DISCUSSION

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The results of the present experimentations are discussed under the following heads:-

Requirement of suitable medium

Out of various media tried, two media i.e. Knop's medium and Murashige & Skoog's medium were found best suited for the present experimentations. Knop's basal medium solidified with 8 gm/l agar was established as the optimal medium for regeneration of rhizome segments in Marsilea minuta and spore germination in Ceratopteris thalictroides. Earlier studies on Ceratopteris pteridoides; Regnellidium diphyllum (Cheema, 1984 and Cheema & Kaur, 1985) and Pteris vittata (Sinha & Jha, 1986) have also reported spore germination and regeneration of rhizome segments on Knop's medium. However, Murashige & Skoog's medium was found favorable for callus induction and its further differentiation. Profuse callus growth on this medium showed that even for ferns the nutritional requirements can be as complex as that of higher plants. Dykeman & Cumming (1985); Camloh, et al. (1989) and Haruzo & Ameri (1989) also used MS medium for in vitro fern culture. In some of the earlier reports simple nutritional medium favored morphogenetic response of ferns (Bristow, 1962; Mehra & Sulklyan, 1969; Mehra & Palta, 1971; Kshirsagar & Mehta, 1978 and Beri &

Bir, 1993). The results of these studies emphasized the simple nutritional requirements of fern system in vitro.

Factors favoring induction and regeneration of vegetative buds

Rhizome segments of fern Marsilea minuta (on Knop's medium) and leaf explants of Ceratopteris thalictroides (on both Knop's + 0.5% S and basal MS medium) regenerated vegetative buds. In case of Marsilea minuta rhizome segments with and without nodal part used in order to study their regenerative ability. Rhizome segments (with node) regenerated a green bud like structure which later on proliferated and developed spatulate leaves. On further subculturing bifoliate and normal quadrifoliate leaves were also regenerated. This heteroblastic development of leaves from spatulate leaf to quadrifoliate leaf stage was in accordance with that observed by Allsopp (1953) who worked on sporeling development in Marsilea. On the other hand, rhizome segments without node turned brown and remained quiescent throughout the period of experimentation without differentiating any organ. This perhaps can be attributed to the presence of meristematic cells in the nodal region which appears to be a pre-requisite for regeneration and differentiation.

In the present experimentation multiple shoot formation was observed after six weeks of culturing on Knop's medium

without sucrose and growth hormones. Earlier, Padhya (1984) also propagated three species of fern Cyathea by using excised apical meristems of rhizomes as explants on modified Knudson's medium supplemented with vitamins, sucrose and auxins-cytokinins in various combinations. These apical meristems regenerated buds, and when these buds were seperated and subcultured, more buds were induced which later on regenerated plantlets. Cheema & Kaur (1985) while working on some heterosporous ferns (i.e. Marsilea minuta and Regnellidium diphyllum) also reported the multiple shoot formation on Knop's medium containing 2% sucrose and 0.5 ppm Kinetin. Caponetti & Byrne (1985) reported that runner tips of Boston fern (Nephrolepis exaltata) when inoculated on Murashige's fern multiplication medium supplemented with Kinetin (5 X 10^{-6} and 5 X 10^{-5} M) and NAA (10^{-7} M) produced maximum shoots. Similarly Dykeman & Cumming (1985) propagated fern Matteuccia struthiopteris utilizing shoot tips derived by forcing lateral buds on the rhizome. Maximum shoot proliferation was attained with Kinetin (1 mg/l) with half strength MS inorganic salts and sucrose, agar, $NaHPO_4$, adenine sulphate, inositol and thiamine. HCl at 30,000, 4000, 85, 40, 100, 0.4 mg/l respectively. Later, Rajarathinam & Padhya (1988) cultured excised stolon segments of in vitro grown Nephrolepis cordifolia on B_5 medium containing IBA (1mg/l). Large

number of buds developed from meristematic growth centres on low concentrations of cytokinins (i.e. Kinetin and BAP) within 4 weeks. Plantlet regeneration was reported when these buds were cultured on basal medium. Further, Haruzo & Ameri (1989) reported that rhizome segments of Asplenium nidus produced green globular bodies on Murashige & Skoog's medium supplemented with 2.2 µM BAP and 43.7 mM sucrose. These green globular bodies regenerated plantlets about two months after culture on BAP free medium. Camloh, et al. in the same year while propagating the fern Nephrolepis exaltata used runner (stolon) apices as explants, reported that maximum number of shoots were produced on modified Murashige & Skoog's medium enriched with dimethylaminopurin (0.5 µM). They also observed that shoot proliferation was maximum on half strength MS medium containing 0.5 µM NAA and 5 µM Kinetin. For rooting half strength MS medium free from hormones was the most suitable medium. In all the above mention reports, medium was supplemented with different hormones as well as sucrose in order to induce buds and their further regeneration. In sharp contrast to these reports, in the present experimentation Knop's medium free from growth regulators and sucrose was found to be the most suitable medium for regeneration of rhizome segments, further growth of plantlets and formation of multiple shoots.

