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Vol. 9(12), pp. 855-862, 25 March, 2015
DOI: 10.5897/AJMR2014.7250
Article Number: 52DCE3851837
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Selenium stress in *Ganoderma lucidum*: A scanning electron microscopy appraisal

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Received 5 November, 2014; Accepted 13 March, 2015

The present investigation explores the effect of selenium supplementation on radial growth and ultrastructural alterations in the hyphae and spores of *Ganoderma lucidum*. A concentration dependent decrease in radial growth was observed on culturing *G. lucidum* on sodium selenate supplemented mushroom minimal agar post 24 h. However, in comparison to control, selenium supplementation slightly increased diameter of radial growth till 10 ppm after 48 h. The Scanning Electron Microscopy (SEM) studies of radial growth showed selenium concentration dependent gradual decrease in hyphal diameter suggesting thinning and extensive branching of mycelia in response to increased Se supplementation. Moreover, SE micrographs also depict selenium concentration dependent decrease in number of spores and spore size. A significant decrease in spore diameter was recorded for mushroom minimal agar supplemented with 20 and 25 ppm (5.64 and 1.26 µm, respectively) as compared to control (10.04 µm). The spore were morphologically altered from spherical to oval or oblong and inflated to constrict deformed on increasing selenium concentration. SEM-energy dispersive X-ray spectroscopy (SEM-EDS) studies of intact mycelia showed no traces of selenium. However, crushed mycelia samples exhibited Se signals probably due to presence of selenium as integral component of cytosolic moieties like selenoproteins. Atomic absorption spectroscopy (AAS) of mycelia showed an increasing trend in the uptake of selenium with increased selenium supplementation. Percentage absorption was found to be in range of 7.2 - 9.9% with maximum absorption at concentrations of 15 and 25 ppm. Hence, sodium selenate supplementation at 10 ppm (with maximum 15 ppm) can be used for Se fortification as *Ganoderma* can grow rapidly without significant alteration in structure and morphology to enhance its biomedicinal properties.

Key words: *Ganoderma*, scanning electron microscopy (SEM), SEM-energy dispersive X-ray spectroscopy (SEM-EDS), selenium, selenoproteins.

INTRODUCTION

Medicinal mushrooms such as *Ganoderma*, *Auricularia*, *Flammulina* and *Lentinus* have great potential for successful bioprospecting through genetic modification or fortification to enhance their anti-cancer properties.

Medicinal mushrooms are the untapped source of novel pharmaceutical products for cancer therapeutics as these mushrooms consist of several bioactive compounds like glycans, glycoproteins, proteoglycans, quinones,

triglycerides and selenium which exhibit potent anti-cancer properties (Ferreira et al., 2010). Selenium is vital to human health and is regarded as the King of anti-cancer agents by WHO (WHO Report, 2003). It possesses excellent antioxidant properties and hence functions as a cellular protector against free radical oxidative damage. Selenium rich diet is thus beneficial and can be obtained from selenium enriched mushrooms in daily diet. The mushroom selenium content can be increased by incorporating selenium fortified substrate to increase its level up to 30 or 110 µg Se/g dw (*Ganoderma* 72 µg Se/g dw) (Falandysz 2008). Moreover, anti-mutagenic activities of selenium involve identification and elimination of cancer cells by activation of the tumor suppressor genes. It forms an integral component of methyl selenol, and inhibits formation of new blood vessels thereby, curbing blood supply to tumor tissue and eventually causing tissue senescence. Selenium also forms a conjugated complex with mushroom polysaccharides that further enhances its anti-tumor, anti-proliferative and scavenging properties (Shi et al., 2010).

The anti cancer therapies must include mushroom selenoprotein and polysaccharide to effectively treat cancer (Zhao et al., 2008). Mushrooms normally contain selenium, however the selenium content could be enhanced by biotransformation of inorganic selenite in substrate to selenoprotein, selepolysacchride and other compounds (Zhao et al., 2004). Organic selenoproteins particulary the water and alkali soluble proteins (molecular mass ≥16 kDa) are the major repositories for storage of organic selenium. Incidentally SeCys forms the active centre of several antioxidant selenoproteins - glutathione peroxidase, thioredoxinreductase, 15 kDa selenoprotein etc. (Rayman, 2005).

