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ORIGINAL ARTICLE

Effect of Heavy metal stress on spore germination of *Pteris confusa* T. G. Walker and *Pteris argyraea* T. Moore

Irudayaraj V., Johnson M.*, Priyakumari A.S., Janani Prabha A.

Department of Plant Biology and Plant Biotechnology, St. Xavier's College (Autonomous), Palayamkottai – 627 002, Tamil Nadu, India

Tel: + 91 97 86 92 43 34; Fax: + 91 462 2561 765

*E-mail: **ptcjohnson@gmail.com**

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Background: Plants have both constitutive and adaptive mechanisms for coping with the elevated metal concentrations and they are utilized to clean the polluted soil and water. Unlike angiosperms hyperaccumulators, fern hyperaccumulators are equipped with inherent biological characteristics that could be exploited in the phytoremediation strategies aimed at decontaminating polluted sites. Fern spores can be successfully used to screen the hyperaccumualting ferns and also to test the toxicity of the metal contaminated samples.

Purpose of the Study: In the present study, a preliminary attempt was made to compare the tolerance capacity of the spores of two ferns; *Pteris confusa* T. G. Walker and *Pteris argyraea* T. Moore against the heavy metal zinc (Zinc sulphate). Spores of the two ferns were cultured in Knop's liquid medium with various concentrations of zinc sulphate (0-200ppm).

Results: In the case of *P. confusa* normal germination was observed in control, 120 ppm and 140 ppm and the germination of spores were failed in 160, 180 and 200 ppm of zinc supplemented cultures. In contrary, *P. argyraea* showed maximum percentage of spore germination in 140 ppm zinc supplemented cultures and the control and 120 ppm zinc sulphate supplemented cultures were failed to show the germination. The germination percentage and growth rate was decreased in high concentration of zinc sulphate. Rhizoids are showed more tolerance to heavy metal than protonema of *P. argyraea*.

Conclusion: Difference in response of spores to the heavy metal zinc may be due the difference in the hyper-accumulating capacity of the ferns.

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Toxic metal pollution in water and soil is a major environmental problem and most conventional remediation approaches do not provide acceptable solutions. Phyto-remediation, popularly known as

"green clean" is a novel strategy for the removal of toxic contaminants from the environment by using plants. This concept is increasingly being adopted, as it is a cost effective and user friendly alternative to traditional methods of treatment. In India, aquatic vascular plants like *Hydrilla verticillata, Spirodela polyrrhiza, Bacopa monnierii, Phragmites karka* and *Scirpus lacustris* have been used to treat chromium contaminated effluent and sludge from leather tanning industries.

Tolerance of plants to grow in heavy metal contaminated soil is also an evolutionary process. Metal tolerance is one of the best examples of microevolution driven by anthropogenic activities. Humans, in attempt to make life better, have turned into a geophysical force and rapidly destroyed the integrity of the environment by polluting it with heavy metals. A rapid rate of metal pollution of the environment can be string force of selection causing rapid volutionary changes in organisms manifested as metal tolerance occurring over time scales as hundreds of years and even decades (Jules and Shaw, 1994). Plants have both constitutive and adaptive mechanisms for coping with the elevated metal concentrations and they are utilized to clean the polluted soil and water. Plants possess some characteristic features, which enable them to absorb from soil and water, heavy metals such as iron (Fe), manganese (Mn), copper (Cu), molybdenum (Mo) and nickel (Ni), which are essential for their growth and development. Plants also accumulate toxic metals, which may not have any biological function, these include: silver (Ag), cadmium (Cd), chromium (Cr), cobalt (Co), mercury (Hg), lead (Pb) and selenium (Se) etc. the over accumulating capacity of the some plants utilized to clean polluted soil. Unlike angiosperms hyper accumulators, fern hyper accumulators are equipped with inherent biological characteristics that could be exploited in the phytoremediation strategies aimed at decontaminating polluted sites. Fern spores are successfully used to screen the hyper accumulating ferns and also to test the toxicity of the metal contaminated samples. Chinese brake, *Pteris vitata*, exhibits considerable promise in the phytoremediation of arsenic-contaminated sites worldwide due to its unique ability of hyper accumulating arsenic. Currently research is involved in elucidating the physiological and molecular mechanism of arsenic hyper accumulation in Chinese brake. Since ferns have the characteristics of both primitive and land plants, an understanding of biological mechanism of hyper accumulation is necessary (Bondada and Qiying, 2003).

