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Studies in *Atrichum pallidum* and *Campylopus subfragilis* subjected to Cu and Cd toxicity for evaluation of their role in pollution monitoring

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Abstract: **Present study was focused on to appraise the peroxidase activity and phenolic content in two mosses *Atrichum pallidum* and *Campylopus subfragilis* under the different concentrations of two heavy metals Copper and Cadmium for 10, 20 and 30 days. The preferred concentrations for the experiment were 100ppm to 400ppm of Copper and 5ppm to 20ppm of Cadmium. Both the mosses showed significant increase in peroxidase activity and phenolic content when treated with different concentrations of Cadmium and Copper. *C. subfragilis* showed better survival (increased peroxidase activity) with Cu and the maximum increase was 0.61±0.01Kat/sec/mg/protein in 300ppm till 30th day as compared to *A. pallidum* in which maximum increase was 0.31±0.05Kat/sec/mg/protein in 300ppm till 20th day. During the analysis of phenols *Campylopus subfragilis* under Cu stress the maximum increase in content was 28±0.07mg/g/fw in 400ppm on 20th day whereas *A. pallidum* showed maximum increase up to 9.23±0.005 mg/g/fw in 400ppm on 10th day.**

Under Cd stress, *C. subfragilis* showed maximum increase in peroxidase activity in 10ppm as 0.48±0.004 Kat/sec/mg/protein on 10th day and in *A. pallidum* as 0.215±0.03 Kat/sec/mg/protein in 5ppm on 10th day, whereas the maximum increase in phenolic content in *C. subfragilis* in 10ppm was 34±0.03 mg/g/fw on 30th day and in *A. pallidum* was7.8±0.004 mg/g/fw in 5ppm on 20th day. After the maximum increase in activity as well as content both the taxa showed gradual decline. During AAS analysis the net content of absorbed concentration of Cu in *C. subfragilis* was 142.1ppm and in *A. pallidum* was 219.6ppm, and of Cd was 0.80ppm in *C. subfragilis* and 0.87ppm in *A. pallidum*. The regeneration was much better in *C. subfragilis* as compared to *A. pallidum*. All these observations led to conclusion that *C. subfragilis* survived better in both the metals (Cu or Cd) as it has absorbed less content of Cu or Cd as compared to *A. pallidum*. Hence *A. pallidum* can be used as a good bio-monitor (due to more content absorbed of Cu or Cd) as well as bio-indicator (due to its sensitive response to metal stress) of heavy metal pollution.

Keywords: **Mosses *Campylopus subfragilis*, *Atrichum pallidum*, Phytotoxic metals, Copper, Cadium, enzyme peroxidase, secondary metabolite phenols, Impact, AAS.**

1. INTRODUCTION

Copper is the metal released from burning fuel, leakage of engine oils and corrosion of batteries. It is most abundant in mafic, intermediate and carbonate rocks. Being essential micro-nutrient it does not risk the environment. But its higher concentrations become toxic and inhibit the growth of the plant by damaging various cellular processes such as respiration and photosynthesis (Marscher, 1995, Prasad and Strzalka, 1999).

On the other hand, Cadmium is an eco-toxic metal and exhibits highly adverse effects on the health of humans, soil biological activity and plant metabolism. The main source of Cadmium is the Ni/Cd batteries and automobile industries. Anthropogenic accumulations of cadmium in the environment is a matter of concern in the industrialised countries (Kabata-Pendias, 2001).

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Few moss species are considered to be very sensitive to heavy metal pollution as they show noticeable marks of injuries on their plant body, whereas some are known to accumulate heavy metals without any noticeable marks of injuries (Martensson and Berggreu[8], 1954).

Bryophytes seem to have evolutionary background to tolerate environmental stress, though very little is known about physiological responses and oxidative stress damages in bryophytes during heavy metal pollution (Panda, 2003).

