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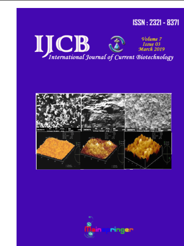

Studies on Effect of Chromium on Biochemicals and Protein Profiles in *Amanita Muscaria* and *Laccaria Ohiensis*

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A B S T R A C T

The cultures of *Amanita muscaria* and *Laccaria ohiensis* were grown in different concentrations of Cr (0.5, 1.0, 1.5, 2.0 and 2.5 M). The effects of Cr in all the physio-chemical and biochemical activities such as biomass, total soluble sugars, total amino acids, total phenolic content, total lipids intracellular and extracellular protein content, polypeptide changes, intracellular and extracellular enzyme activity (acid phosphatase and alkaline phosphatase) *Amanita muscaria* showed higher activities than *Laccaria Oheinsis*

Introduction

Heavy metal pollution in ecosystems has long been considered as a serious public and environmental concern. Due to the modern industrial activities such as mining, smelting, and creation of synthetic compounds, has caused an exponential accumulation of heavy metals into the environment. This accumulated heavy metal causes a serious threating to human health and biotic ecosystem. Among the different kinds of heavy metals copper (cu), chromium (cr), Lead (Pb), mercury (Hg), and cadmium (Cd) are common heavy metal toxins causes oxidative stress related various chronic diseases in human beings. Most essential and non-essential metals exhibit toxicity above a certain concentration, which will vary depending on the organisms physico-Chemical properties of the metal and environmental factors (Gadd, 1993). Pollution of the biosphere with toxic metals due to man-made activities poses a major environmental and human health problem. Acidification of forest soil produced by atmospheric pollution may increase the availability of heavy in some soils (Xiang 1998). Burning of fossil fuels, mining and smelling of metalliferous ores, municipal wastes, industrial effluents, fertilizers, pesticides has rich sources of heavy metals; it causes cytotoxic, carcinogenic and mutagenic.

Chromium is abundantly present in all the parts of environment including air, water and soil. Chromium and its derived compounds are having very high toxicological potential. Chromium often occurs in the trivalent (+3, chromic) or hexavalent (+6, chromate) states under natural environmental conditions. It was predominantly used in the electroplating industry as anticorrosive and antibio-foulent agents and also in steel production. Acute systemic poisoning can result from high exposure to hexavalent chromium (Ulmanu *et al*., 2003). A recent epidemiological studies strongly points to Cr (VI) as the agent of carcinogenesis. Studies have shown that chronic exposure of Cr (VI) leads to respiratory cancer in human being. It also shown that subcutaneous administration of chromium can cause oxidative damage, genotoxic effect and alteration of antioxidants s status in experimental animals. It can also cause chromosomal abnormalities, DNA strand breaks, DNA fragmentation and oxidative stress in human liver cancer cells. Therefore industries using or manufacturing chromium chemicals must provide suitable control on such operations. (Ilo, 1983).

Before consuming a mushroom proper identification of the target mushroom is must. Literature on taxonomy and identification of Amanita mushrooms which can be referred for correct identification: (Abraham and Kachroo, 1989; Bhatt and Bhatt, 1996; Bhatt and Lakhanpal, 1988; 1989; Bhatt et al., 1989; 1999; 2003; 2007, 2017). New technologies are needed to address the eradication of heavy metal accumulation in the environment. In the last three decade, phytoremediation has more attention and is offering an efficient way to reclaim the heavy metal contamination. Rhizofiltration is a subset technique of

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phytoremediation which refers to the method of using fungi biomass for removing contaminants, primarily toxic metals, from polluted water (Chaudhry*et al*., 1998). It is gaining popularity in reducing metal load in the contaminated medium as it is eco-friendly less destructive to soil biota and cheaper. Extensive research studies revealed that, heavy metals are directly and indirectly involved in fungal growth metabolism. Davies *et al*. (2002) revealed that arbuscular mycorrhizal fungi increase the chromium uptake in sunflower plant. Another in vitro study suggests that the co-incubation of mycorrhizal fungi with pepper plant were increase the Cr tolerance and modifying amino acid metabolism. It was reported that mycorrhizal fungi increase the chlorophyll content and biochemical composition in maize leaves. However there are no scientific literatures are not found the beneficial phytoremediation role of ectomycorrhizal fungi against chromium mediated stress. The aim of the present work this research is to investigate the effect of different concentrations of chromium on the ectomycorrhizal fungi of *Amanita muscaria* and *Laccaria ohiensis*. The changes in the biomass, total soluble sugars, total amino acids, total phenolic content, total lipids and alkaline phosphatase activity due to the stress are to be recorded. Special attention will be paid to the separate the total soluble protein content of these fungi, using gel electrophoresis.

Materials and Methods

General laboratory techniques recommended by Purvis *et al*.(1966), Tuite (1969) and Booth (1971) were followed for the preparation of media, inoculation and maintenance of cultures. Heavy metal, chromium (Cr) was selected for the study, Different concentration of Chromium viz., 0.5, 1.0, 1.5, 2.0 and 2.5 µM were filter sterilized and added to the medium aseptically. Harvests were made at 15, 30 and 45 day intervals. On 15, 30 and 45 day incubation, the mycelia duft was transferred to a pre-weighed, pre-dried filter paper and washed repeatedly with distilled water to make it free from any trace of adherent medium and dried in an oven at 80°C to constant weight for 24 h.

