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Termitomyces holomorph benefits from anomalous Sulphur content in teleomorph

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Abstract: **An attempt was made to investigate the possible Sulphur source in Termite-*Termitomyces* mutualistic system using SEM-EDX and ICP-AES and assess its role for production of healthy *Termitomyces* holomorph. Our study successfully indicated the *Termitomyces* substratum called fungus comb is the rich source of Sulphur for production of Sulphur rich mushroom fruitbodies.**

***Keywords: Termitomyces*, Sulphur, SEM-EDX, ICP-AES, holomorph, teleomorph.**

I. INTRODUCTION

Termitomyces Heim a basidiomycetes genus (Lyophyllaceae, Agaricales) has a symbiotic relation with fungus grower termites (Macrotermitinae) (Heim 1977; Mossebo *et al.*, 2017). Currently there are 57 distinct species and 93 taxa indicated in Index Fungorum (http://www.indexfungorum.org/). These are widely distributed throughout Asian and African continents. *Termitomyces* consist of two distinct stages: hypogeal sporodochia –the mitospore producer anamorph and epigeal sporophores –the basidiospore producer teleomorph. Recently, de Souza and Kamat (2017) described holomorph of *T. bulborhizus* species, making first such attempt to understand the linkages between the strictly sporodochial hypogeal anamorph and ecologically vulnerable, seasonal, ephemeral, epigeal teleomorph. Mound building termites are known as soil ecosystem engineers due to their role in foraging litter, soil composition, nutrient flow rates and localization (Dangerfield *et al.*, 1998; Chen *et al.*, 2018). Several studies have been conducted on termite-*Termitomyces* mutualistic system (TTMS) which is extensively reviewed (Sands 1969; Wood and Thomas 1989; Darlington 1994). This symbiotic fungus grows on special substrate called fungus comb inside the termite mound formed from partially digested plant litter foraged and passed through termite gut (Sieber and Leuthold 1981). In the entire material transport budget in TTMS from external environment to the fungus comb scanty attention has been paid to biogeochemical cycling of elements like Sulphur. Sulphur is known as fifth most common element on the earth and present in form of elemental Sulphur, sulphides and sulphate minerals. It enters the biological system from the earth's crust and soils. Soils are reported to have total Sulphur content in the range 30-400 mg/kg with C:N:S ratio 100:8.9:1. Plants take up Sulphur in form of SO^{4-} (Prasad and Shivay 2018). Sulphur found in hot springs, mineral waters has been researched for its biological importance as it is known to have anti-inflammatory, antibacterial and antifungal properties (Nunes and Tamura 2012). Sulphur containing amino acids (Cysteine and Methionine) are ubiquitous in biological systems. Sulphur is third most abundant mineral in human body and known as healing mineral having antioxidant properties. Several bacteria, archaea and eukaryotes (fungi) are involved in oxidation of sulphur. Besides several mushrooms have been tested for mineral compositional studies for various macro and micronutrient and these have indicated presence of Sodium, Potassium, Phosphorus, Calcium, Magnesium, Aluminium, Chlorine and Sulphur (Bernas *et al.*, 2006). Mound building termites play major role in turn-over of forest plant litter so there are possible chances for Sulphur to play a role in termite-*Termitomyces* mutualistic system. Recently de Souza *et al.*, (2018) reported a Sulphur rich melanin in *Termitomyces* raising important questions regarding the source and role of Sulphur in TTMS. Hence the present study was undertaken to understand the role of Sulphur in genus *Termitomyces*.

II. MATERIALS AND METHODS

A. Collection and processing of samples

Termite mound soil: Local ethnomycological knowledge helped to identify the fertile termite mounds. Termite mound surface soil samples from local habitats (1 Kg) were powdered and sieved through brass sieves with mesh sizes 100 µ.

Termites: Sampling of termites was limited to extrication of adult workers involved in tending the sporodochia. The rationale behind this was based on previous records in literature mentioning passage of soil, chewed plant litter, the old comb material and the harvested sporodochia through their complex digestive system (Wood and sands, 1978; Collins, 1983). About 100 – 200 adult workers were scanned under the stereomicroscope and then were lifted with forceps. The worker termites isolated from the fungus comb were dried on the hot plate of magnetic drier at 60 ^{o}C for half an hour.

Fungus comb**:** About 10 g of fresh fungus comb retrieved from fungus gardens of active, intact termite mound was ground using mortar and pestle and sieved through 100 µ sieve.

