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In vitro spore germination and gametophyte growth assessment of a critically endangered fern: *Pteris tripartita* Sw.

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Abstract
In this present investigation, we have studied the effects of both MS medium and heavy metals concentration on spore germination and developmental gametophyte stages of *Pteris tripartita* Sw. Among four different concentration of MS medium, the spore germination rate was significantly increased in half strength MS medium (80%) followed by full strength MS medium (70%) and significant gametophyte growth was observed in ½ strength MS medium in 45 days. The effects of heavy metals such as $ZnSo_4$, $CuSo_4$, $HgCl_2$, $CdCl_2$, Na_3AsO_4. $7H_2O$ on spore germination were also studied. The heavy metals culture medium swiftly caused the spore germination percentage and its protonemal cells. The *in vitro* spore germination, protonemal growth appraisal could be used to evaluate the fern tolerance on heavy metals and also its toxicity on environmental pollution.

Keywords: spore germination, heavy metals, *Pteris tripartita,* protonemal cells

Introduction

From conservation point of view, ferns are becoming endangered and spores have difficulty to germinate under natural condition due to environmental factors. So interest on developmental patterns of both gametophytes and sporophytes in heavy metal culture condition has been investigated in recent decades (Amoroso, 1990; Amoroso and Amoroso, 1998, 2003; Aspiras, 2010). In general, Pteridophyte spores are easy to obtain, also can be stored in large quantities, and can germinate rapidly in simple media (Dyer, 1979). The spore germination and protonemal length in *Funaria hygrometrica* studied to account metal toxicity (Coombes and Lepp, 1974; Lepp and Roberts, 1977). Francis and Petersen (1983) determined the effect of Cu, Cd, and Zn on the spore germination of two ferns, *Osmunda cinnamomea* and *Onoclea sensibilis*. The effect of copper on spore germination, growth and ultrastructure of *Polypodium cambricum* L. gametophytes was studied by Muccifora (2008). In this connection, heavy metals are significant elements of mining and industrial pollution. Spore germination and protonema growth were significantly selected to evaluate both water and soil pollution investigations in the directions of study of environmental pollution (Coombes and Lepp,1974; Lepp and Roberts 1977; Petersen and Francis, 1980). *P. tripartita* Sw. (PT) is a terrestrial herb with short scaly rhizome and grow on the stream banks under the shaded regions. Sori are dark brown, tetrahedral and arranged continuously along each side of the lobes or the margin from base to midway or sometimes to apical part of segments. Moreover, they attain fertility on September to December. Baskaran and Jeyachandran (2010) reported that *P. tripartita* fronds possess antioxidant activity. Our present investigation focused on the effects of MS medium and heavy metals on spore germination and protonemal growth of a critically endangered fern, *P. tripartita.*

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Materials and Methods

Collection of plant materials

PT spores were collected from Alagar hills of Madurai. The reserve forests of Alagar hill are 22 km Northeast of Madurai city (Lat. 12°18° N; Long. 76°42°E; Alti. 600m above mean sea level) (Shumuga Sundari et al., 2012).

Collection and storage of spores

Matured spores of *P.tripartita* were harvested from sporophytic plants. The spores lose their viability if stored at room temperature. So that, collected spores were preserved at low temperature (4^{o}C).

Spore germination

The spores were sown in culture medium within one month of their collection. Before inoculation process, spores (5 mg) were immersed in water for 2 h. Then spores were sterilized with commercial bleach solution (NaClO, 0.5% v/v) containing double distilled water for 10 min. All the spores were rinsed at least three times with sterile double distilled water and then they were centrifuged at 3,000 rpm for 3 minutes. After that, spores were collected in a sterile condition and cultured in 25 ml culture tubes (Borosil, India) containing 10 ml of Murashige and Skoog basal medium (1962) and heavy metals in separate culture. Both medium were augmented with 2% (w/v) sucrose and 0.7% (w/v) agar and pH was adjusted to 5.7 with 0.1N NaOH and 0.1N HCl. Finally, all the cultures were maintained at 25^{o}C under cool-white fluorescent light (40 μmol $m^{-2}s^{-1}$) with a 16 h artificial photoperiod (Philips, India). After two weeks, hundred spores were scored per treatment to study spore germination rate and ten prothalli were observed for the measurement of gametophyte growth in each treatment.

Microscopical studies

The prothalli of *P. tripartita* were grown on MS and heavy metals culture medium to measure its growth. Furthermore, morphological studies of the gametophytes were measured with the help of stereomicroscope (Nikon SMZ800, Japan) after 45 days and photographed using fluorescence microscope (Nikon Eclipse E200, Japan). The effects of MS medium on spore germination and protonemal growth were photographed in light microscope (Deep vision, India) after 15 days of germination.

Statistical analysis

All the results were represented as Mean±Standard Error. Each percent germination value and prothalli growth represents the average of three replicates. The analysis was carried out with one way ANOVA test with Duncan's multiple range test (DMRT) along with $p<0.05$ as the limit of significance.