Western Ghats in India is rich in fern diversity; about 250 ferns are growing in variety of edaphic conditions. The tropical genus Pteris to which the Chinese brake fern belongs, is with about fifteen species on the Western Ghats (Manickam and Irudayaraj, 1992). The tropical genus Pteris to which the Chinese brake fern belongs, is with about fifteen species on the Western Ghats (Manickam and Irudayaraj, 1992). They are cytologically diversified (Manickam and Irudayaraj, 1988). The presence of cytological diversity which is the indication of genetical diversity provides opportunities for the biologist to find out the cytotype / genotype with more of hyper accumulating potentiality. It was also known that, like the sporophyte of the ferns, the gametophytes are also show the hyper accumulating capacity. Before the application of a plant in phyto-remediation program, it should be thoroughly checked up for its hyper accumulating capacity. Since ferns are with independent sporophytic and gametophytic generation, it is necessary to carry out such studies in both sporophytic and gametophytic generations. With this knowledge the present study was aimed to study the effect of heavy metal Zinc

on spore germination and growth of *Pteris confusa* T. G. Walker and *Pteris argyraea* T. Moore. Since Zinc is also a micronutrient and vast amount of fertilizer " Zinc sulphate" is used in Tea and Coffee estate leading to soil pollution, it has been selected for the present study. *P. confusa* and *P. argyraea* are common terrestrial ferns growing in and around the tea estate in high altitude of the Western Ghats. So these species have been selected to know the tolerance capacity of the spores during germination.

MATERIALS AND METHODS

Spores of *Pteris confusa* T. G. Walker and *Pteris argyraea* T. Moore were collected from the natural habitats at Kothayar Hills, Tirunelveli, Tamil Nadu, India. The collected spores were passed through nylon mesh (40 µm) to remove the sporangial wall materials and cleaned spores were collected and used for culture initiation. The pH of the medium was adjusted to 5.8 using pH meter with 1 N NaOH or 1 N HCl before autoclaving at 121°C for 15 min. Since the present study is to know the toxic effects of zinc on spore germination, the unsterilized spores were used as explants. For spore germination, the spores of *Pteris confusa* and *Pteris argyraea* were cultured on liquid Knop's medium supplemented with various concentration of Zinc sulphate (0, 120, 140, 160, 180 and 200 ppm). After the inoculation, the cultures were incubated at 25° ± 2°C under 70% relative humidity and 12 h photoperiod/day in a culture room. All the cultures were kept in 1200-1500 lux light intensity provided by cool white fluorescent tubes (Phillips India Ltd, Mumbai). After 15 days, spore samples were taken from the culture vials, mounted on a slide and examined under microscope for the presence of protonema and rhizoid and the development was observed carefully. All the micro-morphological developments were observed and recorded using the research microscope.

Table 1 Characteristics of Spore germination in *Pteris confusa* and *Pteris argyraea* in different concentration of Zinc sulphate

Conc. Zn So_4 in ppm	Mean % of Spore germination	Rhizoid (Present / Absent)	Protonema		
			Number of Cells	Uniseriate / Biseriate	Colour of the cells
Pteris confusa					
Control	45	Absent	10	Uniseriate	Dark green
120	25	Absent	6	Uniseriate	Dark green
140	18	Absent	9	Uniseriate	Pale green
160	0	Germination Nil			
180	0	Germination Nil			
200	0	Germination Nil			
Pteris argyraea					
Control	0	Germination Nil			
120	0	Germination Nil			
140	42	Present	11	Biseriate	Dark green
160	32	Present	3	Uniseriate	Dark green
180	28	Absent	2	Biseriate	Pale green
200	10	Present	Absent		

RESULTS

Both in *Pteris confusa* and *Pteris argyraea*, the spores are normal and brown in colour. They are trilete with minutely reticulate exine. The fern spore is unicellular with a solitary, usually centrally placed(s) nucleus surrounded by vacuolated cytolplasm in which are suspended chloroplastids or leucoplastids and food material, commonly in the form of oil globules. Spores started to germinate after ten days of inoculation. The spore germination is preceded by swelling of contents of spores by absorption of water, intine expands, but the exine is pushed open at the laesura. The germination was seen as the formation of pale green filaments in the liquid medium. At the initial stage the spores started to germinate by cracking the spore wall and the intine of the spores comes out as a germ tube. The germ tube continues to grow as single uniseriate filament. Each cell is filled with many chloroplasts. A series of cell division take place in a definite sequence resulting in the formation of the protonema. The sequence of cell divisions and differentiation leading on to development of the characteristics adult form of the prothallus from the unicellular spore varies among the ferns. Commonly on germination the spore produces a primary rhizoid followed by an elongated uniseriate germ filament. The spore germination is of vittaria type resulting in a uniseriate germ filament that develops a prothallial plate (Nayar and Kaur, 1970).