A. Termitomyces heimii specimens: The flushes of fresh fruitbodies (Fig. 1) were then spotted on mound, collected, transported to the laboratory and processed immediately for further studies. The specimens were cleaned by using camel hair bushes gently and then sorted manually according to the maturity, sizes and morphological variation. Standard taxonomic keys were used for specimen identification (Heim, 1942; 1951; 1977). Only 100 healthy mature mushroom specimens were selected and umbo, stipe and pseudorrhiza were dissected and separated. These samples were separately dried in a Stockli dehydrator at 65 ^{o}C till they turned brown, crisp and crumbly. Dried samples were then powdered using a clean dry mortar and pestles and these powdered samples were stored in a dessicator and used within a week for further analysis.

Figure 1: A typical local natural habitat of *Termitomyces heimii*.

Basidiospores**:** Spore prints of taxonomically identified mature and healthy specimens were obtained by decapitating several pilei from stipes and placing these over a clean overhead projector sheets (Garware) with gills on lower side (Kaur and Kamat 2004). The spores obtained after 24 hours were scrapped from deposit, were dried and pulverized in a clean sterile mortar and pestle carefully to obtain fine powder

B. Instrumental analysis of processed samples

SEM- EDX analyses: Scanning electron microscopy coupled with energy-dispersive X-ray spectrometry (SEM-EDX, Carl Zeiss SEM model No. EVO18) was performed to examine the chemical nature of dry samples of termite mound soil and fungus comb at 20 kV.

ICP-AES of samples from TTMS: Various powdered samples such as termite mound soil, termites, fungus comb, *Termitomyces heimii* fruitbodies parts (umbo, stipe, pseudorrhiza) and basidiospores were used to estimate sulphur content. The powdered samples (0.2 g) were processed and analysed for sulphur content using SPECTRO Analytical Instruments GmbH, Germany model ARCOS, Simultaneous ICP Spectrometer at SAIF-IIT Bombay.

III. RESULTS AND DISCUSSION

Sulphur in TTMS: Several edible mushrooms have been subjected for proximate qualities, mineral and amino acid compositions. Present study indicated presence of Sulphur in *Termitomyces* species. Our study indicates that the fungus comb, the basal substrate for teleomorphic *Termitomyces* fruitbodies is rich in Sulphur (Table 1).Previously not much attention was given on estimation of Sulphur content for genus *Termitomyces* in spite of it being the major element found in wild growing mushrooms (Kalac 2009) but other elemental compositions has been estimated (Aletor 1995; Adejumo and Awosanya 2005; Gbolagade *et al.*, 2006; Atri *et al.*, 2014; Devi *et al.*, 2014; Ijioma *et al.*, 2015; Woldegiorgis *et al.*, 2015; Ijeh *et al.*, 2016). SEM-EDX studies on *Astraeus hygrometricus* indicated that Potassium is highest followed by Phosphorous and Sulphur. The Sulphur content ranged from 440.9–509.5 mg/100 g and Sulphur containing amino acids - methionine with 1.66-1.68 g/100 g protein and cysteine 0.70-0.74 g/100 g protein were also reported from uncooked dry mass of edible A. *hygrometricus* (Pavithra *et al.*, 2018). According to Turfan *et al.* (2018) elemental analysis of Sulphur was reported to be in range 952.41–12486.63 mg/kg, for selected wild and cultivated mushrooms found in Turkey. Sulphur was found to be second major mineral element in mushrooms and *Boletus edulis* contained highest Sulphur value 12486.63 mg kg^{-1}. Bernas *et al.* (2006) indicated Sulphur content for *Pleurotus ostreatus* (210 mg/100 g), *Agaricus bisporous* (450 mg/100 g) and *Boletus edulis* (450 mg/100 g). Sulphur content depends on the composition of substratum on which mushroom grows. Our SEM-EDX studies of termite mound soil and substrate i.e. fungus comb indicates presence of various elements such as Aluminium, Barium, Carbon, Calcium, Cadmium, Copper, Gold, Iron, Lead, Magnesium, Manganese, Oxygen, Sulphur, Silicon and Zinc (Fig. 2). Thus, termite mound soil and fungus comb material are possible nutrient sources for uptake by *Termitomyces* teleomorph via pseudorrhiza. Sulphur is also known to impart aroma for mushrooms along with other elements such as Nitrogen, Phosphorous, Potassium, Iron and Zinc. In a mature termite nest of *Macrotermes* sp. with 2 –5 million individuals we find fungus comb biomass of 10- 37 kg with turnover rate of 5 – 8 weeks. Fungus combs are also reported to have Sulphur (0.1 % wt/wt) and a Sulphur containing amino acid methionine (2.5 µM/g) has been detected (Arshad and Schnitzer 1987). Fungus combs are known to be formed from mixture of soil, plant material and fungus tissue (Cmelik and Douglas 1970). Sulphur found in termite mound soils, plant litter, dead termites and dead fungus tissue could also contribute to net Sulphur influx in making fungus comb and thus increase bioavailability of Sulphur for *Termitomyces* mushrooms. Present study indicates that the Sulphur pool in the basal fungus comb is large enough to drive forward biochemical reactions in *Termitomyces* without depletion of Sulphur content in teleomorphic fruitbody. Taxonomists have described *Termitomyces* fruitbodies occurring in grey to black specially at perforatorium / umbonal region, pileus and pseudorrhiza but occasionally stipe is pigmented due to injury (Heim 1977; Piearce 1987; Pegler and Vanhaecke 1994; de Kesel 2011; Tibuhwa 2012 Karun and Sridhar 2013; Aryal nd Budathoki 2016). Recently this grey- black pigment found in *Termitomyces* species was reported as Sulphur containing melanin possibly a pheomelanin (de Souza *et al.*, 2018).

