Agricultural Research Station, Ummedganj Farm, Kaithoon Road, Kota (Rajasthan) as per standard methods.

SOIL SAMPLING PROCEDURE

1. *For making composite sample, small portions of soil up to the desired depth (0-15 cm or more) was collected by means of suitable sam3pling tools such as khurpi, soil tube (tube auger)etc. from 15 to 20 well distributed spots, moving in a zigzag manner from each individual sampling site after scrapping off the surface litter.*
2. *The soil collected from various spots covering the entire area was mixed thoroughly by hand on a clean piece of cloth or polythene sheei.*

3. *The bulk was reduced to about 500 gm by quartering process in which the entire soil mass is spread, divided into four quarters, two opposite ones are discarded and the remaining two remixed. This process continued until about 500 gm of soil was left.*
4. *A label of thick paper with identification mark and other details was put inside the sample bag and another label carrying some detail tied outside the bag.*

Bacterial and fungal isolation from the soil sample

Bacterial and fungal species were isolated from the collected soil samples by serial dilution and agar plating method (Figure 7) wherein the soil sample was diluted from 10^{-1} to 10^{-5} dilutions and the diluted soil samples were spread on sterile Nutrient agar plates. The inoculated plates were incubated at 37°C for 24 hours. The purity of cultures was cross checked by gram staining procedure.

VAM association

For VAM association tryphan blue and lactoglycerol were used.

Preparation of Lacto glycerol

Mix 10 ml lactic acid, 20 ml glycerol and 10 ml of distilled water.

Preparation of stain tryphan blue

Dissolve .05 gm tryphan blue in 100 ml of distilled water.

Method

1cm root segments washed with tap water and clean in 10% KOH at 90°C in an oven for 60 minutes. Material was washed with distilled water several times at room temperature and stained with .05% tryptophan blue. Material was kept for 15 minutes at 60°C after cooling the stain was drained and kept in lacto glycerol for destain. Temporary slides were prepared for microscopic observations.

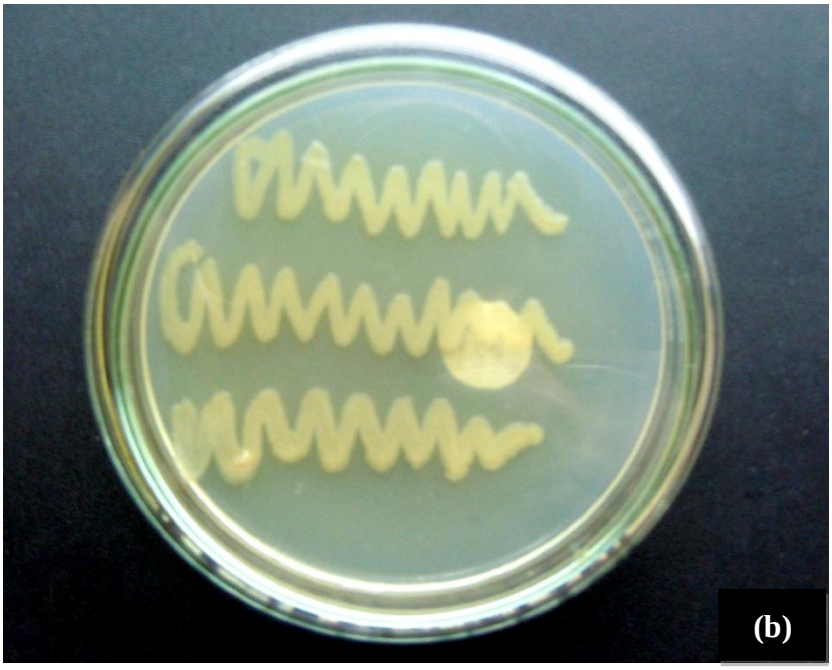
Figure-7

Spread plates (sample spread on solidified agar)

(a) Mid plate sample

(b) Edge plate sample

Figure-7



ECO-MORPHOLOGY

Morphological observations were carried out on fresh as well as herbarium material. All measurements were recorded in metric scale.

Details of habitat, soil and commonly associated flora of the aquatic as well as the terrestrial habitat were noted during regular survey of the localities.

Besides this regular field visits were arranged to the localities in order to study the area of distribution of various species of *Marsilea*, any seasonal variation, species and population interaction, as well as the possibilities of their survival under constant biological pressure.

PHENOLOGY

Phenological observations of selected populations were recorded at study sites in nature/ under cultivation every month (Jan-Dec) indicating active vegetative growth, initiation of sporocarp production, maturation of sporocarp, drying of sporocarp, dehiscence of sporocarp, withering of leaves, formation of dormant buds and resting propagules, formation of new sporophytes, sprouting of resting buds. These stages are represented in the form of phenograms.

ANATOMY

Sections of root, rhizome, and petiole of these populations of *M.minuta* and *M.cf coromandelina* were cut at 10-15 μm thickness using microtome. Safranin-fast green combination was employed for staining and mounting was done in DPX following Johansen (1940). Some hand cut sections were also observed under the binocular microscope and photographed.

CYTOLOGY

Root tips of the selected population of *Marsilea* were collected early in the morning just before sunrise. These were pretreated with a saturated solution of 8-hydroxyquinoline at 4°-5° C

for 4-6 hours prior to fixation. Fixation was done in acetic-alcohol (1:3) for 3 hours at low temperature (4°-5° C). Squashes were made by judicious warming of root tips in a mixture of aceto-lacmoid: NHCl (9:1) and stained with aceto-lacmoid only. Permanent preparations were made by the usual McClintok method (1928).

PHYTOCHEMISTRY

Phytochemical studies including quantitative estimation of total soluble sugars, total phenol, starch, proteins, ascorbic acid, total free proline, and photosynthetic pigments organwise root, rhizome, petiole and leaves were carried out as per standard methods-

Total soluble sugars (Yem & Willis, 1964)

Reagent: 200 mg of Anthrone was dissolved in 100 ml ice cooled 70% H_2SO_4. The solution was stirred well. Fresh solution was used every time.

200 mg dried material of each organ was homogenized in 10 ml of 80% alcohol and the two supernatants were combined and made up to a particular volume. An appropriate amount of aliquot and 4 ml of anthrone reagent were mixed well and placed in boiling water for 8 minutes. Anthrone was used as a blank. Optical density was recorded at 800 nm.

Standard curve was prepared using glucose.

Total phenols (Farkas & Kirlay, 1962)

Reagents:

(1) 30 % sodium carbonate (Na_2CO_3) solution: 30gm of Na_2CO_3 was dissolved in distilled water and made up to 100 ml.

(2) Follin's reagent: Commercially available reagent was used after two times dilution with distilled water.

200 mg of dried material of each organ was homogenized in 10 ml of 80% alcohol. After centrifugation, residue was again extracted with 10 ml of 80% alcohol. The supernatants were combined and made up to a specific volume and used as a source of total phenols.

To suitable amount of aliquot was added 3ml of Na_2CO_3 solution and 0.5 ml of Follin's reagent. In blank aliquot was replaced by an equal amount of distilled water. Test tubes were placed in boiling water for 1minute and centrifuged to clear the turbidity. Optical density was recorded at 760 nm.

Standard curve was prepared using caffeic acid.

Total starch (Chinoy, 1939)

Reagents: 0.2% iodine (I_2) in 2% Potassium iodide (KI)

A suitable amount (200 mg) of dried material of each organ homogenized in 10 ml of 80% ethanol (C_2H_5OH). After centrifugation, the residue was boiled in 10 ml of 1% KOH for 30 minutes. After centrifugation supernatant was used for the estimation of starch.

To the suitable of aliquot was added 1 ml of reagent. In blank aliquot was replaced by equal amount of distilled water. Optical density was recorded at 600 nm. Standard curve was prepared using starch.

Total soluble proteins (Lowery et al., 1951)

Reagents:

(1) 2% Sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH): 2 gm of sodium carbonate and 4 gm of sodium Hydroxide dissolved in water and made up to 100 ml.

(2) 0.3 % Copper sulphate in 1% sodium Potassium tartarate: 300 mg of Copper sulphate ($CuSO_4$) and 1 gm of sodium Potassium tartarate dissolved water and made up to 100 ml.

(3) Folin's reagent: Commercially available reagent used after two times dilution with distilled water.

Reagent (1) and (2) were mixed in a ratio of 50:1 shortly before use.

A suitable quantity of dried material (200 mg) was homogenous in 10 ml of Phosphate buffer (0.2 Molar, pH = 6.1). After centrifugation, supernatant was used as a source of soluble proteins.

A suitable amount of supernatant was mixed with 0.5 ml of 10% Trichloroacetic acid and left at 4°C for 6h. After centrifugation 3 ml of mixed reagent (1) and (2) and 0.1 ml of phenol (Folin's reagent) were added. In blank aliquot was replaced by distilled water. After 10 minutes optical density was recorded at 800 nm. Standard curve was prepared by using Bovine Serum albumin.

Ascorbic Acid (Jenson W. A, 1962)

Fresh and healthy roots, shoots and fruits of selected plants collected from Bikaner district were dried and homogenized in a mortar with 2% metaphosphoric acid (MPA)(10 mg powder: 100 ml MPA) and allow to macerate for one hour. The mixtures were centrifuged at low speed (2500 rpm) and supernatants were used for estimation of ascorbic acid following the colorimetric method **Jenson W.A (1962)**. Absorbency of each of the sample was measured on a spectronic-20 colorimeter (Bausch & Lamb) set at 546nm against blank.

Proline (Bates et al., 1973)

Reagents:

(1) 3% aqueous sulphosalicylic acid.

(2) Ninhydrin reagent: 1.25 gm Ninhydrin, 30 ml glacial acetic acid and 20 ml of 6 molar orthophosphoric acid mixed and heated gently.

(3) Toluene

200 mg of plant material was homogenized in sulphosalicylic acid. After centrifugation supernatant was used for free proline estimation.

A suitable amount (1 ml in each case) of aliquot was mixed with 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent. The test tubes were placed in boiling water for 45 minutes and then transferred to ice bath. 4 ml of toluene was added to each test tube which was then thoroughly shaken. The upper pink coloured organic phase was removed by separating funnel. Optical density was recorded at 540 nm.

Standard curve was prepared using pure proline.

Pigments

200 mg of fresh leaves of mature plants were homogenized in 15 ml of 80 % acetone. After centrifugation quantitative estimation of total chlorophylls (Chl a and Chl b) and total carotenoids was estimated according to the method suggested by Robbelen (1957).

Optical densities were recorded at 645 nm for chlorophylls and at 430 nm for total carotenoids content using spectrophotometer.

Amounts of chlorophylls and carotenoids were calculated as per the following formulae:

Chl a (mg/g fresh wt) = $\frac{12.3 \times OD\ 663 - 0.86 \times OD\ 645}{a \times 1000 \times W} \times V$

Chl a (mg/g fresh wt) = $\frac{19.3 \times OD\ 645 - 3.6 \times OD\ 663}{a \times 1000 \times W} \times V$

Total chlorophylls (mg/g fresh wt) = $\frac{20.2 \times OD\ 645 - 8.02 \times OD\ 663}{a \times 1000 \times W} \times V$

Total carotenoids (mg/g fresh wt) = $\frac{4.75 \times OD\ 430 - (Chl\ a + Chl\ b) \times 0.226}{a \times 1000 \times W} \times V$

V= volume of solvent (acetone) in which the fresh material was crushed

A= length of light path in the cell which is usually 1 cm.

W= fresh weight in gm which was crushed

REPRODUCTIVE BIOLOGY

Freshly collected sporocarps as well as those stored in paper bags belonging to all the populations were put for germination studies. A small cut on one side of the ventral edge with the help of a sharp scalpel was done. The sporocarps were kept in a petridish containing tap water in order to permit liberation of the sorophore. Sporocarp contents including number of sori per sporocarp, number of megaspores per sorus, total number of mega and microspores per sporocarp of both *M.minuta* and *M.cf coromandelina* have been counted to evaluate the reproductive capacity. An average of two replicates was taken for each species and five sporocarps of each species were scarified and kept for germination in a petridish. Using the following parameters-

(a) Hot water/cold/cold water

(b) Saline water/soil water

(c) Sugar solution/coconut milk

(d) Ordinary water/rain water

(e) Using different wavelengths- Red, Yellow, Green and blue.

Observations using stereoscopic binocular microscope included time taken for sorophore extrusion to sporelings formation were carried. Reproductive capacity was calculated by the following formula:

$$\textit{Reproductive capacity per five sporocarps} = \textit{Total no. of megaspores germinated } x \frac{100}{\textit{Total no. of megaspores}}$$

EXPERIMENTAL STUDIES

Comparative SEM, spore germination and in vitro studies of the two species was done using M.S media

Composition of M.S.medium

Components	**Mg/l**
Potassium nitrate	1900.00
Ammonium nitrate	1650.00
Calcium chloride.$2H_2O$	440.00
Magnesium sulphate.H_2O	180.69
Potassium phosphate monobasic	170.00
Magnesium sulphate.H_2O	16.90
Boric acid	06.20

Potassium iodide	0.83
Molybdic acid (sodium salt).$2H_2O$	0.25
Zinc sulphate.$7H_2O$	8.60
Copper sulphate.$5H_20$	0.025
Cobalt chloride.$6H_20$	0.025
Ferrous sulphate.$7H_20$	27.80
Na2-EDTA	37.30
Myo-inositol	100.00
Thiamine hydrochloride	0.10
Pyridoxine hydrochloride	0.50
Nicotinic acid	0.50
Glycine	2.00
Sucrose	30000.00
Agar	8000.00
Total gm/l	42.54
PGR's & vitamin	**Concentration**
BAP	1-5 mg/l
Kinetin	1-5 mg/l

The medium was sterilized by autoclaving at 121^0C under 18 lb pressure for 20 minutes. For tissue culture study isolated rhizome apices of *M.minuta* and *M.cf.coromandelina* with intact apical bud, devoid of any leaves and roots were taken. The explants were surface sterilized with antibacterial and antifungal component. Thereafter they were treated with $HgCl_2$ for 30 sec in

laminar air flow. Then explants were inoculated in MS medium supplemented with growth regulators. Inoculation was done under aseptic natural conditions.

GENOMIC ANALYSIS

Genomic DNA isolation (CTAB DNA isolation method)

Fresh leaves obtained from cultivated plants were used for extraction of DNA. The non transformed DNA serves as control DNA. Since the leaves of *Marsilea* are rich in polysaccharide and secondary metabolites, the isolation and purification of DNA sample becomes tough. These contaminations can also cause downstream problems in PCR by inhibiting the enzymes. Hence forth, genomic DNA extraction protocol outline was evaluated with minor modifications to isolate intact genomic DNA from transformed leaves.

1 gm of leaves were harvested and grinded in 2 ml of extraction buffer containing (6% Cetyl trimethyl ammonium bromide, 1 M Tris – HCl pH 8.0, 0.5 M EDTA pH 8.0, 5M NaCl, 0.5 % PVP and 100 µl of β- mercaptoethanol) in a pestle and mortar to fine powder and transferred in eppendorf tube. It was then incubated for 1 hr at 65°C temperatures in dry bath with gentle shaking after each 20 min during incubation. The tube was centrifuged at 5000 rpm for 5 min. The solution was cooled and 800 µl of pre-chilled chloroform: isoamyl alcohol (24: 1) was added and mixed well for 10-20 times. Again centrifuged at 6000 rpm for 16 min. The supernatant was pipette out into new eppendorf tube. The procedure was repeated to get clear supernatant. 5µl RNase was added in to supernatant. 100 µl NaCl (2M) added in supernatant. After than equal amount of pre-chilled isopropanol was added to precipitate the DNA. If needed the tube was incubated at 4°C for overnight. The tube was centrifuged at 10,000 rpm for 9 min. The supernatant was pipette out and pellet was taken out and. incubated for 30 min at 50°C in dry

Table- 2

List of RAPD Primers (From operon technologies)

S. No.	Primer code	Sequence
1.	OPA-01	5’-CAGGCCCTTC-3’
2.	OPA-02	5’-TGCCGAGCTG-3’
3.	OPA-03	5’-AGTCAGCCAC-3’
4.	OPA-04	5’-AATCGGGCTG-3’
5.	OPA-05	5’-AGGGGTCTTG-3’
6.	OPA-06	5’-GGTCCCTGAC-3’
7.	OPA-07	5’-GAAACGGGTG-3’
8.	OPA-08	5’-GTGACGTAGG-3’
9.	OPA-09	5’-GGGTAACGCC-3’
10.	OPA-10	5’-GTGATCGCAG-3’

bath. The pellet was washed with 100µl of 70% ethanol, dried at 50°C for 20 min in dry bath. Pellet was dissolved in 50 µl TE buffer. Tube was incubated at 55°C for 10 min.

PCR amplification

The PCR mixture (25 µl) contained 50 mg of DNA prepared from normal leaves respectively as the template, 1X PCR buffer, 25 pmoles of each primer, 2.5 mM of dNTPs and 1 unit of Taq DNA polymerase (Bangalore Geini). PCR was carried out by amplifying with initial denaturation at 94ºC for 5 min followed by 35 cycles of 1 min denaturation at 94ºC, 1 min annealing at 55ºC and 1 min extension at 72ºC with a final extension of 72ºC for 10 min using a thermal cycler. Amplification was achieved in a thermal cycler (Bio-Rad, La Jolla, CA) programmed for 35 cycles. List of RAPD primers used in PCR amplification is given in Table 2.

Agarose Gel Electrophoresis

The integrity of DNA isolated was determined by agarose gel electrophoresis. The electrophoresis was carried out in a horizontal gel system on 1% agarose in TAE buffer and 4.5 µl of Ethidium bromide at constant voltage of 100 V for 1 to 2 hrs and visualized under Gel Documentation System (Bio-Rad, La Jolla, CA). The purity of the DNA was checked spectrophotometrically. Electrophoresis pattern of RAPD profile was studied on 1 per cent agarose gel. Only those fragments which consistently amplified were considered for analysis.

Data Analysis

Each RAPD band was assumed to represent a single locus and data were scored as presence of bands (1) and its absence as (0). The similarity coefficients were utilized to generate dendrogram by using UPGMA (Unweighted Pair Group Method of Arithmetic means).

CHAPTER IV

COMPARATIVE OBSERVATIONS

Soil analysis-

Soil is the mixture of minerals, organic matter,gases, liquids and a mynad of organisms that can support life. The present study include the soil analysis of the soil samples collected from the selected localities of Kota (Anantpura, Talwandi, Borawas and Mandana). The data collected has been shown in Table-3.

The pH of the soil sample varied from 6.43-8.10. The maximum pH of the soil sample was recorded from Mandana locality and minimum pH was recorded from Talwandi locality.

The total soluble salt content of the soil samples was expressed as electrical conductivity (EC). The values of EC varied from 0.45 ds/cm to 0.58 ds/cm in the area. The maximum EC was of the sample collected from Borawas while minimum of Anantpura locality.

The organic carbon content of the soil samples varied from 0.45 % to 0.96 %. Total nitrogen content (%) of soil samples varied from 240 kg/ha (Mandana) to 522 kg/ha (anantpura).

Available phosphorous (P) (Kg/ha) content of the soil samples ranged from 14.5 kg/ha - 49.5 kg/ha. Available Potassium (K) content of soil samples varied from 395 Kg/ha to 530 Kg/ha. The maximum value was recorded from Mandana locality and minimum value was recorded from Borawas locality.

Similarly inorganic ion content (Zn, Cu, Mn, Fe) recorded in table-3 The water holding capacity was recorded to be the highest (45.79) in Mandana locality while lowest (30.0) in Anantpura locality.

SOIL MICROFLORA

Soil culture of Borawas locality indicate the presence of gram positive and negative bacteria along with fungi like *Fusarium* and *Aspergillus* which may be responsible for affecting the growth of the plant. (Fig-8).

Table- 3

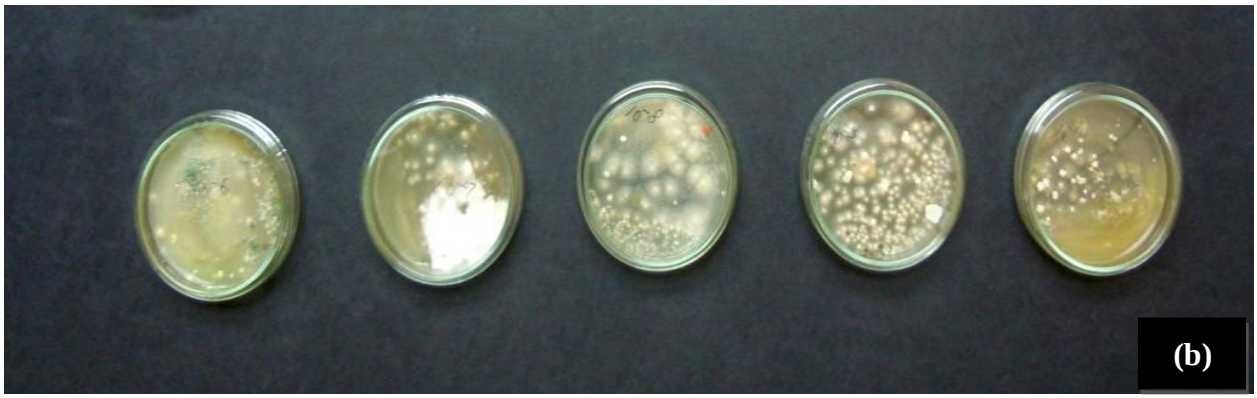
Analysis of soil samples from selected *Marsilea* localities

S.No.	Parameters/Sample number	Anantpura	Borawas	Mandana	Talwandi
1.	pH (1:2)	7.45	7.01	8.10	6.43
2.	Electrical conductivity, EC (dS/m)	0.45	0.58	0.56	0.50
3.	Total Organic carbon, OC (%)	0.96	0.75	0.45	0.68
4.	Available N (Kg/ha)	522	450	240	500
5.	Available P (Kg/ha)	49.5	14.5	24.3	48.4
6.	Available K (Kg/ha)	465	395	530	462
7.	Available Zn^{+2} (ppm)	4.11	1.52	1.28	3.12
8.	Available Cu^{+2} (ppm)	2.20	1.01	0.68	1.00
9.	Available Mn^{+2} (ppm)	3.30	9.21	3.07	4.12
10.	Available Fe^{+2} (ppm)	8.47	15.80	4.65	8.10
11.	Water holding capacity	30.0	42.23	45.79	35.82

Figure- 8

a) **Soil culture (Agar plate) of Borawas locality showing gram positive and gram negative bacteria.**
b) **Soil culture (Agar plate) of Borawas locality showing fungal colonies (*Aspergillus* & *Pencilium*).**

Figure-8



VAM association

Mycorhizosphere includes the region around the mycorhizal roots. The concept of mycorhizosphere is based on the fact that mycorhizal exert a strong influence on the microflora in the rhizosphere. There are two types of mycorhizal – ectomycorhiza and endomycorhiza. The former survives on the surface of the host plant while the later lives inside the tissue system. The mycorhizal may be arbuscular mycorhizal (AR), vesicular mycorhizal (VR), or vesicular Arbuscular mycorhizal (VAM).Arbuscular mycorhizal fungi, AM symbiosis is the most widespread mycorhizal association type with plants that have true roots ie, Pteridophytes, Gymnosperms and Angiosperms (Read et al, 2000). An arbuscular mycorhizal fungus is a type of mycorhizal in which the fungus penetrates the cortical cells of the roots of a vascular plant. AM fungi (AMF) help plants to capture nutrients such as phosphorus, sulfur, nitrogen and micronutrients from the soil. The development of the arbuscular mycorhizal symbiosis played a crucial role in the initial colonization of land by plants and in the evolution of the vascular plants.

Present study is made on the roots of *M.cf.coromandelina*. The Vesicular arbuscular mycorhizal (VAM) fungus association has been observed with the roots of *M.cf.coromandelina.* (Fig-9). Hyphal and vesicular stages of colonization were seen in the roots. The shape of the vesicles varied from spherical to elliptical to irregular (fig 9, a, b, d). Formation of ellipsoidal and spherical vesicles indicates the presence of Fungus belonging to the genus *Glomus*.. The fungus penetrates the roots by developing appressoria (Fig 9,c). It spread forming inter and intra cellular hyphae (fig 9, f) through the epidermis and the outermost cortical layers, where it formed vesicles, hyphal coils-like and arbuscules. The mycorhizal association was about 50-60 % in the roots.

Figure- 9

M.cf coromandelina **roots association with Vesicular arbuscular mycorhizal (VAM) fungus**

a) Arrow pointing spherical vesicles.

b) Arrow pointing irregular vesicles.

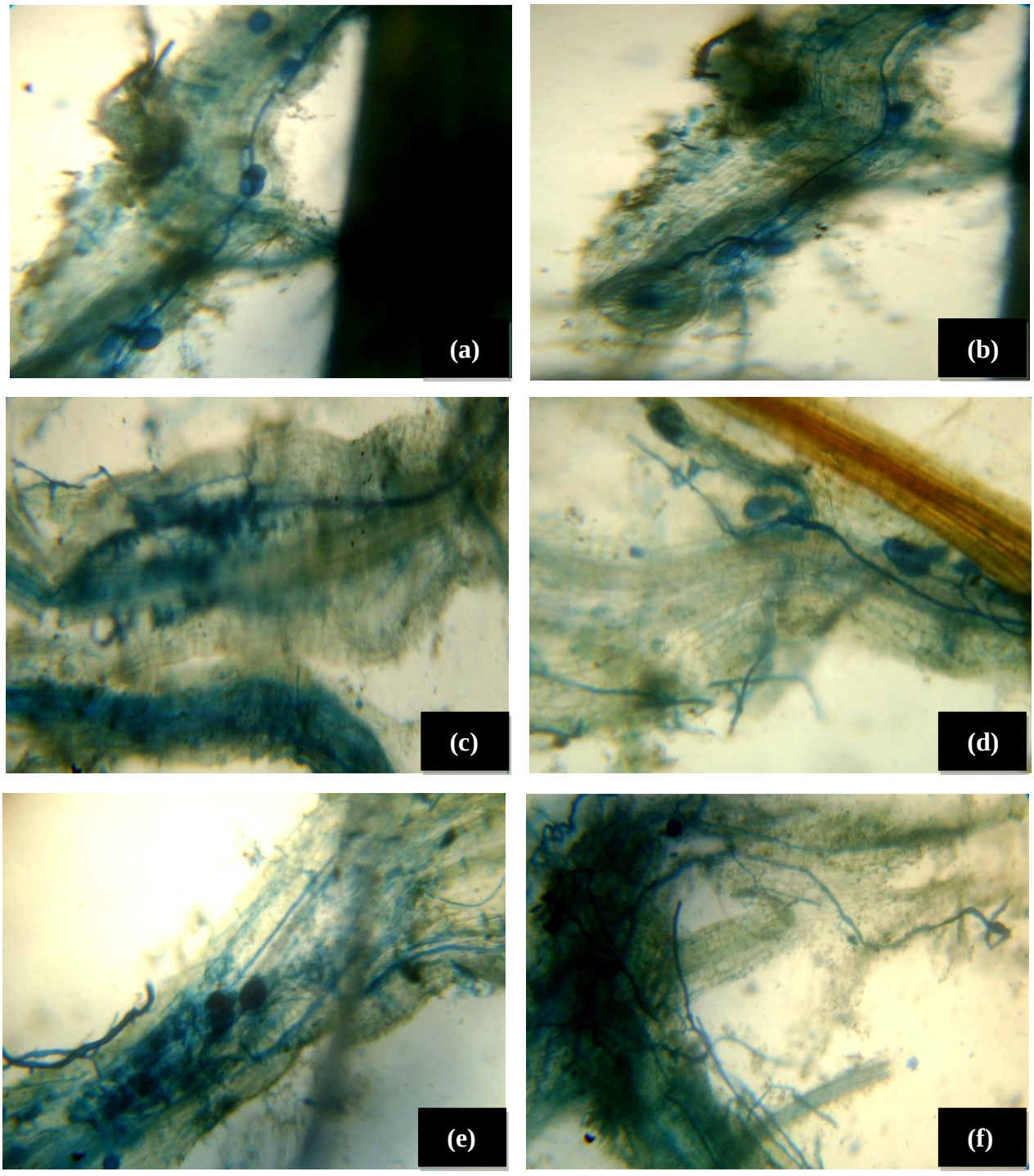
c) Fungus penetrating the roots and forming appressoria.

d) Arrow pointing elliptical vesicles.

e) Cluster of spherical vesicles.

f) Fungal hyphae in the roots.