Extraction of fungal mycelium

Amanita muscari and *Laccaria Oheinsis (*Fungal mycelium),harvest were made at 15,30 and 45 from various concentration of Chromium viz., 0.5, 1.0, 1.5, 2.0 and 2.5 µM 5 g were cut into small pieces (1-2 cm), plugged in boiling ethyl alcohol for 10 min. It was cooled and homogenized for 5-10 min in a glass mortar and pestle, the homogenate was filtered through cheese cloth and the residue was re-extracted with 2-3 mL of 80% alcohol. The content of total soluble sugars, total amino acids, total phenolic content and total lipids were estimated from the alcohol extract.

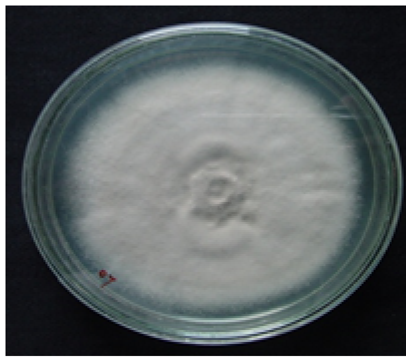
Biochemical changes

The total soluble sugars were estimated by according to the method of Mahadevan and Sridhar (1996). Alcohol interferes with colour development during anthrone-sugar reaction; hence alcohol was evaporated by using hot water bath. The residue was dissolved in distilled water and the final volume was adjusted to 1 mL for every 100 mg of fungal mycelium. The amount of total soluble sugars in the extract was estimated by anthrone method. Amino acid content was estimated by according to the method of Mahadevan and Sridhar (1996) The alcohol extract, 1 mL was pipette out into a clean test tube, to which a drop of methyl red indicator was added and the sample was neutralized with 0.1N NaOH. 1 mL of ninhydrin reagent was added, mixed thoroughly and a glass marble was placed on top of each tube. The tube with the content was heated for 20 min in boiling water. Blanks with 1 mL of distilled water were maintained. The tubes were removed from water bath and cooled under a running tap water. The contents were mixed thoroughly, until it turns into purple colour. The contents ware mode upto 5 mL with diluting solution and the absorbance was measured at 750 nm. The amount of total amino acids present was calculated using a standard curve prepared from glutamic acid.. The total phenolic content was estimated by (Alhakmani*et al*., 2013) One mL of the alcohol extract was pipette out into a graduated test tube, 1mL of Folin-Ciocatteu (Folin-Phenol) reagent was added followed by 2 mL of 20% Sodium Bicarbonate. The tubes were shaken well and heated in boiling water both for 1 min and cooled under running tap water. The volume was made upto 25 mL with distilled water and the colour intensity was read at 650 nm in Beckman Du-40 Spectrophotometer, blank was prepared with reagents and alcohol. The total phenolic content was calculated by using a standard graph made of catechol.

The total lipid content was estimated by the method described by Barnes and Blackstock, (1973). In a test tube, 20 µL of the extract and 0.5mL of H_2SO_4 were mixed well and kept in a boiling water bath for 10 min. 5 mL of Phosphovanillin reagent was added and left for 30 min. The colour intensity was read at 520 nm in a spectrophotometer and quantified with cholesterol standard.

The total soluble proteins were extracted by Raorane*et al*., (2016) Protein was extracted in 800 µL of extraction buffer. Samples were maintained at 0ºC for 10 min and equal volume of water saturated phenol was added. The solution was mixed well and maintained for 1 h at 4ºC and centrifuged at 14,000 × g for 15 min at 4ºC. The phenol layer was washed trice with equal volume of extraction buffer and cooled. The protein was precipitated overnight at -20°C by adding 800 µL of 0.1 M ammonium acetate in methanol to 200 µL of the phenol layer. The sample was centrifuged for 10 min at 14,000 × g and the precipitate was washed twice with 400 µL of methanol and once with 200 µL acetone. The pellet was solubilized in Laemmli's buffer (Laemmli, 1970) for SDS-PAGE. and the phenolic content was extracted by the method of Burgess *et al*., (1995).

The protein content was estimated by the method of Bradford, (1976). The protein extract (0.5 mL) was precipitated with equal volume of 10% Trichloro acetic acid for overnight at 4ºC. The mixture was centrifuged at 14,000 × g for 20 min. the pellet was dissolved in 1 mL of 0.1N NaOH and 5 mL of Coomassie Brilliant Blue solution. After incubation of 30 min the OD value was read at 595 nm. The protein content was measured by using BSA standard curve. And phosphatase enzymes, acid phosphatase and alkaline phosphatase activity was estimated by the method of Prokicka, (1992). The crude enzyme fraction/culture filtrate was used for determination of total acid phosphatase activity. The medium contained 0.4 mL of 15mM p nitrophenyl phosphate in 0.01 M acetate buffer and 0.2 mL of crude enzyme and the volume of reaction mixture was made upto 3.0 mL with distilled water. It was incubated at 35ºC for 30 min and adding 1.0 mL of 0.1 M NaOH stopped the reaction. Absorbance of free p-nitrophenol was determined at 410 nm in VIS Spectrophotometer (BeckmanDU-40). The enzyme activity was expressed in terms of µmoles mg^{-1} protein/min.



