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CONSERVATION OF VEGETABLE FERN (*DIPLAZIUM ESCULENTUM*) OF WESTERN GHATS THROUGH *IN VITRO* SPORE CULTURE

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Abstract: *Diplazium esculentum* (Retz.)Sw. (Dryopteridacea), commonly called "Churuli" is considered of high value nutritive fern amongst the paniya and chetti tribes of Western Ghats, of kerala, India. But due to indiscriminate collection and destruction of habitats, it is facing the verdict of being endangered. So the present study was intended to produce a protocol for the conservation of this vegetative fern of southern Western Ghats of India using *in vitro* spore culture. In addition, this study reports spore germination, gametophyte development, changes in the reproductive phases and sporophytes formation of the vegetative important fern *Diplazium esculentum* (Retz) SW. The spores sown on the Knop's basal agar medium showed the highest percentage (57.41±2.21) of germination compared to Knudson and Murashige and Skoog's medium. Multiplication of Prothallus was successful in Knop's medium supplemented with Benzylaminopurine (BAP) (1- 4 mg L^{-1}) and Indole 3-acetic acid (IAA) (0.1- 0.5 mg L^{-1}). Differntiation occurred maximum when prothallus was transferred explant on Knop's media containing various concentration of Sodium dihydrogen phosphate (NaH_2PO_4) along with Indole 3-acetic acid (IAA) and Benzylaminopurine (BAP) at the concentration of 3 mg L^{-1} and 0.5 mg L^{-1} each, respectively. Addition of Sodium dihydrogen (250 mg L^{-1}) phosphate in Knop's medium plays a significant role in differentiation from gametophyte stage to sporophytic stage. Rooting was optimal at concentration of 1.0 mg L^{-1} Indole Butyric acid (IBA). Best material for acclimatization was found to be garden sand and river soil.

Key words: Biodiversity, Vegetative, Extinction, Germination, Prothalli, Sporophyte, Multiplication

INTRODUCTION

Pteridophytes, a lower group of plants possess long evolutionary history. Fern has proved to be an excellent material for the study of the alternation of generation. The regular alternation of generation can also be altered by various factors as light, nutrition and physical factors, which play a regulating role in the various stages of the orderly development of gametophytic and sporophytic generation during the life history of fern. Moreover, it is well known that apogamy and apospory circumvented through the occurrence of cyclic alternation of generation. The economic value of the ferns has been enumerated by various authors. Today, serious threats like habitat loss, habitat degradation and increasing exploitation of natural resources caused life diversity of plant in Western Ghats. Western Ghats in India is rich in fern diversity; about 250 ferns are growing in variety of edaphic conditions. Numerous research works have been carried out in higher groups of plants (angiosperms and gymnosperms), while work in the lower groups of plants in general has not been carried out seriously and systematically (Chaudhary, 1998). Some of studies done in fern gametophytes include investigation of growth correlation, regeneration and hormonal effects (Albaum, 1938 a,b); the effect of environmental gradients (Naf, 1953); the induction of sex organ formation (Naf, 1956, 1958); the effect of the inhibition of protein synthesis on growth (Hotta and Osawa, 1958); induction of apogamous sporophyte development (Whittier and Steeves, 1960, 1962; Mehra and Sulklyan, 1969; Kato, 1970); sucrose effect on heteroblastic leaf development (Sussex and Clutter, 1960; Caponetti, 1970) and many more. Several researchers were done on Pteridophytes morphogenetic studies. Morphogenesis in pteridophyte deals with the development of organisms and their parts from a single cell.