Mushroom selenium supplementation is strictly a selenium concentration dependent phenomenon (Stajic et al., 2002). However, selenium concentration in mycelia is not proportional to selenium supplementation in the growth medium. Higher dosage can lead to decrease in hyphal growth due to selenium toxicity. A low concentration of Se (<100 µg/g) in the substrate facilitate the synthesis of total protein and amino acids in *G. lucidum*, but a high concentration of Se (>150 µg/g) played a reverse role (Zhao et al., 2004).

Selenium, hence is an integral component of the mushroom Ionome (that is mineral nutrient and trace element of an organism that involves application of high-throughput techniques to quantitatively and simultaneously measure the elemental composition and changes in response to physiological stimuli, developmental state, and genetic modifications). Electron microscopy (EM) along with Energy Dispersive Spectroscopy (EDS) is a useful tool to decipher altered morphology, topography and ultrastructural changes in a biological sample as well as elemental composition of the sample surface and can provide the relative localization of different elements by X-ray mapping technique. Thus, the aim of our research was to investigate the growth rate, ultrastructural changes in the hyphae and spores of *Ganoderma lucidum* and ability of mycelia to store selenium as a result of selenium supplementation in form of sodium selenate.

MATERIALS AND METHODS

G. lucidum culture was procured from the Mushroom Research Complex, Department of Microbiology, Punjab Agricultural University, Ludhiana on agar slant and was maintained on mushroom minimal agar (MMA) medium at 30°C ± 1°C. MMA was enriched with selenium with different concentrations of 5, 10, 15, 20 and 25 ppm of inorganic sodium selenate (Na_2SeO_3) salt. The Se-MMA plates were inoculated with 5 mm mycelium disc punched from the fresh culture.(5 replications) and incubated at 30°C ± 1°C for 24, 48 and 92 h and radial diameter was measured in centimeter scale on Leica Macroviewer MZ 60.

The mycelia growth was scrapped from the agar surface and the the fungal hyphae were fume fixed in 1% OsO_4 and processed as per standard protocol of Bozzola and Russel (1999) for SEM. The samples were placed on aluminium stub using double sided carbon tape and sputter coated with gold using Hitachi Ion Sputter Coater model E-1010 for 30 s. The samples were then viewed under SEM (Hitachi S-3400N) at 15 kV accelerating voltage in secondary electron (SE) mode. The SEM-EDS was performed to ascertain the extracellular presence of selenoproteins or organic selenium in the cytosol by using Thermo Noran EDS System Six module attached to SEM. The significance of the data collected was checked statistically by calculating critical difference at 5% level using SPSS 20 software.

The concentration of absorbed Se was measured with a graphite generation atomic absorption spectrophotometer (model Avanta GBC GF 3000 system) at various working concentrations of sodium selenate. Fungal sample (0.5 g) was digested with 10 mL of di-acid (conc. HNO_3: perchloric acid in 3:1 ratio). After cooling, the obtained samples were diluted with distilled water to a final volume 50 mL and filtered through Whatman filter paper no. 42.

RESULTS AND DISCUSSION

Effect of selenium on radial growth

G. lucidum strain GL-II was grown on selenium fortified with sodium selenate mushroom minimal agar MMA at different concentrations (5 to 25 ppm). Stereomicroscopic studies revealed a decrease in radial hyphal growth of the mycelium in MMA. After 24 h of incubation, a decrease in radial diameter at the given concentrations of selenium was observed (Table 1 and Figure 2). However, selenium supplementation resulted in similar growth in radial diameter with respect to control at concentration of5 and 10 ppm at 48 h (Figure 1). There was a slight increase

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Table 1. Effect of selenium on radial growth (in cm) of *Ganoderma lucidum*

Incubation time (h)	Selenium Concentration							
	Control	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm	Mean	R^2
24	1.375	1.175	1.10	1.25	1.15	1.05	1.18	0.511
48	2.95	2.95	2.93	2.73	2.48	2.35	2.72	0.884
92	6.15	6.45	6.55	6.425	6.15	5.75	6.24	0.303
Mean	3.49	3.52	3.52	3.46	3.25	3.05		
CD @ 5%: A (Selenium concentration) -0.0752, B (Incubation duration) – 0.0532, AB (Interaction) – 0.130								