Figure-9



MORPHOLOGY (FIGURE 10-13)

Marsilea is an extremely plastic and ecologically complex genus and requires caution in taxonomic studies (Bhardwaja, 1975). The range of morphological plasticity is considerable so pronounced that populations of a species growing under diverse habitat conditions appear to be distinct species. This has led to species polymorphism having wide ecological amplitude with a wider range of tolerance to habitat features and which has been responsible for greater adaptability resulting into hydrophytic, amphibious and xerophytic populations. The populations representing varied taxonomic categories in Hadauti region do exhibit great variation in the length, thickness and branching of rhizomes, the number of roots formed at the node and internode, the thickness and height of the petiole and the margin of leaflets under aquatic and terrestrial conditions.

Eco-morphological observations of selected *Marsilea* populations of the investigated localities viz. Talwandi, Anantpura, Borawas, Mandana and Borawas pond have been recorded in the table-4.

1. **Roots**

   Johnson (1986) has emphasized the presence or absence of internodal roots as an important taxonomic character. In the present study surprisingly internodal roots were found in the *M.minuta* populations growing in Borawas pond locality (Fig 10). In *Marsilea* populations growing at various investigated localities, the number and length of roots vary according to the habitat and soil type. The longest roots were observed in *M.minuta* growing at Talwandi locality (15.12 ± 3.18) while the smallest were those of populations growing at Anantpura (9.14 ± 3.13 cm).

Figure 10

a) Internodal roots in *M.minuta* (Borawas pond) population.

b) Another view of *M.minuta* (Borawas pond) population showing Internodal roots

c) Internodal roots between two nodes.

Figure-10

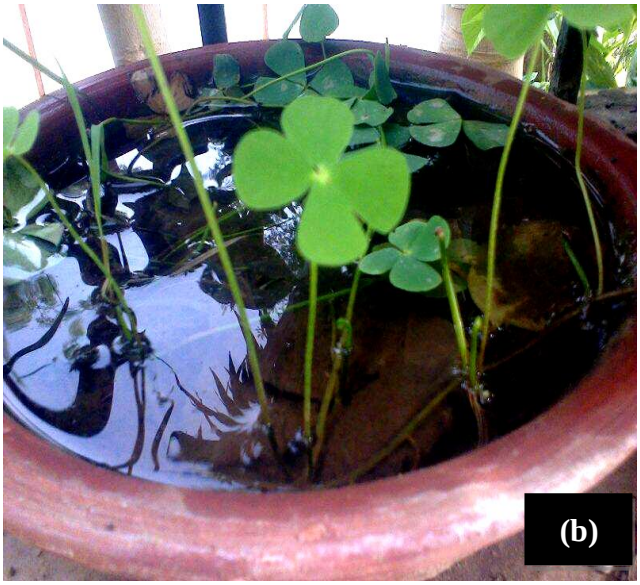


Table 4

Eco-morphological variations in *Marsilea* populations of surveyed localities

S. No.	Parameters	*M.minuta* (Talwandi) [A]	*M.minuta* (Anantpura) [B]	*M.minuta* (Mandana) [C]	*M.cf.coromandelina* (Borawas) [D]	*M.minuta* (Borawas pond) [E]
1.	Petiole length (cm)	20.42±4.1	12.14±4.6	18.6±4.6	15.62±6.4	20.14±6.3
2.	Leaflet size (LxB)	2.64x1.82	1.34x1.41	2.12x1.82	1.46x1.86	3.14x1.80
3.	Leaflet margin	Almost entire	Almost entire	Slightly crenate	Crenate	Entire
4.	No. of roots/node	5-8	6-8	6-8	7-8	4-6
5.	Length of root (cm)	15.12±3.18	9.14±3.13	10.11±1.14	9.20±2.13	14.32±4.32
6.	Internodal length	8.14±3.18	2.13±0.14	3.14±2.13	3.14±1.12	10.60±2.48
7.	No.& position of sporocarp	1-2 sporocarps at the base of petiole	3-4 sporocarps attached to lower portion of petiole	2-3 sporocarps at the base of petiole	2 sporocarps attached to lower surface in a erect manner above the ground.	4-5 sporocarps at base
8.	Shape of sporocarp	Oval	Obliquely ovoid	Oval	Squarish with prominent ridges	Oval
9.	Sporocarp size (LxB)	0.42±0.1x0.27±0.3	0.41±0.2x 0.28±0.2	0.40±0.2x 0.27±0.4	0.36x±0.3x0.51±0.2	0.43±0.2x0.26±0.3

Number of roots/node was found to be highest (7-8) in *M.cf.coromandelina* (Borawas) while lowest were those of populations growing at Borawas pond (5-6).

2. Petiole

The petioles are comparatively much longer and equal in Borawas pond and Talwandi (aquatic form) as compared to other localities. It is recorded to be smallest (12.14 ± 4.6) in *Marsilea* populations (Anantpura) followed by *M.cf.coromandelina,* Borawas (13.62 ± 6.4), Mandana (18.6 ± 4.6), respectively.

3. Internodal length

The internodal length varies greatly in the investigated populations of *Marsilea* at various localities. *M.minuta* populations growing in Borawas pond has been found to be the maximum internodal length (10.60 ± 2.4) followed by populations at Talwandi (8.14 ± 3.18), Mandana (3.14 ± 2.13), Borawas (3.14 ± 1.12) and Anantpura (2.13 ± 0.14) respectively.

4. Leaves

Leaves have large petioles terminating into four leaflets. Leaflets are obovate with smooth or crenate margins. Young leaves arise in a circinate manner. The mature leaves of *Marsilea* are quadrifoliate i.e. two leaflets being slightly higher than the lower two and are inserted in alternate fashion. The leaflets exhibit sleep movement *Marsilea* and *Regnellidium* are the only cryptograms showing these movements. At night the pinnae become folded upwards drooping at petiole apex.

Size of leaflets and their margins show characteristic habitat and interspecific features. The leaflet margin varies with the habitat. It is observed that the leaflet margin in land form is deeply crenate (Fig.11, c) while it is almost entire in aquatic form. (Fig.11, a) Hairs are seen

Figure- 11

***Marsilea* population of Kota Plateau showing morphological features**

(a) ***M.minuta*** **–[Talwandi] Population A showing entire leaves.**

(b) ***M.minuta*** **– [Anantpura] Population B showing almost entire leaves**

(c) ***M.minuta*** **(hybrid) – [Mandana] Population C-arrow pointing crenate leaf margin and massive growth of roots.**

(d) ***M.cf.coromandelina*****–[Borawas] Population D showing squarish sporocarp**

(e) ***M.minuta*** **–[Borawas pond] Population E showing entire leaflets**

Figure- 11

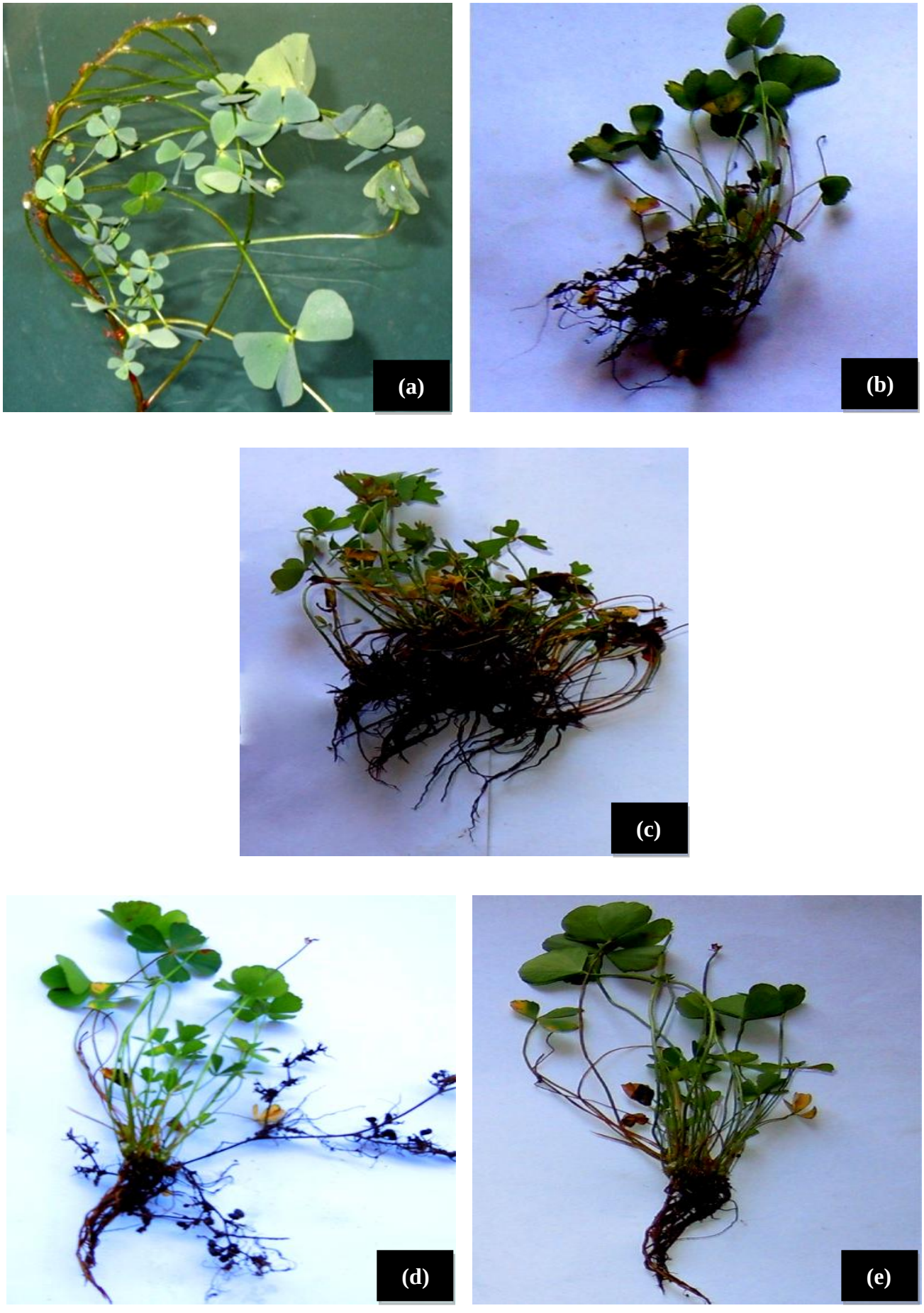
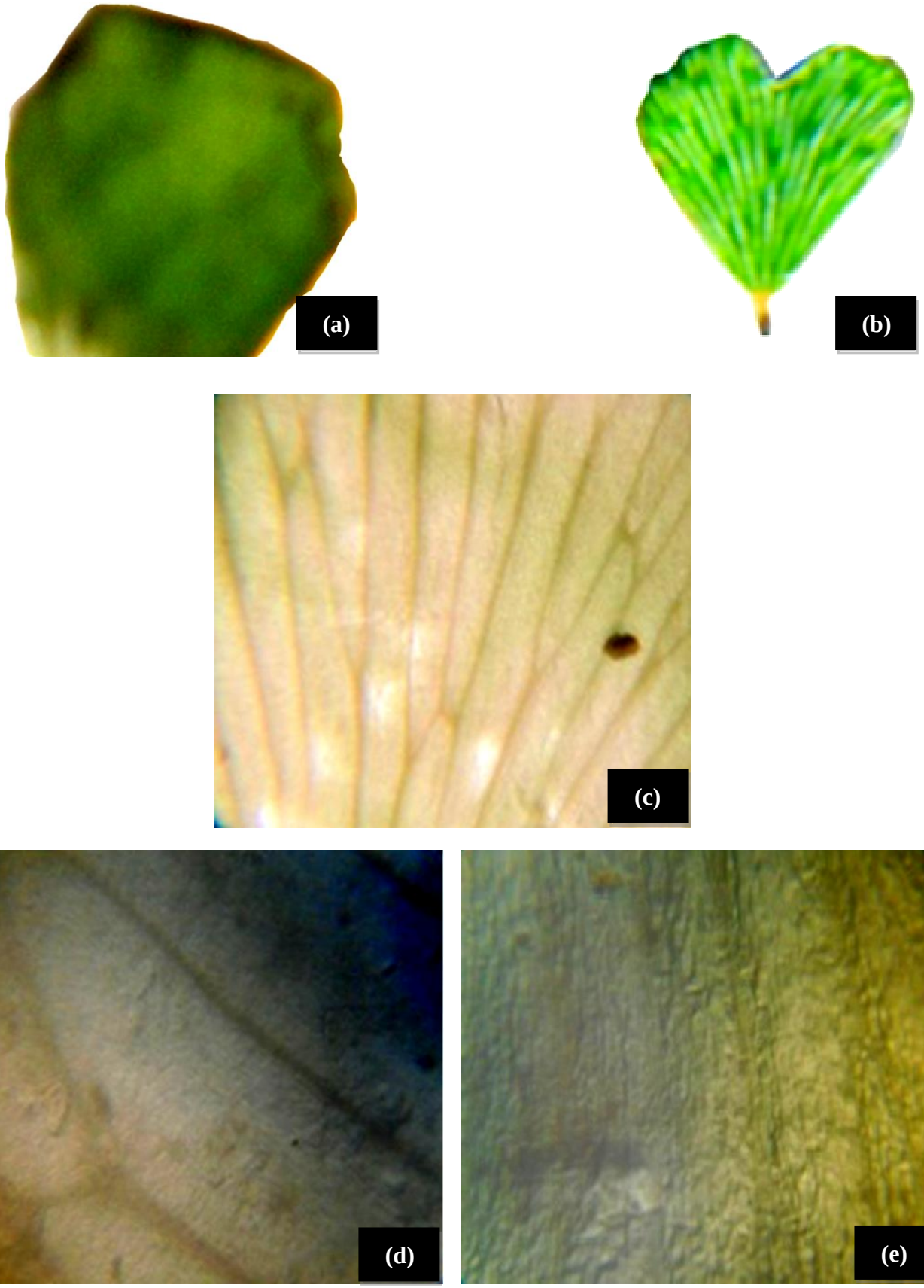


Figure-12

(a) No streaks observed in *M.minuta* leaves
(b) Characteristic pellucid streaks and veins shown by the *M.cf.coromandelina* leaves
(c) General venation pattern shown by *M.minua* leaves.
(d) Leaflet of M.minuta showing unevenly tuberculate veins
(e) Leaflet of *M.cf.coromandelina* showing sclerotic veins

Figure-12



in the land form which may be a conservative and characteristic feature for decreasing transpiration rate. Fig-12 represents the venation pattern of *Marsilea* leaves.The leaflet margin is lobed, with prominently shinning pellucid streaks present between the veins showing the distinctive feature in *M.cf.coromandelina* (Fig-12.b)

The leaflet size is shown to be maximum in Borawas pond population of *Marsilea* (3.14 x .80 cm) followed by Talwandi population (2.64 x 1.82cm), Mandana locality (2.12 x 1.82), Borawas M.cf.coromandelina locality (1.46 x 1.86) and smallest in Anantpura locality (1.34 x 1.41 cm)

5. Sporocarp (Fig-13)

The sporocarp of *Marsilea* is a unique, unparalleled structure among. pteridophytes. The sporocarps are stalked. The shape size and contents of the sporocarp and the attachment of its pedicel to the petiole of the leaf vary considerably (Fig. 13)

The sporocarp differs slightly in size and surface features in land and aquatic populations of *Marsilea* in various investigated localities. It also differs in phenological appearance. The sporocarps appear ovoid in shape in almost all investigated localities except Borawas locality where the sporocarps of *M.cf.coromandelina* appear squarish in shape [Fig.13 (d)]. The sporocarp measures 0.40 ± 0.2 x 0.27 ± 0.4 cm in Mandana locality of *M.minuta* while the sporocarp of Talwandi, Anantpura, and borawas pond measures 0.42±0.1x0.27±0.3, 0.41±0.2x 0.28±0.2, and 0.43±0.2x0.26±0.3cm respectively. In land forms of *M.minuta* 3-4 sporocarps remain attached to lower portion of petiole in a linear fashion. Each sporocarp is ovoid in shape with an upper blunt horn. It is hairy when young but almost bald at maturity. In contrast to this the sporocarps of *M.cf.coromandelina* differs from *M.minuta* in the former having a central bulge and characteristic depression (ridges) which is visible externally. The

sporocarp of this species is squarish in shape with a prominent blunt horn. Sporocarps are mostly two in number at each node, rarely one in many drying plants with condensed rhizomes. It is densely covered with hair when young and also at maturity. Sporocarps measure 0.36 ± 0. x 0.30 ± 0.01 cm.

Figure – 13

***Marsilea* sporocarps of the surveyed localities**

(a) *M.minuta* –Population A (Oval sporocarp with blunt horn)
(b) *M.minuta* (hybrid) –Population B (Ovoid with upper prominent & lower obtuse horn)
(c) *M.minuta* (hybrid) – Population C (Ovoid with a prominent upper & lower blunt horn)
(d) *M.cf.coromandelina* – Population D (Rectangular and centrally ribbed with upper blunt & lower obtuse horn)
(e) *M.minuta* –Population E (Obliquely ovoid with two protuberances)

Figure – 13

(a)

(b)

(c)

(d)

(e)

PHENOLOGY

Climatologically the year in Rajasthan has been divided into three major seasons- Hot weather season (March to mid June), Rainy season (Mid June to September), Cold weather season (October to February)

The climatic factors have the greatest effect on the vegetation distribution in different parts of the state. The inhabiting populations of *Marsilea* have to experience the stress of an extreme of climatic conditions from humidity and moisture to extreme dryness.

Simultaneous phonological observations of the selected populations in nature and under cultivation regarding active vegetative growth, initiation of sporocarp production, maturation of sporocarp, drying of sporocarp, withering of leaves, formation of dormant buds and resting propagules, formation of new sporophytes and sprouting of resting buds have been represented as phenograms (Text fig-1)

Population A [Talwandi locality]

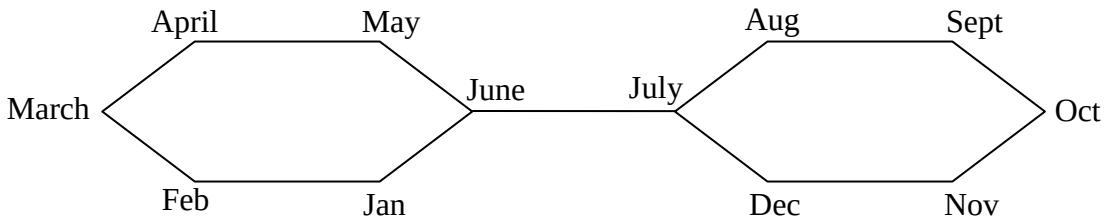
This population of *Marsilea* is situated in the centre of Kota city. The plants remain in water all through the year from the drain. The population was seen in the vegetative phase in most parts of the year with morphologically expanded fronds floating on the surface of water and enormously elongated petioles as compared to the xerophytic populations. Sporocarps were seen in bunch at the base of petiole in the month of November. The sporocarps readily germinate to form new plantlets.

As this population is aquatic and remains in water all round the year, it mainly reproduces through vegetative propagation rather than sporocarp.

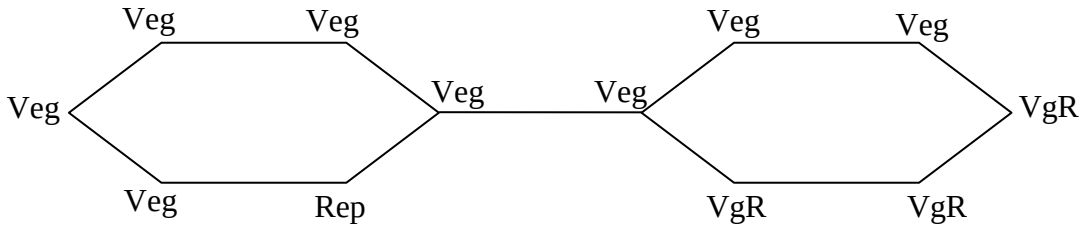
Text Figure 1

Comparative phenograms of *Marsilea* populations

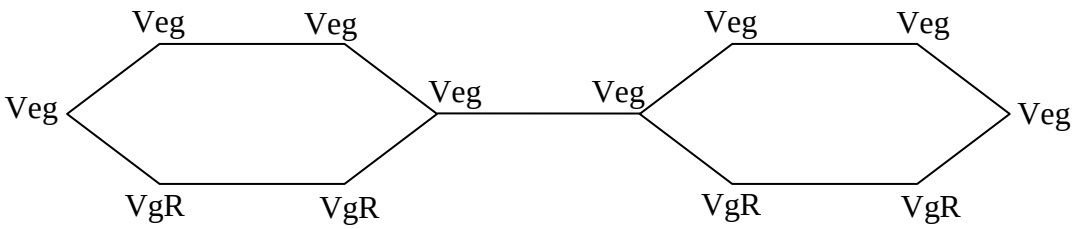
GENERAL PHENOGRAM REPRESENTING SEASONS OF A YEAR



a) TALWANDI LOCALITY (*M.minuta*)



b) ANANTPURA LOCALITY [*M.minuta* (hybrid)]



Population B [Anantpura locality]

M.minuta was found abundantly growing in this locality. Here both the xerophytic and hydrophytic populations of this species were seen. Population was found in vegetative-reproductive stage in the month of January to February producing oval shaped sporocarps. Sporocarps dried and liberate spores which again germinated to form young leaves. The plant then would come in vegetative phase up to the month of October after which it entered in reproductive phase producing oval shaped sporocarp. It was seen that the hydrophytic population in the same locality hardly produce sporocarp and mostly spreads through the vegetative method.

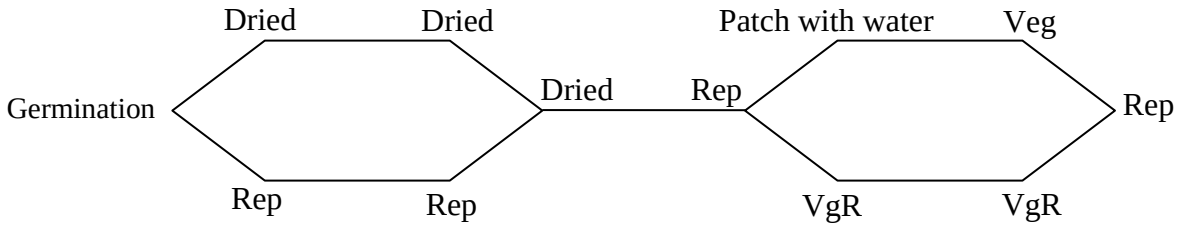
Population C [Mandana locality]

The plant enters the reproductive phase in the month of October after the rainy season. It then produces sporocarps which germinate and form new plantlets. Some sporocarps remain buried in soil. Since there is no water source surrounding this locality, the dormant sporocarps germinate only in the month of July after getting rain.

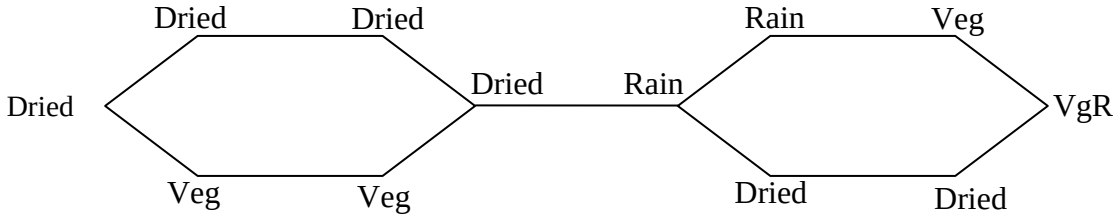
Population D [Borawas locality]

The phenology of this population is very specific as this population appears in nature twice in a year completing both reproductive and vegetative phases in a short duration. A dried patch of *M.coromandelina* (complex) at borawas was also found sprouting by forming small young green leaves from old dark brown coloured ripe sporocarps characteristic of xeromorphic forms of *Marsilea.* It was observed that the soil was dry and also barren in the vicinity of rocky

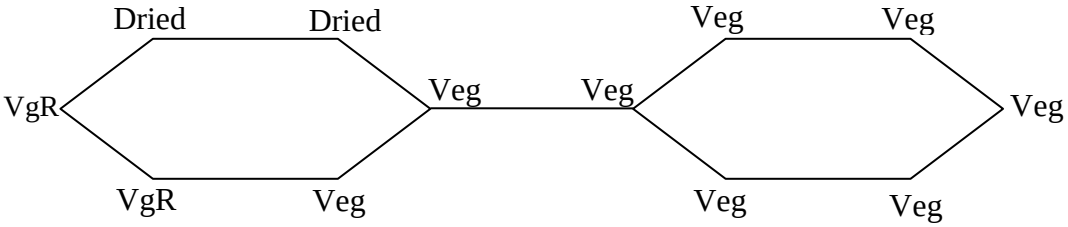
c) MANDANA LOCALITY [*M.minuta* (hybrid)]



d) BORAWAS LOCALITY (*M.cf.coromandelina*)



e) **BORAWAS POND LOCALITY (*M.minuta*)**



Veg - Vegetative phase

VgR - Vegetative+Reproductive phase

mountainous region and the dried plants were practically placed far off from the direct source of water and there had been little or no rain. The life cycle of this population starts from the month of September when the sporocarps get submerged under water and germinate to form small clover shaped leaves with distinct morphology. The patch of this endemic population of *M.cf.coromandelina* remains vegetative in the month of September. It then comes in reproductive phase in the month of October producing squarish sporocarps which appear to be raised above the ground. The leaves start drying in the month of November and towards the end of month the population completely disappears while the sporocarps mix in the soil. As Rajasthan is situated in westernmost part of India it receives rain in winter due to retreating monsoon called mavath. After getting this rain this population is again seen in January completeing its life cycle in a short span and soon it disappear until September. The patch is completely dried up (Figure-5,g) with only hard nut shelled sporocarps in the soil. During rainy season this patch gets completely filled with water for two months i.e. July and august. When the rain water dries up, clover shaped leaves are seen with distinct morphology. Thus, the phenology of this endemic population of *Marsilea* is very specific.

Population E [Borawas Pond locality]

The population is seen throughout the year growing both in xerophytic and hydrophytic habitats. During rainy season (July-sept) the plant shows enormous growth producing expanded fronds floating on surface of water (Fig-6,c). The rhizome trails to several metres under water. The population remain in this state until water gets evaporated and drained away. It begins to produce sporocarp in months of February which again germinate to form new plantlets on getting rain.

ORGAN ANATOMY

A comparative anatomical study of various organs of the selected *Marsilea* populations of Hadauti region has been carried out. This study was aimed at evaluating the degree of variations in relative development of aerenchyma and stellar tissues in the wake of habitat variations. This study has revealed that the basic anatomical plan is retained in all the populations while significant variations have been observed in these populations pertaining to their morphological plasticity.

