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Mechanism of copper tolerance in *Trichoderma asperellum* (MH593785): A key component in the consortium of Copper-Chitosan- *Trichoderma*

Akansha Singh, Erayya and J Kumar

Abstract
In this study, electron microscopy of copper tolerant and copper sensitive *Trichoderma asperellum* isolates revealed that *T. asperellum* (MH593785) had copper tolerance properties as copper was adsorbed in/on the fungal cell wall along with some morphological alteration such as hyphal swelling, while mycelia of *T. asperellum* strain TCMS 5 (copper sensitive isolate) got collapsed along with ultra-structural changes such as shrinking of cytoplasmic material, destruction of mycelia due to penetration of copper ions into the cell membrane and increase in the vacuole size. EDX studies of CuOH (500 ppm) amended *T. asperellum* (MH593785) isolate showed 1.78% of copper peak area while in control copper peak was not detected. Fatty acid profiling showed that octadecenoic acid derivatives (9.67%), octadecadienoic acid derivatives (17.46%), hexadecanoic acid (4.37%) and petroselinic acid (2.51%) were found to be significantly higher in copper treated *Trichoderma* as compared to control. petroselinic acid peak was found in CuOH (500ppm) amended *T. asperellum* culture only. Phospholipids play an important role in metal ions movement across the plasma membrane. In our study, high amount of phospholipid was observed in control (3.73%) while in CuOH-500 (ppm) treated *T. asperellum* (MH593785) less amount (1%) of phospholipid was observed. Fatty acid profiling of CuOH tolerant *T. asperellum* (MH593785) proved that modulation in total fatty acid content in presence of CuOH (500ppm) made the plasma membrane stable in copper amended PDB media.

Keywords: copper, *Trichoderma asperellum*, fatty acids, Plasma membrane

Introduction
A novel consortium of Cu $(OH)_2$, Chitosan and copper tolerant *Trichoderma asperellum* (MH593785) has been formulated at Biocontrol Lab of G.B. Pant university of agriculture and Technology, Pantnagar (Eryya, 2014) [1] whose efficacy is comparable to the commercially available fungicides although in the consortium reduced copper dose is compensated by an elicitor Chitosan and biocontrol agent *T. asperellum* (MH593785). It is a biointensive disease management approach to overcome dependency on chemical pesticides and emergence of new pathogenic races. *Trichoderma* spp. are the most successful bio-control agents as more than 60% of the registered bio-fungicides used in today's agriculture belong to *Trichoderma*-based formulation. Copper is a potentially toxic metal which, at low concentrations, can act as an essential micronutrient for microbial growth, serving as a cofactor for certain enzymes and, as a result of its ability to undergo Cu(I)-to-Cu(II) transitions, playing a role in cellular redox reactions (Yruela, 2005) [20]. Exposure of fungi and yeasts to elevated copper concentrations can lead to a rapid decline in membrane integrity, which is generally manifested as leakage of mobile cellular solute (eg. K^+). *Trichoderma* strains can persist in ecosystems with high concentrations of heavy metals. Copper tolerance in fungi has also been ascribed to diverse mechanisms involving trapping of the metal by cell-wall components, altered uptake of copper, extracellular chelation or precipitation by secreted metabolites, and intracellular complexing by sulphur compound *viz.* metallothioneins and phytochelatins (Cerventas and Corona, 1994; Scheck, 1996) [8, 15]. Cu^{++} can bind to the cell wall surface of *T. viride*, a mechanism of metal tolerance making it less available in the medium (Anand *et al*., 2006) [3]. Extensive metal-induced disruption of membrane integrity inevitably leads to loss of cell viability. However, even relatively small alterations in the physical properties of biological membranes can elicit marked changes in the activities of essential membrane-dependent functions, including transport protein activity, ion

Impermeability (Keenan *et al*., 1982; Borel and Simon, 1996) [7, 13] and Phagocytosis (Avery *et al*., 1995) [5]. The physical properties of a membrane are largely determined by its lipid composition. Microbial membrane fatty acid composition is highly variable and is influenced by both environmental and intrinsic factors. For example, the unsaturated fatty acid contents of microorganisms generally increase at low temperatures. The low melting temperatures and large physical volumes occupied by unsaturated fatty acids are thought to partially compensate for the lipid-ordering effect of chilling (Cossins, 1994; Hazel, 1990) [9, 11] composition between microbial groups. Indeed, microbial fatty acid profiles have proven to be useful taxonomic criteria (Thompson *et al*., 1993) [17] and can be indirectly correlated with other phenotypic characteristics, including pathogenicity (Harbige and Sharief, 2007) [12]. The purpose of the present investigation was to examine the relationship between modulation in plasma membrane fatty acid composition and copper tolerance in *T. asperellum* (MH593785).