Ammanita muscaria

Laccaria ohiensis

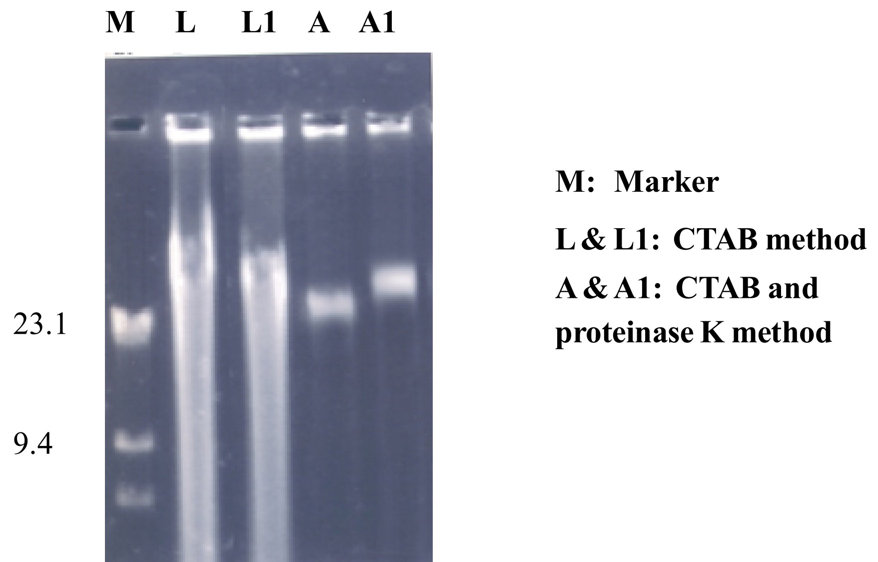


Figure 1: Extraction of DNA from *A. muscaria* and *L. ohiensis*

Fig.2. Changes in total phenolic content in *A. muscaria* and *L. ohiensis* grown with different concentrations of Cr at different day intervals.

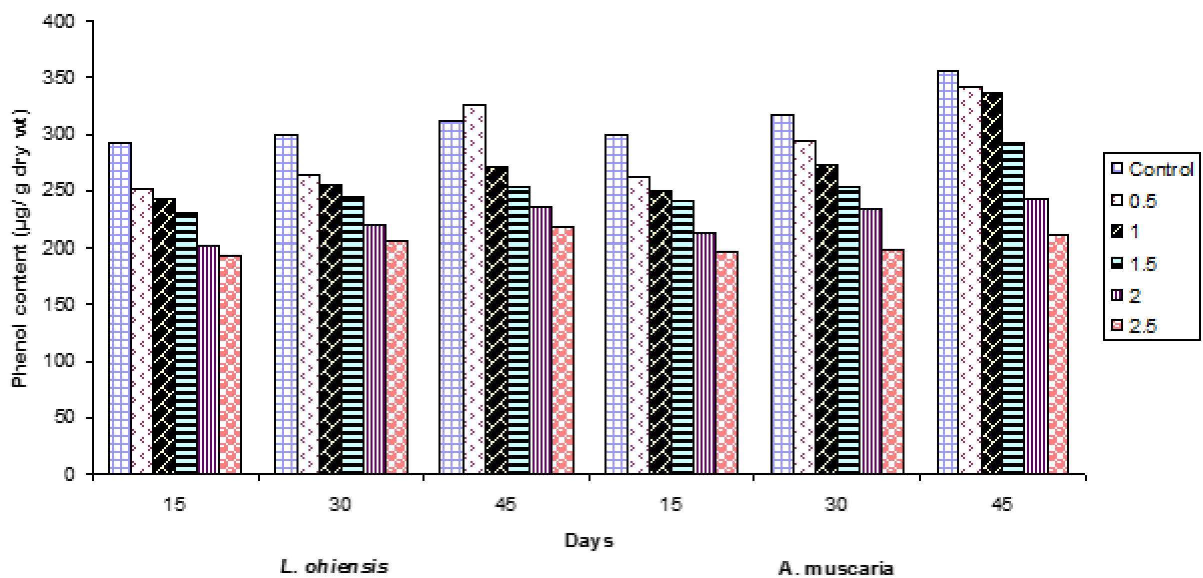


Table 1. Changes in biomass of *L. ohiensis* and *A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc.of Cr (µM)	Dry weight of the mycelium (g)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	0.72	1.32	1.53	0.81	1.39	1.55
0.5	**0.84**	**1.44**	**1.62**	**0.93**	**1.46**	**1.63**
1.0	0.42	1.06	1.41	0.76	1.31	1.51
1.5	0.31	0.93	1.01	0.61	1.23	1.36
2.0	0.26	0.81	0.94	0.32	0.91	1.15
2.5	0.21	0.43	0.81	0.21	0.66	1.01