Mosses have earlier been used in metal pollution monitoring studies, due to their extensive surface volume ratio, very simple anatomy, absence of cuticle and ectohydric nature, all these characters help them to hold on the metals (Samecka-Cymerman *et al*., 2002; Zechmeister *et al*., 2003; Sun *et al*., 2007). The process of absorption of pollutants by the mosses takes place either from the atmosphere by crude trapping, through ion exchange or by the solutes present in substrate through capillary or both (Kapila *et al*., 2005).

As we know metals are non-degradable and after they get released into the ecosystem they get inherent to that ecosystem. And this intrusion of heavy metals in the ecosystem leads to variations in the environment by changing the physiological responses of the community (Saxena and Afreen, 2009). Bryophytes are considered to be more tolerant to heavy metals in comparison to vascular plants (Govindapyari, 2010).

The impact of two heavy metals Cu and Cd on the antioxidant, photosynthetic activity and productivity of moss *Racomitrium crispulum* was studied by Saxena and Afreen (2009). They concluded that higher concentrations for prolonged period create an impact on oxidative enzymes as well as on photosynthesis.

The major advantage of the heavy metal absorption by mosses is possibly the cleanest and economical method in the remediation of selected hazardous areas.

Present study was focused on to appraise the impact of the different applied concentrations of the two metals Copper or Cadmium on the activity of peroxidase and the content of phenols in two acrocarpic mosses *Atrichum pallidum* and *Campylopus subfragilis* and to investigate the potential of both the mosses to absorb these metals.

2. MATERIALS AND METHODS

The studied moss taxa along with their substrata were collected from non-polluted sites of Haripurdham (Distt. Sirmaur) and Dalhausie (Distt. Chamba). The specimens are deposited in herbarium of Department of Botany, Panjab University, Chandigarh (PAN).

The plant material was separated from the substratum, washed several times with water and then observed under binoculars to remove any other plant material and attached soil particles. Then these plants were grown in clean petri-dishes and sprayed with 2 ml of different concentrations of Cu or Cd after every fifth day. To make the extract of metal treated plants, the mosses were washed with double distilled water and then air dried under room temperature. Peroxidase activity was determined by methodology given by Egley *et al.* (1983) and phenolic content according to Swain and Hillis (1959).

The net content of Copper and Cadmium absorbed by these two mosses was detected by using AAS technique.

3. RESULTS

Table 1: Response of peroxidase activity in *C. subfragilis* and *A. pallidum* when treated with different concentrations of Copper and Cadmium (Figs. 1, 2):

Name of Taxon	Locality and substratum	Herbarium Reference No.
Campylopus subfragilis	Haripurdham (Distt. Sirmaur), on soil gathered on rocks.	PAN 6318
Atrichum pallidum	Dalhausie (Distt. Chamba), on soil gathered on rocks.	PAN 6321

Cu: *C. subfragilis* with the external applications of Cu (100, 200, 300, 400ppm) for 10, 20 and 30 days respectively showed significant increase in the peroxidase activity as compared to control (0.45±0.002 Kat/sec/mg/protein) up to 400ppm (0.56±0.22 Kat/sec/mg/protein) till 10th day, up to 300ppm (0.62±0.1 Kat/sec/mg/protein) till 20th day and up to 200ppm (0.61±0.001 Kat/sec/mg/protein) till 30th day. Whereas *A. pallidum* showed increase in peroxidase activity up to 400ppm (0.3±0.04 Kat/sec/mg/protein) on 10th day, up to 300ppm (0.31±0.05 Kat/sec/mg/protein) till 20th day but the increase was minor. After that, decline in activity in all the concentrations was noticed on 30th day (Fig. 1).

It becomes evident that *C. subfragilis* has better capacity to survive in extended period of Cu stress as compared to *A. pallidum*.