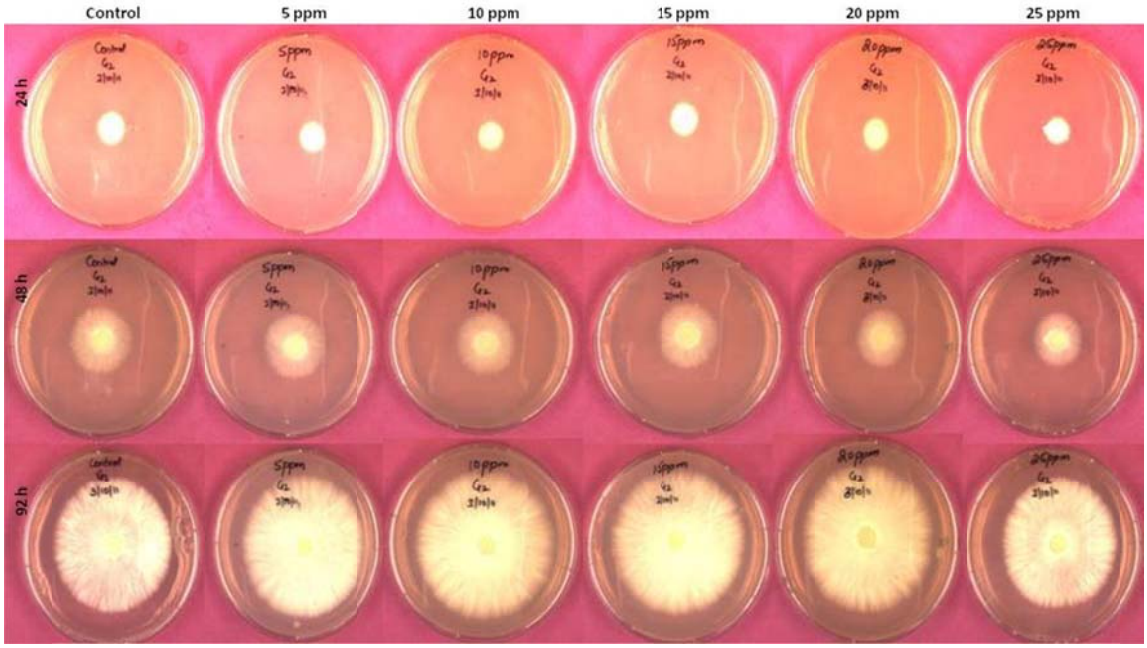

Figure 1. Effect of selenium on radial growth (in cms) of *Ganodeirma lucidum.*

in radial growth at concentrations from 5 to 15 ppm followed by a decreasing trend at higher concentrations at 92 h of incubation (Figure 1). A significant absorption was observed at 48 h at 10 and 15 ppm Se concentrations (R^2 value 0.0885). Thus, 10 and 15 ppm sodium selenate concentrations can be utilized for fortification without any significant change in radial growth. At concentrations higher than 15 ppm, the growth decreased significantly, probably due to enhanced toxic effect of selenium.

Stamets (2000) stated that *G. lucidum* grows radially without making aerial hyphae, and later develops mycelia running parallel to the surface and gained an intensive cover. The culture exhibited initial white color pigmentation than turns to golden yellow. It has been reported that Se concentrations were 4.6 and 9.3 µg/g (d.w) at 5 and 10 µg/g of added Se in the media, respectively (Stajic et al., 2002; Werner and Beelman, 2002). Se accumulation occurs approximately linear in relation to levels of this element added to the substrate.

Mushrooms grown on substrates supplemented with 10 ppm would provide 81.4 µg of Se, representing 116.3% of the daily value (DV) and according to the FDA, a serving size of fresh *P. eryngii* mushrooms produced in Na_2SeO_3 supplemented substrates should be considered an excellent source of Se because it provides more than 20% of the DV (Estrada et al., 2009).

Effect of selenium on spore count

Scanning electron microscopy studies were carried out to decipher the alterations in the spore, hyphal morphology and topography of *Ganoderma lucidum* in response to added selenium. A significant decrease in the spore count with respect to control was recorded with increase in selenium concentration (Table 2, Figure 2). At 25 ppm, SE micrographs showed absence or presence of very sparsely spaced spores while at lower concentration (5 ppm), the spore count was relatively better than