Table 2. Changes in total soluble sugars of *L. ohiensi s*and *A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc. of Cr (µM)	Total soluble sugars (µg / g dry wt.)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	288	410	386	296	421	391
0.5	291	401	375	310	431	385
1.0	315	431	366	315	451	387
1.5	318	440	333	304	420	351
2.0	215	386	318	281	405	325
2.5	205	372	301	246	386	311

Table 3. Changes in total amino acids of *L. ohiensis* and *A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc. of Cr (µM)	Total amino acids (µg / g dry wt.)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	943	1212	1336	961	1240	1341
0.5	1012	1248	1276	1051	1251	1360
1.0	1119	1261	1281	1129	1272	1382
1.5	1124	1276	1293	1136	1281	1390
2.0	915	1121	1130	1008	1112	1116
2.5	856	1008	1091	941	1003	1102

Table 4. Changes in total phenolic content of *L. ohiensis*and*A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc. of Cr (µM)	Total phenolic content (µg / g dry wt.)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	292	298	312	299	316	356
0.5	251	263	326	262	294	341
1.0	242	254	271	250	272	337
1.5	230	245	252	241	253	292
2.0	202	219	236	212	233	243
2.5	193	205	218	197	199	210

Table 5. Changes in total lipids of *L. ohiensi s*and *A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc. of Cr (µM)	Total lipids (µg / g dry wt.)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	130	143	136	141	179	151
0.5	124	136	128	139	143	142
1.0	133	153	134	143	199	169
1.5	128	129	131	130	156	121
2.0	104	109	105	121	132	115
2.5	93	101	99	115	122	99

Table 6. Changes in intracellular protein content of *L. ohiensis* and *A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc. of Cr (µM)	Intracellular protein content (µg / g dry wt.)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	86	93	101	87	97	112
0.5	73	69	72	79	72	86
1.0	76	64	71	83	71	77
1.5	79	61	64	85	76	65
2.0	74	56	62	73	64	59
2.5	69	41	43	56	53	41

Table 7. Changes in extracellular protein content of *L. ohiensis* and*A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc. of Cr (µM)	Extracellular protein content (µg / mL culture filtrate)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	33	42	55	39	49	56
0.5	37	49	59	43	54	73
1.0	41	51	61	47	59	75
1.5	46	54	63	52	66	81
2.0	38	43	48	48	51	83
2.5	34	38	41	37	42	63

Table 8. Changes in intracellular acid phosphatase activity of *L. ohiensis* and *A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc.of Cr (µM)	Intracellular acid phosphatase activity (µmoles PNP released / mg protein / min)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	296	141	131	221	199	142
0.5	318	288	291	386	272	298
1.0	386	293	299	392	269	299
1.5	295	215	242	398	241	262
2.0	293	208	219	346	218	131
2.5	105	104	91	172	96	119

Table 9. Changes in extracellular acid phosphatase activity of *L. ohiensis* and *A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc.of Cr (µM)	Extracellular acid phosphatase activity (µmoles PNP released / mg protein / min)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	327	421	489	329	434	483
0.5	412	586	593	416	587	594
1.0	405	493	499	412	515	513
1.5	386	405	412	391	420	466
2.0	341	372	387	352	375	396
2.5	291	296	342	305	315	361

Table 10. Changes in intracellular alkaline phosphatase activity of *L. ohiensis* and *A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc. of Cr (µM)	Intracellular alkaline phosphatase activity (µmoles PNP released / mg protein / min)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	79	64	59	87	72	64
0.5	83	73	70	84	75	72
1.0	96	92	85	99	94	86
1.5	91	83	79	104	93	84
2.0	74	72	68	82	86	83
2.5	69	61	43	73	79	70

Table 11. Changes in extracellular alkaline phosphatase activity of *L. ohiensis*and*A.muscaria* grown with different concentrations of Cr at different day intervals.

Conc. of Cr (µM)	Extracellular alkaline phosphatase activity (µmoles PNP released / mg protein / min)					
	L. ohiensis			*A. muscaria*		
	15 d	**30 d**	**45 d**	**15 d**	**30 d**	**45 d**
Control	41	43	45	43	47	49
0.5	44	49	50	46	55	56
1.0	51	56	62	52	61	68
1.5	52	58	65	56	66	69
2.0	56	61	69	58	69	73
2.5	59	64	71	61	72	76

RESULTS AND DISCUSSION

Changes in Biomass

Mycelial dry weight of *A.muscaria* and *L.ohiensis* were presented in Table 1. The mycelial dry weight was decreased in all the concentrations of Cr with increasing day intervals except at 0.5 µM, when compared to control. In *A. muscaria,* the maximum dry weight (0.93, 1.46 and 1.63 g) was observed in 0.5 µM concentration and the minimum dry weight (0.21, 0.66 and 1.01 g) was noticed in 2.5 µM concentration of Cr when compared to control (0.81, 1.39 and 1.55 g). on the other hand, The maximum dry weight (0.84, 1.44 and 1.62 g) was noticed in 0.5 µM concentration and the minimum dry weight (0.21, 0.43 and 0.81 g) was noticed in 2.5 µM concentration of Cr when compared to control (0.72, 1.32 and 1.53 g) in *L.ohiensis*. The mycelial dry weight of *A. muscaria*was higher than the *L ohiensis*.