Cd: *C. subfragilis* when subjected to external applications of Cd stress (5, 10, 15, 20ppm) the activity increased up to 10ppm (0.48±0.004 Kat/sec/mg/protein) on 10th day as compared to control (0.02±0.002 Kat/sec/mg/protein). The activity started to decline gradually and reached to (0.01±0.1 Kat/sec/mg/protein) in 20ppm on 30th day.

On the other hand, in case of *A. pallidum* increase was noticed as compared to control (0.2±0.02 Kat/sec/mg/protein) only in 5ppm (0.215±0.03 Kat/sec/mg/protein) on 10th day and after that gradual decrease in all the concentrations was observed till 30th day (Fig. 2).

These observations indicate that Cd is toxic even at low concentrations and even *C. subfragilis* has shown less survival in this case.

Response of phenols in *C. subfragilis* and *A. pallidum* when treated with different concentrations of Copper and Cadmium (Figs. 3, 4):

Cu: When *C. subfragilis* subjected to varied concentrations of Cu, signicant increase in phenolic content was observed up to 400ppm (21.9±0.009 mg/g/fw on 10th day, 28±0.07 mg/g/fw on 20th day). In 300ppm Cu, (24±0.02 mg/g/fw) increase was observed till 30th day as compared to control (19.6±0.002 mg/g/fw).Whereas *A. pallidum* showed lesser increase in phenolic content i.e. in 400ppm (9.23±0.005 mg/g/fw) till 10th day, in 300ppm (8.2±0.003 mg/g/fw) till 20th day and in 200ppm (7.8±0.004 mg/g/fw) till 30th day as compared to control (5.4±0.004 mg/g/fw) (Fig. 3).

Cd: Under Cd stress *C. subfragilis* showed increase in phenolic content up to 20ppm (23.2±0.06 mg/g/fw on 10th day, 23.7±0.01 mg/g/fw on 20th day) till 20th day and up to 15ppm (22.8±0.001 mg/g/fw) till 30th day as compared to control (19.6±0.002 mg/g/fw). *A. pallidum* showed increased phenolic content (6.4±0.003 mg/g/fw) in 15ppm till 10th day, (7.8±0.004 mg/g/fw) in 5ppm till 20th day and no increase was noticed after this. The content decreased gradually in all the concentrations on 30th day as compared to control (Fig 4).

These observations showed that *C. subfragilis* has survived better under stress of Cu or Cd and has shown better results in peroxidase activity as well as phenolic than the *A. pallidum*. In addition to this, it has also been noticed that both the peroxidase and phenols (Antioxidants) increased simultaneously under stress but the decrease in phenols was delayed as compared to peroxidase.

Uptake of Cu by *C. subfragilis* and *A. pallidum* when exposed to varied concentrations 100, 200, 300 and 400ppm (Fig. 5):

The content of Copper found in control plant of *C. subfragilis* was 6.95 ppm. On exposure of Cu in 100 ppm, the absorbed content was 20.51 ppm on 10th day, 29.08 ppm on 20th day and 41.00 ppm on 30th day. Under 200 ppm exposure the absorbed content of Cu increased to 40.11 ppm on 10th day, 71.50 ppm on 20th day and 83.51 ppm on 30th day. In case of 300 ppm the absorbed content was 60.06 ppm on 10th day, 101.60 ppm on 20th day and 116.10 ppm on 30th day. In case of 400 ppm the absorbed content of Cu was maximum i.e. 77.13 ppm on 10th day, 127.5 ppm on 20th day and 142.1 ppm on 30th day.

The content of Copper found in control in *A. pallidum* was 6.99 ppm. In case of 100 ppm of Cu exposure the absorbed content was 42.52 ppm on 10th day, 57.07 ppm on 20th day and 62.00 ppm on 30th day. In case of 200 ppm the absorbed content increased to 80.06 ppm on 10th day, 95.13 ppm on 20th day and 104.5 ppm on 30th day, in case of 300 ppm the absorbed content was 97.11 ppm on 10th day, 149.03 ppm on 20th day and 183.0 ppm on 30th day, whereas in case of 400 ppm the content absorbed was highest i.e. 114.5 ppm on 10th day, 200 ppm on 20th day and 219.6 ppm on 30th day.