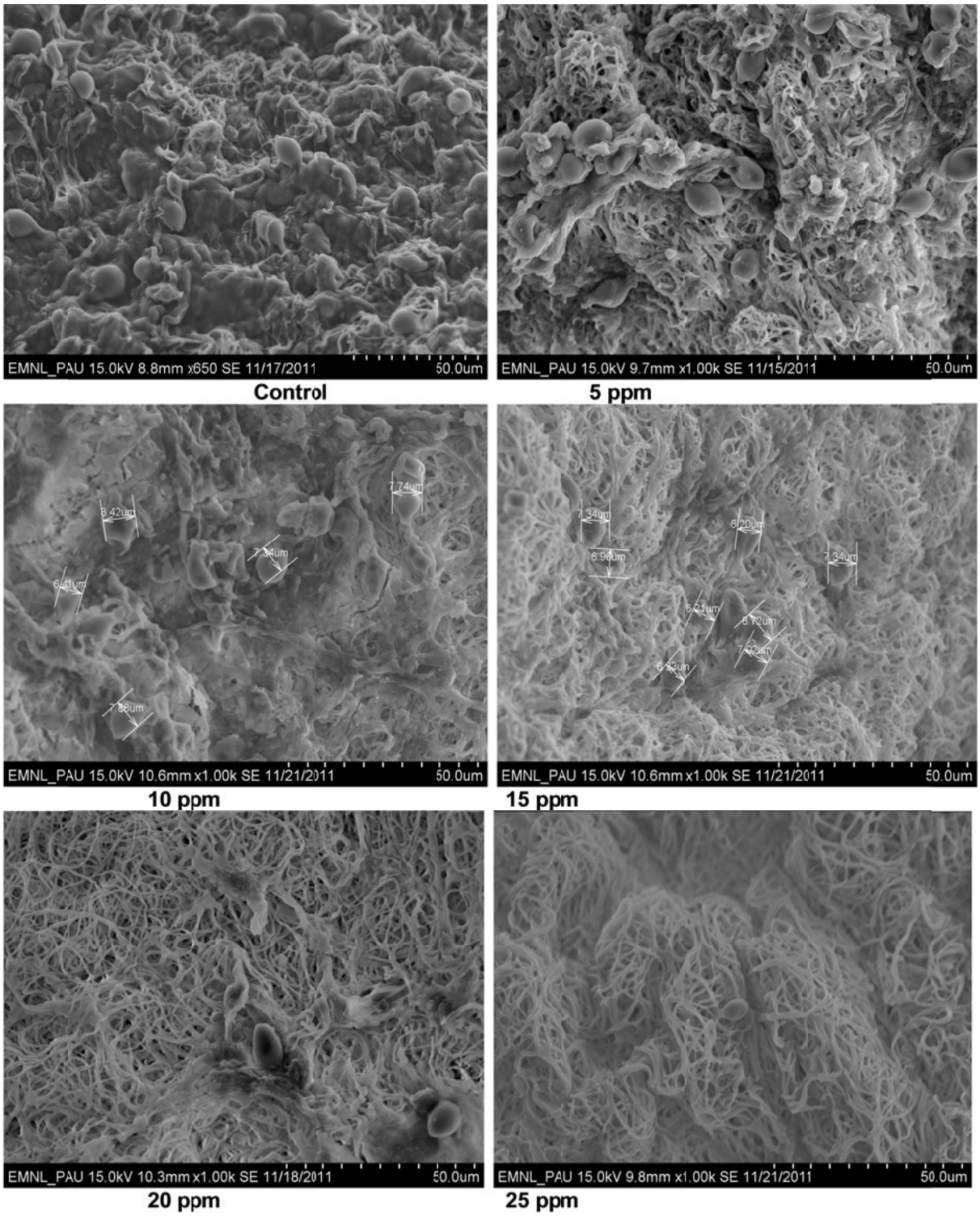

Figure 2. Effect of selenium on spore count of *Ganoderma lucidum.*

consecutive higher concentrations.