Results and Discussion

Effects of MS media on spore germination

Habitually, spores could be germinated by rupturing their spore-coat and division of its cell. Moreover, spores underwent only one asymmetric cell division giving rise to rhizoid and protonemal cell, which continued to divide to form a gametophyte (Chang et al., 2007). Spore germination percentages were calculated over 100 spores, in which 10 protonema were randomly selected in each treatment to study the morphological development of prothalli. A spore was defined as germinated if the first rhizoid was emerging and formation of chlorophyll pigments within the spore wall. In our present investigation, spores of *P. tripartita* was germinated only in light but not in the dark field which indicate that light is mainly required for spore germination. Because, light is one of the most important factors that affect fern life cycle and also awaken the dormant fern spore. It has been reported in early literature that spore germination involves a light sensitive pigment phytochrome (Raghavan, 1992). At the end of 15 days, greatest spore germinability was observed at half (80%, Fig. 1B) and full strengths MS medium (70.66%). The rhizoid was formed only in lowest concentration likely, ½ (26.33%), ¼ (5.33%) and 1/8 strengths MS medium (2.66%), respectively. In previous literature also, the highest germination occurred on ½ strength MS basal medium supplemented with 2% sucrose in spores of *Pteris cretica* and *P. umbrosa* (Lijuan, 1999), *Osmunda japonica* (He et al., 2004), *Pteris cretica* (Xu et al., 2005), *D. fortunei* (Chang et al., 2007), *A. nidus* (Khan et al., 2008), *Pteris wallichiana (*Zhang et al., 2008), *Pyrrosia lingua* (Du et al., 2009), *Drynaria roosii* (Zhang et al., 2009), and some ferns (Kyte and Kleyn, 1996). Therefore half strength medium was selected for further study to evaluate the effects of heavy metals on spore germination and its prothalli growth.

Table 1: Spore germination and protonemal growth of *P. tripartita* Sw. in MS medium

MS medium concentration	Spore germination (%)	Rhizoid formation (%)	Protonemal Length (µm)	Protonemal Width (µm)	Protonemal Shape
Full strength	70.66±1.45	-	394.40±8.00	644.37±31.10	Spatulate
Half	80.00±1.15	26.33±0.88	513.27±1.92	1269.86±133.15	Heart
1/4 strength	59.00±1.15	5.33±0.88	394.40±27.77	895.46±153.90	Spatulate
1/8 strength	56.00±2.51	2.66±0.88	349.96±10.71	687.70±37.77	Filament

All the values are represents as Mean±SE of three replicates

Table 2: Effects of heavy metals on spore germination and protonemal growth of *P. tripartita* Sw. after 15 days

Heavy metals (mg/100ml)	Spore germination (%)	Rhizoid formation (%)	Protonemal Numbers	Protonemal Length (µm)	Protonemal Width (µm)
$ZnSo_4$					
5	60.00±1.15	-	5.14±0.04	86.56±0.86	66.17±3.16
10	57.33±1.20	-	3.97±0.12	80.17±1.64	51.83±1.69
20	47.66±1.20	-	3.97±0.08	63.67±6.45	45.00±0.00
50	35.66±2.02	-	3.73±0.03	51.17±2.24	46.00±1.00
100	12.33±0.88	-	3.07±0.06	47.00±1.00	45.50±0.50
$CuSo_4$					
5	12.66±1.45	-	4.47±0.08	78.17±1.59	51.00±0.86
10	7.00±0.57	-	3.77±0.24	81.17±1.92	49.50±0.86
Mercuric chloride	-	-	-	-	-
$CdCl_2$	-	-	-	-	-
Arsenic	-	-	-	-	-

All the values are represents as Mean±SE of three replicates

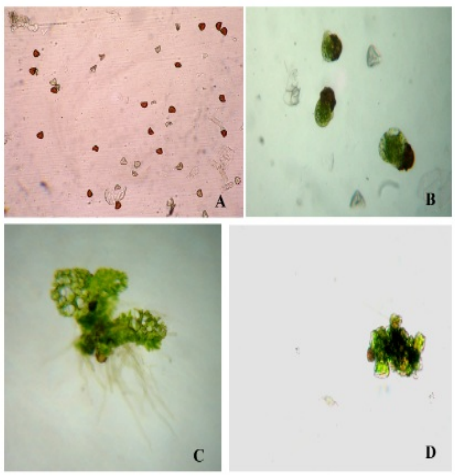
Effects of MS media on Gametophyte growth