Uptake of Cd by *C. subfragilis* and *A. pallidum* when exposed to varied concentrations 5, 10, 15 and 20ppm (Fig. 6):

The content of Cadmium found in *A. pallidum* control (plant body) was 0.45 ppm. In case of 5 ppm the absorbed content of Cd was 0.66 ppm on 10th day, 0.68 ppm on 20th day and 0.71 ppm on 30th day. Under 10 ppm of Cd exposure the absorbed content of Cd was 0.70 ppm on 10th day, 0.73 ppm on 20th day and 0.77 ppm on 30th day, under 15 ppm the absorbed content was 0.74 ppm on 10th day, 0.78 ppm on 20th day and 0.82 ppm on 30th day, and rising to 0.77 ppm on 10th day, 0.82 ppm on 20th day and 0.87 ppm on 30th day in 20ppm exposure.

The content of Cadmium found in *C. subfragilis* control (plant body) was 0.35 ppm. In case of 5 ppm the absorbed content of Cd was 0.61 ppm on 10^{th} day, 0.64 ppm on 20^{th} day and 0.68 ppm on 30^{th} day. With 10 ppm exposure the absorbed content increased to 0.65 ppm on 10^{th} day, 0.70 ppm on 20^{th} day and 0.74 ppm on 30^{th} day. In case of 15 ppm the absorbed content was 0.69 ppm on 10^{th} day, 0.72 ppm on 20^{th} day and 0.78 ppm on 30^{th} day, finally under 20 ppm the content absorbed was 0.73 ppm on 10^{th} day, 0.77 ppm on 20^{th} day and 0.80 ppm on 30^{th} day.

The obtained data show that *A. pallidum* absorbed more content of Cu and Cd as compared to *C. subfragilis.* In both the species, uptake of Cu increased with increase in number of days of exposure. Cd uptake did not increase significantly with prolonged exposure (Figs. 5,6). Due to much absorbed content of Cu or Cd, *A. pallidum* has shown poor regeneration.

Regeneration (Figs. 7, 8, 9):

Under Cu stress: *C. subfragilis* showed the regeneration in all the concentrations of Cu (100-400ppm) till 25^{th} day (Fig. 7). *A. pallidum* also showed regeneration in all the concentrations of Cu but till 20^{th} day (Fig. 9).

Under Cd stress: *C. subfragilis* showed regeneration in all the concentrations till 20^{th} day (Fig. 8), whereas *A. pallidum* showed regeneration only in 5ppm till 15^{th} day (Fig. 9).

Statistical analysis: t-test and paired t-test, One way ANOVA and Post hoc test (Dunnett test) for multiple comparison were applied to compare the control with each concentration at 10^{th}, 20^{th} and 30^{th} day (significans value < .05=significant).

4. DISCUSSION

The increased peroxidase activity and phenolic content in both the presently studied moss taxa under Cu or Cd stress as compared to control might be due to the characteristic feature of mosses to sustain in metal toxicity for prolonged periods. Earlier, Dazy *et al*. (2009) reported that the aquatic moss *Fontinalis antipyretica* treated with various heavy metals (Cd, Cu, Pb and Zn) at different concentrations 0, 0.1, 1, 10, 100 and 1000µM showed an increase in lipid peroxidation and enzymatic activity as compared to control. The antioxidant defense process which activates a great number of peroxidase enzymes inhibits ROS by decomposing hydrogen peroxide, a toxic intermediate produced during metal stress and protects the plants against the stress (Reddy *et al*., 2005). It has been found that both peroxidases and phenols are interlinked and play important role during stress conditions. As the number of peroxidases increase, the phenols also increase, especially flavonoids and phenylopropanoids, and are oxidized by enzyme peroxidase, which may act as H_2O_2- scavenging or phenolics/POX system. It has also been noticed during the studies that fall of phenolic content was delayed as compared to peroxidase activity and this was due to the capacity of phenols to resist the ROS even after the decrease of enzymatic activity. Like peroxidases, phenols also act as antioxidants due to their capacity to chelate the metals. Hydroxyl groups may lead to inactivation of iron ions by chelating and by suppressing the superoxide-driven Fenton reaction, considered to be the most important source of ROS (Michalak, 2006).