The roots, rhizomes and petioles of the five investigated populations of these selected localities have been studied. The results of the anatomical study are depicted in table-5 and figures 14, 15, and 16. The comparative anatomical features observed thus displays inter-populational variations to some extent.

1. **Petiole:** (Table-5, fig-14)

   The comparison of the petiolar anatomy among the five populations (Fig10- and table-5) has revealed that while the epidermal cells and cuticle is almost round and well defined in all the populations cuticle of *M.cf.coromandelina* population (borawas) is not well defined. The shape and width of air chambers also show variation. They are irregularly round in 'A' 'B' and 'C' while squarish in 'D' and large and rectangular in 'E'.

   The thick walled zone in the middle cortex is single layered in 'A' while 2-3 layered in 'C'. However it is not significant in 'B', 'D' and 'C' populations. The width of vascular zone is found to be maximum in population 'C' (Mandana locality) while minimum in population 'E' (Borawas pond). Thus, the petiolar anatomy seems to provide some dependable anatomical features for taxonomic comparison (Johnson, 1986)

Table-5

Study of anatomical variations among selected localities of *Marsilea* population of Hadauti plateau

Anatomical parameters	Locality				
	Talwandi (aquatic) A	Anantpura B	Mandana C	Borawas D	Borawas pond E
PETIOLE **Epidermis** Shape of cells	Round	Almost round	Oval	Oval	Round
Cuticle	Well defined	Well defined	Well defined	Not well defined	Well defined
Cortex Air chamber	Irregularly round	Roundish in shape	Irregularly round	squarish	Large and rectangular in shape
Thick walled zone in middle cortex	Single layer of thick walled cells	Not significant	2-3 layer of thick walled cells outside vascular zone	Not significant	Not demarcated
Width of vascular zone	Average	Average	Maximum	Average	Minimum
RHIZOME **Epidermis**	Continuous single layered	Continuous single layered	Continuous single layered	Continuous single layered	Continuous single layered
Air chamber	Irregularly round	Small obliterated	Squarish	squarish	Large and rectangular
Cortex	Middle zone thick walled 3-layered	Middle zone thick walled 2-3 layered	Middle zone thick walled 2 layered	Middle zone thick walled 2-3 layered	Middle zone thick walled 3 layered
Vascular zone **Xylem**	Single layered ring	Single layered ring	Single layered ring	Single layered ring	Single layered ring
Tracheidal cells	Tracheidal cells medium size	Tracheidal cells large	Tracheidal cells medium size	Tracheidal cells medium size	Trachedial cells smaller
Phloem	2-3 layered with larger seive tubes	2-3 layered with some larger seive tubes	3 layered middle layer of larger seive cells	3-4 layered larger seive tubes	2-3 layered with medium size seive cells
Pith	Sclerotic more than 20 cells	Sclerotic 27-30 cells	Sclerotic 20-25 cells	Sclerotic 28-30 cells	Sclerotic 15-20 cells
ROOT **Cortex**	Thickened zone 2 layered thick	Thickened zone 3 layered thick	Thickened zone 2 layered thick	Thickened zone 3 layered thick	Thickened zone 2-3 layered thick
Stele	Diarch, exarch	Diarch, exarch	Diarch, exarch	Diarch, exarch	Diarch, exarch

Figure- 14

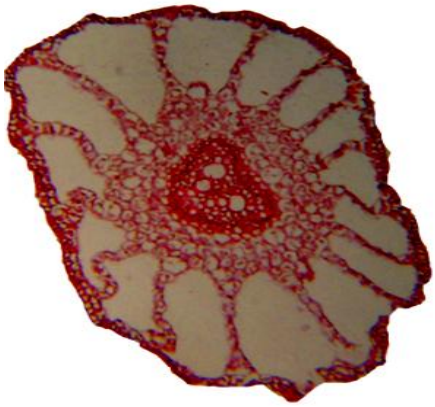
Petiole anatomy (T.S.) of selected *Marsilea* populations of Hadauti Plateau

(a) *M.minuta* –Population A (Irregular round air chambers in cortex)
(b) *M.minuta*– Population B (Almost round cells of outer cortex)
(c) *M.minuta* (hybrid) – Population C Presence of aerenchymatous inner cortex)
(d) *M.cf.coromandelina* – Population D (well developed arenchyma and typical V shaped xylem
(e) *M.minuta* –Population E (large air chambers in cortex)

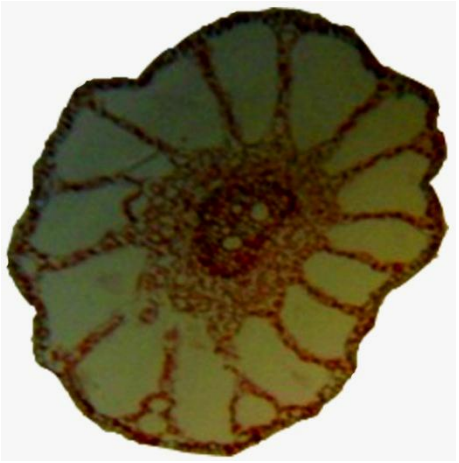
Figure- 14

(a)

(b)



(c)



(d)

(e)

2. **Rhizome** (Table-5 , Fig15)

   The epidermis of all the populations is continuous and single layered. Air chambers shows variation in shape and size. These are irregularly round in 'A', small and obliterated in 'B', squarish in 'C' & 'D' while large and rectangular in 'E'. The cortical region consists of a middle zone which is 2-3 layered thick in all the populations. The xylem ring is single layered in all populations, while the tracheidal cell size varies. Tracheidal cells are larger in 'B', medium in 'A', 'C' and 'D' while these are smaller in 'E'. Phloem is 2-3 layered and has wider sieve cells in all these populations of *Marsilea* while it is exceptionally 3-4 layered in population 'D' (*M.cf.coromandelina* locality). It is clear from table that rhizomes of all populations show sclerotic pith, the number of cells varying between 15-30 with the maximum number being found in population of 'B' and 'D' locality.

3. **Root**- (Table-5, Fig16)

   Root anatomy reveals a zone of thick walled cells in the inner cortex which is 2-layered in 'A' and 'C' populations while it is 3-layered in 'B', 'D' and 'E' populations. The stele is diarch and exarch in all the investigated populations. It may be stated by way of a general conclusion that the comparative study of root anatomy of the five populations' does not provide any distinctive morphological criteria of diagnostic relevance in interspecific delimitations in different species of *Marsilea*.

Figure- 15

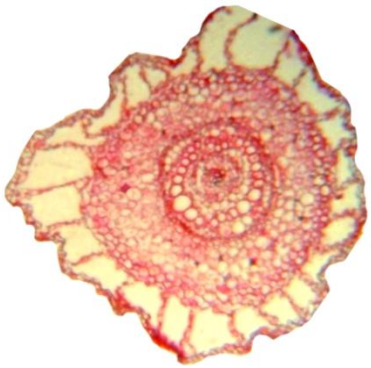
Rhizome anatomy (T.S.) of *Marsilea* populations of Hadauti Plateau

(a) *M.minuta* –Population A (middle zone thick walled cortex)
(b) *M.minuta* (hybrid) – Population B (Small scattered air chambers)
(c) *M.minuta* (hybrid) – PopulatioC (Irregular air chambers in cortex)
(d) *M.cf.coromandelina* – Population D (Vascular bundle showing amphiphloic siphonostele)
(e) *M.minuta* –Population E (Aerenchymatous outer cortex and less sclerotic pith)

Figure 15

(a)

(b)



(c)

(d)

(e)

Figure- 16

Root anatomy of *Marsilea* populations of Hadauti Plateau

(a) *M.minuta* –Population A (Thick walled inner cortical cells surrounding well developed vascular tissue)

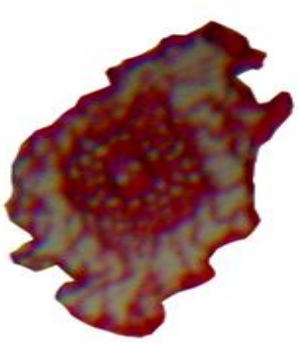
(b) *M.minuta* (hybrid) – Population B (Plate like xylem occupying the centre of stele)

(c) *M.minuta* (hybrid) – Population C (Inner cortex delimited by endodermis)

(d) *M.cf.coromandelina* – Population D (outer piliferous layer consisting of compactly arranged biconvex cells)

(e) *M.minuta* –Population E (No medullary tissue or pith in the stele)

Figure- 16



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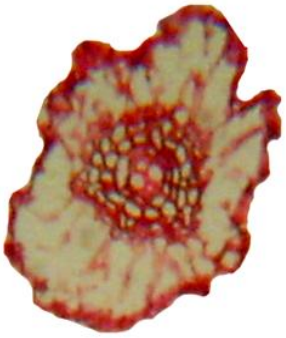

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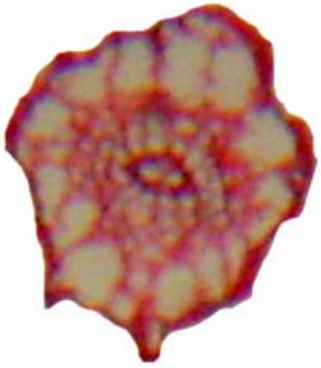

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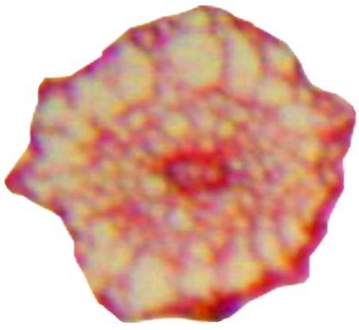

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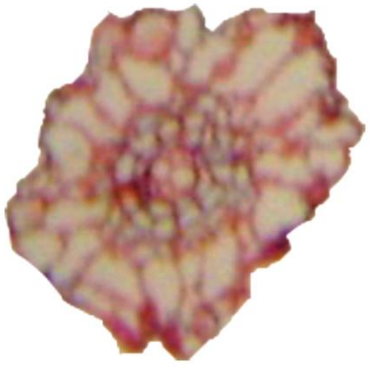


(e)

CYTOLOGY (Fig-17)

Chromosomal variation is widespread in plants and animals. It often contributes to the genetic barriers to gene flow that exist between species and hence its role in species diversification has been heavily debated. When the list of plant chromosome numbers were published by A & D.Love (1948) and Tischler (1950), the chromosome numbers of many pteridophytes were uncertain or wholly unknown. During the past eight years, however, some very important contributions to pteridophyte cytology have been made especially in England. For the present studies only mitotic studies based on root tip squashes of the two populations of *Marsilea* viz. *M.minuta* (Talwandi) and *M.cf.coromandelina* (Borawas) have been undertaken.

The mitotic metaphase chromosomes of the two taxa are presented in fig-17; clearly establish the diploid nature of *M.minuta*. The chromosome count in both the populations could be ascertained to be 2n=40 for *M.minuta* and 2n=42 for *M.cf.coromandelina* (Fig-17). Somatic chromosome analysis revealed that the mitotic chromosomes in *M.cf.coromandelina* were small, they ranged in length from 1.2 to 1.8 µm in length while *M.minuta* mitotic chromosomes measured 1.4 to 2.0µm in length. Morphological details of the chromosomes were not very clearly visible.The haploid chromosome number n=21 in *M.cf.coromandelina* shows this species to be of aneuploid origin and is cited as evidence of the role of aneuploidy in species diversification in the genus. (Kuriachan, 1991).

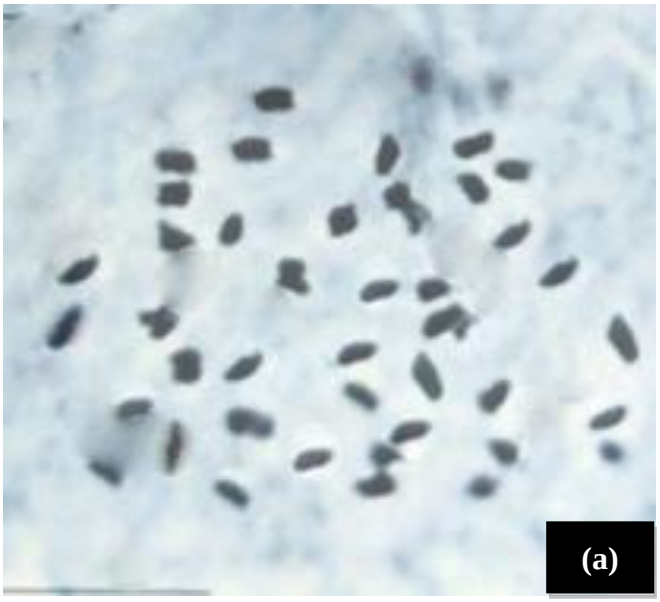
Somatic chromosome analysis of *M.minuta* is shown to be diploid having chromosome number 2n =40 in squashes of root tips. Other cytotypes are however also present in this species, notably a sterile triploid with 2n = 60 which is otherwise very like the diploid and frequently intermixed with it. (Mehra &Loyal, 1956). Nevertheless, the cytological basis of hybridity can

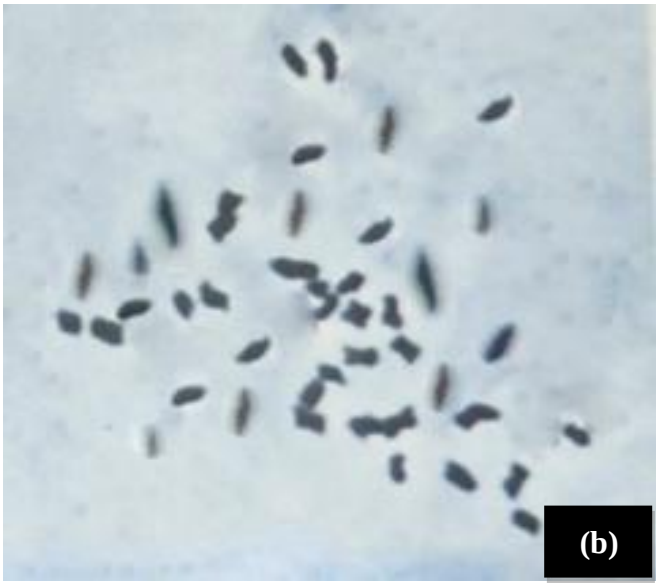
Figure – 17

Mitotic phase of *Marsilea* species

a) **Root tip cells of *M.minuta* showing 2n=40 chromosomes.**
b) **Root tip cells of *M.cf.coromandelina* showing 2n-42 chromosomes.**

Figure- 17





certainly be deduced from the circumstances of the *M.minuta* population growing contiguously and interacting with each other and the hybrid populations evolving as a result of their interaction at Hadauti Plateau.

CHAPTER V

EXPERIMENTAL STUDIES

PHYTOCHEMISTRY

Medicinal value of pteridophytes is known to man for more than 2000 years. In recent years, various types of herbal medicines have been used as anxiolytic drugs in different parts of the world. The genus *Marsilea* is the common water fern found in wet and flooded lowland. Common vernacular names of *M.minuta* L. are Sunishannaka, Parnaka, Vastika-parnika, Swastika, Chatushpatri, sunsunishaak, chaupaitra etc. The plant as a whole is used as sweet, astringent, cooling, digestive, diuretic, hypnotic and expectorant. Treatment for psychopathy, diarrhoea, cough, bronchitis, skin diseases and fever also has been reported in ayurveda. The use of *Marsilea minuta* as sedative has been referred in many text of Ayurveda for the treatment of insomnia and other mental disorders. The decoction of this plant was fed to the patients suffering from mental disorders along with their meal as a routine procedure in some mental clinics.. Chatterjee et al., 1963 isolated marsiline (macro cyclic ketone) from chloroform extract of *Marsilea minuta* leaves (yield- 0.1 to 0.05%) and from whole plant (yield-0.03 to 0.04%) and reported its sedative and anticonvulsant properties.

The present phytochemical investigation includes the quantitative estimation of total soluble sugar, total phenol, chlorophyll and proline contents of the two *Marsilea* species namely *M.minuta* and *M.cf. coromandelina* occurring commonly in Kota. The methodology employed for these studies has been described in chapter III. The data recorded for the above mentioned parameters have been presented as average values.

Interspecific as well as intraspecific differences of these substances in *Marsilea* population of these two species have provided interesting data (Table-6).

Total soluble sugar (mg/gdw) [Table-6, Text figure-2]

Higher sugar content for the entire plant was recorded in *M.minuta* (6.47) as compared to *M.cf.coromandelina* (4.86). Sugar content was found to be 0.35 in roots of *M.minuta* while 0.40 mg/gdw sugar content was recorded in *M.cf.coromandelina*.

In rhizome maximum amount of sugar content (10.12 mg/gdw) was recorded in *M.cf.coromandelina* as compared to 1.40 mg/gdw found in *M.minuta*.

In petiole higher sugar content (8.14mg/gdw) was recorded in *M.minuta* compared to 4.45 mg/gdw recorded in *M.cf.coromandelina*.

In entire plant higher sugar content was recorded in *M.minuta* (6.47 mg/gdw) while it was 4.86 mg/gdw in *M.cf.coromandelina* respectively.

Among various organs, sugar content was found to be highest in rhizome and lowest in root for both the two taxa.

Total Phenol contents (mg/gdw) [Table-6, Text figure-3]

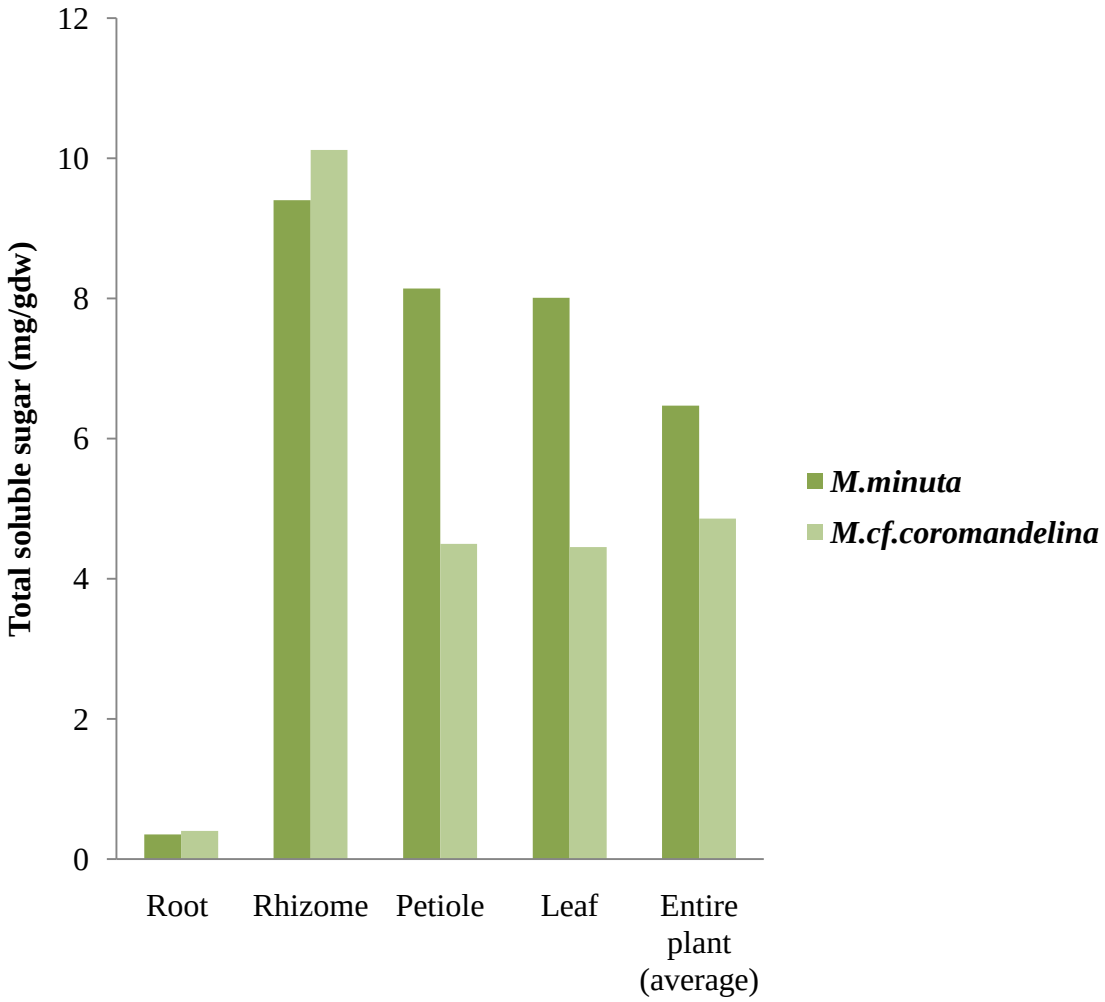
Higher phenol content was recorded in roots of *M.cf.coromandelina* (4.12 mg/gdw) as compared to *M.minuta* (3.00mg/gdw). Similarly phenol content in rhizome of *M.cf.coromandelina* was found to be 15.13 mg/gdw which is higher than *M.minuta* (14.14 mg/gdw)

In petiole, phenol content was recorded to be comparatively higher (13.52) in *M.minuta* and this value was recorded to be (12.32) in *M.cf.coromandelina.*

Leaflets of *M.minuta* recorded higher phenol content (12.48) than those of *M.cf.coromandelina* (9.32) Organ wise comparison has revealed that rhizome of both the species possess maximum phenol content while minimum amount was recorded in roots of both the species.

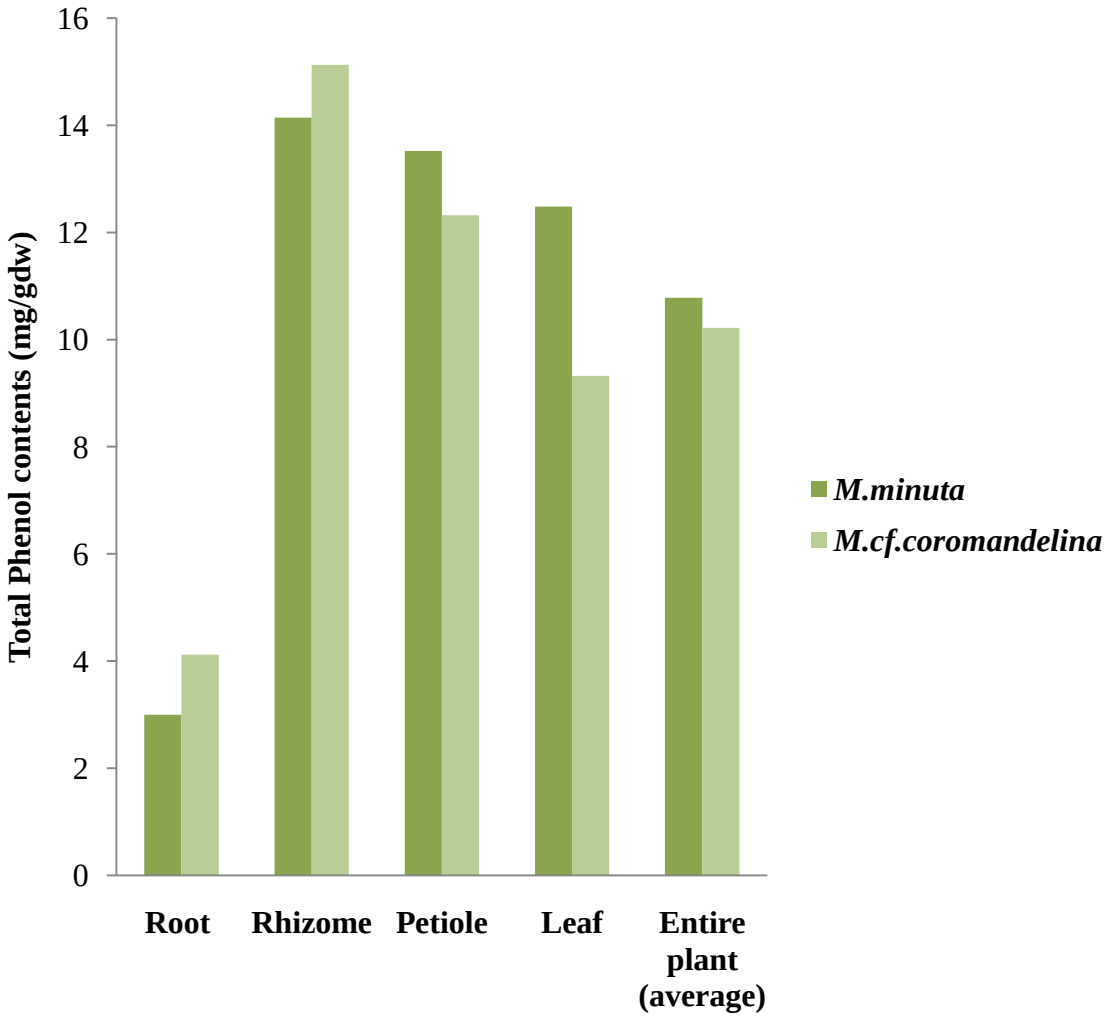
Text Figure 2

TOTAL SOLUBLE SUGAR



Text figure 3

TOTAL PHENOL



Total starch contents (mg/gdw) [Table-6, Text figure-4]

Total starch content of entire plant of *M.minuta* was higher (3.94) than that of *M.cf. coromandelina* (3.08). In roots, a higher quantity of starch (3.3) was observed in *M.minuta* than *M.cf. coromandelina* (2.6). Similarly, in rhizome starch content has been found to be significantly higher in M.minuta (4.43) than *M.cf. coromandelina* (3.18).

Starch content of petiole was recorded to be 2.42 in *M.cf. coromandelina* while it is 2.94 in *M.minuta*. The leaflets of *M.minuta* revealed 5.12 mg/gdw of starch with a slightly lower quantity (4.13 mg/gdw) being present in *M.cf. coromandelina.*

Total soluble proteins (mg/gdw) [Table-6, Text figure-5]

Among the two species, *M.minuta* and *M.cf. coromandelina* maximum protein content (12.81 mg/gdw) was observed in the entire plant of *M.minuta* while a lesser quantity (9.74 mg/gdw) was observed in *M.cf.coromandelina*.

Protein content was found to be 19.12 mg/gdw in roots of *M.minuta* while 14.14 mg/gdw protein content was found in *M.cf. coromandelina*.