In case of fern Ceratopteris thalictroides development of adventitious buds was also observed from the leaf margin and notch of the leaf explants (both intact and excised). Leaf explants (excised) when cultured on Knop's + 0.5 % S medium produced only a few of buds i.e. 2-3 buds per leaf, while basal MS medium was found to be optimal medium for bud induction as 6-9 buds per leaf were formed on the leaf margin of excised leaf explants. Maximum bud differentiation was observed from the intact leaf explants on basal MS medium. Interestingly, all the buds regenerated complete sporophytes on further subculturing on the respective media.

Intact leaves when inoculated on MS medium containing variable concentrations of sucrose i.e. 0.5 %, 1 % and 1.5 % S also produced buds but these buds on further subculturing on the same medium regenerated leafy structures inspite of regenerating complete sporophytes. Previously, Cheema (1984) has also reported the development of buds from the vegetative leaves (both intact and excised) of fern Ceratopteris pteridoides. Buds produced on juvenile leaves regenerated only vegetative leaves when intact with the parent leaf but when the plantlings were excised and inoculated on fresh medium, fertile fronds were regenerated. Padhya (1984) while working on three species of tree fern (Cyathea) reported that young excised leaves of these three ferns when cultured on modified Knudson's medium

supplemented vitamins, sucrose, Kinetin (2 mg/l) and low concentration of NAA (0.2 mg/l) showed high degree of autonomy in their development. These excised organs finally developed complete plants. Caponetti (1990) also propagated fern Tectoria gammifera using frond buds. According to him, frond bud when placed on water agar media produced well developed leaves and roots but these plantlets showed mineral deficiency. Frond buds placed on MS medium containing 10^{-5} M Kinetin or with NAA produced small inhibited leaves and roots, but did not show nutritional deficiencies.

Factors favoring gametophyte development and sex expression in Ceratopteris thalictroides

Spores of Ceratopteris thalictroides germinated after 5-6 days of inoculation. Earlier, Endress (1974) reported that the spores of Ceratopteris thalictroides germinate in 8-12 days. Knop's medium solidified with 8 gm/l was found to be the most suitable medium for spore germination and gametophyte development. Sucrose and growth hormones did not affect germination. Camloh (1993) reported that sucrose has no promotive effect on either spore germination or early gametophyte growth of fern Platycerium bifurcatum. Gupta & Bhambie (1992) observed that spores of Adiantum capillus germinate earlier on the auxins rich media than on control. Whittier (1990) while working on Psilotum reported that

nitrate and nitrite inhibited germination at low concentrations and almost completely prevented it at high concentrations No such type of inhibition of spore germination was observed in the present course of investigation.

Germination of spores and further gametophyte development was found to be best in fully illuminated conditions of light. Whittier (1995) reported that the best development of Huperzia species was in dark.

In the present experimentation no apical cell formation was observed and three types of gametophytes were obtained i.e., I spatulate, II cordate symmetrical and III cordate asymmetrical. Regarding the sex expression, spatulate prothalli bore antheridia and remained antheridiate throughout while, cordate symmetrical and cordate asymmetrical were hermaphodite bearing both archegonia and antheridia. Javalgekar (1960) and Nayar & Kaur (1969) described two types of gametophytes in Ceratopteris thalictroides i.e. cordate bisexual and ameristic spatulate. Pal & Pal (1963) reported that most of the gametophytes were bisexual but some which were small, unbranched and spatulate bore antheridia only. Klekowski (1970) observed two kinds of ontogenic patterns of gametophyte development in Ceratopteris thalictroides. The ameristic prothalli developed only antheridia and remained antheridiate through

out. Those with faster growth initiate a notch meristem in lateral position and became cordate and bisexual.

Factors favoring callus cultures

Basal MS medium was found optimal for callus studies. Callus was induced from different plant parts of both the ferns used viz; Marsilea minuta and Ceratopteris thalictroides on basal MS medium with different growth regulators (i.e. 2,4-D; NAA; Kinetin, and BAP, alone). In case of Marsilea minuta, rhizome segments with node when cultured on basal MS medium containing growth hormones induced healthy and proliferating callus. Optimal callus was obtained on Kinetin (0.5 mg/l to 2mg/l). It was nodular and heterogeneous. Rhizome callus induced on NAA (0.5 mg/l to 2 mg/l); Kinetin (0.5mg/l to 3 mg/l) and BAP (0.5 mg/l to 2 mg/l), alone differentiated shoots on same medium as well as on transference to basal MS medium. Maximum shoot differentiation was observed on 0.5 mg/l NAA. Sporadic differentiation of shoots was observed from the rhizome callus induced on 0.5 mg/l 2,4-D but it differentiated many roots on basal MS medium.