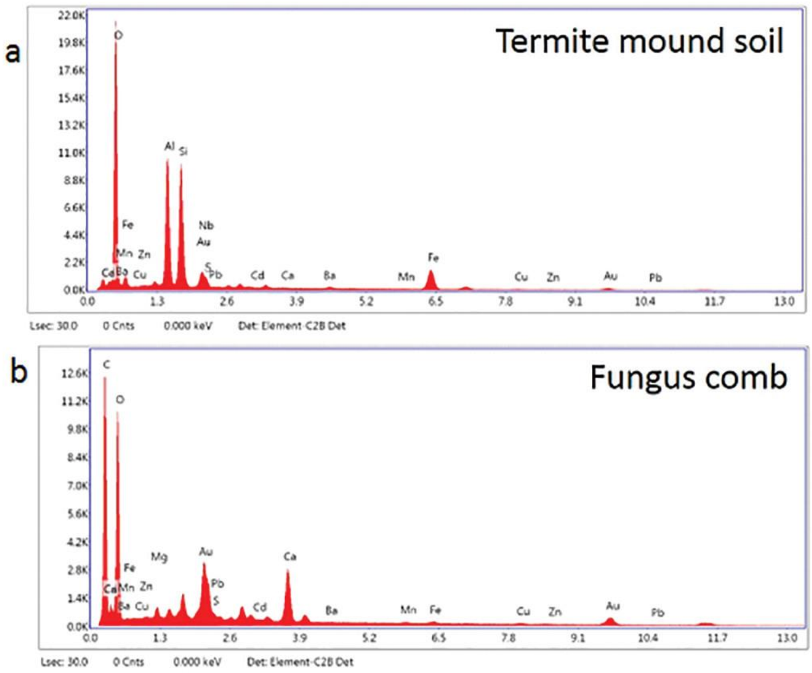

Figure 2: SEM-EDX profile of a) Termite mound soil b) Fungus comb.

Anomalous Sulphur in *Termitomyces*: Our work showed that there is relatively high Sulphur content in umbo (0.28 %) followed by pseudorrhiza (0.187%), basidiospores (0.172%) and least in stipe (0.153%) (Table 1). Our results show that the reduction of Sulphur content from pseudorrhiza to stipe at hypogeal stage is about 0.034% (w/w) indicating less pheomelanin in colourless stipe. The Sulphur content in most of the mushrooms range from 1000 – 3000 mg/kg dry matter (Kalac 2009). The Sulphur content of the termitarium soil which may indicate Sulphur in regolith showed 2.23 – 24.57 mg/kg. They also reported increase in macro/micronutrient contents with respect to termitarium sizes (Joseph *et al.*, 2012). Sulphur containing compounds are known to be radioprotective (Foye 1992) and have been well understood in shiitake mushrooms (Sneeden *et al.*, 2004). Hence, *Termitomyces* mushrooms could be subjected to similar studies to get more insights towards Sulphur containing compounds. Melanin is also known for several biochemical activities such as photo/ mechanical protection, antioxidation, antiradiation (Casadevall *et al.*, 2017). We can postulate that the Sulphur rich melanin / Sulphur bound to melanin might be adding flexibility with strength to the melanized organs, an effect similar to vulcanization of natural rubber, imparting protection against frictional effect during the critical drilling process when the hypogeal stage needs to transit through the overlying hard surface. Sulphur rich melanization may enhance survival chances of the hypogeal stage before it transits into a mature epigeal teleomorph (Fig. 3). Since incorporation if Sulphur in melanin supramolecular matrix affords higher mechanical rigidity, we speculate that any depletion of Sulphur in fungus comb may impact the *Termitomyces* holomorph. The only exception is teleomorph of *T. microcarpus* which grows strictly epigeal and lacks a clear pseudorrhiza. It has not escaped our attention that perhaps this unique adaptation in *T. microcarpus* which lacks the hypogeal stage may also be due to poor Sulphur content of the basal fungus comb and other complex factors making the fungus growing termites to collect and mechanically scatter the fragments of the basal fungus comb material on ground. We propose that the incorporation of Sulphur in melanin supramolecular scaffold indicates the favourable evolutionary adaptation of TTMS in Sulphur rich regolithic environments. Without reinforcement provided by Sulphur rich melanin the perforatorium as a soil piercing driller head and pseudorrhiza as a tubular and nutrient translocating organ would not function finding it difficult to emerge through hard soils during teleomorph production.