The responses of spores in Knop's medium supplemented with various concentrations of zinc sulphare were observed two times at the intervals of two weeks. At the initial stage there was an indication of spore germination by the change of colourless medium into light green colour medium by the formation of green protonema. There were differences in responses of spore germination in different concentrations of zinc sulphate in different species. The percentage of spore germination in both the species, in different concentration of zinc sulphate, after 15 days of culture, has been given in the Table -1(Fig. 1 A, B, D,E, G, H, K, N and Q).

In *Pteris confusa* spore germination was observed in the culture medium without zinc sulpahte and medium supplemented with 120 and 140 ppm of zinc sulphate. The Knop's medium supplemented with 160, 180 and 200 ppm zinc sulphate supplemented culture tubes were failed to show the germination (Fig. 1 K, N and Q). Highest percentage (45%) of spore germination was observed in the Knop's medium without zinc sulphate (Fig. 1 A). The percentage of spore germination in 120 and 140 ppm zinc supplemented cultures showed 29% and 18% respectively (Fig. 1 D,E, G and H). A reverse trend has been observed in spores of *Pteris argyraea*. Thus there was no germination of spores in the control and 120 ppm zinc sulphate supplemented spore cultures (Fig. 1 C and F). In all the cases where germination is present the protonema is with uniseriate filament only (Fig. 1. I, J, L, M, N, O, P and R). Multiseriate protonema was not observed even after 30 days. It indicates that the growth of the protonema is slow. Rhizoids are rare and they are long, slender and translucent. The protonema is with dark green cells in control and 120 ppm while they are green in 140 ppm zinc augmented cultures. Individual cells are longer and narrow.