Presently, the uptake of Cu as well as Cd was more in *A. pallidum* as compared to *C. subfragilis*. This may be due to the broad leaf surface area, fast cation exchange mechanism and capacity to hold the metal ions by *A. pallidum* for longer period. It has also been observed that both the moss taxa survived to good extent under the stress of Cu as compared to that of Cd. The uptake of Cd created more toxicity in both the taxa as compared to Cu since Cd is a non-essential micro-element and is more toxic even at lower concentrations and leading to death of the plant. The changes in the plant colour were not apparent with low concentrations of Cu up to 10-20 days, but prolonged exposure led to change in colour from green to pale yellow to brown.The change in colour was prompt in case of Cd. The change in the chlorophyll content in plants exposed to higher concentrations of heavy metals is considered as first visible symptom for photosynthetic damage in plant tissues (Doganlar*et al*., 2012). Both the moss taxa showed good regeneration under all the concentrations of Cu up to 25 days as compared to Cd. This may be due to the fact that Cu is an essential micro-nutrient and Cd is toxic even at lower concentrations. But higher concentrations of both the metals for prolonged periods are toxic in both the moss taxa. Inhibition of protonemal growth and bud formation in two species of *Bryum bicolour* and *B. argenteum* exposed to Cd, Cu and Pb were observed by Saini (1995). Retardation in growth (Gilbert, 1969) and photosynthesis (Sommer and Winkler,1982) of many mosses have been recorded during prolonged exposer to metal stress. Seregin *et al*. (2001) reported various metals lead to 50% inhibition of enzymatic activities in the following molar concentrations Ag^{+}, Cu^{2+} (10^{-7} to 10^{-5}) > Cd^{2+} (10^{-6} to 3×10^{-5}) > Zn^{2+} (10^{-5} to 10^{-4}) > Pb^{2+} (10^{-5} to 2×10^{-4}) > Ni^{2+}(10^{-5} to 6×10^{-4}) > Co^{2+}(2×10^{-4} to 3×10^{-4}).

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Presently observed sensitive response and high capacity of *A. pallidum* to accumulate the metals make it a good bio-indicator as well as bio-monitor, and *C. subfragilis* a bio-monitor and phytoremediator due to its potential to survive under prolonged stress conditions of toxic concentrations of metal pollution.

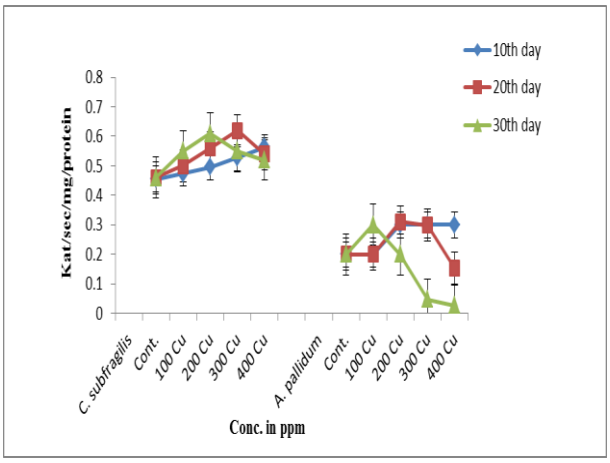

Fig 1: *C. subfragilis* and *A. pallidum* showing the response of peroxidase activity when subjected to Cu stress.