Effect of selenium on spore diameter

SE micrographs also revealed the ultrastructural alterations in spore size (diameter) and shape with increase in selenium concentration (Figure 3). The spore structures were altered from spherical to oval or oblong, inflated to constricted deformed and deflated on increasing Se concentration (5 to 25 ppm). Similar trend

Table 2. Effect of selenium on hyphal and spore diameter and number of spores of *Ganoderma lucidum*

Selenium Concentration	Hyphal diameter (nm)	Spore diameter (µm)	Number of spores
Control	861.2 (919 – 756)	10.04	31
5 ppm	632 (728 – 428)	8.87	22
10 ppm	608.2 (705 – 456)	7.7	12
15 ppm	518 (580 – 452)	5.99	9
20 ppm	508.4 (566 – 449)	5.64	8
25 ppm	525 (577 – 434)	5.60	2
CD @ 5%	95.52	1.26	-

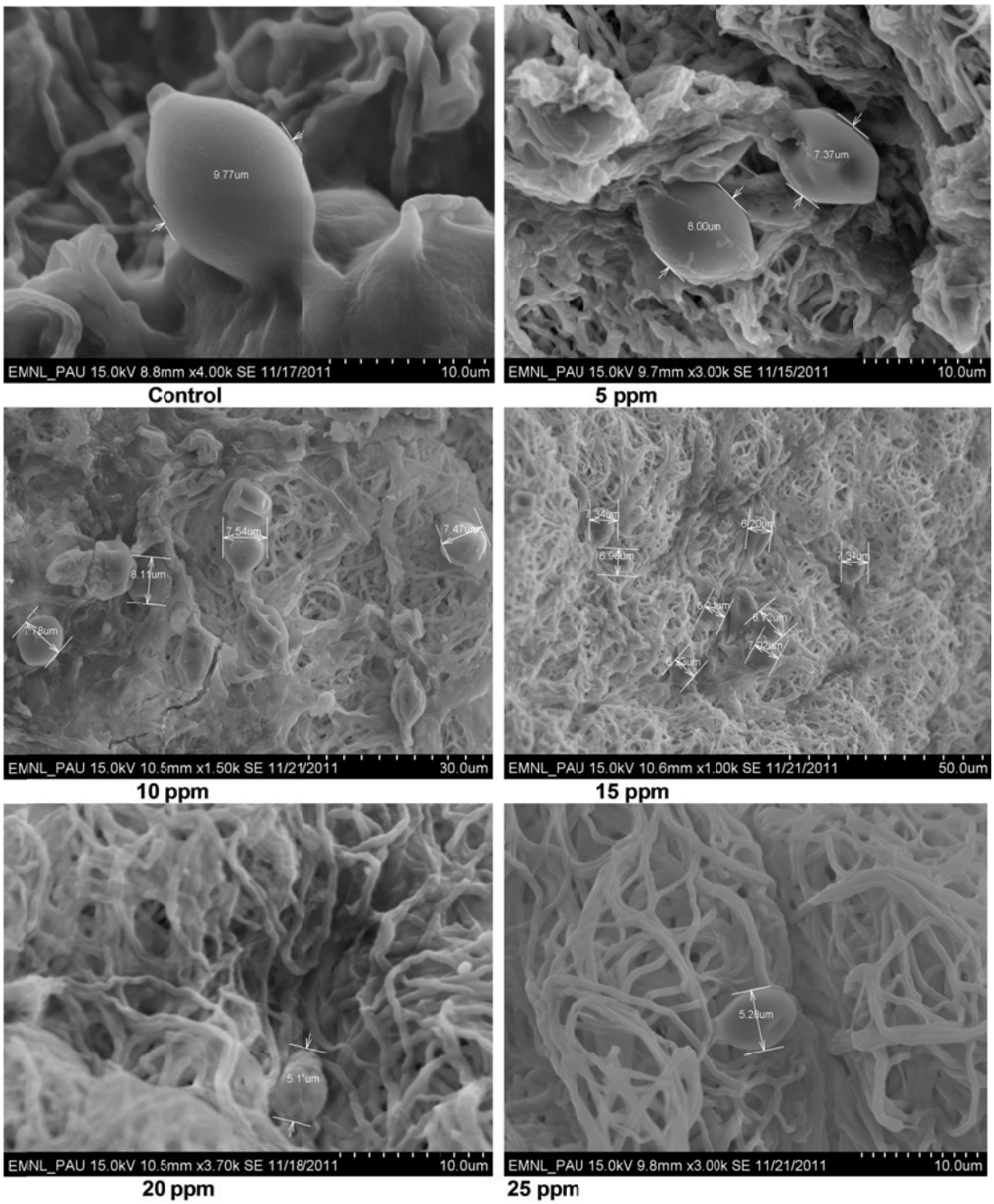

Figure 3. Effect of selenium on spore diameter of *Ganoderma lucidum.*

was recorded in spore diameter studies. A significant decrease in spore diameter was observed in concentration of 20 and 25 ppm (5.60 and 1.26 µm, respectively) as compared to control (10.04 µm). Earlier SEM studies performed by Guler et al. (2011) report presence of septate mycelium and non-smooth spores surface with average size of 92.5-108 µm x 301-331 µm with beaked germinating point in *G. lucidum.*