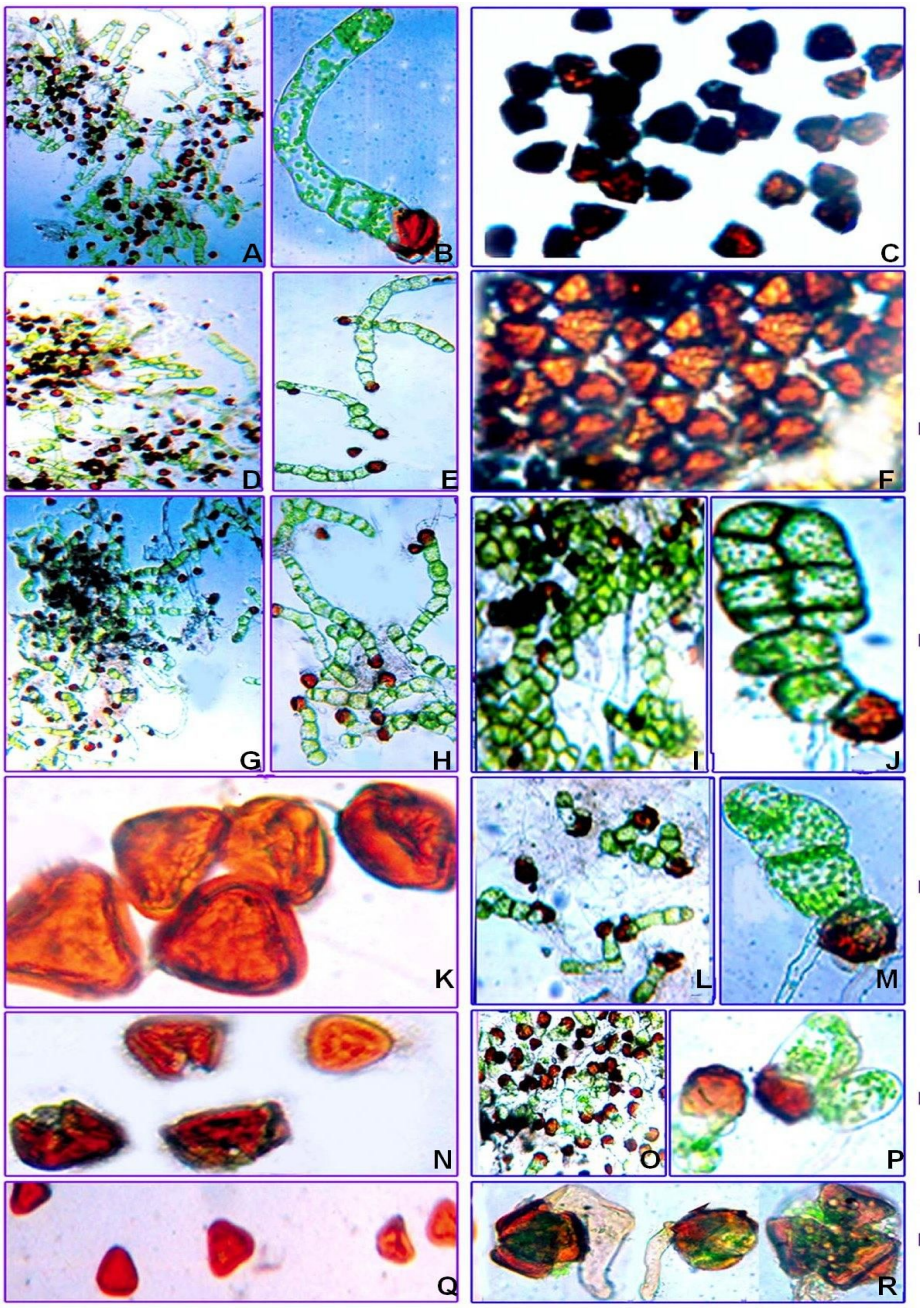

Figure 1. Effect of Heavy metal stress on spore germination of *Pteris confusa* T. G. Walker and *Pteris argyraea* T. Moore

A. P. confusa spores cultured on Knop's basal medium; B. P. confusa spores cultured on Knop's basal medium – filamentous stage; C. P. argyraea spores cultured on Knop's basal medium; D & E. P. confusa spores cultured on Knop's basal medium supplemented with 120 ppm of Zinc sulphate; F. P. argyraea spores cultured on Knop's basal medium supplemented with 120 ppm of Zinc sulphate; G & H. P. confusa spores cultured on Knop's basal medium supplemented with 140 ppm of Zinc sulphate; I & J. P. argyraea spores cultured on Knop's basal medium supplemented with 140 ppm of Zinc sulphate; K. P. confusa spores cultured on Knop's basal medium supplemented with 160 ppm of Zinc sulphate; L & M. P. argyraea spores cultured on Knop's basal medium supplemented with 160 ppm of Zinc sulphate; N. P. confusa spores cultured on Knop's basal medium supplemented with 180 ppm of Zinc sulphate; O & P. P. argyraea spores cultured on Knop's basal medium supplemented with 180 ppm of Zinc sulphate; Q. P. confusa spores cultured on Knop's basal medium supplemented with 200 ppm of Zinc sulphate; R. P. argyraea spores cultured on Knop's basal medium supplemented with 200 ppm of Zinc sulphate

DISSUSSION

The present observation shows that zinc sulphate from 160 ppm and above is lethal to the spores of *Pteris confusa* and the maximum germination in control without zinc sulphate shows the presence of endogenous zinc in spores of *Pteris confusa*. But in the related species *Pteris argyraea* spores do not germinate in Knop's basal medium and Knop's fortified with 120 ppm of zinc sulphate. The differences in germination of spore without zinc sulphate shows the presence of endogenous zinc in different amount in spores of different species, which in turn may depend upon the absorptive and storage capacity due to the indication for the presence of enough endogenous zinc in the sporophyte and spores they are able to germinate even without zinc in the external medium. In contrast spores of *P. argyraea* require external supply of zinc for germination due to the presence of low amount of endogenous zinc. The low adsorptive and storage capacity of *P. argyraea* is attributed to its morphology. It is well known that zinc deficiency causes chlorosis of the older leaves. In the case of *P. argyraea* the presence of broad white band along either side of the midrib of the pinnae is the salient feature in contrast to *P. confusa* in which the pinnae are uniformly green. So the total chlorophyll in the leaves of *P. argyraea* will be less when compared to the leaves of *P. confusa*. It is evidenced from the phytochemical study of three taxa of *Pteris* by Jesudass et al., (1993). Thus the total chlorophyll in the leaves of *P. argyraea* is only 1.97 mg/g DW in contrast to 2.52 mg/g DW in P. confusa. So less amount of zinc is enough for *P. argyraea* and they may absorb and store only required amount of zinc. During spore germination they need external supply of zinc. The amount of minerals such as potassium, calcium and sodium in the leaves is also higher in *P. confusa* than *P. argyraea*. Thus the foliar mineral content of potassium, calcium and sodium in *P. confusa* is 0.052, 0.428 and 0.05 respectively in contrast to 0.02, 0.374 and 0.04 in *P. argyraea* (Jesudass *et al.*, 1993). It is also substantiated that the plant with high nutritional status follows agamospory (Whittier and Steeves, 1960; Whittier, 1965; Sulkyan and Mehra, 1977; Von Aderkas, 1984). Thus the diploid *P. confusa* with high nutritional status follows agamosporous mode of reproduction in contrast to the diploid sexual *P. argyraea* (Manickam and Irudayaraj, 1992) with low nutritional status.