vitro spore germination are best applied and commercially exploited in fern species (Fay, 1994). However, spore propagation is generally a time-consuming procedure (Thentz and Moncousin, 1984), while *ex vitro* spore cultivation is vulnerable to attack by phytopathological agents (Lane, 1981). Application of this *in vitro* spore germination technology for large–scale multiplication of certain species of ferns from the Western Ghats has been demonstrated (Sara *et al*., 1998; Manickam *et al*., 2003; Johnson *et al*., 2005; Sara and Manickam, 2005; Johnson and Manickam, 2006; Sara and Manickam, 2007; Johnson and Manickam, 2007; Johnson *et al*., 2008). It has been possible to induce apogamous sporophytes and aposporous gametophyte *in vitro* by suitably manipulating the cultural condition, without the intervention of fertilization and sporogenesis. Thus, pteridophytes offer a vast scope of morphogenetic studies. Such studies have been found useful for several works on experimental pteridology. Endangered ferns such as *Diplazium cognatum, Histiopteris incisa, Hypodematium crenatum, Pteris vittata*, *Metathelypteris flaccida, Thelypteris confluens, Athyrium nigripes, Pteris gongalensis, Pteris confusa, Cyathea crinita, Cheilanthes viridis, Pronephrium articulatum, and Nephrolepis multiflora* have been multiplied through *in vitro* spore culture as a part of *ex situ* conservation (Sara, 2001; Johnson, 2003; Manickam *et al*., 2003; Irudayaraj *et al*., 2003; Vallinayagam, 2003). *Diplazium esculentum* (Retz.) SW is found in the rural areas of Western Ghats, growing on the bank of streams and rivers. Stems erect, scales brown, linear-lanceolate and margins dentate. The stalks are 20-50 cm long, are green and somewhat smooth. The fronds are 2 or 3 pinnate, 50-80 cm long, and about half as wide as long. The pinnules are broad ovate, 2-3 cm long and rather coarsely toothed. The clusters of spore on the side of the veins or veinlets. Despite the great nutritional value (Archana *et al*., 2012) and physiological importance of *D.esculentum* leaves, very little information has been documented for its *in vitro* multiplication. Since this edible fern is at the merge of extinction, there is an urgent need for its mass multiplication. So, in the present study *in vitro* culture of *Diplazium esculentum* has been attempted as an effort to conserve this species of great nutritional value.

MATERIALS AND METHODS

***Fern material*:** Matured fertile fronds of the selected species were collected from the wild of the Western Ghats and established in the green house attached to the Department of Botany, University College, Trivandrum, India.

Spore collection: The mature fronds were washed in running tap water for a few minutes. The fronds were cut into small pieces and dried over absorbent paper at room temperature (25°C). After drying the fronds at room temperature for 24 hr, the liberated spores were passed through 40 mm nylon mesh to remove the sporangial wall materials and the clean spores were collected and stored in a refrigerator at 5° C.

Spores sterilization: Spores of *Diplazium esculentum* were surface sterilized with 0.1 % $HgCl_2$ solution for 5 min and washed with sterile distilled water for 10 min.

***In-vitro culture medium*:** The surface sterilized spores were inoculated onto different media viz., Knops (1906), Knudson C (1946) and Murashige & Skoog's medium (1962) in petridishes devoid of sugar and plant growth regulators and incubated at 25° C±2°C. The *p*H of the media was adjusted to 5.8 before adding agar 0.5% (w/v) and autoclaved at 121°C for 15 min. Both liquid and agar nutrient media were used for spore germination. Gametophytes regenerated from spores were sub–cultured on different basal media (Knops, Knudson and Murashige and Skoog's medium) for sporophyte formation.

For multiplication, prothalli were transferred on to media containing Knop's or Knudson C or Murashige and Skoog's medium with growth regulators Kinetin (1-5 mg L^{-1}) or BAP (1-5 mg L^{-1}) and 2,4-D (0.1-0.5 mg L^{-1}) or NAA (0.1-0.5 mg L^{-1}) or IAA (0.1-0.5 mg L^{-1}) each respectively with 3% sucrose. Cultures were incubated in the growth room for four week at 24 ± 2°C, at 2000 lux for 16 h photoperiod. There were at least five replicates per formulation. Data were recorded every week for average mean length and width of prothalli.