Tiqiang et al. (1996) studied micro-morphology using optical microscope (OM) and SEM of intact and wall-cracked spores of log-cultivated *G. lucidum*. Optical Micrographs depicted the spore to be ovate-oblong or ovoid with truncated apex or blunt taper and a size of 6.53 - 8.04 × 9.55 -12.56 µm while SEM micrographs provided some sinuous depressions or minute holes on the surface of the spore. The spore size and shape were observed to be similar to our findings (Figure 3).

Effect of selenium on hyphal diameter

SE micrographs exhibited gradual decrease in hyphal diameter with increase in selenium concentration. The hyphae branched out more oftenly with smaller internodes as the selenium concentration increased. At higher concentration, the networking of mycelia was enormous, a phenomenon might be used by growing culture to overcome stress conditions. At 10 ppm (Figure 4, Table 2) concentration no prominent Se stress could be observed and culture grew rapidly without any significant alteration in structure and morphology.

Scanning electron microscope-energy dispersive X-ray spectroscopy study

The SEM-EDS studies were performed to observe the elemental composition of sample surface and to reveal the presence of selenoproteins or organic selenium on the surface of hyphae, however; no selenium was recorded on the hyphal surface thus suggesting selenoproteins or other forms of selenium to be cytosolic moieties. On the contrary, the SEM-EDS results of the samples crushed with liquid nitrogen showed the presence of selenium in samples indicating the uptake of the selenium by the hyphae and sequestration as cytosolic proteins (Table 3).

SEM-EDS studies of the hyphal mass showed difference in the % weight and % atom carbon and oxygen composition. Thus it is noted that a trend of an initial decrease in % carbon till 10 ppm followed by increasing trend with respect to control with maximum % weight carbon was recorded at 15 ppm. On the contrary % weight oxygen exhibited a decreasing trend. Similar observations were observed in % atom C and O composition (Table 4).

Since mushrooms contain relatively high protein levels, and can accumulate large amounts of selenium, it is reasonable to expect that selenium could be incorporated into proteins. The growth of mycelia and fruit body formation of *Pleurotus ostreatus* (Hk-35 and P70) over wide range of concentrations of inorganic form of selenium was examined and showed stimulatory effects (in concentration of 1-50 mg/l) and toxic effects in higher concentration (Savic et al., 2009). It has been reported that the low concentration of Se (<100 µg/g) in the medium leads to enhanced synthesis of total protein and amino acids in *G. lucidum,* but high concentration of Se with more than 150 µg/g was found to have played a reverse role and was fatal (Zhao et al. 2004).

Atomic absorption spectroscopic studies

Atomic absorption spectroscopy of fungal samples exhibited an increasing trend in the uptake of Se by *Ganoderma* hyphal network or hyphae with increased concentration of sodium selenate (Table 5). Percentage of absorption was found to be in range of 7.2% - 9.9% with maximum absorption at concentrations of 15 and 25 ppm.

The Se-enriched Champignon mushroom could contain up to 30 or 110 µg Se/g dw, while the Varnished Polypore (*Ganoderma lucidum*) could contain up to 72 µg Se/g dw). An increasingly growing database on chemical forms of Se-enriched mushrooms indicates that seleno-compounds identified in carpophore include selenoysteine, selenomethionine, Se-methylselenocysteine, selenite, and several unidentified seleno-compounds; though their proportions vary widely (Falandysz, 2008).

The overall study indicated that selenium absorption increased with increasing concentration of sodium selenate. However, selenium uptake induces stress conditions and ultrastructural changes in hyphae and spores of *G. lucidum.* A decrease in hyphal and spore diameter occurred with increase in concentration though a statistically significant change was recorded at 20 ppm and 25 ppm concentrations. Finally, it can be concluded that Se-supplementation till 15 ppm could be considered to be safe and selenium is absorbed as intracellular component in form of organic selenium in selenoproteins.

The above studies conclude that Se supplementation (10 and 15 ppm) is useful for Se fortification in *G. lucidum* to enhance its medicinal properties (by enhanced production of cancer suppressor and antioxidant selenoproteins) without having significant effect on ultra-architectural features on the fungal hyphae and spores.