In vitro culture of gametophyte growth was developed in MS medium concentration after 45 days, in those observation different stages of gametophyte was observed such as filamentous, spatulate and heart shaped (Figure 1). The heart shaped prothalli (Fig.1C) was observed in half strength MS medium with significant length (513.27µm) and width (1269.86µm), while full strength MS medium showed average growth of protonemal length (394.40µm) with protonemal width (644.37µm). In earlier reports, induction of gametophytes and its significant growth was noticed on half strength MS medium in spores of *Pityrogramma calomelanos* (Martin et al., 2006), *Osmunda regalis*, *Cyrtomium falcatum*, *Asplenium ruta-muraria*, *Dryopteris dilatata* (Soare and Andrei, 2005; 2006; 2007b), *Dryopteris fortunei* (Chang et al., 2007), *Pteris vittata* (Yang et al., 2007), *Phegopteris connectilis* (Soare et al., 2007a), *D. varia* (Ouyang et al., 2008), *Pteris wallichiana (*Zhang et al., 2008), *Pyrrosia lingua* (Du et al., 2009), *D. affinis* (Soare et al., 2010) and some selected *Blechnum* sp., *Cibotium* sp., *Cyathea* sp., *Dicksonia* sp. (Goller and Rybczynski, 2007). In both ¼ and 1/8 strength MS medium, minimum growth were studied with spatulate and filament shape gametophytes (Table 1).

Effects of heavy metals on spore germination

Spore germination was significantly inhibited by high concentration of heavy metals namely, $ZnSo_4$, $CuSo_4$, $HgCl_2$, $CdCl_2$, Na_3AsO_4. $7H_2O$ (Table 2). Among various heavy metals concentrations (5-100mg/100ml), highest spore sprouting rate was studied in both 5mg, 10mg/100ml of Zinc sulphate (60%, 57.33%

respectively). Moderate germination capability was observed in 20, 50mg of Zinc sulphate (47.66%, 35.66%). Furthermore very lowest amount of spore germination was observed in 100mg/100ml of $ZnSo_4$ (12.33%). In $CuSo_4$ lowest concentrations also rapidly inhibited the germination of *P.tripartita*. In 5mg/100ml of $CuSo_4$ only 12.66% and 7% in 10mg/100ml were observed. The present result directly coincide with the previous result that $CuSo_4$ is more toxic than $ZnSo_4$ (Francis and Petersen, 1983; Biesinger and Christensen 1972). According to Irudayaraj et al. (2011), germination percentage and growth rate of both *Pteris confusa* and *Pteris argyraea* were decreased in high concentration of zinc sulphate. Due to heavy toxic effects, there was no germination occurred in mercuric chloride, cadmium chloride and arsenic. The effect of $CdCl_2$ on spore germination and development of the gametophytes of *Ceratopteris thalictroides* (L.), *Drynaria quercifolia* (L.), *Christella parasitica* (L.), *Pteris ensiformis* Bum., *P. vittata* L., *Thelypteris augescence* (Link.), *Ampelopteris prolifera* (Retz.), and *Adiantum lunulatum* Burm. were assessed in which percentage of spore germination was significantly affected at high concentration (Gupta and Devi, 1992). However, *P.tripartita* spores were more sensitive to $CuSo_4$, $HgCl_2$, $CdCl_2$, Na_3AsO_4. $7H_2O$ than $ZnSo_4$.



A. Spores of *P. tripartita* B. Germinated spores in ½ strength MS medium
C. Heart shaped prothalli in half strength MS medium after 45 days
D. Unusual shape of prothalli in $ZnSO_4$ (100mg/100ml) after 45 days

Fig.1: Effect of MS medium and heavy metal concentration on spore germination and gametophytes growth of *P.tripartita*

Effects of heavy metals on gametophyte growth

Gametophyte growth rate was assessed after 15 days (Length and width of gametophyte, number of protonemal cell). The heavy metals concentration affected the prothallus size negatively. Gametophyte growth was normal in MS medium, but with heavy metals, protonemal growth was affected. Among germinated spores of PT, the highest protonemal number was attained in both low concentration of $ZnSo_4$ (5.14) and $CuSo_4$ (4.47). Significant protonemal lengths (86.56µm) along with widths (66.17µm) and 80.17µm length and 51.83µm of protonemal width were noticed in 5 and 10mg/100ml of $ZnSo_4$, respectively (Table 2). At 20, 50, 100mg/100ml of $ZnSo_4$ (Fig.1D), least growth was observed and unusual shape of prothalli was noticed. In $CuSo_4$, more or less similar growth was assessed in both 5mg/100ml (protonemal length- 78.17µm; width- 51µm) and 10mg/100ml (length- 81.17µm; width- 49.50µm), respectively. The essential micronutrient for plant growth is copper, which mainly used in structural and catalytic cofactor of enzymes and a component of many of the electron carriers involved in oxidative phosphorylation and photosynthesis. But, excess absorption of copper is toxic which alters chromatin structure, cytoplasmic damage, vacuolar compartment, protein synthesis, enzyme activity, photosynthesis, respiration and membrane damage (Vinit Dunand et al., 2002; Peng et al., 2005; Muccifora, 2008).

Conclusion

Due to high toxicity of heavy metals, spores of *P. tripartita* could not able to germinate on $HgCl_2$, $CdCl_2$ and Na_3AsO_4. $7H_2O$. But, the spores are having tolerance and capacity to grow in low concentration of metals in the culture medium. However, germinated spores did not show any anomaly or malformations under the influence of MS medium. But cultures with heavy metals, shrinkage and disruptions in chlorophyll pigment were observed.

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