For differentiation into sporophytes, clumps of prothallus were cultured in Knops or Knudson C or Murashige and Skoog's medium with varying concentrations of NaH_2PO_4 (100-300 mg L^{-1}). Sodium dihydrogen phosphate (NaH_2PO_4) is routinely included in tissue culture media and it affects on plant maturation and growth (Kyte and Kleyn, 1996). To this media growth regulators Kinetin (1-5 mg L^{-1}) or BAP (1-5 mg L^{-1}) and 2, 4-D (0.1-0.5 mg L^{-1}) or NAA (0.1-0.5 mg L^{-1}) or IAA (0.1- 0.5 mg L^{-1}), 3% sucrose was added in all combinations at *p*H 5.8. Cultures were incubated in the growth chamber for four weeks. A layer of liquid media was suspended above the solid media to facilitate the fertilization process. Data was recorded every week for average number of plants, number of leaves, average length and width of plants.

For rooting, differentiated plants were then transferred on solid medium, Knops or Knudson C or Murashige and Skoog's medium containing NaH_2PO_4 (100-300 mg L^{-1}), with NAA or IAA or 2, 4- D or IBA concentration ranging from 0 - 2.5 mg L^{-1} was used.

For acclimatization, the plants with well developed roots were removed from culture tubes, washed in running tap water and planted separately onto a 10cm diameter plastic cup filled with various supporting media like garden sand, farm yard manure, river sand and combination of all three were used. The plantlets established in pots were transferred to a shade net house for 3–4 weeks and then repotted in larger pots (20 cm diameter) with one plant in each pot.

RESULTS

The surface sterilized spores were inoculated onto solid and liquid cultures of different media viz., Knops (1906), Knudson (1946), and Murashige and Skoog's medium (1962) devoid of sugar.

***Prothalli formation*:** After 35 days, the spores started to germinate in solid knops agar medium (Fig. 1A). Liquid nutrient media gave no response on spore germination. In the liquid media, the germination of inoculated spores failed due to the high incidence of microbial contamination. The prothallial plate was formed by repeated divisions of the cells undergoing different stages (Fig. 1B). The thallus was dorsiventrally flat with an apical notch (Fig. 1C). The Mature prothalli were cordate type (Fig. 1D). A high percentage of prothalli formation (48.55±0.24) was observed in Knop's medium (Table 1). Glandular hairs were present on the margin and central areas of the gametophyte (Fig. 1E). Adult prothallus is large; heart shaped

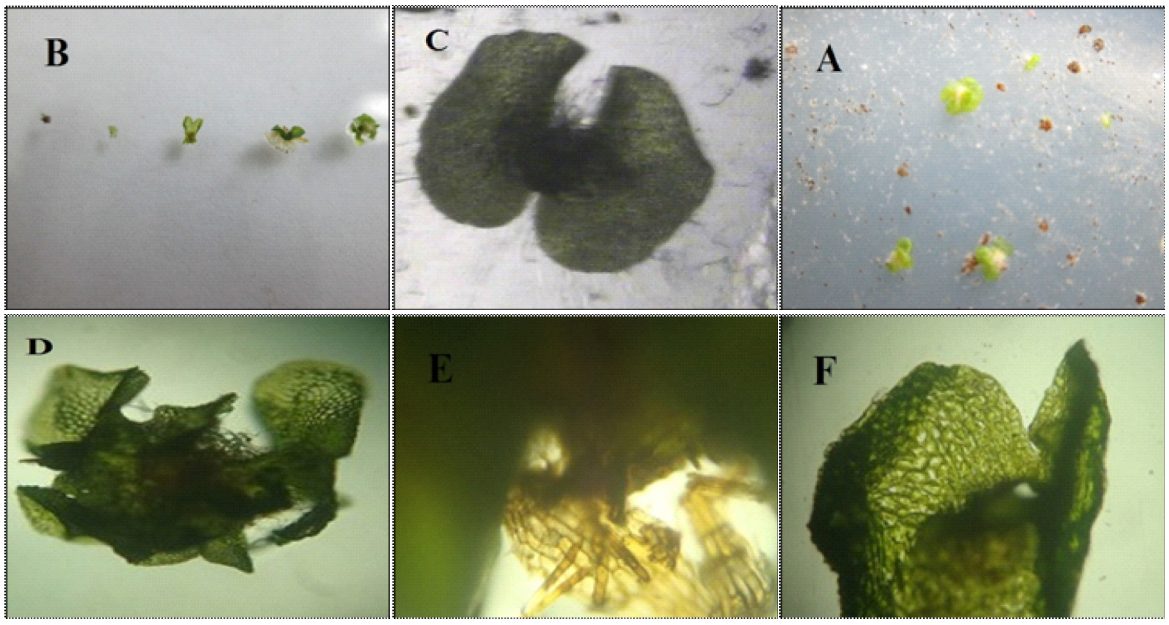

Fig. 1. *In vitro* spore culture: Different morphological changes in prothallus of *Diplazium esculentum*