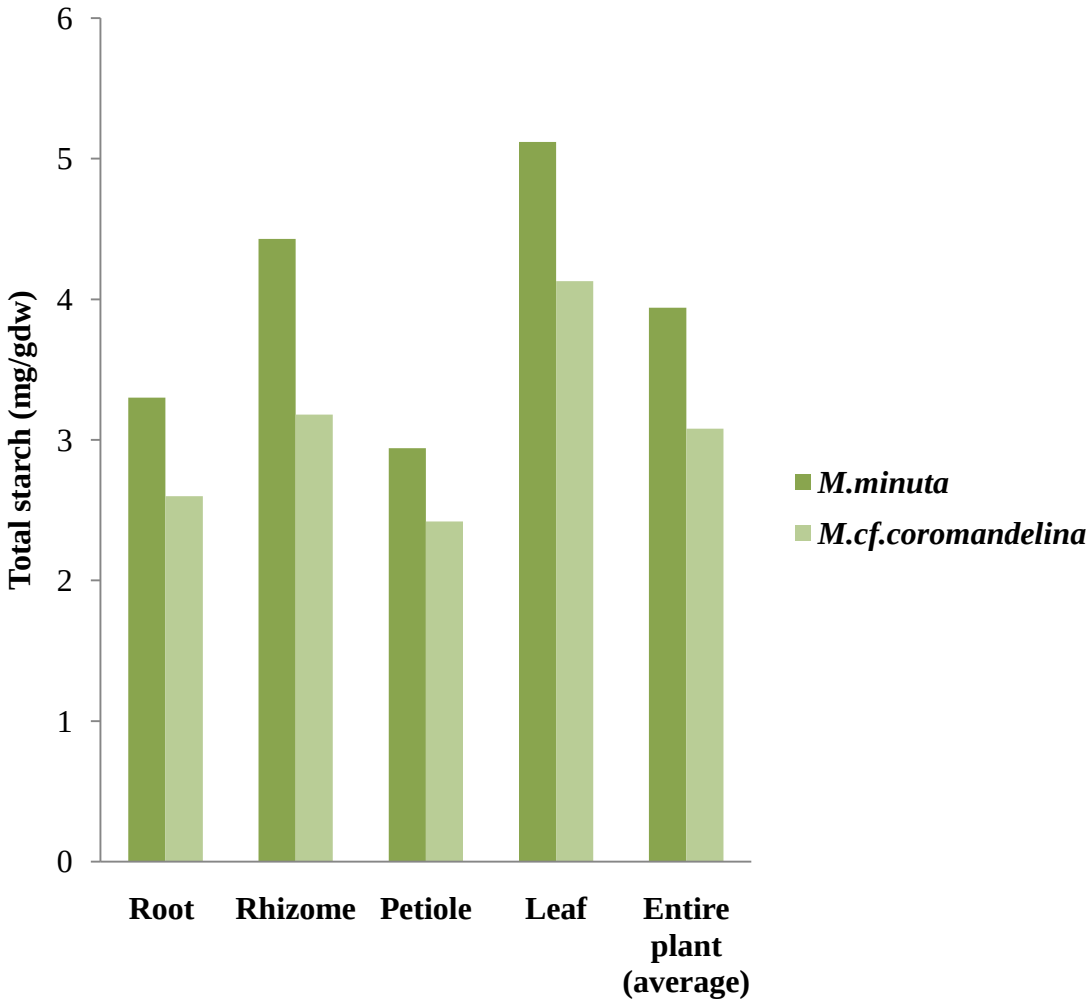
In rhizome, maximum amount of protein content (12.20 mg/gdw) was recorded in *M.minuta* as compared to 10.62 mg/gdw found in *M.cf. coromandelina*.

In leaf lamina, maximum protein content (9.33 mg/gdw) was recorded in *M.minuta* with 8.73 mg/gdw being noted in *M.cf. coromandelina*.

Petioles of *M.minuta* again revealed a higher protein content (10.60 mg/gdw than *M.cf. coromandelina* which contained 5.48 mg/gdw.

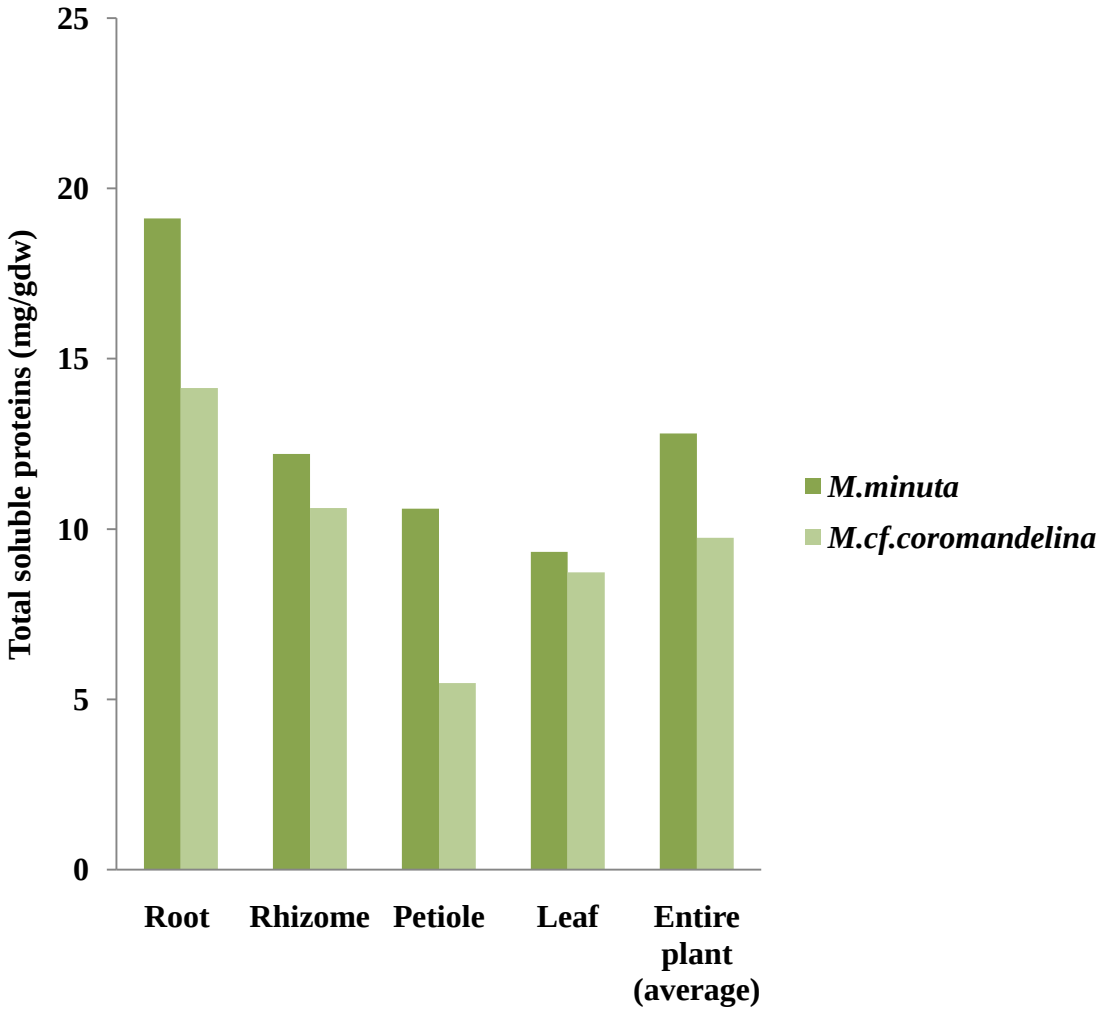
Text figure – 4

TOTAL STARCH



Text figure – 5

TOTAL SOLUBLE PROTEINS



Ascorbic acid (mg/gdw) [Table-6, Text figure- 6]

Higher ascorbic acid content for the entire plant was recorded in *M.cf. coromandelina* as compared to *M.minuta*. Thus, 19.07 mg/gdw ascorbic acid was recorded in former while 18.21 mg/gdw was observed in *M.minuta*. Roots of *M.cf. coromandelina* revealed a higher ascorbic acid content (18.22 mg/gdw) than *M.minuta* which contained 15.30mg/gdw. In rhizome higher ascorbic acid content (19.43 mg/gdw) was noted in *M.cf. coromandelina* while it was less (18.30 mg/gdw) in *M.minuta*.

In leaves ascorbic acid content was found to be comparatively higher 24.02 mg/gdw in *M.minuta* while this value was recorded to be 22.21 in *M.cf. coromandelina.*

In terms of ascorbic acid content in petiole, it was recorded to be 16.45 mg/gdw in *M.cf. coromandelina* while lower content (15.23 mg/gdw) was recorded in petioles of *M.minuta*.

E) Total free Proline (mg/gdw) [Table-6, Text figure-7]

Total free proline content for the entire plant was recorded to be much higher in *M.cf. coromandelina* as compared to *M.minuta*. Thus, proline content was observed to be 0.61 mg/gdw in *M.cf. coromandelina* while in *M.minuta* the proline content was 0.36 mg/gdw. The roots of *M.cf. coromandelina* were found to have a higher proline content (0.40 mg/gdw) as compared to 0.11 mg/gdw proline content observed in *M.minuta.*

In rhizome, 0.81 mg/gdw proline content was observed in *M.cf. coromandelina* while its amount in *M.minuta* was 0.44 mg/gdw.

The leaves of *M.cf. coromandelina* showed 0.65 mg/gdw proline content while it was 0.60 mg/gdw. In petiole, maximum proline content (0.62 mg/gdw) was recorded in *M.cf. coromandelina* while 0.34 mg/gdw being noted in *M.minuta.*

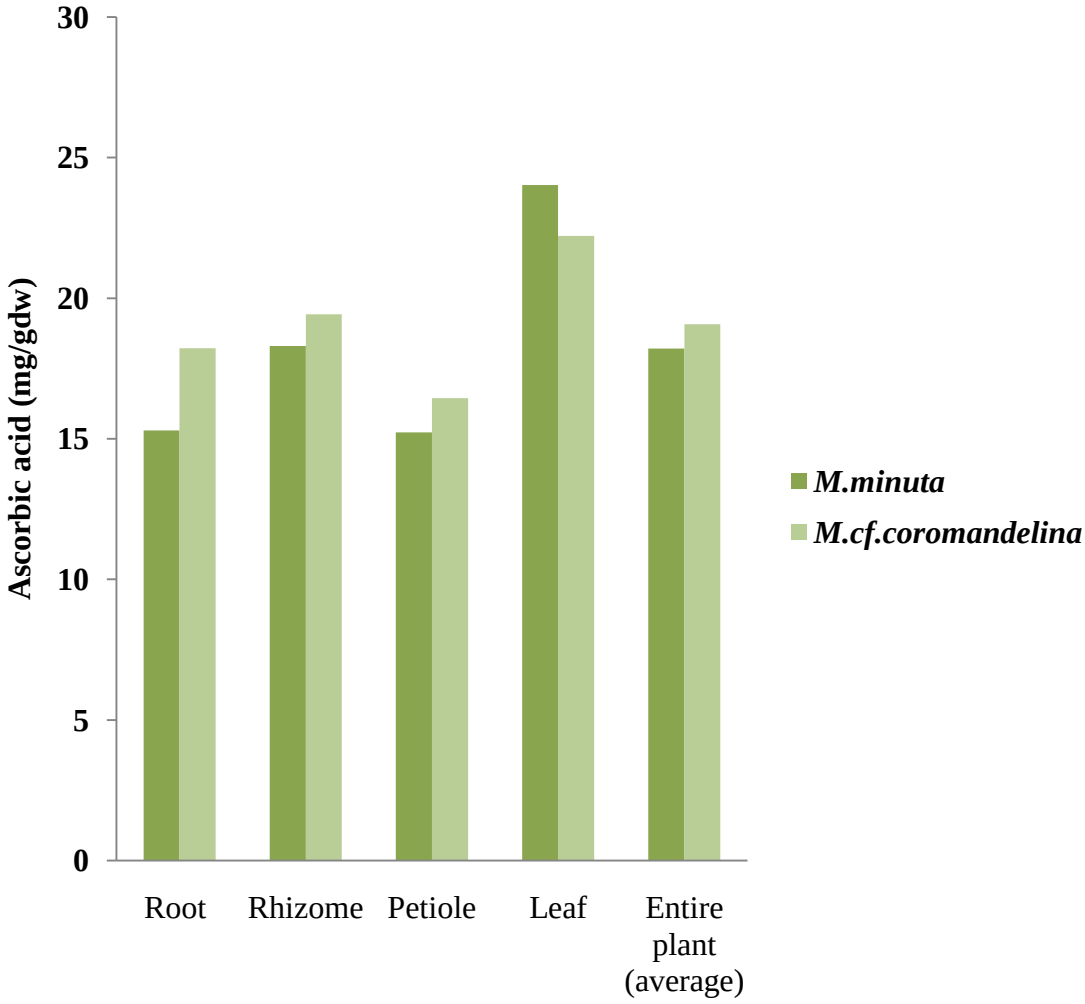
Table 6

Organ wise estimation of total soluble sugars, phenols, starch, proteins, ascorbic acid, and proline of the two species of *Marsilea* found in Hadauti region

Parameters (mg/gdw)	***M.minuta***					***M.cf.coromandelina***				
	Root	Rhizome	Petiole	Leaf	Entire plant (average)	Root	Rhizome	Petiole	Leaf	Entire plant (average)
Total soluble sugar	0.35	9.40	8.14	8.01	6.47	0.40	10.12	4.50	4.45	4.86
Total Phenol contents	3.00	14.14	13.52	12.48	10.78	4.12	15.13	12.32	9.32	10.22
Total starch	3.3	4.43	2.94	5.12	3.94	2.6	3.18	2.42	4.13	3.08
Total soluble proteins	19.12	12.20	10.60	9.33	12.81	14.14	10.62	5.48	8.73	9.74
Ascorbic acid	15.30	18.30	15.23	24.02	18.21	18.22	19.43	16.45	22.21	19.07
Total proline contents	0.11	0.44	0.34	0.60	0.36	0.40	0.81	0.62	0.65	0.61

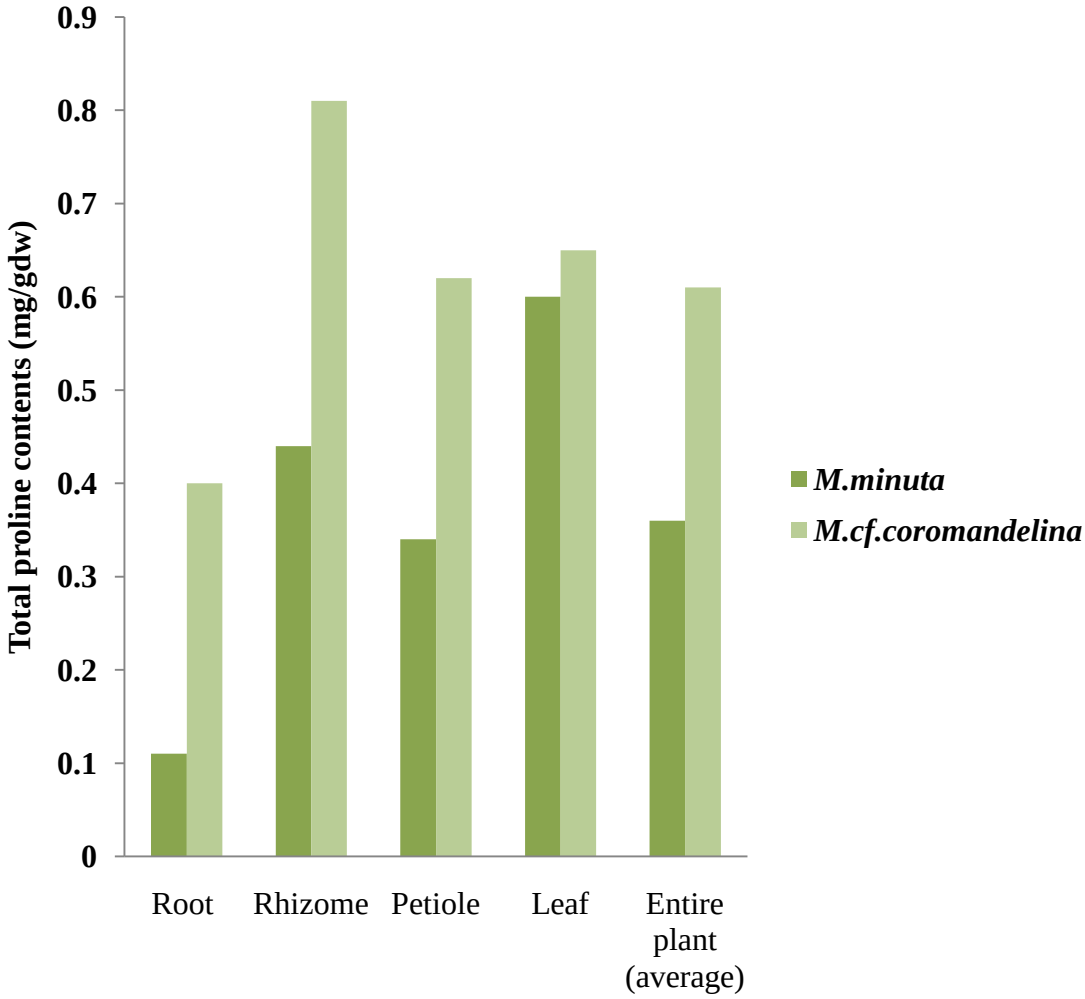
Text figure 6

ASCORBIC ACID



Text figure – 07

TOTAL PROLINE



Photosynthetic pigments- (mg/gdw) [Table-7, Text figure-8]

Total chlorophyll content of *M.cf.coromandelina* leaves was higher than that of *M.minuta*. The leaves of *M.cf.coromandelina* were seen to possess a higher amount of chlorophyll (5.78 mg/gdw) while a lower amount was recorded in *M.minuta* (4.43 mg/gdw).

Chlorophyll a content was found to be more in both the investigated species as compared to chlorophyll b. *M.cf.coromandelina* possess a higher chlorophyll a content (3.84 mg/dw) while the quantity of chlorophyll a in *M.minuta* was found to be 3.32 mg/gdw.

Likewise, chlorophyll b in *M.cf.coromandelina* was found to be 1.94 mg/gdw while a slightly lower quantity (1.21 mg/gdw) was noted in *M.minuta*.

Similarly, higher carotenoid content was recorded in *M.cf.coromandelina* (2.10 mg/gdw) as compared to *M.minuta* (1.93 mg/gdw).

Quantitative organ wise estimation of various metabolites- soluble sugars, phenols, starch, proteins, ascorbic acid and proline have been carried out in the two species. An overall higher trend of these metabolites has been recorded in *M.minuta*. Proline content, which is an important parameter of drought resistance, reveals that it is slightly higher in the entire plant parts of *M.cf. coromandelina.*

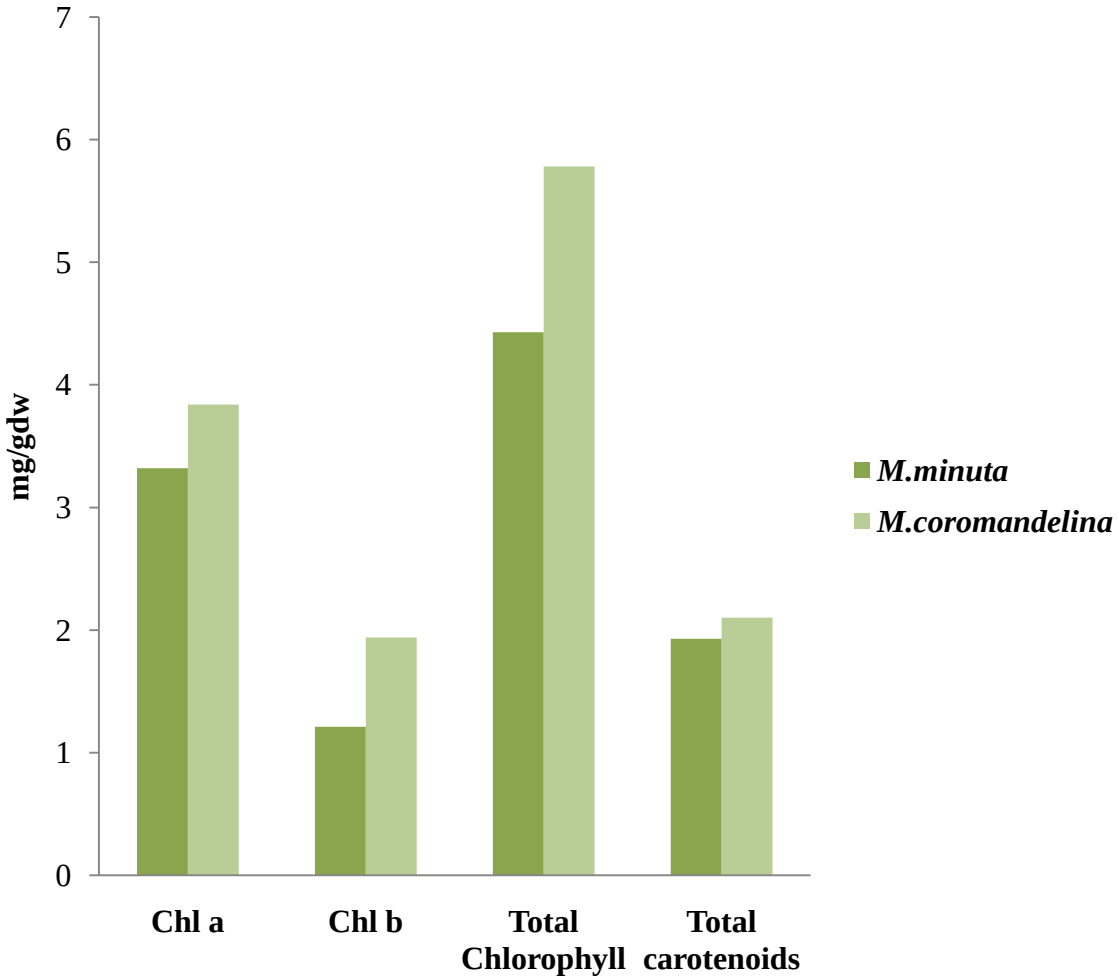
Table 7

Chlorophyll and carotenoid content of *Marsilea* species

S.No.	Population	Chlorophyll a (mg/gdw)	Chlorophyll b (mg/gdw)	Total Chlorophyll (mg/gdw)	Total carotenoids (mg/gdw)
1.	*M.minuta*	3.32	1.21	4.43	1.93
2.	*M.cf.coromandelina*	3.84	1.94	5.78	2.10

Text figure – 8

PHOTOSYNTHETIC PIGMENTS



REPRODUCTIVE BIOLOGY

Sporocarp contents including number of sori per sporocarp, number of megaspores per sorus number of microsporangia per sorus, total number of mega and microspores per sporocarp of both *M.minuta* and *M.cf. coromandelina* have been counted to evaluate reproductive capacity.

SPOROCARP GERMINATION

Freshly collected ripe sporocarps as well as those stored in paper bags belonging to two species were put for germination studies. After applying a small cut on one side of the ventral edge with the help of a sharp scalpel, with 3 sporocarps of *M.minuta* and 10 sporocarps of *M.cf.coromandelina* were kept in a petridish containing tap water in order to permit the liberation of sorophore (Fig-18). Detailed observations regarding spore liberation, extrusion of sorophore, sporelings initiation, and initiation of first leaf have been recorded in table-8. Various stages of release of micro-megaspores from sori of the two species have been shown in fig-19, 20. Interspecific differences regarding the above aspects of reproductive biology were observed in these species.

As the sporangia mature the parenchymatous tissue next to sporocarp wall become gelatinous. The outer thick and hard rind is resistant to drying and injury and stands in the way of its early dehiscence. The sporocarp imbibes water through the cut end and sorophore protrudes out. It is observed that the sorophore extrusion takes place rather rapidly in *M.cf coromandelina* in about 1-2 hours after placing the scarified sporocarps in water while it takes about 2-3 hr for sorophore extrusion in *M.minuta* . Thus sorophore extrusion is quicker in *M.cf coromandelina* among these two species.

Figure – 18

a) **Scarified sporocarps (Three) placed in water-*M.minuta***
b) **Scarified sporocarps (Ten) placed in wate- *M.cf.coromandelina***
c) **Sporeling growth after five days- *M.minuta***
d) **Sporeling growth after five days-- *M.cf.coromandelina***

Figure- 18

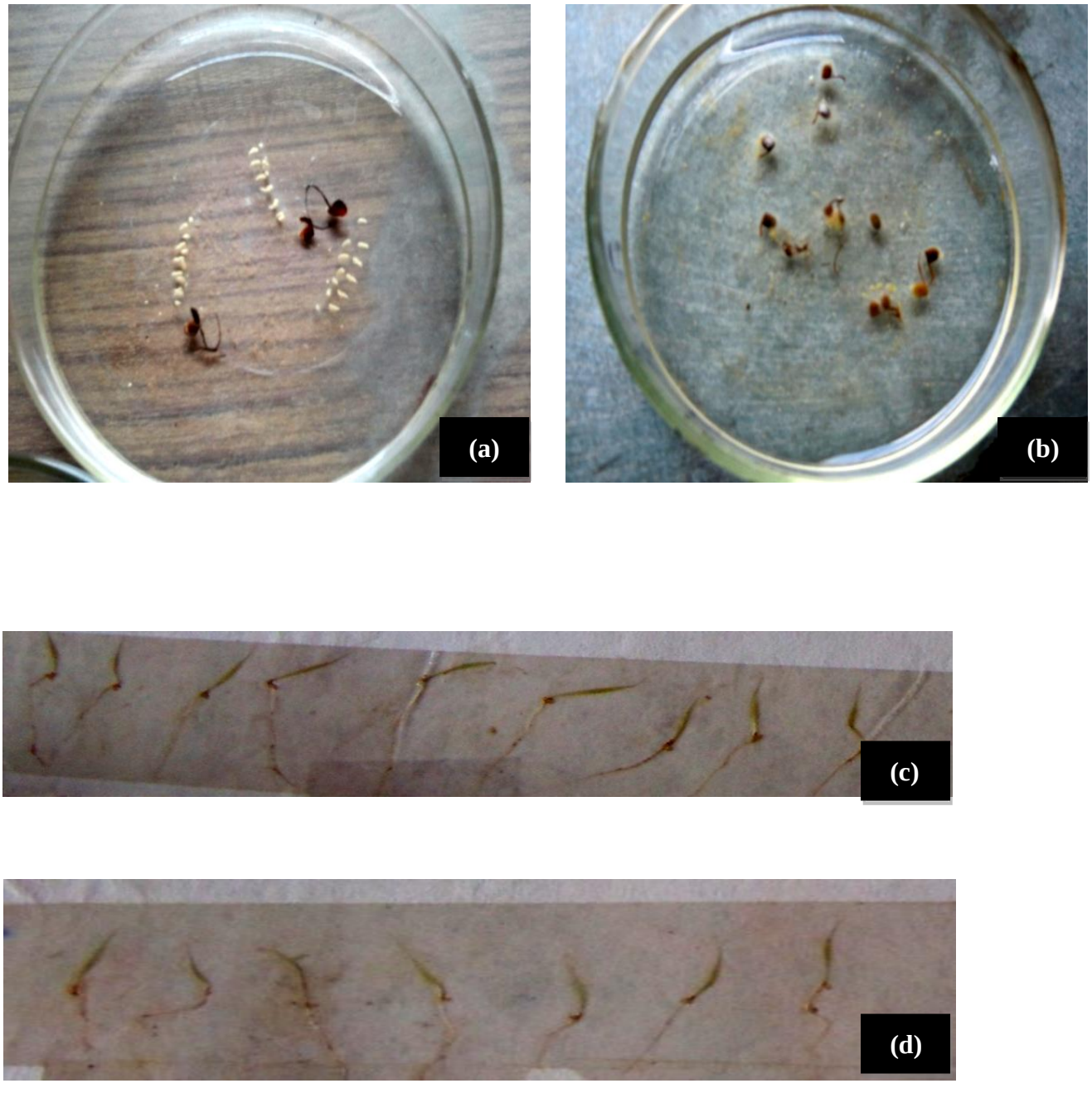


Table-8

Comparative time taken (in hrs) for sorophore extrusion, spore liberation, sporelings development and calculation of reproductive capacity of *M.minuta and M.cf.coromandelina*

Parameter	***M.minuta***	***M.cf.coromandelina***
Extrusion of sorophore (h)	1-3	1-2
Release of spores (h)	9-10	8-9
Initiation of sporelings (h)	32-36	20-26
Initiation of first leaf (h)	40-45	40-42
Initiation of first root (h)	46-65	45-60
Sporelings with 2mm long leaf (h)	72-80	80-90
No. of megaspores/sporocarp	14	12
No. of germinated megaspores	11	10
No.of sporelings formed	10	9
Reproductive capacity (%)	78.5	83.2
No. of microspores/microsporangium	16-18	7-9
No. of sori/sporocarp	8-10	6-8