Changes in Biochemical content

Total soluble sugars

The effect of different concentrations of Cr on total soluble sugars content of *A. muscaria* and *L. ohiensis* were shown in Table 2. The total soluble sugars content significantly increased in lower concentrations of Cr whereas significantly decreased in higher concentrations when compared to control. On the 15th day of *A. muscaria*, the maximum total soluble sugars content (304 µg/g) was noticed in 1.5 µM concentration and minimum content (246µg/g) was found in 2.5 µM concentration of Cr when compared to control (296 µg/g). On 30th day, the higher amount of total soluble sugar content (420 µg/g) was observed in 1.5 µM concentration whereas lower content (386 µg/g) was found in 2.5 µM concentration when compared to control (421 µg/g). However, the total soluble sugars content gradually decreased in the increasing concentrations of Cr when compared to control. Nevertheless, the maximum total soluble sugars content (318 µg/g dry wt. Of mycelium) was noticed in 1.5 µM concentrations of Cr and minimum content (205 µg/g) was found in 2.5 µM concentration when compared to control (288 µg/g) in 15th day of treatment of *L. ohiensis*. On 30th day, higher total soluble sugar content (440 µg/g) was observed in 1.5 µM concentration whereas lower content (372 µg/g) was found in 2.5 µM concentration when compared to control (410 µg/g). On 45th day, the total soluble sugars content gradually decreased in the increasing concentrations of Cr when compared to control. The total soluble sugars higher content in *A. muscaria.*

Total amino acids

The changes in total amino acids content of *A. muscaria* and*L. ohiensis* are presented in Table 3. In Cr amended medium, the total amino acids content increased in lower concentrations and decreased at higher concentrations in all the day intervals. With increasing day intervals, the total amino acids content increased in all the treatments (Fig. 5). The maximum total amino acids content (1136 µg/g) was noticed in 1.5 µM concentrations and minimum content (941 µg/g) was found in 2.5 µM concentration when compared to control (961 µg/g) in 15th day of *A. muscaria* treatment. On 30th day, the higher amount of total amino acids content (1281 µg/g) was observed in 1.5 µM concentration whereas lower content (1003 µg/g) was found in 2.5 µM concentration when compared to control (1240 µg/g) and 45th day of treatment, 1.5 µM concentration of Cr showed the maximum amino acid content (1390 µg/g) and the 2.5 µM concentration of Cr showed the minimum content (1102 µg/g) when compared to control (1341 µg/g). However, in *L. ohiensis* treatment, the maximum total amino acids content (1124 µg/g) was noticed in 1.5 µM concentration and the minimum content (856 µg/g) was found in 2.5 µM concentration when compared to control (943 µg/g). In 30th day, the higher amount of total amino acids content (1276 µg/g) was observed in 1.5 µM concentration whereas lower content (1008 µg/g) was found in 2.5 µM concentrations when compared to control (1212 µg/g). On 45th day, 1.5 µM concentration of Cr showed the maximum total amino acids content (1293 µg/g) and the 2.5 µM concentration of Cr showed the maximum content (1091µg/g) when compared to control (1336). The total amino acids content of *A. muscaria* was higher than the *L. ohiensis*.

Total phenolic content

The total phenolic content of *A. muscaria*and*L. ohiensis*are presented in Table 4 (Fig. 6). The total phenolic content decreased in all the concentrations when compared to untreated control in different time intervals. It shows that, the increasing time intervals leads to increase in phenolic content. The maximum total phenolic content (356 µg/g) was observed in control on 45 d, and the minimum content (197 µg/g) was found in 2.5 µM concentration of Cr on 15th day of *A. muscaria*treatment. On the other hand, the maximum total phenolic content was observed in control (312 µg/g) on 45 d, and the minimum content (193µg/g) was found in 2.5 µM concentration on 15th day of *L. ohiensis*treatment. The total phenolic content of *A. muscaria* was higher when compared to *L. ohiensis*.

Total lipids

The total lipids content of *A. muscaria* and *L. ohiensis* are presented in Table 5 (Fig. 7). The total lipids content decreased in all the concentrations of Cr except 1.0 µM concentration in all the day intervals when compared to control. On 30th day of *A. muscaria*treatment, the maximum total lipids content (199 µg/g) was found in 1.0 µM concentration and the minimum content (99 µg/g) was observed in 2.5 µM concentrations on 45th day. However On 30th day of *L. ohiensis*, the maximum total lipids content (153 µg/g) was found in 1.0 µM concentration and the minimum content (99 µg/g) was observed in 2.5 µM concentrations on 45th day. The total lipids content of *A. muscaria*was higher than the *L. ohiensis*.