with raised cushion 4-6 celled thick on ventral side. Cells are polygonal (Fig. 1F) and are lined by numerous chloroplasts. The notch is deep with 4-7 meristematic cells lie in it. The male and female sex organs formed after 120 and 150 days respectively. Hermaphrodite prothallus with antheridia appears on posterior part of cushion near the edges of midrib region (Fig. 2A). Mature antheridium is large and spherical. It is raised on 3- 4 celled stalk and bears a cap cell with spermatocytes. At maturity, many archegonia are found on the anterior part of cushion (Fig. 2B). Archegonia are long with 4-6 celled neck bearing egg and are turned obliquely towards posterior. So, from the present study, it was confirmed that highest percentage (57.41 ± 2.21) of spore germination was observed in the Knop's basal agar. Germination was not observed on the Murashige and Skoog's basal medium. The highest frequency of gametophyte formation and multiplication (69.13 ± 0.52) were observed in Knop's media compared to Knudson C basal medium and M S media (Table 1).

***Multiplication*:** A profound effect was observed on the multiplication of plant in Knop's media containing growth regulators BAP and IAA (Fig. 2C, D), Knudson and MS medium showed no significant result. Knop's medium (K8) with concentration of 3 mg L^{-1} and 0.5 mg L^{-1} respectively showed the significant increase in multiplication of plant (Table 2) with maximum plant length of 0.62 ± 0.012, where as low concentrations of BAP and IAA showed less number of plantlets and at higher concentration, prothallus started to show browning which confers the detrimental effect of the dose. This indicates that plants need both auxins and

Table 1. Effect of medium on spore germination, development of gametophyte and sporophyte and field establishment in *Diplazium esculentum*

Medium & *p*H	% of Germination ± SE	% of Prothalli formation ± SE	% of Sporophyte formation ±_SE	% of establishment in field ± SE
KN Solid 5.8	57.41±2.21	57.11±1.55	48.55±0.24	69.13±0.52
KC Solid 5.8	11.02±0.22	23.47±0.41	-	-
MS Solid 5.8	-	--	-	-
KN liquid 5.8	-	-	-	-
KC liquid 5.8	-	-	-	-
MS liquid 5.8	-	-	-	-