Figure – 19

***M.minuta*:-Stages in release of mega-microspores**

a) Sporocarp releasing gelatinous chord

b) Sorus showing enveloped micro-megasporangium

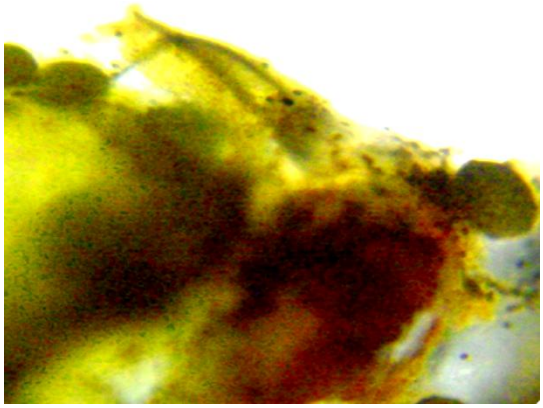
c) Mega- microsporangia showing released spores

d) Dispersed mega-microspores

e) Single enlarged megaspore

f) Megagametophyte- initiation of first leaf and rhizoids at papillar end

Figure- 19



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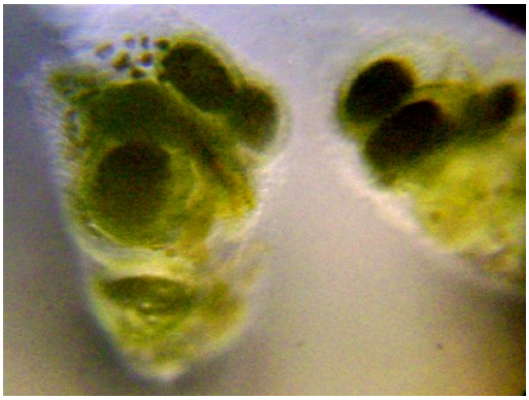

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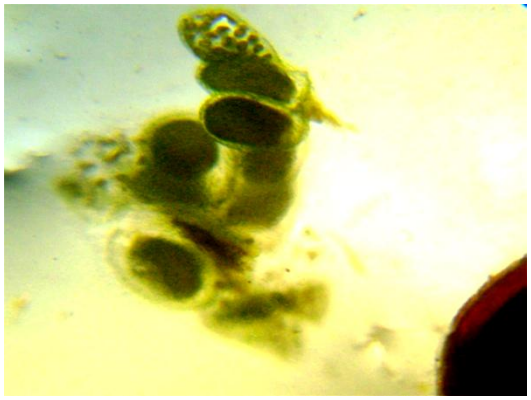

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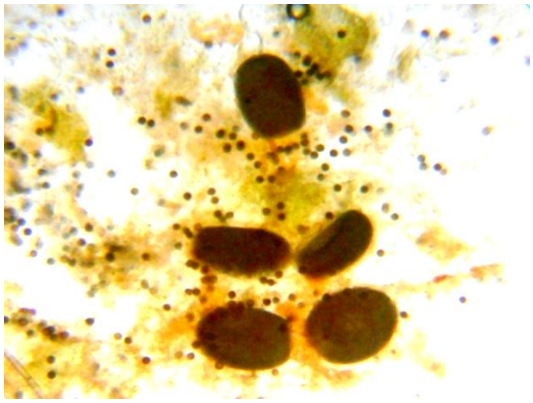

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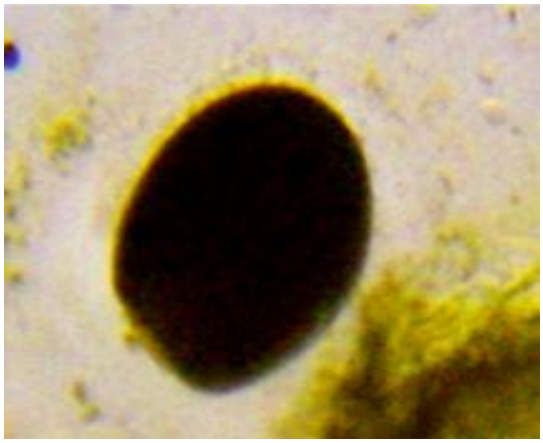


Figure – 20

***M.cf.coromandelina*:-Stages in release of mega-microspores**

a) Gelatinous matrix releasing from sporocarp.

b) Soral release

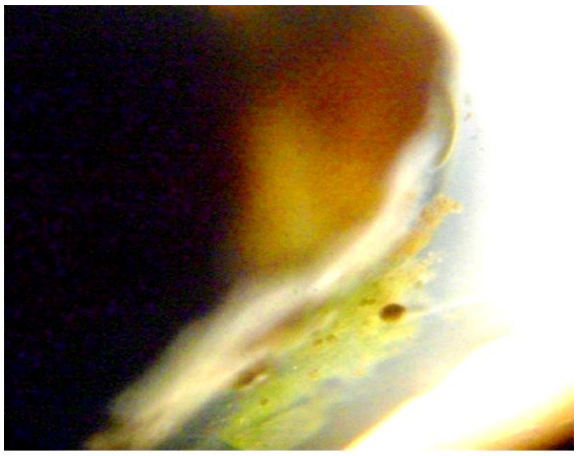
c) Microspores enclosed in sporangium.

d) Dispersed mega-microspores

e) Megaspore enveloping matrix

f) Megagametophyte sporeling development at papillar end.

Figure- 20



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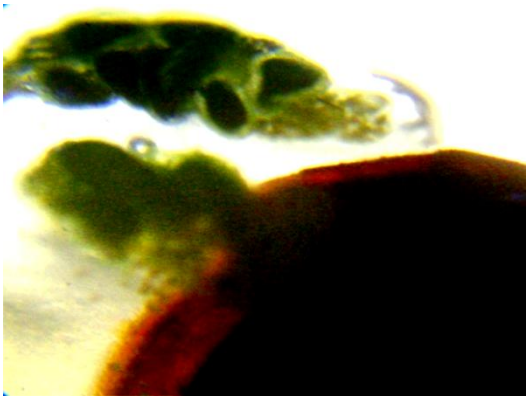

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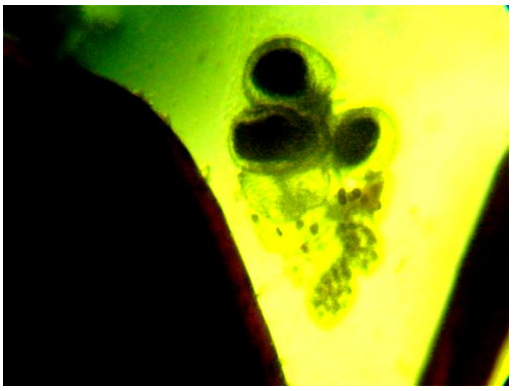

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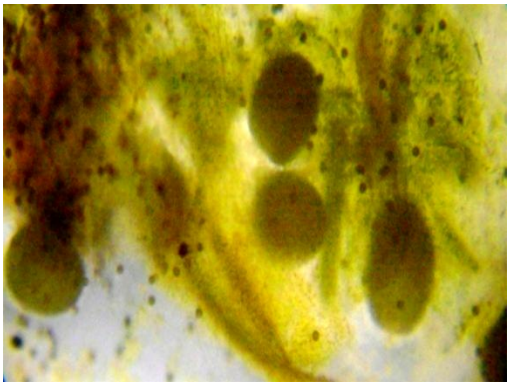
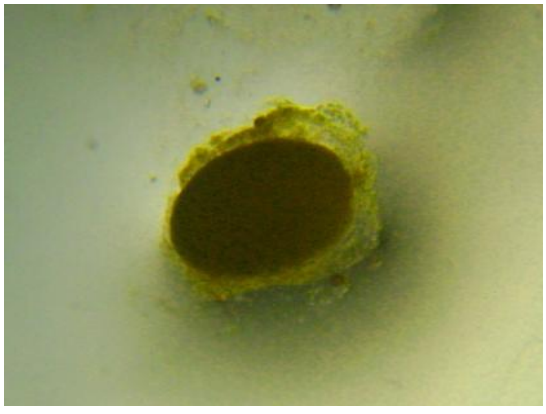
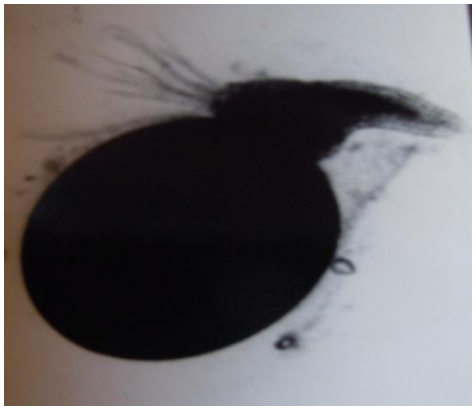


(d)





Sporelings initiation is quicker in *M.cf coromandelina* i.e. within 20-26 h in comparison to *M.minuta* which takes about 32-36 h. Subsequent development of the first leaf takes 40-42 h in *M.cf coromandelina* while in *M.minuta* it takes about 40-45 h. Sporelings with about 2mm long leaf have been formed in 72-80 h in *M.minuta* while it takes about 80-90 h in *M.cf coromandelina*.

Thus it is evident from this table that *M.cf coromandelina* requires a greater time for sporelings formation though the spores are released early as compared to *M.minuta*. It is therefore, evident that interspecific differences do occur in the time period requirement for various aspects of reproductive biology of the investigated species.

Among the two investigated species, *M.cf coromandelina* has got greater reproductive potential (82.20 %) in comparison to *M.minuta* whose reproductive capacity was calculated to be (78.50 %).This may be due to the fact megaspore germination is dependent upon reserve food material available in the megaspore itself. Lesser the number of megaspores per sporocarp better is the nutrition available for their development and storage.

An attempt was made to assess the relative leaf versus rhizoid growth under varying medium conditions in *M.minuta* (Fig-21)

Figure 21 (a-h)

M.minuta **showing root/leaf length in germination sporelings under varied medium conditions**

a) Cold water- Almost equal leaf/Rhizoid length

b) Hot water- Elongated leaf compared to rhizoid

c) Rain water-Leaf longer than rhizoid

d) Distilled water- Almost equal leaf/Rhizoid length

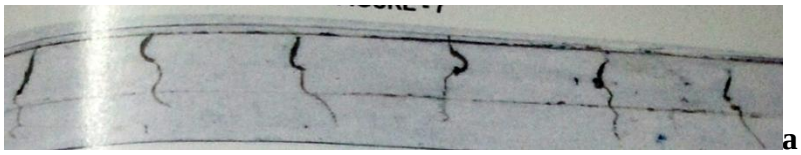
e) Coconut milk - Leaf elongated than the rhizoid

f) Soil water –Almost equal leaf/Rhizoid length

g) Sugar solution - Leaf longer while rhizoid rather suppressed

h) Saline solution-Leaf and rhizoid both suppressed

Figure 21

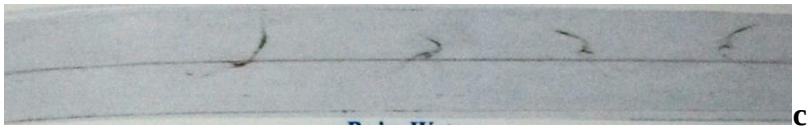


a

Cold water

b

Hot water



c

Rain water

d

Distilled water

e

Coconut milk

f

Soil water

g

Sugar solution

Light is the most important requirement to hydration hence it has been taken up as important parameter for spore germination studies. Spore germination has been reported to be a phytochrome mediated process in ferns operating under red and far red regions of light spectrum (Raghvan, 1971). The most favourable photoperiod condition for germination and sporelings formation has been found to be continous light. Experimental studies were now taken up to observe the effect of spore germination under electromagnetic radiations using blue, green, yellow and red light. Spore germinations has been found to be species specific character and interesting variations among these two species of *Marsilea* namely *M.minuta* and *M.cf.coromandelina* was observed.

Table-9 shows the percentage spore germination per four sporocarps under various light intensities in these two species of *Marsilea.* By and large the sporeling formation shows a greater percentage of germination in blue and yellow light in both these species with some variability in germination and early sporelings growth under various light intensities. It is quite obvious in the two species of *Marsilea*. Thus percentage sporeling formation is more pronounced in all the four light intensities- red, yellow, green and blue in *M.cf.coromandelina* as compared to *M.minuta*. D'Souza reported that the germination is sequentially enhanced in Red-Green-Yellow-Blue light however, the Kota populations of Marsilea have been found to follow this enhancement in the order of Blue-Yellow-Green-Red light. This exceptional behavior may not be attributed to any physical factor and must be regarded as species specific feature.

Another features concerning relative leaf versus rhizoid growth under various light intensities of blue, green, yellow and red was also taken up in the present studies. The relative leaf/rhizoid length under various light intensities of *M.minuta* and *M.cf.coromandelina* has been shown in

Table-9

Percentage spore germination of *Marsilea* species under various light intensities

Light intensity	Number of sporelings per four sporocarps	
	M.minuta	*M.cf.coromandelina*
Blue	26	34
Green	8	10
Yellow	21	29
Red	8	12

fig-22 and in fig 23 for observation of relative leaf and rhizoid elongation under varied light quality also show quite interesting features. Thus while in *M.minuta* leaf elongation is more pronounced under blue light (fig 22) in comparison to *M.cf.coromandelina*. (Fig-23) The same is the case in these species under green light. Under yellow light however, the rhizoidal growth is more pronounced than the leaf in *M.minuta* while in *M.cf.coromandelina* leaf and rhizoid are almost equal in length. Under red exposure rhizoid elongation is more pronounced as compared to leaf in *M.minuta* (Fig22) while leaf and rhizoid are almost equal in length in *M.cf.coromandelina* (fig23). Thus comparative leaf and rhizoid elongation also seems to be a species specific character.

Figure 22

M.minuta **showing relative leaf/rhizoid length under various light intensities**

Figure 22

Blue light

Green light

Yellow light

Red light

Figure 23

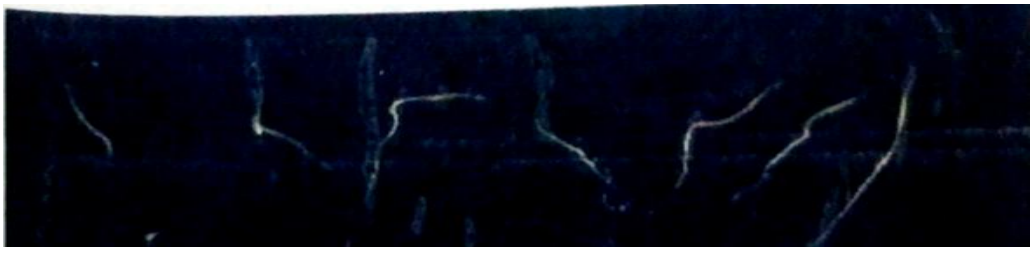
M.cf.coromandelina **showing relative leaf/root length under various light intensities**

Figure 23

Blue light

Green light

Yellowlight



Red light

TISSUE CULTURE AND IN-VITRO MICROPROPAGATION

Tissue culture technology is potent and has opened extensive areas of research for biodiversity conservation. The present study was undertaken with a view to evaluate intra specific variations induced by varying carbohydrate nutrition (glucose) concentrations in culture of *M.minuta* and *M.cf.coromandelina.* Isolated rhizome apices of both the species with intact apical bud, devoid of any leaves and roots were taken and surface sterilized and cultured aseptically on M.S media supplemented with different concentrations of glucose. Observations were recorded after 20 days of setting the experiment.

Various parameters of growth including root length, petiole length, internodal length and leaflet size are recorded in table 10-12 and have been illustrated in figures (24-28)

a) Effect of carbohydrate nutrition on isolated rhizome apices of *M.minuta* and *M.cf.coromandelina* (Table-10, fig-25, 26, text fig-9)

It is known that morphological and anatomical characteristics in different populations of *Marsilea* may also be induced under aseptic conditions in response to quantitative variations of an organic carbon source such as glucose. However, plants obviated from glucose fail to develop such characteristics. The present study was undertaken with a view to evaluate the growth of rhizome apices in different concentration of glucose in cultures of *M.minuta* and *M.cf.coromandelina.* A perusal of the data in table-9 reveals the effect of different concentration of sugar (glucose) on shoot regeneration in *Marsilea*.

Variation in root length and leaflet size of *M.minuta* and *M.cf.coromandelina* was observed in different concentrations of glucose and it was found that moderate concentration (2-3%) of glucose enhance the root and petiole growth. It was also observed that shoot response of explants decline when sugar concentration is increased.

Figure – 24

***Marsilea In-vitro* culture**

a) Explants inoculation in laminar air flow chamber

b) Inoculated explants in culture (M.S media)

Figure- 24

Table 9

Effect of carbohydrate nutrition on isolated rhizome apices of *M.minuta* and *M.cf.coromandelina*

	Glucose conc.	Root Length (cm)	Petiole Length (cm)	Internodal Length (cm)	Leaflet Size; LxB (cm)
M. minuta	**1%**	1.6	4	1	0.4X0.3
	2%	2	8.2	1.8	0.6X0.4
	3%	5	4.5	2.6	0.7X0.6
	4%	1.5	3.7	1	0.6X0.4
	5%	2.25	3.5	0.3	0.6X0.5
M.cf.coromandelina	**1%**	1.5	5.2	1.3	0.5X0.5
	2%	2	6.8	0.6	0.5X0.4
	3%	4.12	3.1	0.4	0.4X0.3
	4%	3.12	3	1.2	0.5X0.3
	5%	3.7	3.4	0.5	0.3X0.2

The root length was found to be maximum at 3% glucose concentration in both species of *Marsilea*, the length of root noted to be 5.00 cm in *M.minuta* while 4.12 cm in *M.cf.coromandelina*. Thereafter at higher concentration a sharp decline is noted. Almost similar pattern of petiole length was noted. Here the longest petiole was produced at 2% glucose concentration in *M.minuta* (8.20cm) while the petiolar length is 6.60 cm in *M.cf.coromandelina* at this concentration. Thus, there was a gradual decline in petiolar length. In *M.minuta* the longest intermodal length (2.60 cm) was observed in cultures having 3% glucose concentration while on the contrary it was maximum (1.20 cm) at 4% concentration in *M.cf.coromandelina*.

Similarly leaflet size was observed to be maximum at 3% glucose concentration in *M.minuta* (0.7x0.6 cm) while it was almost same (0.6x0.4cm) at 4% and 5% glucose concentration. On the contrary in *M.cf.coromandelina*, 2% glucose in the culture resulted in maximum size of leaflets (0.5x0.6cm). At higher concentrations there was a gradual decrease in leaflet size.

Figure – 25

Morphogenetic effect of carbohydrate nutrition (glucose) on isolated rhizome apical segments of *M.minuta*

a) **Culture provided with 1% glucose showing elongated roots and petioles**
b) **Culture provided with 2% glucose showing longer roots and petioles**
c) **Culture provided with 3% glucose showing shorter roots and petioles**
d) **Culture provided with 4% glucose showing reduction in size of roots and petioles**
e) **Culture provided with 5% glucose showing shorter roots.**

Figure- 25

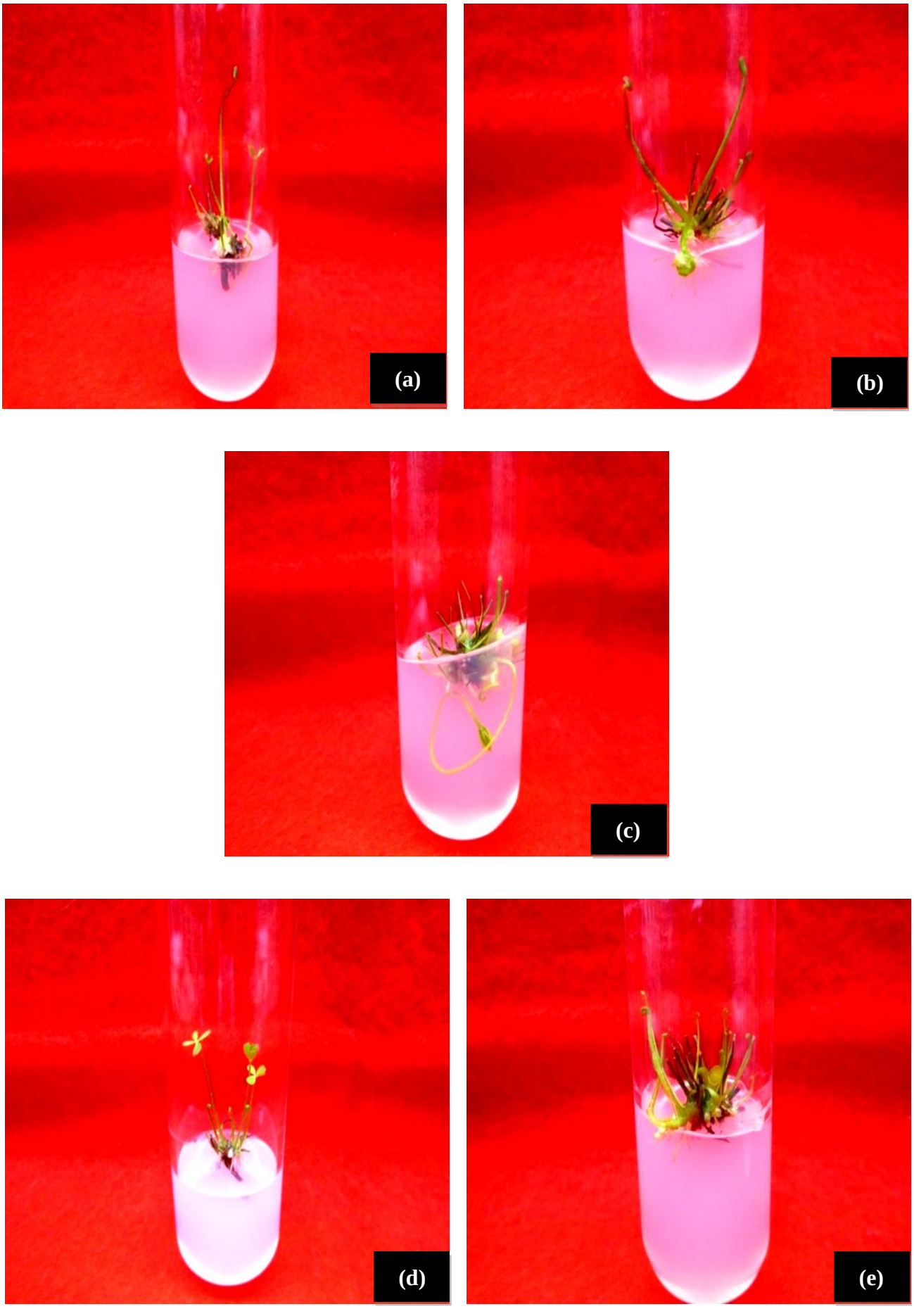
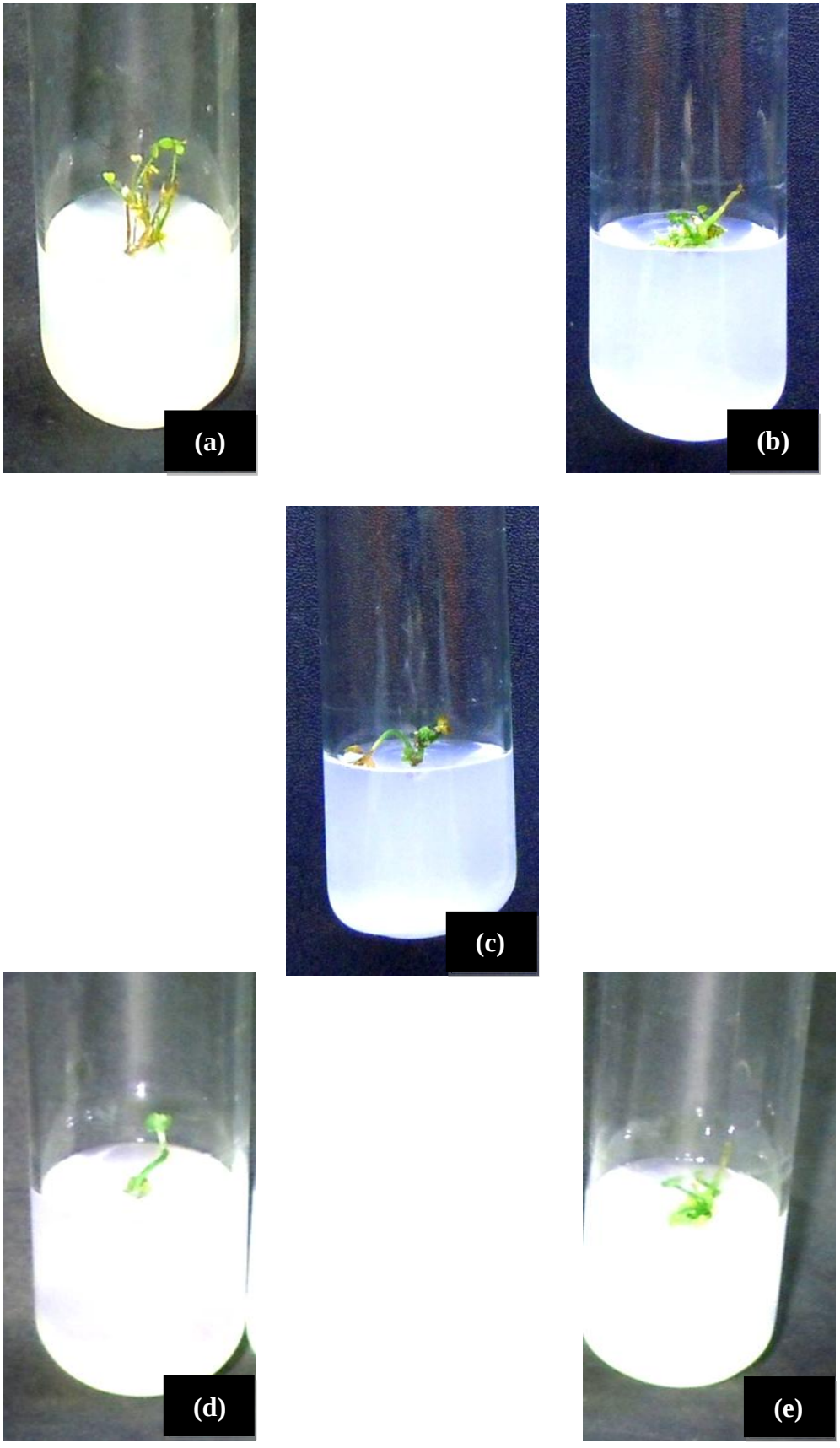


Figure – 26

Morphogenetic effect of carbohydrate nutrition (glucose) on isolated rhizome apical segments of *M.cf.coromandelina*

a) **Culture provided with 1% glucose showing elongated roots and petioles**
b) **Culture provided with 2% glucose showing longer roots and petioles**
c) **Culture provided with 3% glucose showing shorter roots and petiole**
d) **Culture provided with 4% glucose showing shorter roots**
e) **Culture provided with 5% glucose showing reduction in size of roots and petioles.**