Material and Methods

Poisoned food technique

Culture of *T. asperellum* (MH593785) and Trichoderma spp. TCMS 5 were obtained from Bio control Lab, GBPUA&T, Pantnagar. Twenty ml of CuOH (technical grade from Spiess Urania a.i.62.3%) amended medium (250ppm, 500ppm, 750ppm and 1000ppm) were poured in each sterilized Petri plates. Suitable checks were maintained without addition of CuOH. Five mm diameter disc of mycelia growth were cut out from 5 days old culture plate by sterile cork borer and placed in the centre of the poisoned medium of CuOH at different concentrations and incubated at 26±2ºC for 5 days. Three replications were maintained for each treatment. After the desired incubation, the colony growth of both the *Trichoderma strains* was recorded as percent inhibition of the colony over control by using following formula given by Vincent (1947) [18] and data were statistically analyzed.

$$\text{Percent inhibition (I)} = \left(\frac{C - T}{C}\right) * 100$$

Where,
C = Growth of the pathogen in control plate (mm)
T = Growth of the pathogen in treatment plate (mm)

Electron microscopy

Copper tolerant *T. asperellum* (MH593785) and copper sensitive Trichoderma strain TCMS 5 were cultured in CuOH 500 ppm amended Potato Dextrose Broth medium and incubated in shaker at 80 rpm; 26±2ºC for 5 days. Microscopic studies *viz*. Scanning electron microscopy from copper amended PDA culture and Transmission electron microscopy and Electron density X ray spectrometry from Broth culture of both stains were analyzed by Zeiss 435 VP SEM (Oberkochen, Germany), 20keV; TEM, Phillips EM300, 60keV; EDX, Bruker, 20 keV voltage.

Scanning Electron Microscopy

Fungal tissue from solid culture media were fixed by 2.5% glutaraldehyde (fixating agent) for 4 hrs followed by three times washing with 0.1% phosphate buffer saline (pH 7.2-7.4). Samples were dehydrated for every 5 minutes by series of ethanol solution as 30%, 40%, 50%, 70%, 90% and absolute ethanol. Samples were mounted on appropriate stub, and thereafter gold-sputtered (using Scan- Coat six equipment—Oxford) and were observed and photographed using Scanning Electron Microscope—Leo Zeiss 435 VP SEM (Oberkochen, Germany), operating at 20 keV.

Transmission Electron Microscopy

The mycelium was harvested separately and fixed in 2.5% glutaraldehyde for 4 hour at room temperature. It was then washed three times with 1ml of 0.1M phosphate buffer (pH 7.2) and fixed in 1% osmium tetraoxide for two hours at 4^0C. Thereafter, it was dehydrated with acetone (100%). Sections of 0.5 µ sizes were cut using an ultra-microtome and viewed on a Transmission Electron Microscope (TEM, Philips EM300, 60keV voltage).

Electron Dispersive X-ray Spectrometery

The elemental analysis was performed for both copper treated and copper untreated *Trichoderma* (MH593785) at Electron Microscope Laboratory, GBPUA & T, Pantnagar. The samples of mycelia collected from PDB media was prepared for energy dispersive X-ray microanalysis using low temperature fixation in isopentane and lyophilized overnight in a tissue drying chamber at -40 ºC in vacuum. Subsequently, samples were mounted on carbon stubs and coated with carbon in an evaporator. Samples were analysed by using energy dispersive X-ray spectrometer (BRUKER) equipped with Cu detector. The analytical conditions was 20 keV accelerating voltage.