Differentiation of callus of Ceratopteris thalictroides was found to be fully dependent on presence or absence of sucrose in the medium. Callus induced from different plant organs of gametophytes of Ceratopteris thalictroides when

transferred on MS medium differentiated gametophytes, while, root/shoot differentiation was observed on basal MS medium. However, gametophytic callus induced on basal MS + 1 mg/l Kinetin on transferring to MS medium with low level of sucrose i.e. 0.4 % S also differentiated gametophytes. Similar interactions of sucrose was observed by Bristow (1962). According to which controlled differentiation of leaf callus of Pteris cretica was depended on sucrose concentrations in the medium. Higher concentrations of sucrose favored sporophytic differentiation while low level of sucrose or complete omission of sucrose favored gametophytic differentiation. Mehra & Sulklyan (1969) demonstrated that controlled differentiation of rhizome segments of Ampelopteris prolifera into gametophytes and sporophytes was conditioned by nutritional status of the medium. On medium containing 0.5% or more sucrose, rhizome segments regenerated sporophytes while at low level of sucrose or on sucrose free medium they gave rise to gametophytes only. In Anogramma leptophylla however, the callus exhibited differentiation of gametophytic structures irrespective of the presence or absence of sugar (Cheema, 1983). Organogenesis of callus (induced from the different plant organs of both ferns) into root/shoot was observed on the medium of callus induction as well as on transference of callus on basal MS medium. Caponetti & Byrne

(1985) while propagating Boston fern (Nephrolepis exaltata) reported that callus was induced from the apical and lateral buds of stolon tips on Murashige's fern multiplication medium with 3% sucrose and 0.5 mg/l 2,4-D. According to them organogenesis into shoots and roots from callus occurred on basal medium with combinations of 5 X 10^{-7} M Kinetin + 5 X 10^{-7} M NAA; 10^{-6} M Kinetin + 10^{-7} M NAA and 10^{-6} M Kinetin + 5 X 10^{-7} M NAA in 12 weeks. Nataraja & Joshi (1985) induced friable callus from the root segments of Microsorium punctatum on Knop's minerals + 2,4-D (1 ppm) + IBA (1 ppm). Callus cells on subculturing on Moore's minerals + Kinetin (1 ppm) + IAA (1 ppm) divided like spore producing protonema and developed into gametophytes. The cells also differentiated embryo like structures and plantlets on the same medium.

The present investigations revealed that in case of gametophytic callus induced on 2,4-D (0.5 mg/l to 2 mg/l); NAA (1 mg/l to 3mg/l); (Kinetin 3mg/l to 6 mg/l mg/l); BAP (0.5 mg/l) and leaf callus induced on Kinetin (0.5 mg/l, 1 mg/l and 2 mg/l) and BAP (0.5 mg/l to 2 mg/l) turned brown. Similarly Zenkteler & Urbaniak (1994) also reported the browning of fern tissue. According to them it was due to the presence of numerous phenolic compounds. Production of phenolics in fern Acrosticum aureum during growth in vitro was also studied earlier by Padhya, et al.

(1980) according to which the total phenolics were increased from young to mature gametophytes and also in sporophytes both produced sexually and apogamously.

Explants excised from young sporophytes of both the ferns used, manifest stronger ability for callus induction while different plant parts taken from adult sporophytes failed to induce callus. In the same way rhizome segments (with node) of fern Marsilea minuta induced callus but was never induced from the rhizome segments without nodal part. It has also been observed that callus was induced from young prothalli of Ceratopteris thalictroides indicating there by that the nature of the tissue is also important for the callus induction. Earlier, Kato (1965) has derived the relationship between the callus inducing ability of the juvenile verses adult leaves and concluded that juvenile leaves of young sporophytes had stronger callus inducing ability. Later on Padhya (1984) while working on three species of Cyathea reported that when young excised leaves of three ferns inoculated on modified Knudson's medium containing vitamins, sucrose, Kinetin and low level of NAA showed high degree of autonomy in their development. Cheema (1984) reported that young gametophytes of Anogramma leptophylla and petioles of the juvenile leaves of Regnellidium diphyllum induced callus while petioles of adult leaves failed to induced callus.

From the cytological investigations it was revealed that the cells of callus obtained from rhizome explants of Marsilea minuta and leaf explants of Ceratopteris thalictroides on basal MS medium containing growth hormones (viz; 2,4-D; NAA; Kinetin; and BAP, alone) were bearing normal diploid set of chromosomes. After maintaining the callus for six weeks on their respective media increase in ploidy level was observed. Tetraploid cells were observed in case of callus induced from rhizome segments (with node) on 0.5 mg/l Kinetin. Takei (1989) reported low chromosomal variability in the leaf calli of Lepisorus thunbergians induced on MS medium with 3% sucrose, 2,4-D (1 mg/l) and Kinetin (1mg/l or 0.2 mg/l). About 15 % of these cells were tetraploid and the others were diploid. Palta & Mehra (1983) also carried out cytological investigations in regenerated sporophytes from the leaf calli of Pteris vittata. They suggested that the whole sporophytes have been regenerated from sporophytic calli without occurrence of the chromosomal change.