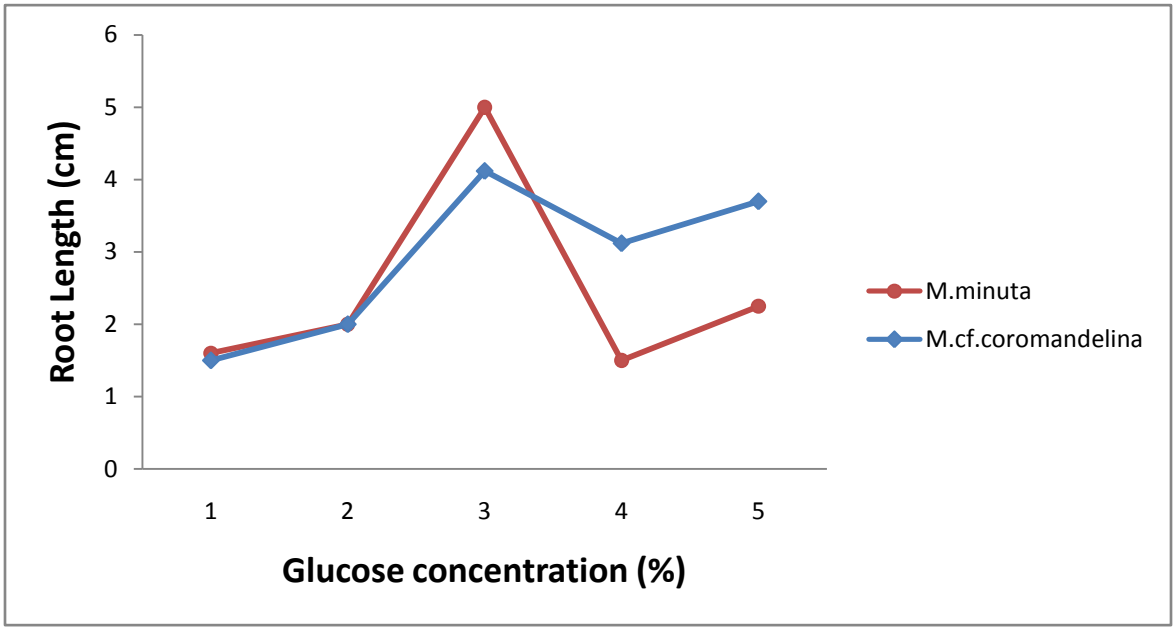
Figure- 26



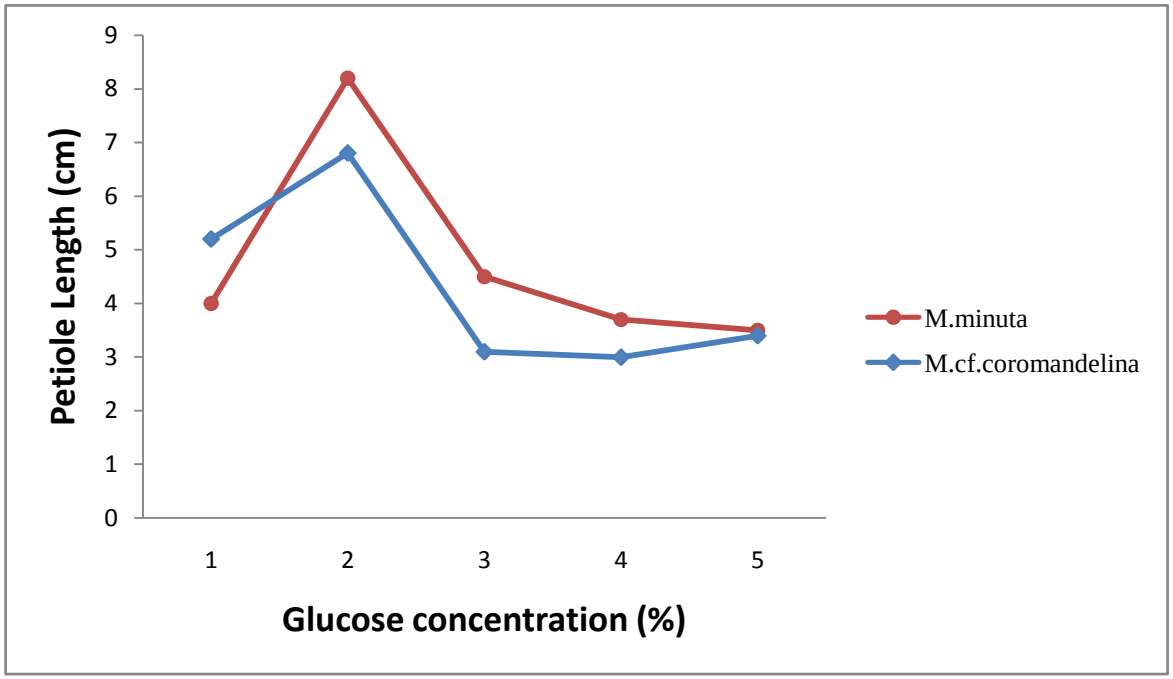
Text figure- 9

Effect of carbohydrate nutrition on isolated rhizome apices of *M.minuta* and *M.cf.coromandelina*

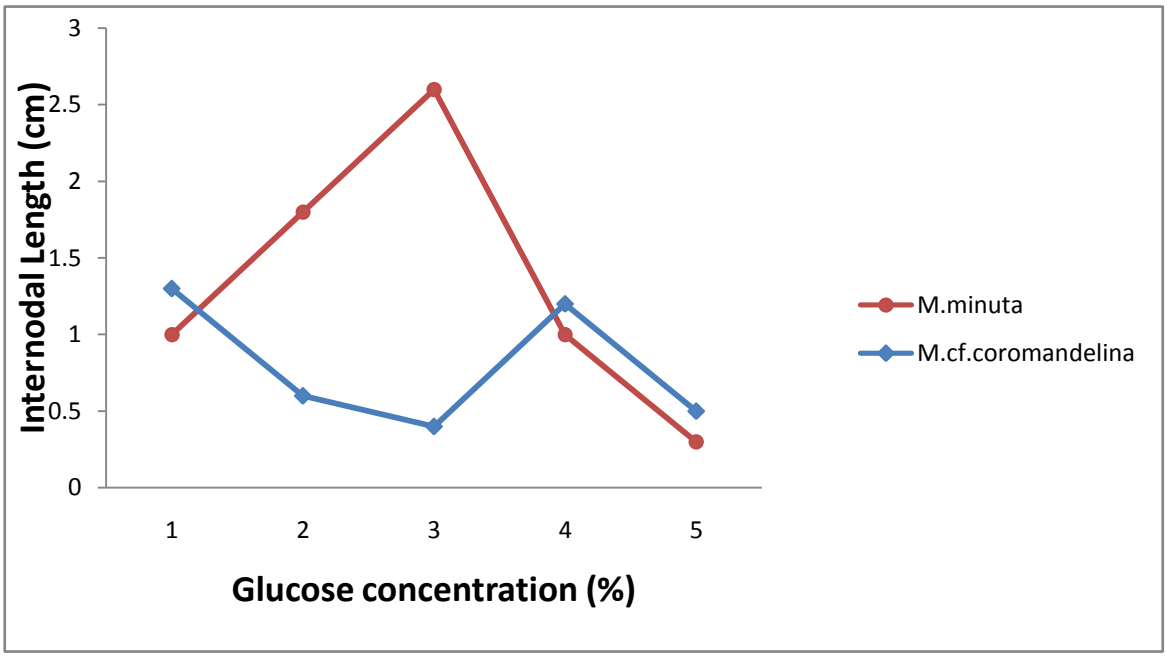
a) Root length



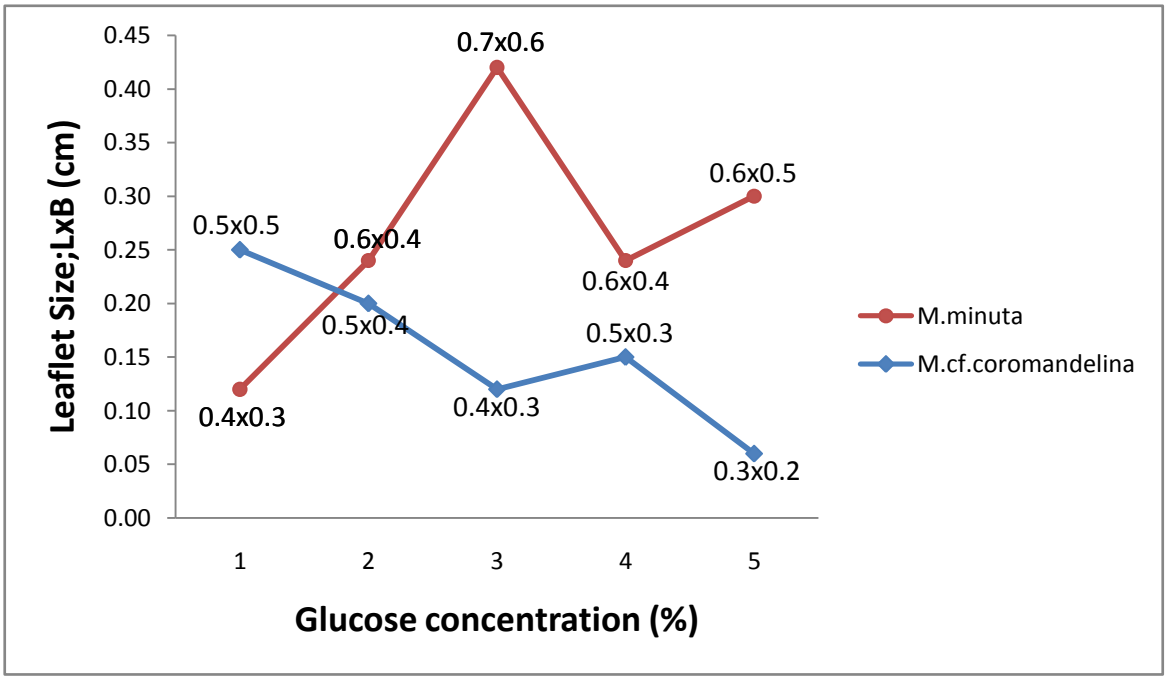
b) Petiole Length



c) Internodal length



d) Leaflet size



b) Effect of cytokinin (BAP & Kn) on shoot proliferation from nodal shoot explants of *M.minuta and M.cf.coromandelina* (Table-11, fig-27, text fig-10)

Study of growth regulators like cytokinin (BAP & Kinetin) has also been undertaken on the growth pattern of *Marsilea* species i.e. *M.minuta* and *M.cf.coromandelina* respectively. Perusal of table-11 shows different concentration of BAP and kinetin regulating the growth of shoot proliferation of explants. The observations were recorded after 4 weeks subsequent to treatment with different growth regulators.

The maximum root length was found to occur at 3.0 mg/l concentration of BAP in both *M.minuta* (2.13 cm) and *M.cf.coromandelina* (2.10 cm) while minimal at 5.6 mg/l concentration i.e. 0.50 cm in M.minuta and 1.14 cm in *M.cf.coromandelina.*

Observation of petiole length showed that shoot length is maximum at 3 mg/l BAP & kinetin and then decline when the concentration of these hormones is increased. It is 6.12 cm in *M.minuta* and 5.10 cm in *M.cf.coromandelina*

In *M.minuta* maximum leaflet size (0.5x0.4cm) was obtained at 3mg/l conc. of BAP while in *M.cf.coromandelina* the maximum leaflet size was obtained at 5mg/l conc. of this hormone.

Effect of kinetin on leaflet size was almost same in both the species of *Marsilea*, the maximum being at 4mg/l concentration. Leaflet size at this concentration of kinetin was reported to be 0.6x0.4 cm in *M.minuta* and 0.5x0.2 cm in *M.cf.coromandelina.*

Table 11

Effect of cytokinin (BAP & Kn) on shoot proliferation from nodal shoot explants of *M.minuta and M.cf.coromandelina*

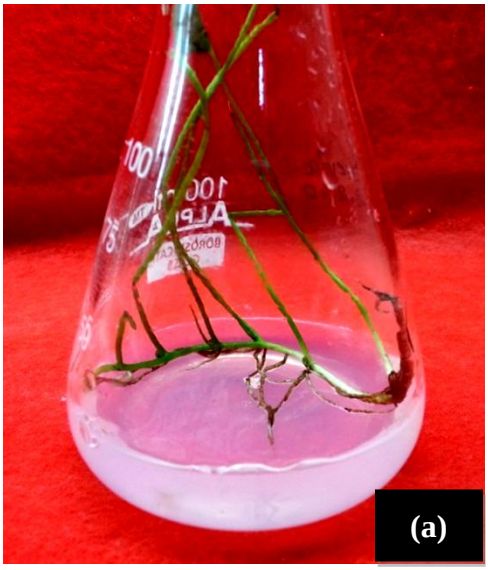
		Hormones concentration									
		BAP conc. (mg/l)					Kinetin (mg/l)				
		1	2	3	4	5	1	2	3	4	5
M. minuta	**Root length (cm)**	1.50	2.00	2.13	1.40	0.50	1.00	1.13	2.14	1.20	1.45
	Petiole length (cm)	4.20	5.13	6.12	5.23	4.15	3.10	4.12	5.10	4.14	3.10
	Leaflet size; LxB (cm)	0.4x0.2	0.3x0.5	0.5x0.4	0.4x0.3	0.3x0.2	0.2x0.1	0.3x0.4	0.5x0.3	0.6x0.4	0.2x0.1
M.cf. coromandelina	**Root length (cm)**	1.40	1.82	2.10	2.00	1.14	0.40	1.10	1.20	1.40	1.80
	Petiole length (cm)	4.00	4.25	5.13	6.30	5.14	4.40	4.60	5.14	5.00	4.14
	Leaflet size; LxB (cm)	0.5x0.4	0.3x0.2	0.2x0.1	0.3x0.2	0.4x0.3	0.2x0.1	0.3x0.2	0.4x0.3	0.5x0.2	0.2x0.1

Figure -27

Effect of cytokinin (BAP & Kn) on shoot proliferation from nodal shoot explants of *M.minuta* and *M.cf.coromandelina*

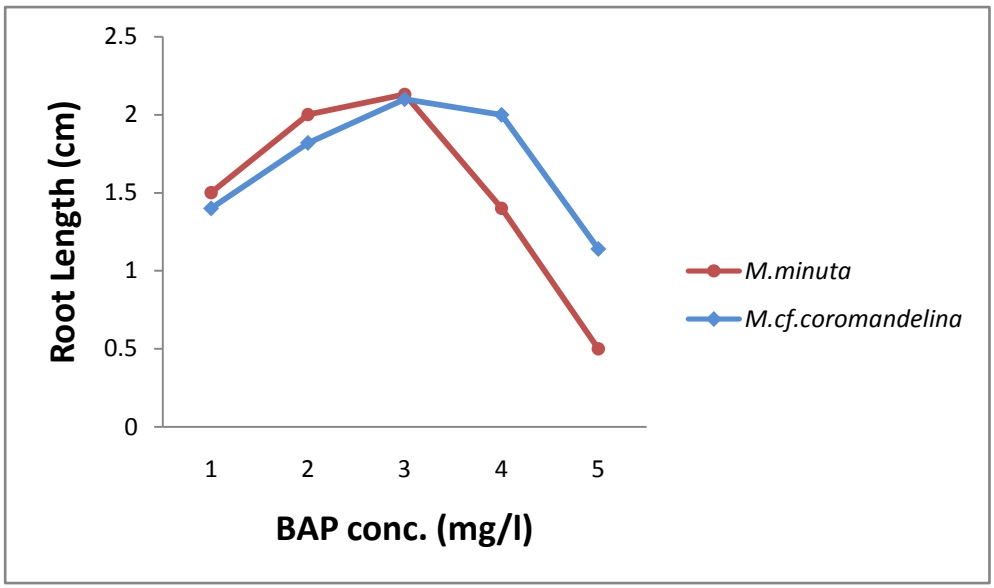
a) ***M.minuta*: Explant raised in 3mg/l BAP concentration shows elongated roots and petioles.**
b) ***M.cf.coromandelina*: Explant raised in 4mg/l shows longer petioles.**
c) ***M.minuta:* Petiolar length maximum at 3mg/l kinetin.**
d) ***M.cf.coromandelina*: culture provided with 3mg/l kinetin showing longest petiole.**

Figure- 27

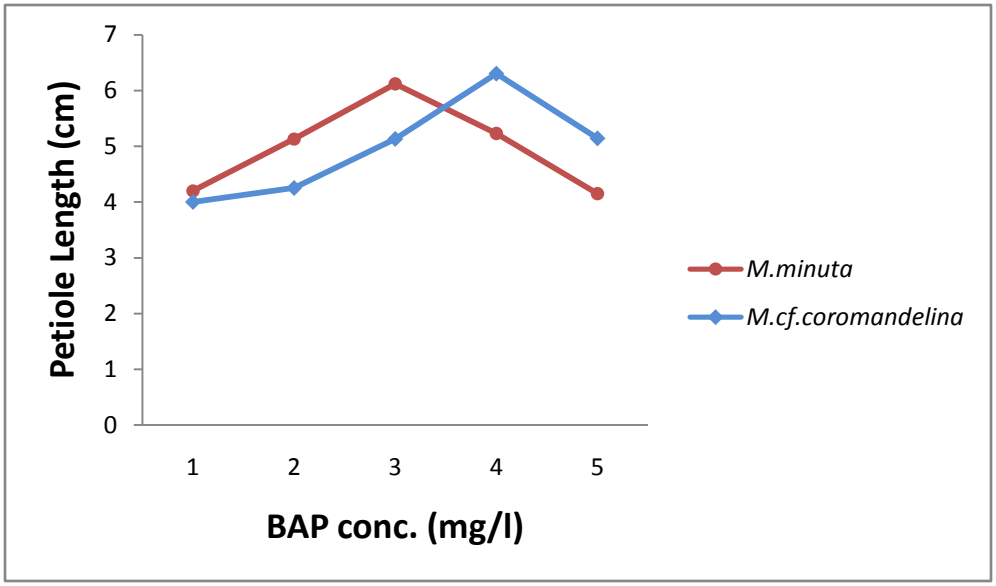


Text figure 10

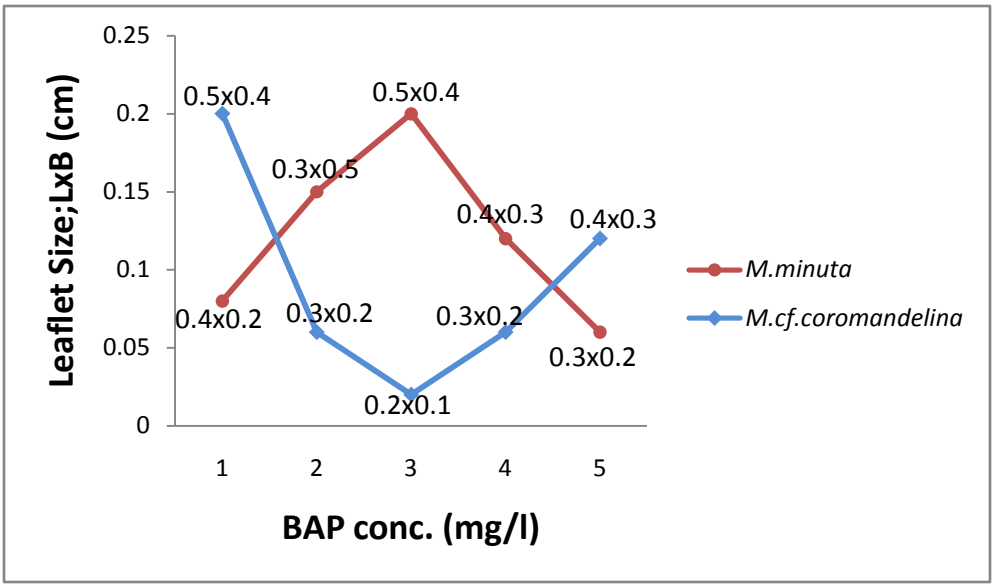
a) Root Length



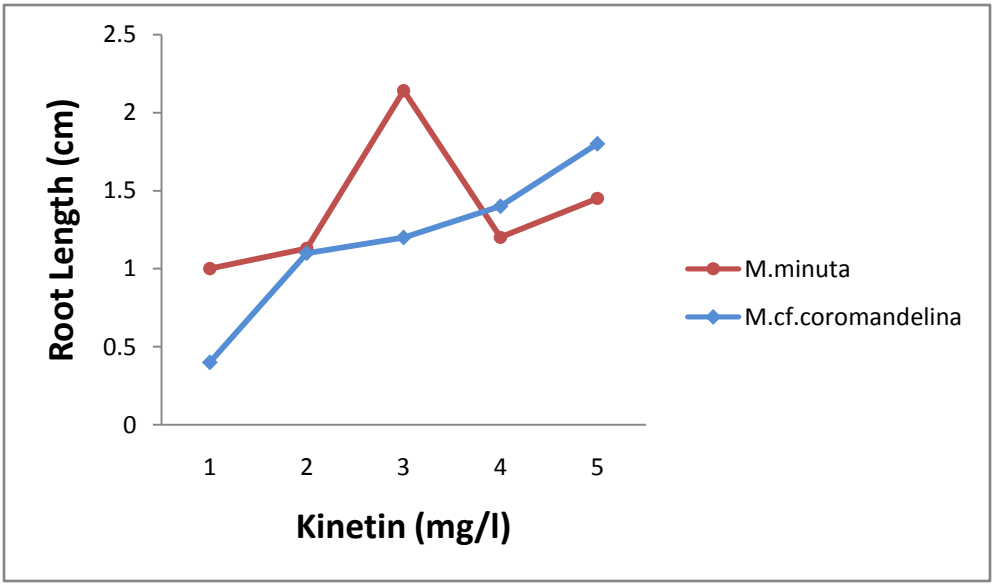
b) Petiole Length



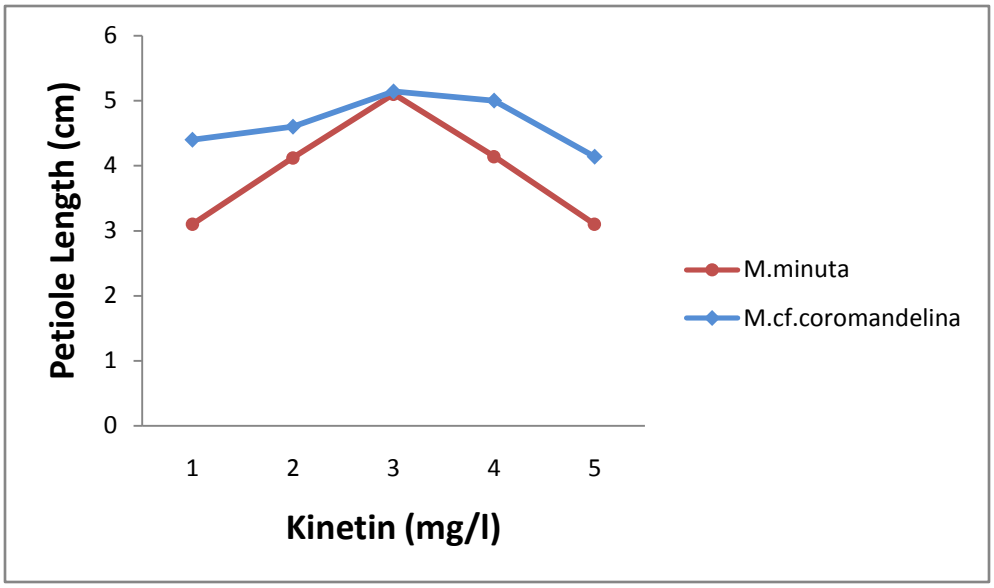
c) Leaflet Size



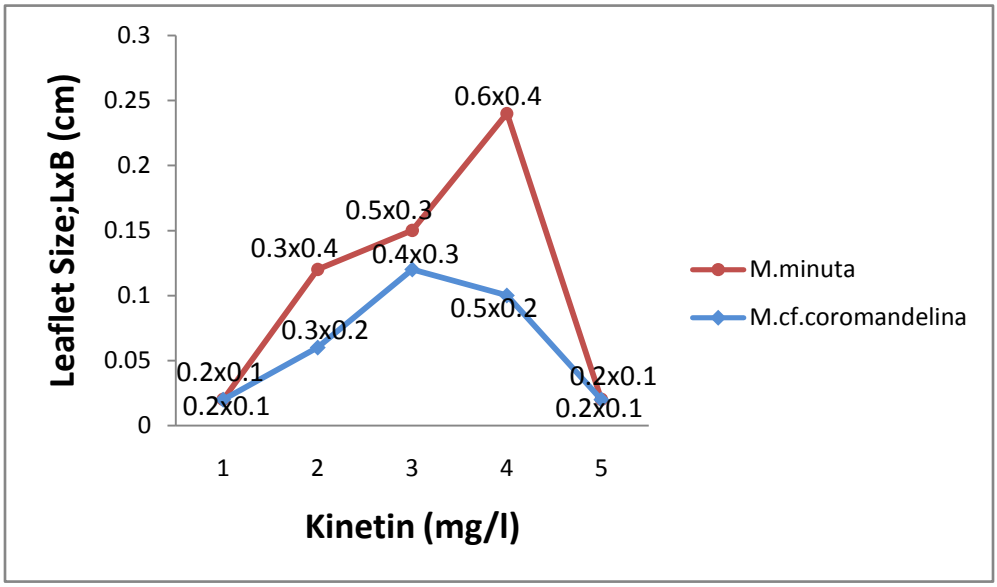
d) Root Length



e) Petiole Length



f) Leaflet Size



c) Interactive effect of cytokinin (BAP+Kn) on shoot multiplication by subculture of shoot clumps of *M.minuta* (Table-12, fig-28, text fig.11)

To study the interactive effect of cytokinin (BAP + Kn) on shoot multiplication by subculture of shoot clumps of *M.minuta*, different concentrations of BAP & Kinetin incorporated media was used. After 40 days of treatment growth parameters like number of shoot per explant, shoot length and shooting response were recorded in Table- 12.

The data pertaining to growth have been graphically represented in text figure-11

Various growth parameters as have been mentioned in table-12 are briefly described below-

In *M.minuta*, at hormone concentration (0.5BAP + 0.5 Kn) no. of shoot/explant observed to be 1.71± 0.38 having length 3.70 ± 0.28 cm showing shooting response 80 %. However, in *M.cf. coromandelina* at this concentration of hormone no. of shoot/explant recorded to be 1.12 ± 0.42 having 2.10 ± 0.14 cm showing shooting response 80%.

Shooting response found to be maximum (90%) at 0.5 BAP + 1.0 Kn in both the species of *Marsilea*. At this concentration, no. of shoots/explant to be observed as 3.42 ± 0.39 having petiole length 7.54 ± 0.31 cm in *M.minuta* while in *M.cf. coromandelina* no. of shoots observed to be 3.12 ± 0.31 having length 5.12 ± 0.32 cm.

Lower concentration (0.5 BAP + 0.5-1.0 Kn) promoted the growth of explants while there is a gradual decrease in no. of shoots and petiolar length when the concentration of these hormones is increased.

In *M.minuta* at 0.5 BAP + 2.0 Kn(mg/l), the no. of shoots/explant noted to be as 2.14 ± 0.51 having petiolar length 4.71 ± 0.29 cm and shooting response 70 %. On the contrary, no. of shoots/explant were found to be 1.42 ± 0.50 having petiole length 4.42 ± 0.13 cm and shooting response 60 %.