Protonemal growth and percent of spore germination of *Polytrichum commune* are used to assess the toxicities of heavy metals Cu, Cd and Zn. Synergistic toxic responses have been generated by the Cu: Cd and Cd:Zn a definite antagonistic response was obtained with spore germination, while an additive to slightly antagonistic response was generated with protonemal growth. Despite the comparability of the results obtained with both parameters, percent of spores germination is preferable for routine use as the data are generated rapidly and the response is easily scored (Francis and Petersen, 1989). In the present study only one heavy metal has been used to study the toxicity effect on spore germination. As concluded by Francis and Petersen (1989) it is easy and rapid method for toxicity study. In the present study the results were scored within thirty days by observing the spore germination.

Cadmium induced abnormal development of protonema in *Pteris vittata* (Gupta and Devi, 2005). In the present study the development of protonema was normal but slow. Kamachi et al., (2005) have observed that spore germination in *A. yokoscense* was more tolerant to lead (Pb), compared to that in other fern species, such as *Pteridium aquilinum*, *Lygodium japonicaum* and *P. vittata*. In addition, the

early gametophyte development of *A. yokoscense* was not much affected by 10 M Pb^{2+}, as evaluates from the prothallial growth and rhizoid development. *Athyrium* gametophytes could accumulate more than 10000 µg of lead, and that lead was localized in the cytosol and vacuole of rhizoidal cells, as determined by a transmission electron micrograph (Kamachi et al., 2005). These results indicate that in *Athyrium* gametophytes the rhizoid has the ability to tolerate more than the protonema and they accumulate lead in the rhizoids. In the present study also the rhizoids are more tolerant to the heavy metal zinc. Even in 200 ppm of zinc sulphate rhizoids develop but there was no development of protonema. Yoshihara et al., (2004) have shown that the Cd tolerance of *Athyrium yokoscense* fern is basically independent of the plant parts and the developmental stages, although the accumulation ability is higher in roots than in the other plant parts. In the present study also in the case of *P. argyraea* the rhizoids are more tolerable than the protonema.

Gametophytes of *P. vittata* hyperacuuumulate arsenic in a similar manner to that in the sporophyte. Gametophytes are able to grow normally in medium containing 20mM arsenate and accumulate 2.5% of their dry weight as As. Gametophytes of the related non-accumulating fern *Ceratopteris richardii*, die at even low (0.1mM) arsenic concentrations. Interestingly, gametophytes of the related arsenic accumulator *Pityrogramma calomelanos* appear to tolerate and accumulate arsenic to intermediate levels compared to *P. vittata* and *C. richardii*. There is considerable genetic variation I abilities of various species to tolerate otherwise toxic amounts of non-essential lead, cadmium, silver, aluminum, mercury, tin and other metals (Woolhouse, 1983). Profile and analysis of gene expression changes during the early development in germinating spores of *C. richardii* show that specific genes are likely to be critical for the germination and subsequent early development of diverse cells and tissues emerging from dormancy (Salmi et al., 2005). Plants have both constitutive and adaptive mechanisms for coping with the elevated metal concentrations (Mehrag, 1994). The adaptive mechanism differs from species to species. In the present study in concentration of zinc sulphate from 160 and 200 ppm the spores of *P. confusa* were failed to germinate. But in the related species of *P. argyraea*, there is spore germination in all the concentrations from140 ppm at least by the formation of rhizoid.

The germination of spores of *P. confusa* in the control without zinc shows the presence of endogenous zinc in the spores in enough amounts. The rare occurrence of rhizoids in the germinated spores and absence of germination in 160, 180 and 200 ppm of zinc sulphate show that the gametophytes of *P. confusa* is less tolerant to the heavy metal zinc. But the sporophytes of *P. confusa* seem to be more tolerable with the indication for the presence of endogenous zinc.

CONCLUSION

The present study along with previous studies shows that ferns in general are good phytoremediators and by testing the response of spores in medium containing heavy metals, one can screen the potential phytoremediator in a simple way more effectively and quickly. From the present study it has also been concluded that to test the tolerance capacity of plant or animal species one should take in to consideration not only the external concentration of the toxic substance, but also the amount present already within the organism.

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