Intracellular protein content

The intracellular protein of *A. muscaria*and*L.ohiensis*are presented in Table 6. The intracellular protein content increased in lower concentration of Cr whereas decreased in higher concentrations. With increasing day intervals, the intracellular protein decreased in all the treatments when compare to control (Fig. 8). In *A. muscaria*treatment, the maximum intracellular protein content (87 mg/g) was observed in control whereas minimum intracellular protein content (56 µg/g) was observed in 2.5 µM concentration on 15th day. On 30th day, the intracellular protein content decreased in the fungus grown in all the concentrations of Cr when compared to 15th day. The intracellular protein content highly reduced in all other concentrations on 45th day when compared to 15 and 30th day. The least protein content (41µg/g) was observed in 2.5 µM concentration of Cr on 45th day. However, in *L. ohiensis*, the maximum intracellular protein content (86 mg/g) was found in control whereas increased concentration of the Cr (2.5 µM) showed minimum protein (69µg/g) on 15th day. On 30th day, the intracellular protein content decreased in the fungus grown in all the concentrations of Cr when compared to 15th day. The intracellular protein

content highly reduced in all other concentrations on 45 d. The lowest protein content (41μg/g) was noticed in 2.5 μM concentration of Cr on 45th day. The intracellular protein content of *L. ohiensis*was higher when compared to *A. muscaria*.

Extracellular protein content

The extracellular protein content of mycelium of *A. muscaria*and*L. ohiensis*were grown in different concentrations of Cr are presented in Table 7 (Fig. 9). The extracellular protein content increased with increasing concentrations of Cr in all the day intervals when compared to control. In *A. muscaria*treatment; on 15th day, the higher amount of extracellular protein content (52 μg/mL) was observed in 1.5 μM followed by 2.0 (48μg/g), 1.0 (47μg/g), 0.5 (43μg/g) and 2.5 (37μg/g). Lower content (39μg/g) was found in untreated control. On 30th day of treatment, maximum extracellular protein content (66 μg/mL) was found in 1.5 μM concentration followed by 2.0 (51μg/g), 1.0 (59μg/g), 0.5 (54μg/g) and 2.5 (42μg/g). Lower content (49μg/g) was found in control. Similarly 45th day of treatment, the maximum extracellular protein content (81 μg/mL) was found in 1.5 μM and the lower content was found in control (56μg/g). Nevertheless, the maximum extracellular protein content (46 μg/mL culture filtrate) was noticed in 1.5 μM concentration it was followed by 1.0 (41μg/g), 2.0 (38μg/g) 0.5 (37μg/g) and 2.5 (34μg/g) on 15 d and the lower content was found in untreated control (33μg/g). On 30th day, 1.5 μM concentration of Cr showed higher extracellular protein content (54 μg/mL), followed by 1.0 (54), 0.5 (49μg/g), 2.0 (43μg/g) and 2.5 (38μg/g). Lower content (42μg/g) was observed in control. Subsequently On 45th day of treatment, the higher amount of extracellular protein content (63 μg/mL culture filtrate) was found in 1.5 μM concentration followed by 1.0 (61μg/g), 0.5 (59μg/g) 2.0 (48μg/g), and 2.5 (41μg/g). Lower content was noticed in control (55μg/g). In all the day intervals, the higher concentration of Cr (2.5 μM) showed lesser amount of extracellular protein content when compared to all other concentrations.

Polypeptide profiles

*A. muscaria*and*L. ohiensis*were grown in 1.5 μM concentrations of Cr showed more number of polypeptides when compared to control in both staining methods. Comparatively, silver staining method showed additional number of polypeptide bands when compared to CBB staining method. One similar polypeptide band was present in both the fungi with the molecular weight of 156.9 KDa in silver staining method (Fig. 10).

Intracellular acid phosphatase

The effect of different concentrations of Cr on intracellular acid phosphatase activity of *A. muscaria*and*L. ohiensis*are presented in Table 8 (Fig. 12). The intracellular acid phosphatase activity increased in lower concentrations of Cr and it decreased in higher concentrations when compared to control. The activity decreased with increasing day intervals. The maximum intracellular acid phosphatase activity (392 μmoles PNP released/mg protein/min) was noticed in the fungus grown in 1.0 μM concentration of Cr on 15th day and the minimum activity (119 μmoles PNP released/mg protein/min) was observed in 2.5 μM on 45th day of *A. muscaria* treatment. On the other hand, the fungus grown in 1.0 μM concentration of Cr showed the maximum intracellular acid phosphatase activity (386 μmoles PNP released/mg protein/min) on 15th day and the minimum activity (91 μmoles PNP released/mg protein/min) was observed in 2.5 μM on 45th day. The intracellular acid phosphatase activity of *A. muscaria*was higher than the *L. ohiensis*.