Table-12

Interactive effect of cytokinin (BAP+Kn) on shoot multiplication by subculture of shoot clumps of *M.minuta*

		Hormones concentration (mg/l)				
		0.5 BAP + Kinetin				
		0.5BAP+0.5Kn	**0.5BAP+1.0Kn**	**0.5BAP+2.0Kn**	**0.5BAP+3.0Kn**	**0.5BAP+4.0Kn**
M. minuta	**No. of Shoot/Explants**	1.71±0.38	3.42±0.39	2.14±0.51	2.70±0.42	2.57±0.13
	Shoot length (cm)	3.70±0.28	7.54±0.31	4.71±0.29	5.70±0.41	6.70±0.39
	Shooting response (%)	80	90	70	60	50
M.cf. coromandelina	**No. of Shoot/Explants**	1.12±0.42	3.12±0.31	1.42±0.50	2.50±0.38	2.82±0.40
	Shoot length (cm)	2.10±0.14	5.12±0.32	4.42±0.13	3.20±0.12	3.42±0.28
	Shooting response (%)	80	90	60	70	50

At 0.5 BAP and 3.0 Kn, no. of shoots/explant was noted to be as 2.70 ± 0.42 having petiole length 5.70 ± 0.41 showing shooting response 60 % in *M.minuta* while in the same concentration of these hormones no. of shoots/explants was noted to be as 2.50 ± 0.38 having shoot length 3.20 ± 0.12 cm showing shooting response 70 %.

At 0.5 BAP + 4.0 Kn (mg/l) the no. of shoots/explant in *M.minuta* were noted to be 2.57 ± 0.13, having shoot length 6.70 ± 0.3 cm showing shooting response to be 50 % while *M.cf. coromandelina* shows shooting response 50% with 2.82 ± 0.40 no. of shoots having 3.42 ± 0.28 cm shoot length.

Figure – 28

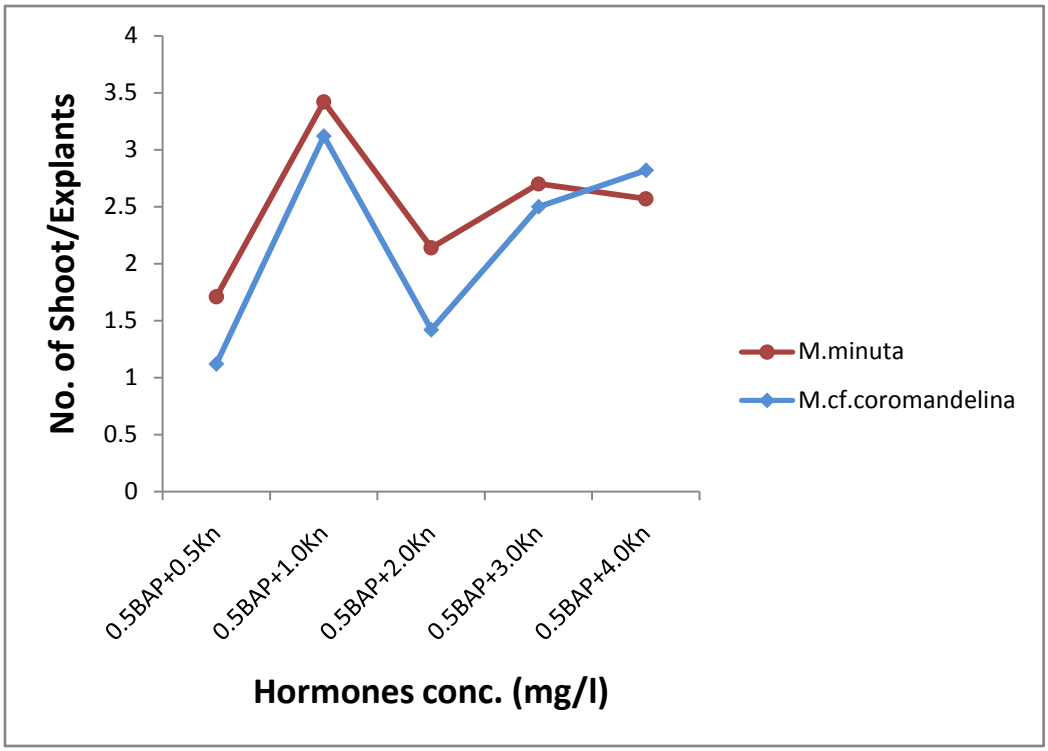
Interactive effect of cytokinin (BAP+Kn) on shoot multiplication by subculture of shoot clumps of *M.minuta*

a) ***M.minuta*: Expanded leaf with elongated petiole in culture raised on 0.5BAP+1.0Kn**
b) ***M.cf.coromandelina*: culture provided with 0.5BAP+0.5Kn showing single petiole with leaf.**

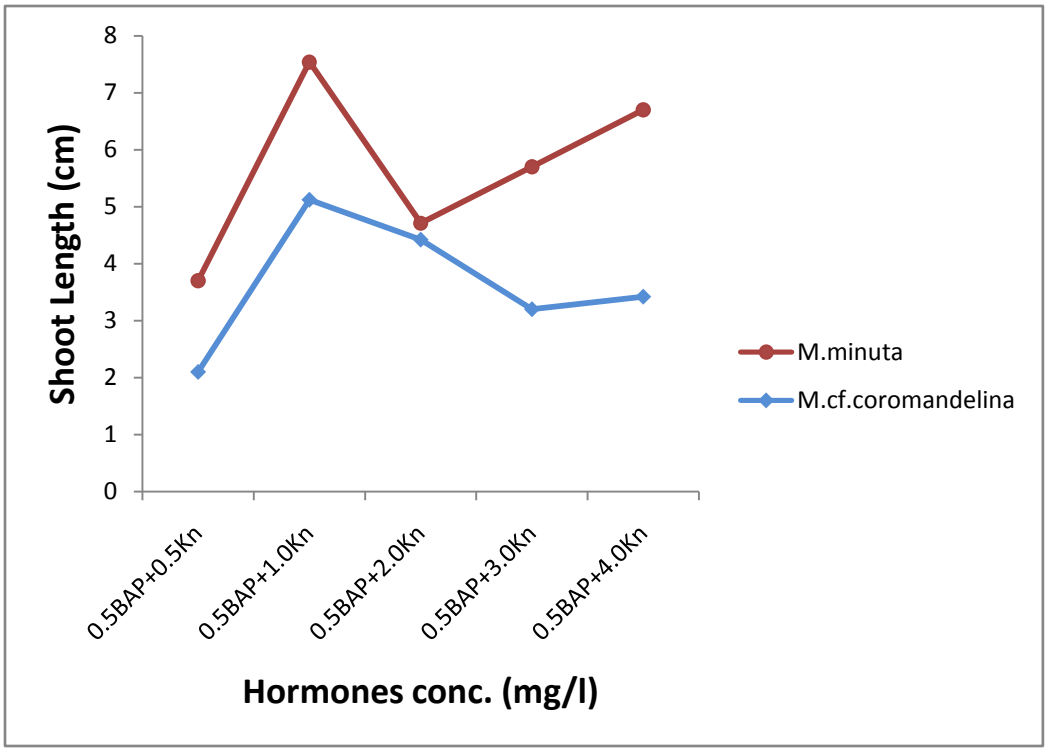
Figure- 28

Text figure 11

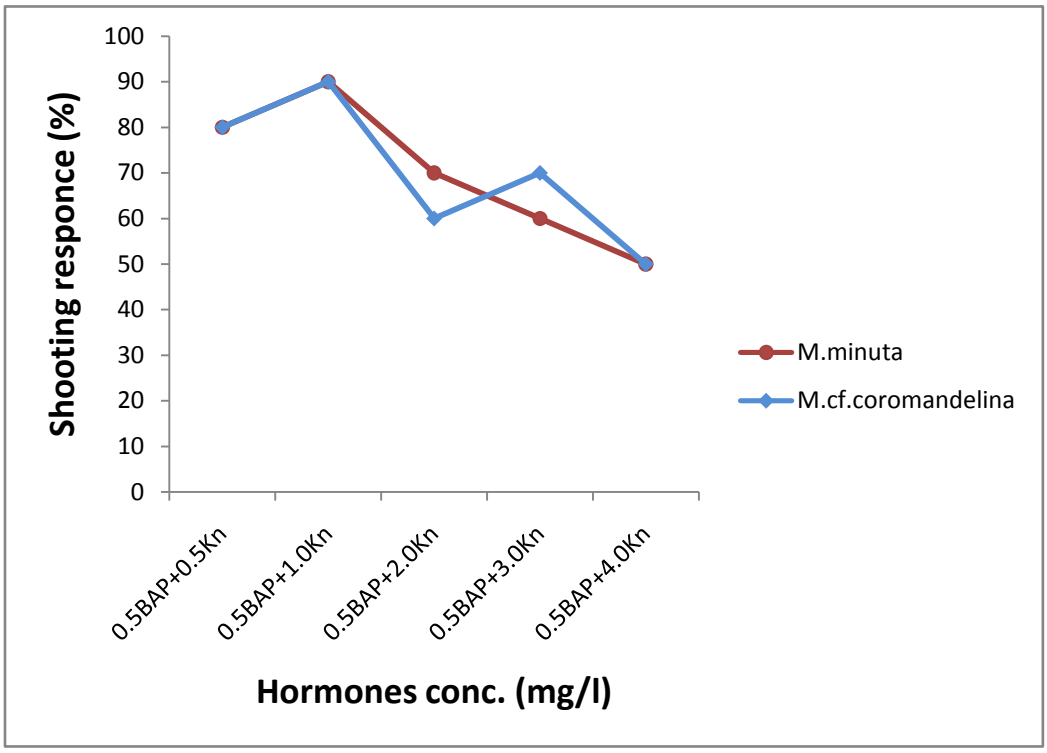
a) No. of Shoot/Explants



b) Shoot Length



c) Shooting response



GENOMIC ANALYSIS THROUGH RAPD

Genus *Marsilea* shows morphological variation within species and as such it is difficult to distinguish species depending on traditional morphology only. Classification of *Marsilea* species based on morphological characteristics has sometimes been difficult because they hybridize easily in nature leading to the formation of hybrid plants with a mosaic of morphological characters between the parental phenotypes. A recent molecular phylogenetic analysis of the genus *Marsilea* has revealed that the rates of molecular evolution are remarkably high. Although many subtle morphological and developmental differences exist between species, few of these differences are phylogenetically useful markers for classifying the species in a way that is consistent with molecular data (Korall and Kenrick 2002, 2004) RAPD markers have been widely used in the analysis of genetic relationships and genetic diversity in a number of plant taxa because of its simplicity, speed and relatively low cost as compared to other DNA based markers (Esselman et al 2000, Rout et al. 2003; Sheng et al 2006 ; Yuzbasioglu et al 2006)

The main aim of the present study was to establish different protocols of DNA isolation and to find out that if the various populations of *Marsilea* are morphologically different due to ecological factor or are they genetically different from one another. The Water-Clovers genetic stability was assessed by random amplified polymorphic DNA (RAPD) analysis. All the selected five population of *Marsilea* growing in Kota and suburban areas were examined for DNA polymorphism using 10 decamer primers (Banglore Genei) showing high (G+C) content. DNA isolation from *Marsilea* leaves from the five surveyed localities has been shown in fig-29.

Figure – 29

Locality wise DNA isolation of *Marsilea* populations

V_1 *M.minuta* – Population A

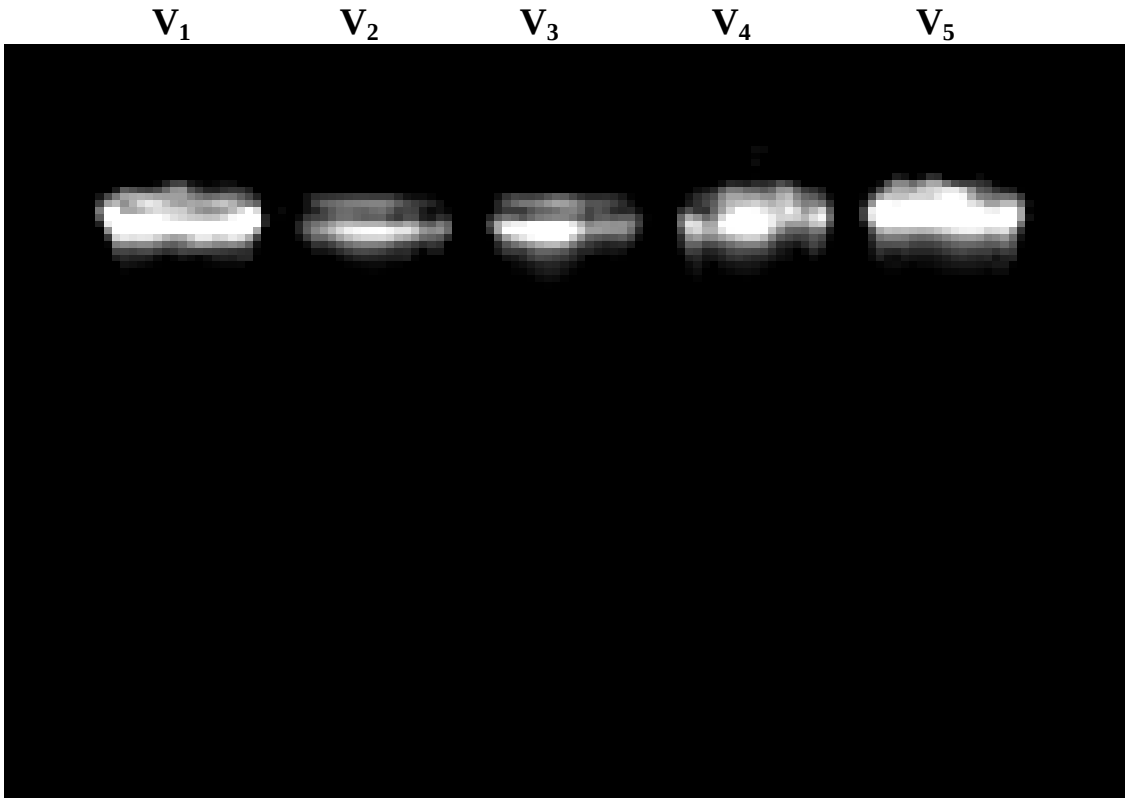
V_2 *M.minuta* - Population B

V_3 *M.minuta* (hybrid) - Population C

V_4 *M.cf.coromandelina* - Population D

V_5 *M.minuta* - Population E

Figure 29



Ten primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-06, OPA-07, OPA-08, OPA-09, OPA-10) were screened for amplification and out of 10 primers only 06 primers (OPA-01, OPA-02, OPA-05, OPA-07,OPA-09, OPA-10) produced amplification and showed variable degree of polymorphism ranging from 0 per cent to 100 per cent. The DNA amplification and polymorphism generated among five selected populations using random primers are presented in Table 13. The representative photographs of electrophoresis gels showing RAPD profiles after amplification are depicted in figure 30,

Six primers on five populations generated a total of 22 bands and all were polymorphic bands (Table 13). The average numbers of polymorphic bands per primer were 3.66 (22/06) (total no of polymorphic band/ no of primers amplified). Overall polymorphism was found to be 100 per cent. Similar results were found by Gupta *et al*. (2008) who observed 84.26% polymorphism in different *Jatropha curcas* genotypes.

The similarity coefficient for different genotypes was in the range of 0.00 to 0.90 (Table-14). The maximum similarity coefficient (0.90) was observed between V_5 and V_1. The results obtained were in conformity with the earlier report of Moura et al. (2005) who selected 93 accessions of Jaborandi to study genetic diversity using RAPD marker with a similarity coefficient of 0.86 while the minimum similarity coefficient (0.00) was observed in V_4 from V_1, V_2, V_3 and V_5.

Table 13

Details of the random primers used for amplification of genomic DNA of five *Marsilea* populations.

Total number of primers	10
Number of primers which show amplification	06
Number of primer which show polymorphism	06
Number of primers which show monomorphism	00
Total number of monomorphic bands	00
Total number of polymorphic bands	22
Total number of bands	85

Figure – 30

RAPD-PCR profiling of *Marsilea* populations [V_1 to V_5]

(M=100bp DNA ladder)

a) Primer OPA-01 (CAGGCCCTTC)

b) Primer OPA-02 (TGCCGAGCTG)

c) Primer OPA-05 (AGGGGTCTTG)

d) Primer OPA-07(GAAACGGGTG)

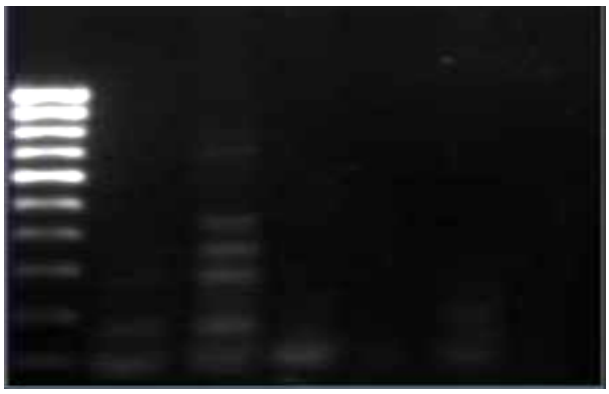
e) Primer OPA- 09 (GGGTAACGCC)

f) Primer OPA-10 (GTGATCGCAG)

Figure – 30

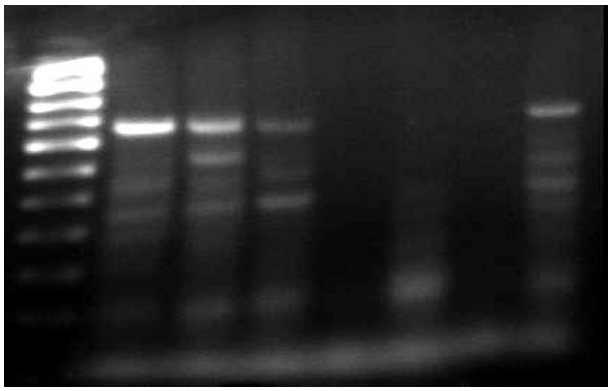
Primer OPA-01

M V_1 V_2 V_3 V_4 V_5



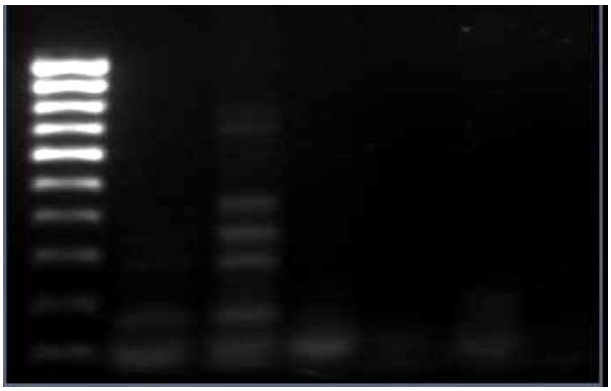
Primer OPA-02

M V_1 V_2 V_3 V_4 V_5



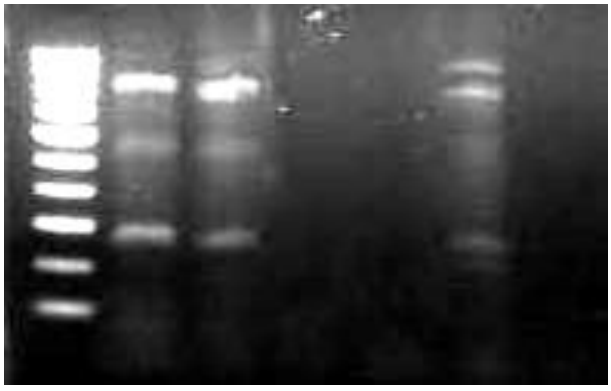
Primer OPA-05

M V_1 V_2 V_3 V_4 V_5



Primer OPA-07

M V_1 V_2 V_3 V_4 V_5



Primer OPA- 09

M V_1 V_2 V_3 V_4 V_5

Primer OPA-10

M V_1 V_2 V_3 V_4 V_5

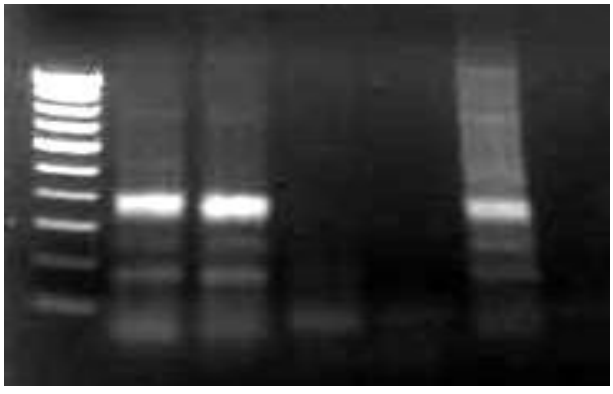


Table 14

Similarity coefficient matrix of five *Marsilea* populations obtained from RAPD markers

Marsilea populations	*M.minuta* (Talwandi)	*M.minuta* (Anantpura)	*M.minuta* hybrid (Mandana)	*M.cf.coromandelina* (Borawas)	*M.minuta* (Borawas pond)
	V_1	V_2	V_3	V_4	V_5
V_1	1.00				
V_2	0.43	1.00			
V_3	0.30	0.19	1.00		
V_4	0.00	0.00	0.00	1.00	
V_5	0.90	0.41	0.27	0.00	1.00

RAPD Dendrogram:

Dendrogram was constructed using similarity matrix value as determined from RAPD data depicting the relationship among the genotypes of 5 selected plants. Fig 12 depicts the genetic relationship between *Marsilea* populations of the surveyed localities. The dendrogram generated on the basis of Jaccard's Similarity Coefficient, clearly indicate two clusters. Ndoye-Ndir et al. (2011) also studied the genetic diversity of 70 individual samples of *Casuarina equisetifolia* subsp *equisetifolia* and *C. equisetifolia* subsp *incana* growing along the northern coast of Senegal which were characterized by RAPD marker. The result suggested sufficient level of genetic diversity.

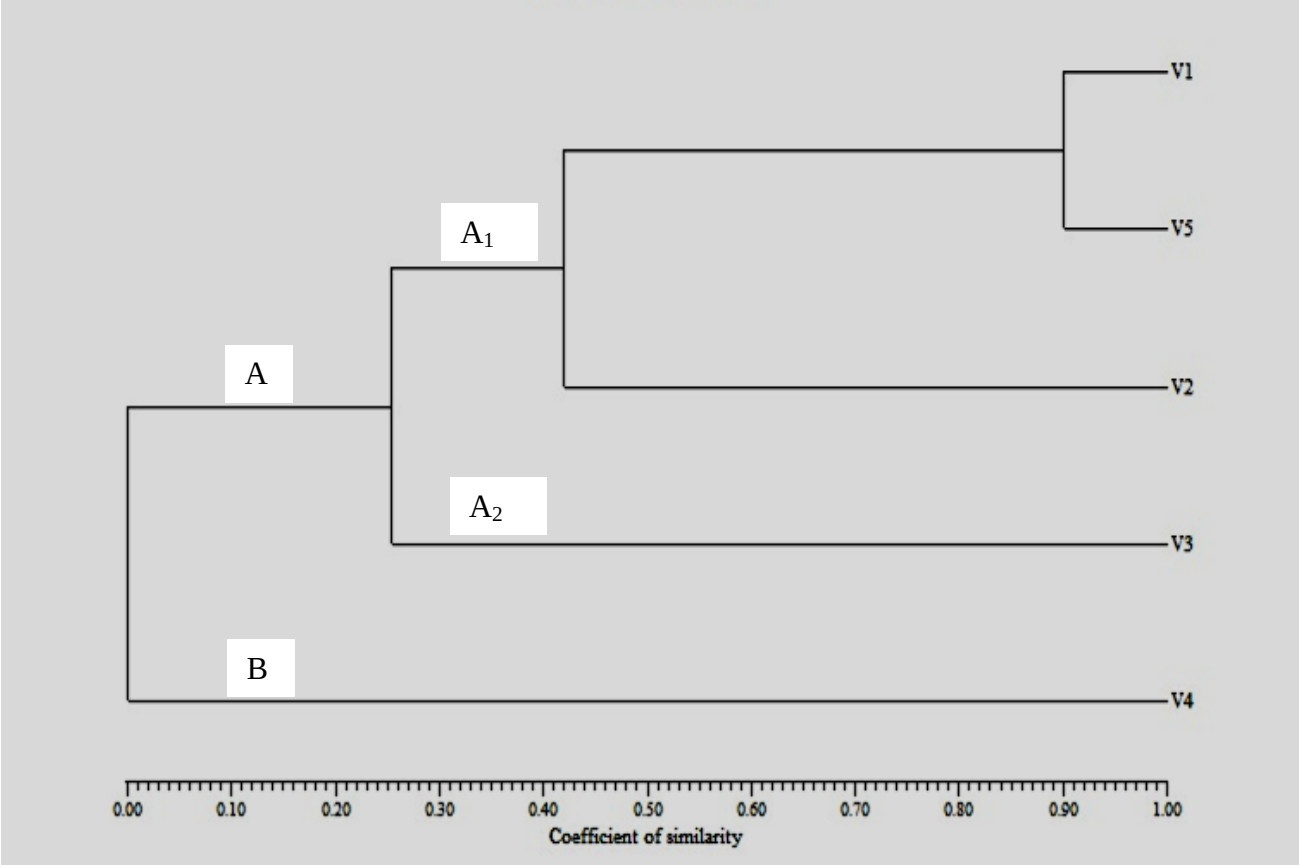
Cluster A include four populations namely. V_1, V_2, V_3, V_5 at similarity coefficient of 0.26. This cluster was divided into two sub-clusters A1, A2 at similarity coefficient of 0.43. Sub-cluster A1 includes 3 populations' viz., V_1, V_5, and V_2. In which V_1 and V_5 were showing similarity coefficient of 0.90 while V_2 separated at similarity coefficient of 0.43. Sub-group A2 consists of one plant V_3 which diverged with cluster A1 at similarity coefficient of 0.27. Sub-group A2 joins A1 at similarity coefficient of 0.27.

Cluster B include one plant V_4. This species diverged from Cluster A at similarity coefficient of 0.00. The diversion of this plant V_4 (*Marsilea coromandelina* complex) from other 4 plants at similarity coefficient of 0.00 indicates that this plant is genetically distinct from other selected *Marsilea* populations.

However this is only a preliminary attempt and needs to be studied in greater details in near future.

Text figure 12

Dendrogram showing common ancestral linkage between *Marsilea* populations as revealed by UPGMA cluster analysis of Jaccard's coefficients based on RAPD markers.



CHAPTER VI

DISCUSSION & SUMMARY

DISCUSSION

The observation recorded during the present investigation of the *Marsilea* population of Hadauti Plateau may now be discussed with specific reference to what is already known about these aspects through the researches of earlier workers in this context. Chapter-wise discussion therefore now is being provided.

SOIL ANALYSIS

To detect the nutritional preference of various *Marsilea* population, soil analysis of some selected localities (Borawas, Anantpura, Talwandi, and Mandana) have been carried out. Edaphic requirements of *Marsilea* species in the hadauti region are clearly differentiated. It is well known that pteridophytic plants prefer very specific habitats where they obtain their life possessing essential nutrients and substratum. It was observed that *Marsilea cf coromandelina* shows very specific habitat requirement. On the basis of these findings it is clear that probably borawas soil is having a rich salt content (Nitrogen-450 kg/ha, Phosphorus-14.5 kg/ha, potassium-395 kg/ha) and allows growing of a very rare species of *Marsilea* i.e. *Marsilea cf coromandelina* which occurs as an isolated patch. It is also recorded that macro and microelement (N, P, K, Zn, Cu, Mn, Fe) content of Anantpura locality shows higher amount and richer nutrition level than other localities (Table-3).

According to Geraldson (1957) conductance of the soil varies inversely with the moisture level. The conductivity of the soil solution can be directly co related with the plant growth. The conductivity increases as the soluble salts in the soil solution are increased. In the present study it has been evident that electrical conductivity of Borawas locality is highest which directly

correlates with the higher growth of the endemic population of *M.cf.coromandelina* in a short growth period.