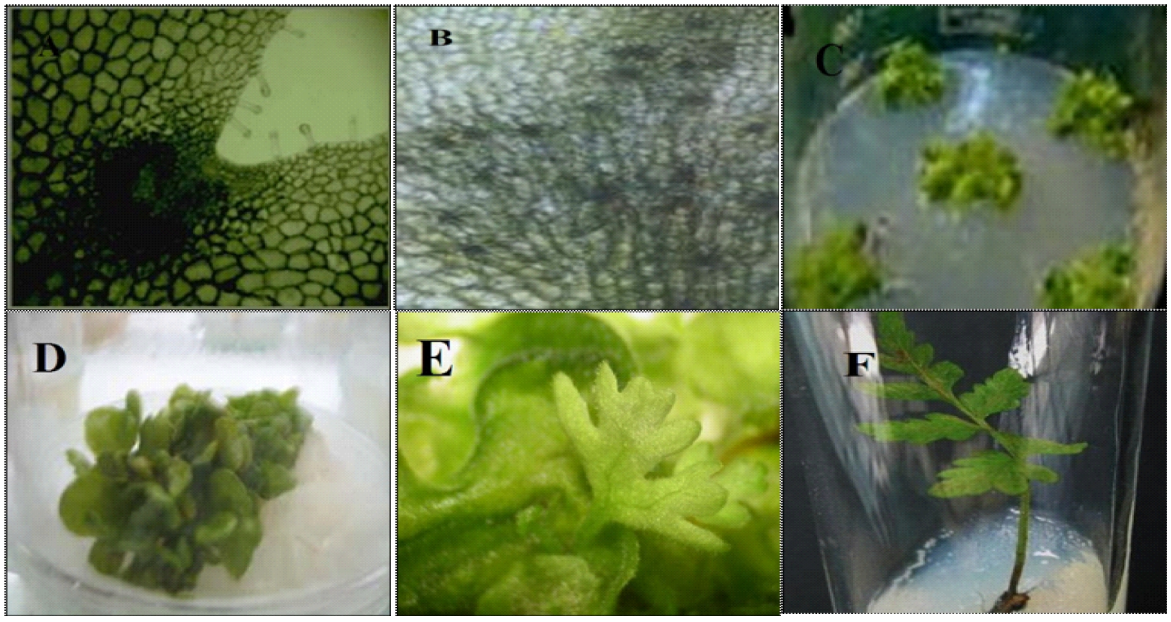

Fig. 2. *In vitro* spore culture: Gametophyte and sporophyte of *Diplazium esculentum*

Table 2. Effect of Knop's medium with BAP in combination with IAA on *in-vitro* multiplication of *Diplazium esculentum* prothallus

Media Codes	BAP(mg L^{-1})	IAA(mg L^{-1})	Mean length + SE (cm)	Mean width + SE (cm)
K1	0	0	0.22 + 0.011	0.20 + 0.015
K2	1	0.1	0.31 ± 0.025	0.27 ± 0.005
K3	2	0.1	0.33 + 0.014	0.35 + 0.011
K4	3	0.1	0.46 ± 0.011	0.44 ± 0.001
K5	4	0.1	0.48 ± 0.007	0.45± 0.017
K6	1	0.5	0.55 + 0.015	0.53 + 0.013
K7	2	0.5	0.59 ± 0.011	0.57 ± 0.001
K8	3	0.5	0.62 + 0.012	0.65 + 0.011
K9	4	0.5	0.52 + 0.009	0.53 + 0.007

SE = Standard Error

cytokinins in the medium and the ratio between auxin and cytokinin seems to be very important for multiplication. There are many reports in which application of growth regulators are indicated that enhanced and suppress the plant growth which directly effect on rate of multiplication (Fernandez & Revilla, 2003).

Differentiation enhanced by Sodium dihydrogen phosphate: After multiplication, successful differentiation stage was optimized by using knop's media KN1, KN2, KN3 and KN4 containing various amount of NaH_2PO_4 along with growth regulator BAP and NAA with the concentration of 3 mgL^{-1} and 0.5 mgL^{-1} respectively. All media containing NaH_2PO_4 showed different degrees of differentiation (Table 3). An increase in axillary shoots was also observed up to a certain concentration of NaH_2PO_4 up to 250 mg L^{-1} at its maximum while reduced growth was observed above the concentration 250 mg L^{-1} (Table 3). This reflects that NaH_2PO_4 plays a significant role in the differentiation process of *Diplazium esculentum*. The sporophyte emergence was noticed in the midrib regions of prothallus (Fig. 2E). The sporophyte formation was observed only in Knop's medium, that too at a high (48.55 ± 0.24) percentage (Fig. 2F); there was no formation of sporophyte in other media. Thus, it was concluded that the NaH_2PO_4 amount in Knop's media is directly related to *in vitro* differentiation of *Diplazium esculentum* up to a certain optimum level.

Rooting: For root induction, experiments were done in which different auxins (IBA, NAA, 2, 4-D and IAA) (Table 4). The plants having multiple shoots were rooted easily on solid medium containing IBA 1.0 mgL^{-1}. Control (medium without IBA) showed browning in differentiated leaves. Above this concentration, the plantlets started to show browning and also decrease in survival rate. The plantlets having roots were acclimatized.