Conflict of interests

The authors did not declare any conflict of interest.

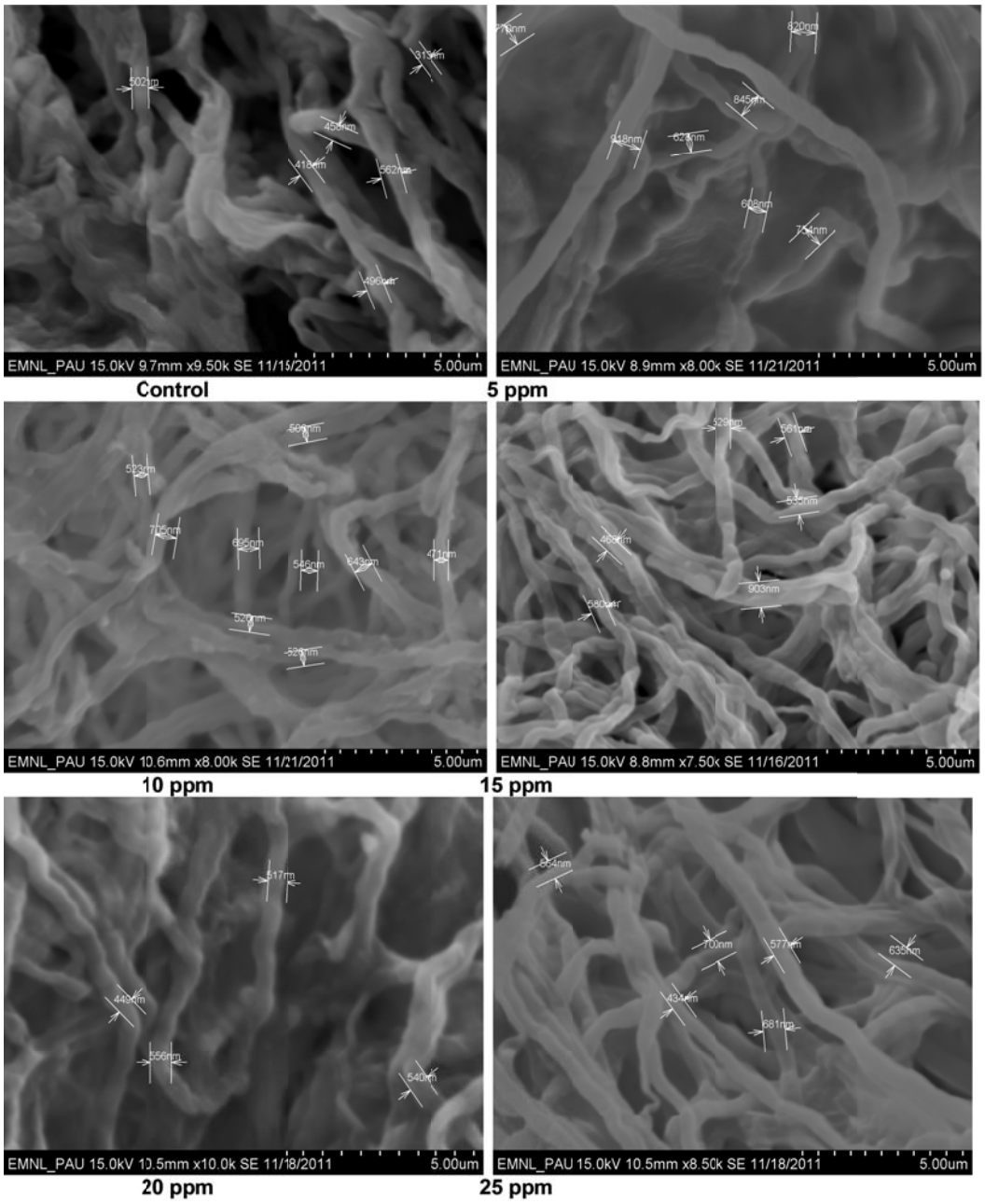

Figure 4. Effect of selenium on hyphal diameter of *Ganoderma lucidum.*

Table 3. SEM-EDS of selenium (%wt) in crushed mycelium of *Ganoderma lucidum*

Parameter	Control	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm	CD @ 5%
% Weight	0.00	0.14	1.14	2.71	0.09	0.30	0.024
% Atom	0.00	0.02	0.21	0.46	0.01	0.05	0.019