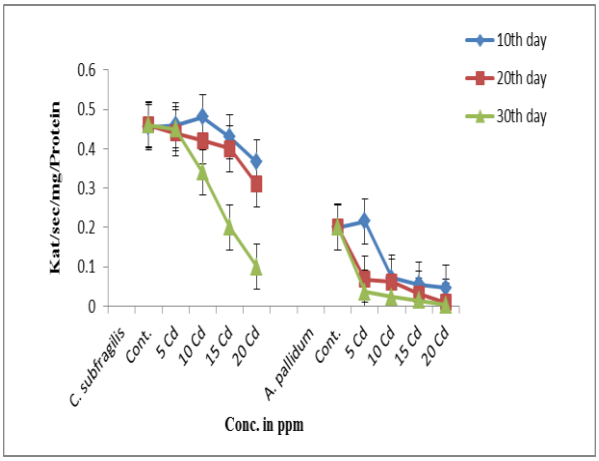

Fig 2: *C. subfragilis* and *A. pallidum* showing the response of peroxidase activity when subjected to Cd stress.

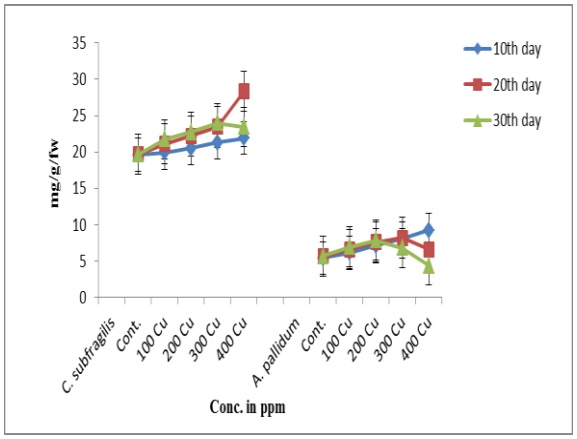

Fig 3: *C. subfragilis* and *A. pallidum* showing the response of phenols when subjected to Cu stress.

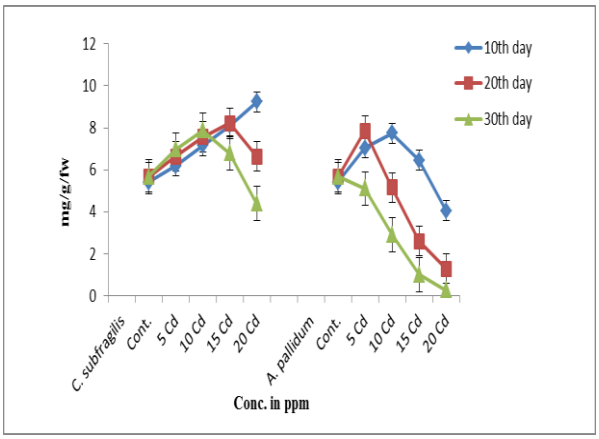

Fig 4: *C. subfragilis* and *A. pallidum* showing the response of phenols when subjected to Cd stress.

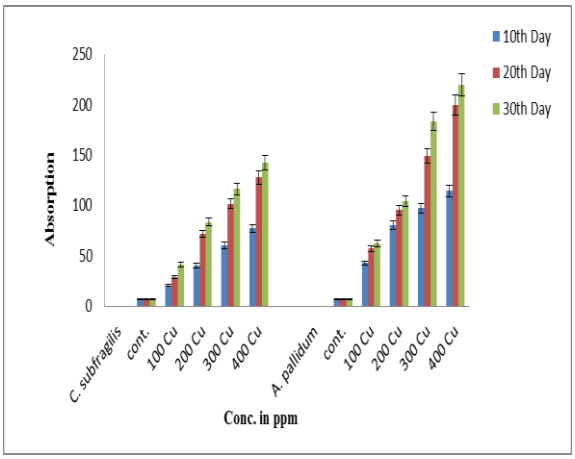

Fig 5: Absorption of varied concentrations of Copper by *C. subfragilis* and *A. pallidum*.