Extracellular acid phosphatase

The effect of different concentrations of Cr on extracellular acid phosphatase activity of *A. muscaria*and*L.ohiensis*are presented in Table 9. The extracellular acid phosphatase increased in the fungus grown in all the concentrations of Cr except in higher concentration (2.5 μM) when compared to control. With increasing day intervals, the activity was also increased (Fig. 13). The maximum extracellular acid phosphatase activity (594 μmoles PNP released/mg protein/min) was noticed in the fungus grown in 0.5 μM concentration of Cr on 45 d. Minimum activity (305) was observed in 2.5 μM concentration of Cr on 15 day of *A. muscaria*. The maximum extracellular acid phosphatase activity (593 μmoles PNP released/mg protein/min) was noticed in the fungus growth in 0.5 μM concentration of Cr on 45 d. Minimum activities (291) was observed in 2.5 μM concentration of Cr on 15 day of *L. ohiensis*. The extracellular acid phosphatase activity of *L. ohiensis* was higher than the *A. muscaria*.

Intracellular alkaline phosphatase

The intracellular alkaline phosphatase activity of *A. muscaria* and *L. ohiensis* were grown in different concentrations of Cr are presented in Table 10 (Fig. 14). The intracellular alkaline phosphatase activity increased up to 1.0 μM concentrations of Cr when compared to control over that concentration it was decreased. Maximum alkaline phosphatase activity (99, 94 and 86 μmoles PNP released/mg protein/min) was observed in 1.0 μM concentration of Cr in 15, 30 and 45 d respectively. Fungus grown in 2.5 μM concentration showed minimum activity (73, 79 and 70) in 15, 30 and 45 d, respectively when compared to control (87, 72 and 64) of *A. mascara*. The intracellular alkaline activity increased up to 1.5μM concentrations of Cr when compared to control. Increased alkaline phosphatase activity (96, 92 and 85 μmoles PNP released/mg protein/min) was observed in 1.0 μM concentration of Cr in 15, 30, 45 d, respectively. Fungus grown in 2.5 μM concentration showed minimum activity (69, 61 and 43) in 15, 30, 45 day, respectively when compared to control (79, 64 and 59) of L. *ohiensis* treatment. The intracellular alkaline phosphatase activity of *L. ohiensis* was higher than the *A. muscaria.*

Extracellular alkaline phosphatase

The effect of different concentrations of Cr on extracellular alkaline phosphatase activity of *A. muscaria* and *L. ohiensi s*are presented in Table 11 (Fig. 15). The activity was increased with increasing concentration of Cr in all the day intervals except 0.5 μM concentration of Cr on 30th day in *A. muscaria*. Significantly increased alkaline phosphatase activity (61 μmoles PNP released/mg protein/min) was found in 2.5 μM concentration of Cr on 15 d. It was followed by 2.0 (58), 1.5 (56), 1.0 (52) and 0.5 μM (46) and lesser activity was found in control (43) were observed in *A. muscaria*. On 30th day, more activity (72 μmoles PNP released/mg protein/min) was found in 2.5 μM concentration followed by 2.0 (69), 1.5 (66), 1.0 (61) and lesser activity (55) was found in 0.5 μM concentration when compared to control (47). However, increased activity (76 μmoles PNP released/mg protein/min) was found in 2.5 μM concentration of Cr on 45 d followed by 2.0 (73), 1.5 (69), 1.0 (68) and 0.5 (56) and control showed significantly low activity (49). In *L. ohiensis*treatment, the level of alkaline phosphatase activity (59 μmoles PNP released/mg protein/min) was found in 2.5 μM on 15 d. It was followed by 2.0 (56), 1.5

(52), 1.0 (51) and 0.5 µM (44) and lesser activity was found in control (41). On 30th day, more activity (64 µmoles PNP released/mg protein/min) was found in 2.5 µM concentration followed by 2.0 (61), 1.5 (58), 1.0 (56) and 0.5 (49) and lesser activity was found in control (49). Successively, 45th day of treatment, 2.5 µM concentration showed the maximum activity (71 µmoles PNP released/mg protein/min) followed by 2.0 (69), 1.5 (65), 1.0 (62) and 0.5 (50) and control showed significantly low activity (51). The extracellular alkaline phosphatase activity of *A. muscaria*was higher than the *L. ohiensis.*

Discussion

Metal uptake and accumulation in the mycelium of ectomycorrhizal fungi have been demonstrated (Colpaert and Van Assche, 1992). Ectomycorrhizal fungi can also solubilize the minerals, including metal containing rock phosphates, by the production of organic acids. In this way, these fungi may increase the availability of the metals in the rhizosphere (Leyvel*et al*., 1997).

In the present study, the ectomycorrhizal fungi of *Amanita muscaria* and *Laccaria ohiensis* showed decreased growth in all the concentrations of Cr except 0.5µM when compared with control in all the day intervals. This is in support of the observations made by Xiang (1995) who observed that the mycelial growth of *A. muscaria*was strongly inhibited by higher concentration of Cu (5-25 mg 1-1) and also Raman *et al*. (2002) reported that the increased dry matter production of *L. laccata* and *Suillus bovinus*. It also proved that *L. laccata* has sensitive to Cu and Al (Cr. Jones and Meuhlchen, 1994).