Table 4 .Effect of selenium on carbon and oxygen (% weight) of *Ganoderma*

Parameter	Control	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm	CD @ 5%
% carbon	58.91	55.24	57.28	62.12	62.58	62.12	0.178
% oxygen	37.65	30.68	29.64	22.35	23.54	21.14	0.179

Table 5. Total Selenium content (in ppm) in mycelia hyphae of *Ganoderma lucidum*

Selenium concentration	Total selenium content hyphae (ppm)	Percentage absorbtion (%)
Control	0.405	-
5	0.380	7.6
10	0.726	7.2
15	1.474	9.82
20	1.950	9.75
25	2.497	9.9
CD @ 5%	0.065	-

REFERENCES

Bozzola JJ, Russell LD (1999). Electron Microscopy. Jones and Bartlett Publishers, Sudbury, Massachusetts.

Estrada AER, Lee HJ, Robert B, Beelman E, Gasco MMJ, Daniel E, Royse J (2009). Enhancement of the antioxidants ergothioneine and selenium in *Pleurotus eryngii var. eryngii* basidiomata through cultural practices. World J. Microbiol. Biotechnol. 25:1597-1607.

Falandysz J (2008). Selenium in edible mushrooms. Environmental Carcinogenesis Reviews-Part C. J. Environ. Sci. Health. C. Environ. Carcinog. Ecotoxicol. Rev. 26:256-99.

Ferreira ICFF, Vaz JA, Vasconcelos MH, Martins A (2010). Compounds from Wild Mushrooms with Antitumor Potential. Anticancer Agents Med. Chem.10:424-36.

Güler P, Kutluer F, Kunduz I, Hacettepe (2011). Screening to mycelium specifications of *Ganoderma lucidum* (Fr.) Karst (Reishi). J. Biol. Chem. 39:397-401.

Rayman MP (2005). Selenium in cancer prevention: A review of the evidence and mechanism of action. Proc. Nutr. Soc. 64:527-42.

Savić MD, Petrović JP, Klaus AS, Nikšić MP, Rajković MB, Filipović NR, Antić MSB (2009). Growth and fruit body formation of *Pleurotus ostreatus* on media supplemented with inorganic selenium. Proc. Nat. Sci. 116: 209-15.

Shi WL, Han H, Chen GZ, Chen X, Hong YK, Chen LK, Chen D, Lu Z (2010). Extraction, characterization of the polysaccharide extracts from Se-enriched *G. lucidum* (Se-GLP) and its inhibition against oxidative damage in ischemic reperfusion mice. Carbohyd. Polym. 80:774-78.

Stajic M, Brceski I, Wasser SP, Nevo E, Vukojevic J, Duletic LS (2005). Ability of selenium absorption by mycelia of *Pleurotus eryngii* (DC.:Fr.) Quel., depending on selenium source in medium. Int. J. Med. Mushrooms 7:469.

Stamets P (2000). Growing gourmet and medicinal mushrooms. 3rd edition. Ten speed press, Berkeley, California.

Tiqiang C, Kaiben L, Yu Z (1996). Micro-Morphology of the intact and wall-cracked spores of log-cultivated *Ganoderma lucidum*. Acta Edulis Fungi 3(1):63-66.

Werner AR, Beelman RB (2002). Growing high-selenium edible and medicinal button mushrooms (*Agaricus bisporus* (J. Lge) *Imbach*) as ingredients for functional foods or dietary supplements. Int. J. Med. Mushrooms 4:167-71.

WHO Report (2003). Report on Diet, nutrition and the prevention of chronic diseases, Geneva. World Health Organisation 2003. http://cancer.gov/clinicaltrials/digestpage/SELECT/

Zhao L, Zhao G, Du M, Zhao Z, Xiao L, Hu X (2008). Effect of selenium on increasing free radical scavenging activities of polysaccharide extracts from a Se-enriched mushroom species of the genus *Ganoderma.* Eur. Fd. Res. Technol. 226(3):499-505.

Zhao L, Zhao G, Zhao Z, Chen P, Tong J, Hu X (2004). Selenium distribution in a Se-enriched mushrooms species of the genus *Ganoderma.* J. Agric. Food Chem. 52:3954-59.