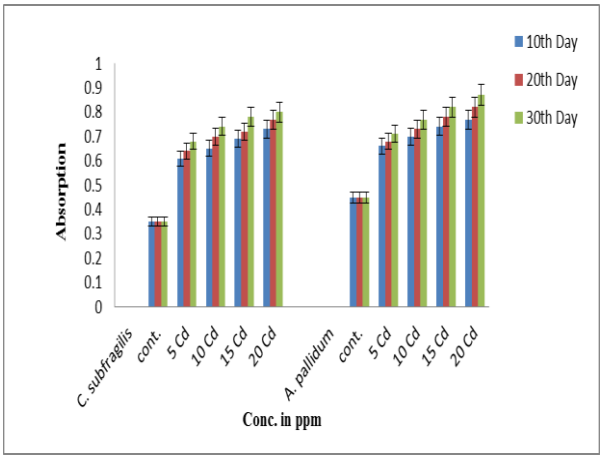

Fig 6: Absorption of varied concentrations of Cadmiumby *C. subfragilis* and *A. pallidum*.

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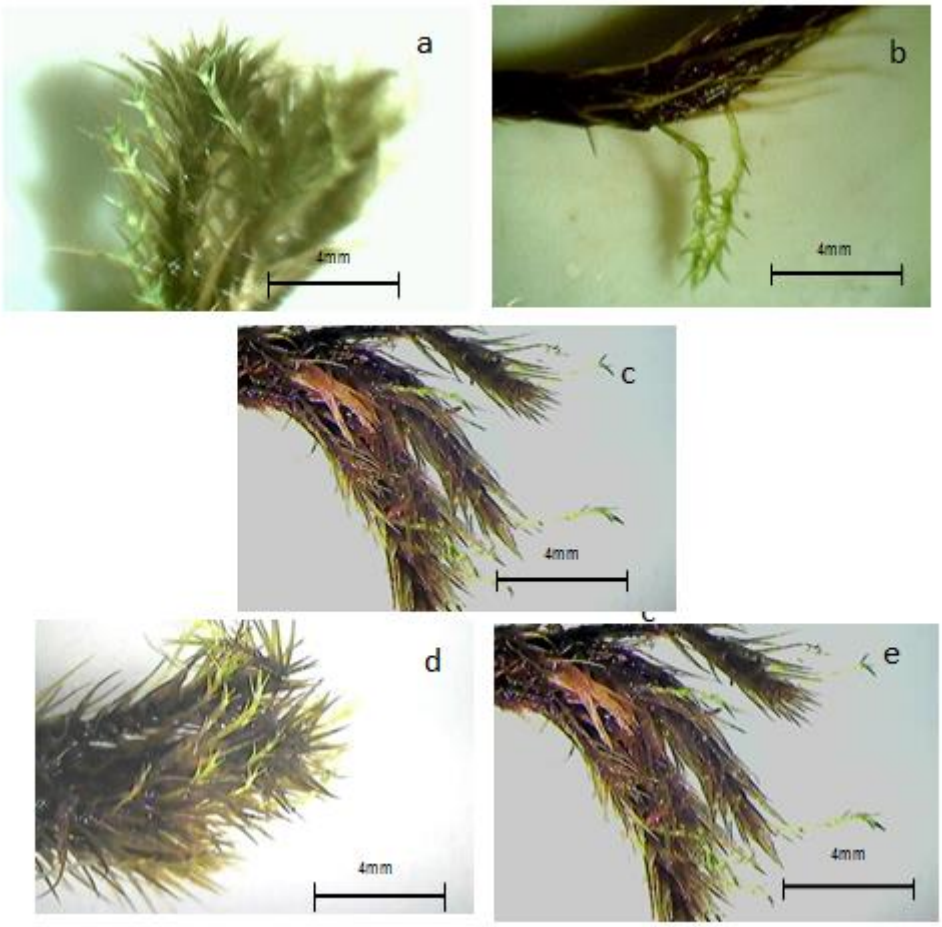

Fig 7: *C. subfragilis* during prolonged exposure to varied concentrations of Cu showing the regenerants: (a) Control, (b) 100ppm, (c) 200ppm, (d) 300ppm, (e) 400ppm.