Water holding capacity is reported to be highest in Mandana soil which probably favours the enormous and continued growth of hybrid population of *Marsilea* for a longer period.

VAM association

Arbuscular mycorhizal fungi (AMF) are associated with 80-85 % of living terresetrial plants. (Pirozynski 1981, Dickson, 2004) and are considered to be ecologically important for most vascular plants because they improve the uptake of specific nutrients by the host, other functions attributed to the fungi include the promotion of plant growth hormone production, protection of host roots from pathogens, and favors the increase of solubility of soil minerals, among other benefits (Remy et al. 1994, Newscham et al. 1995, Merryweather and Fitter 1996, 1998a, 1998b) Blackwell (2000) suggested that symbiotic relationships of AMF and the roots of higher plants were essential in moving plants to land.

The present work has focus attention on VAM association in roots of *M.cf.coromandelina*. Roots are heavily infected with fungus which produce small irregular to distinct arbuscules and vesicles. These observations reasserted to the earlier studies done by P.R. Bhat & K.M. Kaverlappa (2003) which also shows the presence of VAM fungus in *Marsilea minuta*.

MORPHOLOGY

Marsilea is well known for its range of morphological plasticity (Russow, 1972; Shattuck, 1910: Bower, 1928; Tournay, 1951: Gupta, 1955, 1962: Gaudet, 1964: Bhardwaja, 1967b,c, 1975, 1977, 1980, 1987, 1989, 1997; Soni, 1988). Significant intra-specific morphological variations have been recorded during the present investigations on *Marsilea* species grown on land and water habitats pertaining to different soil texture. Johnson (1986) has

asserted taxonomic relevance of internodal roots in *Marsilea*. Internodal roots were observed in Anantpura and Borawas pond population.

Recently, Tai-chung Wu and Wen-Yuan kao (2011) compared the ecophysiological traits of leaves of three *Marsilea* species (*M.crenata*, *M.quadrifolia*, and *M.schelpiana*) in different geographical regions and concluded that the leaves of *Marsilea* species reflect the climate pattern of their habitats. They also suggested that water availability and light intensity are the two important factors contributing to the geographic distribution of the three species.

Significant intraspecific morphological variations have been recorded during the present investigation of *Marsilea* populations. Although an overall increase in size of root, rhizome and leaves is observed in the water forms (Talwandi, Borawas pond) of *M.minuta* as compared to their land forms (Borawas, Anantpura, Mandana), the internode shows a significant increase in length as compared to land forms. (Table-4) Similarly petiolar length also shows a significant increase in water forms of *M.minuta*. *M.cf. coromandelina* shows a high range of plasticity on the basis of its habitat preference. It is a naturally acclimatized terrestrial species growing as an endemic population in a rocky terrain area. *Marsilea cf. coromandelina* has a very short survival span and grows for 15-20 days in the pot with soil of its habitat.

The morphology and the external features of the sporocarp have been the most dependable criteria for taxonomic purposes in *Marsilea*. The squarish sporocarp with a distinct horn and ridge seen in *M.cf.coromandelina* is completely different from the ovoid and non ridged smooth bulging sporocarp of *M.minuta*. (Figure-13, d)

The hair structure of the sporocarp wall has shown rather small differences and the comparison of the hair structure of various taxa of *Marsilea* needs further studies for possible use as a taxonomic criteria.

ANATOMY

Anatomical studies of *Marsilea* have received considerable attention in India from Pande (1923) and Puri & Garg (1953). The present study deals with the aspect of comparing the anatomical differences which has revealed notable habitat based anatomical variations. It is interesting to note that aerenchyma does occur in both the land and water forms of *Marsilea* species but the area occupied by aerenchyma has been found to be more in water forms, maximum to be seen in population growing at borawas pond. Similarly well developed xylem and phloem are observed in the land forms of *Marsilea* (*M.cf. coromandelina*) while the stelar region gets reduced in *M.minuta* (aquatic form). This adaptation shows direct correspondence to habitat factors.

Anatomical studies do not indicate any significant feature of systematic relevance in rhizome, but the petiolar anatomy shows certain features of comparison. The width of the vascular zone was found to be smaller in *M.minuta* as compared to *M.cf.coromandelina*.

Similarly air chambers of rhizome are observed to be rectangular in *M.cf. coromandelina* while they are irregularly round and greater in number in *M.minuta*. The air chambers are separated by thin walled parenchyma cells. It is observed that the parenchyma cells surrounding the air chambers are comparatively thinner in *M.cf.coromandelina* than the single layered or sometimes double layer parenchyma cells in *M.minuta*. (fig.15)

Venation Pattern

The present investigations of vein patterns of the two species of *Marsilea* have brought out interesting morphological features of leaf venation.

Venation is described to be reticulate and multicostate in Marsileaceae in general. The veins divide dichotomously and are joined randomly by cross connections. All the dichotomies

ultimately unite near leaflet margin by loop veins resulting in the formation of a close reticulation of veins and development of a submarginal vein. (Figure-12, c)

It is interesting to observe pellucid streaks in *M.cf.coromandelina* leaves when they are placed in front of direct sunlight while no similar streaks could be observed in *M.minuta* leaves (Fig-12, b). The pellucid streaks provide a characteristic feature observed in leaves of *M.cf.coromandelina* and this feature may serve as an additional parameter for systematic segregation of closely allied species complexes.

Xeric habitat and ecological conditions play a vital role in sporogenesis. Also, there is a definite relationship between microsporal aberrations and leaf morphology. Thus such taxa possess crenulate, crenate lobed or wavy margin of leaflets (Bhardwaja, 1967).

PHENOLOGY

The phenological appearance of *Marsilea* populations at different localities of hadauti region showed a relative difference. The periodical survey in the fields during the present investigation has revealed the sporocarps of *M.minuta* (Anantpura & Talwandi localities) showing injury by insect larvae. The population grown at home in pot also gets attacked by ants and insects due to the presence of a mucilaginous ring inside the sporocarp. However, germination and formation of sorophore was observed only in some cases while the megaspores and microspores were found to be non viable in the infected sporocarp. Loyal and kumar (1977) have mentioned about the contents of *M.minuta* sporocarps being eaten away by insect larva of a weevil (*Echinonemus schonherr*) and other organisms. This may be nature's way to affect entry of water through such injury and consequent bursting of sporocarp due to swelling of sorophore.

It must be pointed out here that *M.cf.coromandelina* population was observed to sprout and resume growth twice in a year post rainfall in months of September and February.

M.cf.coromandelina is found only at a single locality (Borawas) in a small restricted patch. It was reported by Bhardwaja (unpublished) during the survey of this plateau. In general, *M.coromandelina* is a peninsular south Indian taxon while *M.cf.coromandelina* is found growing in Rajasthan exceptionally in Borawas (Kota). Conservation of this particular locality is an urgent need because this species is found in a single localized population in India. It was noticed in the present study that this species exhibited pronounced shrinkage in area each year between 2011-2013 due to constructional activities of the villagers and unless some strict measures are taken to protect this locality there is fear of pushing this species into the "**Rare**" category.

CYTOLOGY

The populations of *Marsilea* are difficult to characterize as species because of the plasticity of their morphological features. Depending on the wetness of the environment, leaf size, shape and hairiness, petiole length and rhizome trichome abundance change, makes consistent identification and classification difficult (Johnson, 1986). Because of these factors, chromosome data could provide useful characters. Cytological comparison of both the investigated species reveals the species diversification of this genus. *Marsilea cf. coromandelina* depicts 42 chromosomes in root tip cells while *M.minuta* showed 40 chromosomes in root tip.

PHYTOCHEMISTRY

Phytochemical studies of *Marsilea* were initiated with a view of its economical exploitation by extraction of Marsilin from *M.minuta* and *M.rajasthanensis* by Chatterjee et al. (1963, b, 1964). The present investigations are an addition to the studies of primary metabolic products of these two species of *Marsilea*. Present investigations have revealed an overall higher trend of accumulation of sugars, phenols, and proteins in *M.minuta* while a higher amount of

starch ascorbic acid and proline contents has been observed in *M.cf.coromandelina*. The amount of sugar, phenol, starch, proteins, ascorbic acid and proline contents of both the species varies distinctly in different parts of the plant body viz. root, rhizome, petiole and leaf.

It was observed that the rhizome of both the species contain maximum amount of sugar (9.40 mg/gdw) and phenol contents.

However, the leaves of *M.cf.coromandelina* contain much higher amount of proline (0.6 mg/gdw) while it is 0.50 mg/gdw in leaves of *M.minuta*. This increase in proline, the most stable amino acid resisting oxidative damage and the least inhibitory, has some correlation with stress metabolism (Palfi et al. 1974). Thus, the present findings indicate a greater stress tolerance capacity due to accumulation of proline contents in leaves of *M.cf.coromandelina*. Sharma (1979), Gena (1980), Kaur (1982), Yadav (1983) and Bhardwaja (1989), Muralidharan nair et al. (2011) in their investigations have concluded that higher amount of proline provides endurance against drought conditions. Higher proline is recorded in rhizome as compared to other organs in both these species of *Marsilea*. (Table-6)

Starch content has shown a significantly higher value in leaf in both the species. Phenols are known to provide resistance against microbial infection and thus higher phenolic content of rhizome makes it resistant to any microbial growth (Farkas & Kirlay, 1962) Total phenol content has shown higher value in *M.minuta* in comparison to *M.cf coromandelina* (Table-6)

An organ-wise comparison regarding the amount of phenol shows that these are maximum in rhizome followed by petiole, leaves and roots of both the species.

Total soluble sugars have shown elaborate variations in both the species. It is almost double in amount in petiole and leaves of *M.minuta* in comparison to *M.cf.coromandelina*. Amount of sugar is found to be maximum in rhizome of both the species.

Quantitative estimation of photosynthetic pigment revealed that chlorophyll content has been recorded to be higher in the leaflets of *M.cf.coromandelina* in comparison to *M.minuta*. In both the species chl b is found to be dominant over chl a. The variation in chl a & b content of leaves of *Marsilea* could be attributed to genetic variations for chlorophyll synthesis as well as variations in soil and environmental effects. However the isolation of constituents and the mechanism of action explained for the observed activities have not been established, and thus further investigations need to be conducted

REPRODUCTIVE BIOLOGY

Reproductive biology of *Marsilea* has received considerable attention due to its possession of a unique reproductive structure, the sporocarp. The sporocarp dispersal and release of spores from the sporocarp of *Marsilea* is specified as a 'paradigm of physiological ingenuity' apart from the sporocarp being considered a morphological enigma. Malone & Proctor (1965) found the resistance of sporocarp wall even after they had passed through intestinal tract of water birds in that the sporocarps could not be digested and contained viable mega- and microspores.

This genus exhibits great variability and plasticity corresponding to the morphological parameter in various aspects of sexual reproduction. As per Bhardwaja (1980) there is practically no limit to spore viability. The wide ecological amplitude of *Marsilea* thus fully concurs with its wide spectrum of reproductive mechanisms (Bhardwaja, 1966; Wadhwani, 1983).

Comparative aspects of spore dispersal in response to the light, studied by Bhardwaja & Mohammad (1967) has been presently included to further observe spore liberation, sorophore emission rates and spore release from mega and microsporangium, sporelings formation in response to segments of the visible spectrum and different media on two *Marsilea* species of Kota. Present observations of spore liberation, sporelings formation in *M.minuta* and

M.cf.coromandelina using various parameters like Tap/Rain water, Hot/Cold water, saline/soil solution, 1966, Wadhwani, 1983). Both the species of *Marsilea* viz. *M.minuta* and *M.cf coromandelina* have shown elaborate variation in the gametophytic structure and sporeling development between the two species. Extrusion of sorophore has been observed to be quicker in *M.cf.coromandelina* (within 2-3 hours after placing the scarified sporocarp in water) while this process takes longer time in *M.cf.coromandelina*. Total number of megaspores and microspores per sporangium is found to be greater in *M.minuta* as compared to *M.cf.coromandelina*. Release of megaspore and microspores is earlier in *M.cf.coromandelina* than *M.minuta*. It may thus be concluded that reproductive capacity is potentially higher in terrestrial species followed by amphibious taxa. These observations indicate that terrestrial conditions lead to enhanced reproductive capacity ensuring survival in harsh xeric conditions.

TISSUE CULTURE AND IN-VITRO PROPAGATION

Marsilea has been a favorite material for morphogenetic and experimental studies. A series of papers were published on morphogenesis in the Australian *M.drummondii* by Allsopp (1952-64). Extensive experimental and analytical studies were done by him under aseptic culture conditions on this taxon including the effect of various sugars on development, reversion of mature sporophyte to juvenile stages, effect of various sugars on development and morphology, apical dominance, anatomical effects of changes in sugar concentration and comparison of effect of growth regulators on sporeling growth with special reference to land and water forms. Sossountzov and her associates (1958-69) also conducted sustained investigations on explants from aseptically raised adult plants of *M.drummondii* with special reference to morphology and growth & development of sporophyte in relation to mineral nutrition in aseptic cultures. Laetsch & Briggs (1961, 1962) studied kinetin induced modification of sporeling ontogeny as well as

photomorphogenetic responses of sporelings in *M.vestita*. White (1965) established a correlation between the apical cell and the heteroblastic leaf series in *M.vestita.*

The present investigations have revealed that differences in glucose concentration induce interspecific variations in the two investigated species. A perusal of table-10 and fig.25-26 shows that a differential effect in morphological and physiological parameters in response to varied glucose concentration is distinctly observed in the plants of the two investigated species which were raised aseptically from rhizome segments with intact apical buds.

Thus, *M.minuta* plants obtained from rhizome apical segments cultured on 2%, 3% glucose concentration show longer roots, elongated petioles, and bigger leaves. On the contrary *M.cf.coromandelina* plant raised similarly shows maximum leaf size at 2% glucose concentration.

Ferns have not been used extensively in studies of plant nutrition. Culturing the gametophytes to maturity is often difficult and slow and growth of the sporelings is relatively slow. Allsopp (1952, 1953) has employed species of *Marsilea* using aseptic methods to study carbohydrate utilization and the effects of starvation upon the morphological and growth responses of the explants. Morphological and anatomical characteristics may be induced under aseptic conditions in response to quantitative variations of an organic carbon source such as glucose. However, plants obviated from an organic carbon source fail to develop such characteristics.

Current models of phytohormonal control of seed plant shoot apical meristems (SAM) emphasize the role of cytokinin (Stahl & Simon 2010, Kurakawa et al., 2005, Kyozuka 200, sablowski 2007, Hurang and Sakakibara 2006, Kepinsiki, 2006). Cytokinin maintains indeterminism and stimulated cell division and apical dominance. They are also involved in a

wide range of responses to abiotic and biotic stimuli including light responses, drought resistance, ion uptake, pathogen defence and symbiotic interaction (Werner & Schmulling, 2009).

Cytokinins regulate a considerable number of different developmental and physiological processes in aerial and subterranean organs, including cell division in shoot and root meristems, chloroplast differentiation, leaf senescence, stress response, and pathogen resistance. They have been proposed as long-range signaling molecules, but since they promote the outgrowth of axillary buds antagonizing the activity of auxin, they may also be considered as local signal for plant development. (Mok 1994, Mok & Mok 2001 & Schmulling 2009). Effect of cytokinin on in vitro culture of some pteridophytes viz. *Osmunda regalis, Phegopteris connectilis, Polypodium vulgare, Cytopteris fragilis* were conducted by Morni (2000), Fernandez, & Revilla (2003), Menendez et al. (2009). Similar studies were carried in the *Marsilea* population of Kota region.

A comparative study of growth regulators (BAP and kinetin) has also been undertaken on the growth of two *Marsilea* species i.e. *M.minuta* and *M.cf.coromandelina.* Various concentrations (1-5 mg/l) of these growth regulators were supplied with the M.S. media to rhizome apices with intact apical bud. Root and petiole length of both the species gradually increased on increasing BAP and Kinetin concentration being maximum at 3mg/l concentration of both the hormones. Interactive effect of cytokinin (BAP & Kn) on morphogenetic changes in the explants of both the species has been recorded. The observations indicate that maximum shooting was observed at 0.5 BAP + 1.0 Kn (mg/l) while minimum shooting was observed in 0.5 BAP + 4.0 Kn (mg/l).

Shoot length also varies considerably in varying different concentration of hormone mixture (BAP + kn). It thus becomes evident that effect of hormones is not uniformly specific for all the

species of *Marsilea*. Such studies may lead to identification of physiological parameters of species delimitation in this genus where morphological plasticity has led to so much systematic confusion especially with regard to interspecific boundaries.

Habitat degradation makes difficult to develop in situ conservation programs, *in-vitro* micro propagation from preformed meristems could be an efficient procedure for ex situ germplasm multiplication and conservation. This technology gives the opportunity to produce a great number of pathogen-free individuals starting from small explants, to control light and temperature, to exclude seasonal changes, and to adjust the supplementation of nutrients and growth regulators. The presence of endemic population of *M.cf.coromandelina* in the borawas is extremely variable year by year according to the water level & seasonal fluctuations. Well developed micro propagated plants were also successfully acclimatized under greenhouse condition. It is therefore suggested that *in-vitro* micro propagation could be useful in the development of ex situ conservation programs of *M.cf.coromandelina,* even in order to possibly reintroduce the plants in their natural environment. *Marsilea* ferns (water clover) are potentially invasive aquatic and wetland plants that are difficult to identify to species because of subtle diagnostic characters, sterile specimens, and unresolved taxonomic problems.

RAPD analysis

Marsilea ferns (water clover) are potentially invasive aquatic and wetland plants that are difficult to identify to species because of subtle diagnostic characters, sterile specimens, and unresolved taxonomic problems. (W.Mark Whitten et al, 2014). Subir Bera et al (2012) provided a simple and efficient protocol for isolating genomic DNA from leaves of *Selaginella* spp. and recorded that the two primers (OPD 5 and OPG 2) yield reproducible amplification of polymorphic fragments of *Selaginella*. A recent molecular phylogenetic analysis of the genus *Selaginella* has

revealed that the rates of molecular evolution are remarkably high. Although many subtle morphological and developmental differences exist between species, few of these differences are phylogenetically useful markers for classifying the species in a way that is consistent with molecular data (Korall and Kenrick 2002, 2004). In the present DNA (RAPD) used to detect genetic diversity in *Marsilea* populations employing 10 primers were found to be useful in detecting genetic purity identification of hybrid varieties. It was observed that RAPD has potential for germplasm conservation. Of the 10 RAPD primers screened for their amplification capacity, 6 primers produced clear and reproducible RAPD bands across all the populations and were chosen and used for the first time to measure genetic relationships among species of *Marsilea*. Among five selected *Marsilea* populations, *M.cf.coromandelina* which was reported as endemic to Borawas (Kota) exhibited species- specific bands which could also be used to identify *Marsilea* species in danger for preservation purposes. In conclusion these results demonstrate the utility of using RAPD markers to characterize interspecific relationships and identify unique bands in *Marsilea* species. Molecular methods were used to enrich this study and testify molecular methods as a tool to find the genetic bases of the inter specific variations of *Marsilea*. The present study is the first molecular study of *Marsilea* population of five surveyed localities in Hadauti plateau. The RAPD-PCR polymorphism and UPGMA study of the genus *Marsilea* have not been reported till date.

In this study, minimum numbers of amplified loci were obtained using primer OPA-02 sequence code (5'-TGCCGAGCTG-3'). The genetic similarity matrix of RAPD data for 5 populations was constructed using the UPGMA (Text fig-09), as described by Chansiripornchai et al. (2000). The dendrogram showed the genetic relationships among species of *Marsilea* and they seem to be very closely related, this similarity between them may be due to their common

geographical localities. However, *Marsilea coromandelina* complex ($V_{4)}$ species diverged from Cluster A at similarity coefficient of 0.00. The diversion of this plant V_4 (*Marsilea coromandelina complex*) from other 4 plants at similarity coefficient of 0.00 is giving indication that this plant is genetically different (species) from the rest of 4 *Marsilea* populations. It was also observed that *Marsilea coromandelina* complex population is morphologically different from other populations having silver shining streaks (pellucid streaks) between the veins of the leaves and squarish ribbed sporocarp. Accordingly to the genetic distance and relationships illustrated, the ability to resolve genetic variation among different *Marsilea* species may relate to the genomic analysis methodology employed in this study.

The PCR-RAPD technology provides the possibility to rapidly evaluate genetic variation in in vitro produced plants. In the present study species specific bands for five selected *Marsilea* populations. In conclusion these results demonstrate the utility of using RAPD markers to characterize interspecific relationships and identify unique bands in *Marsilea* species.(Fig 30) Hence RAPD-PCR patterns of both *Marsilea* species (*M.minuta* and *M.cf.coromandelina* complex) are phylogenetically important which would help in relating them at the genetic and evolutionary level.

It is hoped that these studies will substantially contribute towards an understanding of ecomorphological variations in *Marsilea* with particular reference to inter-specific variation and evolutionary implications in the process of speciation in this genus. It is to be hoped that the present work will inspire future workers to take up extended studies of this interesting fern genus.

FUTURE STRATEGIES

Following immediate aims have been identified for further work -

1. Pteridophytes form the significant vegetation on the earth next to flowering plants and play an important role in the biodiversity of topical and temperate forests. Ferns require specific type of climatic conditions to grow well in nature called as microclimate. Climatic changes from the last decades, high pollution level of soils and water and the degradation of the habitat are the main reasons for threatening the existence of this simple yet evolutionary important group of plants. It is therefore suggested to conserve an endemic population of *Marsilea coromandelina complex* at Borawas (Kota) which has been observed to be decreasing areawise in nature.
2. Phytochemical analysis of pteridophytes makes the bases for the investigations on medicinal uses of this plant. Further research can be carried out towards its various pharmacological properties.
3. *Marsilea* is a very important plant from the pharmacological activity point of view. Many vistas have been explored and many still remain to be looked into. It is a potential target towards future study. Further work will accentuate the isolation and characterization of active principles responsible for bio-efficacy and bioactivity.
4. Further tissue culture and in vitro micropropagation of this highly endemic species of *Marsilea coromandelina* complex will be done with a view of its conservation which seems to be declining at an alarming pace.
5. The RAPD-PCR patterns of this genus would help in relating various populations at the genetic and evolutionary level. These plants can further be evaluated on the basis of proteomic, metabolomics and phytochemistry to a greater extent.

It is hoped that these strategies will contribute substantially to phylogenetic relationships and evolutionary advancement at genetic/species levels and certainly help in comprehending morpho-physiological spectrum of amphibiousness in *Marsilea.* Recurrent variations in natural populations may indicate genomic involvements. The genetic domain of pteridology offers a promise to understand their evolutionary aspects. In fact, the current decade is experiencing the elucidation of several issues through molecular biology. It is to be hoped that the present work will inspire future researchers to take up extended studies of this interesting fern genus towards this end.

SUMMARY

1. The thesis entitled **" Investigations on population variability and experimental studies in some species of the Heterosporous fern *Marsilea* L."** deals with the aspects of the study of the population dynamics and a comparative account of the morphology, phenology, phytochemistry, anatomy, cytology, reproductive biology and tissue culture studies of two *Marsilea* species namely *M.minuta* and *M.cf.coromandelina* in nature and under cultivation. The present investigations have been taken up to evaluate the main causes of threat and ways for the conservation of rare, endangered, threatened and the endemic population of *Marsilea* (*Marsilea cf. coromandelina*)
2. An extensive and up-to-date review of the literature pertaining to the above dimensions of *Marsilea* in India and abroad has been provided for the past three decades.
3. Materials and methods employed for these investigations are described in details in the next chapter.
4. After a brief description of the topography and physical features relating to the climate, soils, rainfall, temperature of Hadauti plateau, a locality wise distribution of *Marsilea* population is given. Exhaustive systemic surveys of all localities of *Marsilea* of Hadauti region during different seasons of the year 2011-2013 have been done.Map illustrating various localities of *Marsilea* populations taken up for the present study has been presented. *Marsilea* is represented by two species in Hadauti plateau. *Marsilea minuta* is frequently found growing in the ditches and ponds; on the other hand *Marsilea c.f. coromandelina* is found in a small patch at Borawas enroute Kota-Rawatbhata road and has been categorized as the **rare** endemic population which needs to be conserved.

5. An in-depth study of morphological range of variations in the *Marsilea* populations of selected localities for present investigations has been carried out. A significant increment in the length of root, internodal length, leaflet size was recorded in water forms of *M.minuta* and *M.cf.coromandelina* over their respective land forms. The shape, size and the attachment of its pedicel to the petiole has been found to be a species specific feature.
6. Quantitative organ wise estimation of various metabolites- soluble sugars, phenols, starch, proteins, ascorbic acid and proline have been carried out in the two species. An overall higher trend of these metabolites has been recorded in *M.minuta*. Proline content, which is an important parameter of drought resistance, reveals that it is slightly higher in the entire plant parts of *M.cf. coromandelina.*
7. A comparative phenological study of various populations of *Marsilea* in nature and their survivability under cultivation and garden conditions has been done. It reveals that *M.minuta* population is found all through the year in ditches and ponds while the land populations get dried up in the absence of rain and again their sporocarp germinate to form new plantlets on getting rain.

   While observations regarding their survivability under pot cultivation reveal that *M.minuta* grows throughout the year on providing the adequate amount of water, *Marsilea cf coromandelina* does not survive in pot cultivation for long period showing its lower survival ability. In nature also it appears only twice a year within a very short period in months of September and January or February depending on rainfall.
8. A comparison of organ anatomy of the five populations has been made. It has been found that although aerenchyma is present in all the populations but it is well developed in cortical regions of rhizome in the population growing in Talwandi, Anantpura (aquatic

form) localities while the relative area occupied is lesser in *M.cf.coromandelina*. But the relative area occupied by stelar region shows a reverse trend. It occupies somewhat higher area in *M.cf.coromandelina* than *M.minuta*.

9. Cytological studies of the two species showed 2n=42 chromosomes in root tip cells of *M.cf.coromandelina* while 2n=40 chromosomes were observed in case of *M.minuta*. The chromosome number n=21 in *M.cf.coromandelina* shows its aneuploid origin from n=20. This provides direct evidence of the role of aneuploidy in species diversification in this genus.
10. Reproductive biology in respect to above population of *Marsilea* is described. This indicates counts of microspores and megaspores per sorus & sporangium, their germination and reproductive capacity as indicated by sporelings formation. It is noted and confirmed that the two species namely *M.cf.coromandelina* and *M.minuta* shows comparative degree of fertility in that the sexual reproductive potential of the former is greater than *M.minuta* which is indicated by the quick greening of the apical mound of germinating spores. It is also observed that fresh sporocarps are incapable of producing embryos in *Marsilea* as the spores seem to require a period of maturation in order to be viable.
11. RAPD analysis of the various populations of *Marsilea* has been done to study their genetic variation and to find out whether the population variations are due to environmental or genetic basis. PCR amplification and Gel electrophoresis shows a clear continuous band of DNA and variation in band pattern of different population of various localities suggest that the variations in the population has a genetic basis in addition to environmental impact.

12. The entire data is discussed with particular reference to evolutionary potential and population variability in Hadauti species of *Marsilea*. The survival and spread mechanism of this amphibious fern in the Hadauti plateau is discussed.

13. The thesis includes exhaustive bibliography on various aspects of the biology of genus *Marsilea* with special reference to population variability, concluding these investigations future lines of research on these species are indicated. The data and observations are adequately supplemented with tables, photographic plates and text figures.

CHAPTER VII

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