Table 1: Total Sulphur content in Termite-*Termitomyces* mutualistic system

Sample	S % by weight
Termite mound soil	0.006
Termites	0.551
Fungus comb	0.204
Termitomyces umbo	0.280
Termitomyces stipe	0.153
Termitomyces pseudorrhiza	0.187
Termitomyces basidiospores	0.172

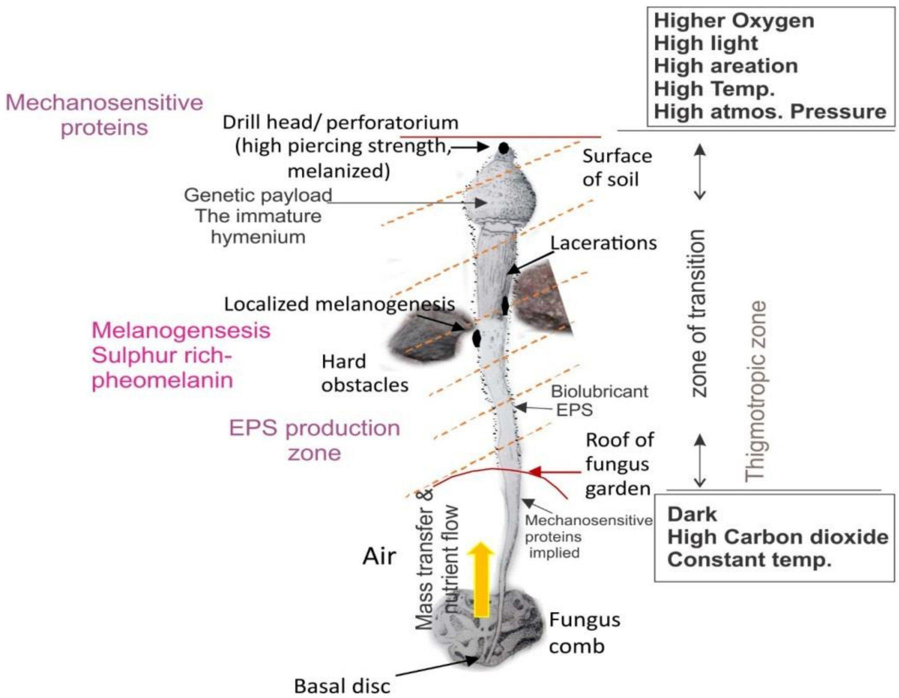

Figure 3: Sulphur rich melanin aiding *Termitomyces* drilling process.

IV. CONCLUSIONS

In this study, the Sulphur content and its role in TTMS was determined and analysed. The results showed that complex organic matrix of the fungus comb could be an excellent source of Sulphur pool for ensuring synthesis of Sulphur rich *Termitomyces* fruitbody melanin. This is the first report describing possible structural and functional role of Sulphur in *Termitomyces* holomorph which may point to unique and favourable evolutionary adaptation to ensure successful fruiting mediated through melanisation process assisted by beneficial incorporation of Sulphur in melanin molecular matrix. Future studies need to be directed at precise quantification of Sulphur flux through TTMS and need to be linked to Sulphur rich and Sulphur poor regoliths and fungus comb matrix material. Besides there is need to shed more light on Sulphur metabolism in Genus *Termitomyces*.

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