Fig 8: *C. subfragilis* during prolonged exposure to varied concentrations of Cd showing the regenerants: (a) 5ppm, (b) 10ppm, (c) 15ppm, (d) 20ppm.

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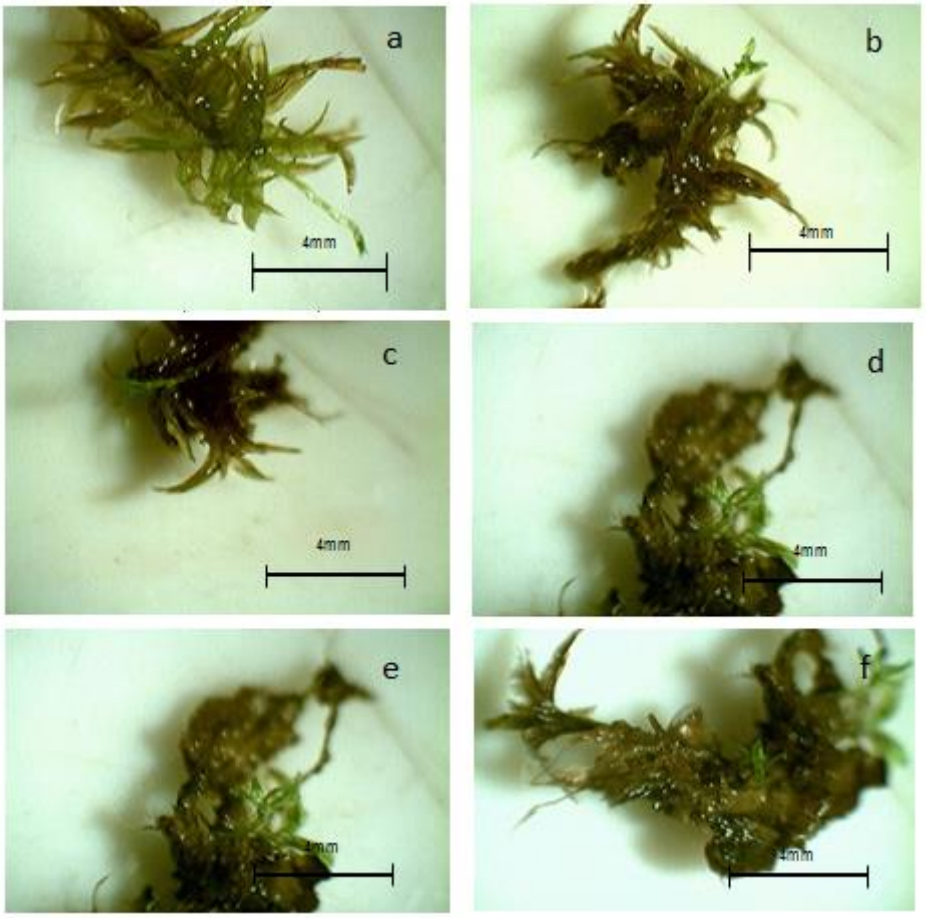

Fig 9: *A. pallidum* during prolonged exposure to varied concentrations of Cu showing the regenerants: (a) Control, (b) 100ppm, (c) 200ppm, (d) 300ppm, (e) 400ppm and (f) under exposure to 5ppm of Cd.

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