Table 3. Influence of NaH_2PO_4 on *In vitro* differentiation of *Diplazium esculentum*

Media Codes (Knop's media)	NaH_2PO_4 (mg L^{-1})	Mean no. plants ± SE (cm)	Mean no. leaves ± SE (cm)	Mean length ± SE (cm)	Mean width ± SE (cm)
KN1	0	1.33 + 0.24	1.57 + 0.47	1.12 + 0.24	1.26 + 0.36
KN2	150	2.52 ± 0.14	3.22 ± 0.63	1.22 ± 0.34	1.35 ± 0.55
KN3	200	2.11 ± 0.31	2.92 ± 0.58	1.03 ± 0.55	1.13 ± 0.37
KN4	250	2.68 + 0.47	3.55 + 0.23	1.46 + 0.63	1.53 + 0.33
KN5	300	2.21 ± 0.53	2.33 ± 0.37	1.34 ± 0.47	1.51 ± 0.52

SE = Standard Error

Acclimatization: For acclimatization, the plants with well developed roots (3–5 cm) were removed from culture tubes, washed in running tap water to remove the remnants of agar and each group was planted separately onto a 10cm diameter plastic cup filled with different potting mixtures: river sand, garden soil and farm yard manures (1:1:1) and sand and soil (2:1) as in Fig 3A. The plantlets established in pots were transferred to a shade net house for 3–4 weeks (Fig 3B) and then repotted in larger pots (20 cm diameter) with one plant in each pot.

Table 4. Effect of IBA on *In vitro* rooting of *Diplazium esculentum*

Media Codes (Knop's media)	IBA Conc. (mg L^{-1})	Root emergence (days)	No. of roots	Root length ± SE (cm)	Field survival %
K11	0.5	30 -35	1	0.35 + 0.036	< 70
K12	1.0	25-29	4	2.57 ± 0.017	< 80
K13	1.5	23-27	1	2.14 ± 0.058	70
K14	2.0	14-17	2	1.68 + 0.047	50
K15	2.5	11-15	3	1.94 ± 0.028	60

SE = Standard Error

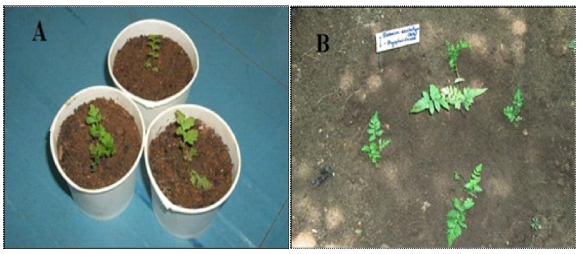

Fig. 3. Hardening and field establishment of *in vitro* raised plantlets of *Diplazium esculentum*

DISCUSSION

Spores are tiny objects which are used by ferns for reproduction. Most of the rare and endangered ferns failed to obtain the optimal growth condition for their development. The present study provided a micropropagation protocol for initiation, multiplication and differentiation of *Diplazium esculentum* is successfully optimized in which spores are used as an explant. Thus, from this micropropagation technique hundreds of plants can be regenerated from a single explant. It is the economical and fastest method for the propagation plants that are hard to propagate vegetatively. However, there are a few published reports on successful germination of spores *in vivo* (Theuerkauf, 1994). Under natural conditions, due to prevalence of unfavourable factors both biotic and abiotic the percentage of spore germination is low. The *in vitro* culture techniques have been used to study different aspects of spore germination, growth and development of gametophytes and sporophytes in ferns (Nester and Coolbaugh, 1986; Hickok *et al.*, 1987). However there are number of factors such as temperature, humidity, light, and nutrient compositions (Raghavan, 1989) which need to be favoured for successful *in vitro* spore culture. A number of workers have studied various physiological and chemical parameters which influence spore germination. In the present investigation also, the spores of the selected species were cultured under varied conditions with the object of developing viable protocols for mass multiplication and conservation. In a comparative perspective, spore cultures are more desirable than tissue culture for rare species conservation as it retains the genetic variability inherent in the genetic make up of a species. Nowadays, accurate recordings of the genetic uniformity of the plants are needed to be verified before conservation.

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