It was found that, the total soluble sugars content of *A. muscaria* and *L. ohiensis* was significantly increased in lower concentrations of Cr whereas decreased in higher concentrations. Blaudez*et al*. (2000a) explored that ectomycorrhizal fungi were utilize the Cd for its energy metabolism, it might be affect glucose utilization. In another study reported that Cd might be inhibit the carbohydrate metabolism, it leads to reduce in polymeric content of the *Cusnnighamellablakesleeana* grown in Cu indicating Cu interfered with the metabolism of cell wall carbohydrates.Due to its storaging property, the total amino acids level of *A. muscaria*and*L. ohiensis*was increased in all the concentration of Cr with increasing day intervals. In axenic culture can influence utilization of amino acids (Anderson *et al*., 2001). Nehls*et al*. (1999) found that amino acids permease from *A. muscaria* act an efficient transporting vehicle of amino acids.

Voluminous of research findings show's that ectomycorrhizal fungi might be degrade the components of plant cell walls, and might be provide access to mineral nutrients complexes with cell wall components. In this study, due to thepolyphenol oxidase activities, total phenolic content of *A. muscaria* and *L. Ohiensis* decreased in all the concentration of Cr. Bending and Read, (1996a, 1996b) has been shows that the depolymerising phenolic compounds was reacted with organic nitrogen and phosphorus compounds and/or detoxifying the soil environment.

In the present study, it has been noticed that the total lipids content decreased in all the concentrations of Cr except 1.0 µM concentration of A. muscari and *L. ohiensis*. Similar findings was observed by monensin and El-zawahry *et al*., (1997). Higher concentration of Al and Ni (Raman *et al*., 1998) reduced the protein content in mycorrhizal fungi. In the present study also, protein content in *A. muscaria* decreased in higher concentration of Cd in the substrate. The metal binding protein such as metal thiolate clusters and protein bound sulphides were found in *Pisolithustinctorius*. In *A. muscaria,* the protein content increased in Cu than Ni treatment. The tolerance of *A. muscaria*to Cu may be due to metal binding protein. This was confirmed by the reports of where *Laccaria laccata* and *Suillusbovinus* growing in Cr amended medium were found to have increased protein content in lower concentrations whereas it decreased in higher concentrations (Raman *et al*. 1998).

In the present study, as revealed by SDS-PAGE, the number of polypeptides increased in *A. muscaria* and*L. Ohiensis* were grown in 1.5 µM concentration of Cr when compared to control. A protein whose biosynthesis is specifically up or down-regulated is identified in several ectomycorrhizal associations. In vitro a system has been documented the simultaneous of symbiosis-regulated acidic 30-35 KDa polypeptides (SRAP) and the appearance of 32 KDa during the interaction of P. tinctorius with E. globulus and E. grandis (Hilbert *et al*., 1991; Burgess *et al*., 1995). Hydrophobin like proteins have been recently detected in the ectomycorrhizal basidiomycetes *Amanita muscaria* (Nelhs, 1998) and *Paxillus involutus* (Chalot, 1999), suggesting that this protein family could be widespread in ectomycorrhizal fungi.

Turnau *et al*. (1995) reported that the phosphatase activity of *A. muscaria* played an important role in heavy metal detoxification. In the present study, intracellular acid and alkaline phosphatase activity of *A. muscaria*and*L. Ohiensis* grown in Cr amended medium enhanced in 15 day and declined in 30 and 45 d when compared with control. In contrast, extracellular acid and alkaline phosphatase activity of *A. muscaria*and*L. ohiensis*were increased in all the concentrations of Cr on 45 d when compared to 15 and 30 day intervals. High acid phosphatase activity and tolerance to high concentrations of Cu and Ni were reported in *Laccarialaccata* (Periasamy and Raman, 1995) and *A. muscaria*(Raman *et al*., 1998). Raman *et al*. (2002) have reported acid phosphatase activity was increased in the increasing concentrations of Cr in L. *laccata*and *Suillusbovinus*whereas in *A. muscaria,* the activity of acid phosphatase declined as Mn concentration was higher (Xiang, 1995).

Turnau and Dexheimer (1995) shown that increased acid phosphatase activity in *P. arrhizus* in the presence of Cr dust. The same line of observation described by Macaskie and Dean (1984) in *Citrobacter* spp. Successful reforestation of acid mine spoil, heavy metal metal polluted soils and area affected by acid precipitation may be enhanced through the introduction of *A. muscaria*(Raman *et al*., 1998).The phosphatase activities in *Rhizopogon* and *Piloderma* bicolor were much lower. While alkaline phosphatase activity of both intra and extracellular was very scanty, the weak phosphatase activity of fungus may account for ineffective phosphorus uptake in such circumstances (Ho and Zak, 1979).In *A. muscaria*and*L. ohiensis*are having maximum phosphatase activity and tolerance to high concentrations of Cr. These fungi are potential in the usage for reclamation of polluted soils. However, further work on the Cr accumulation in plants associated with *A. muscaria* and*L. ohiensis*are needed before field application.

Conclusion

Comparatively, in all the physio-chemical and biochemical activities such as biomass, total soluble sugars, total amino acids, total phenolic content, total lipids intracellular and extracellular protein content, polypeptide changes, intracellular and extracellular enzyme activity (acid phosphatase and alkaline phosphatase) *Amanita muscaria* showed higher activities than *Laccaria oheinsis*.

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