Fatty acid analysis

Cultures of *Trichoderma asperellum* (MH593785) were maintained in without copper (control) and with CuOH-500ppm amended potato dextrose broth (PDB) and incubated at 26±2 °C for 25 days. 0.33 g mycelia were harvested and lyophilized at-40 ºC for vacuum freeze drying. Tissue was homogenized in liquid nitrogen. Homogenized tissue was kept in chloroform: methanol solution for 24 hr. Lipid content was recovered by 3 times repeated centrifuged @ 4500rpm for 10 minutes and phasing out the organic phase. Finally chloroform phase containing lipid is evaporated under vaccum rotary evaporator. Residues suspension in chloroform: methanol (1:2 v/v) solvent was used for FAME analysis by Gas-chromatography equipped with a QP-2010 Ultra mass selective detector. GC-MS analyses were done with ionization energy of 70 eV. FAME analysis done by RTX-5 MS.QGM software. Searched library: WILEY8.LIB, Carrier gas: Helium (column flow rate 1.21 ml/min.). Oven Temperature program: 140 ºC (static for 5 min) then gradually increasing (at a rate of 4 ºC /min) up to 280 ºC (static for 15 min).Injection temperature 260 ºC. The chromatographs were compared and individual peaks were identified by comparing mass spectra to the WILEY8.LIB library references.

Result and Discussion

Bioassay by food poison technique showed that *T. asperellum* (MH593785) tolerated the CuOH concentration upto 500ppm and beyond 500ppm copper toxicity symptoms appeared. Mycelial growth inhibition percent were observed as 54.125% and 65.83% at 750ppm and 1000 ppm respectively (Table 1.1). *Trichoderma* TCMS 5 initiated the copper toxicity symptoms at 500ppm as 72.92%, 85.00% and 100% mycelial growth inhibition were observed at 500 ppm, 750ppm and 1000ppm respectively (Table 1.1). These results indicated the copper tolerance ability in *T. asperellum* (MH593785) strain and Copper sensitivity in TCMS 5 strain.

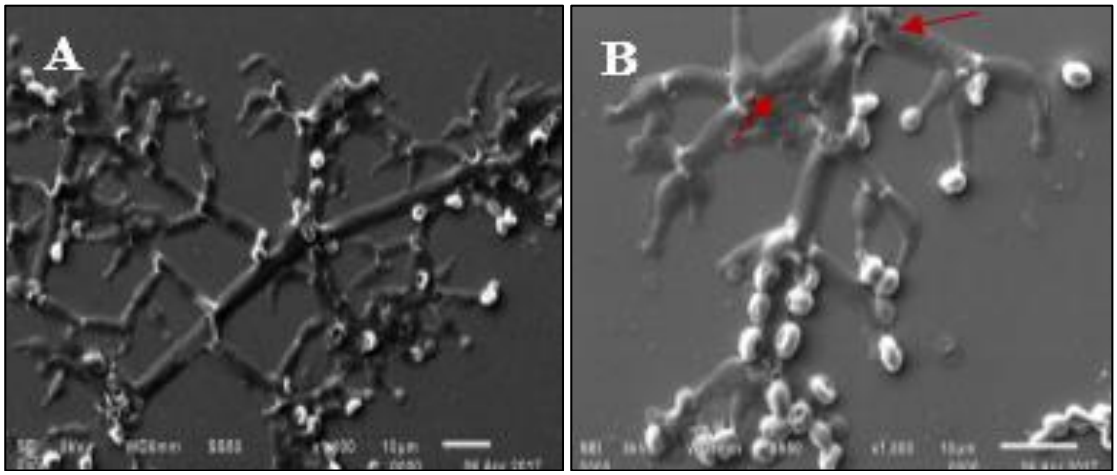
Table 1: Percent inhibition of mycelium growth at different concentration of copper hydroxide

Cu OH Concentration (ppm)	*T. asperellum* (MH593785)		*T. strain* TCMS 5	
	Growth diameter	Percent inhibition	Growth diameter	Percent inhibition
250	8.00	0.00	8.00	0.00
500	5.67	29.13	2.17	72.92
750	3.67	54.125	1.20	85.00
1000	2.73	65.83	0.00	100.00

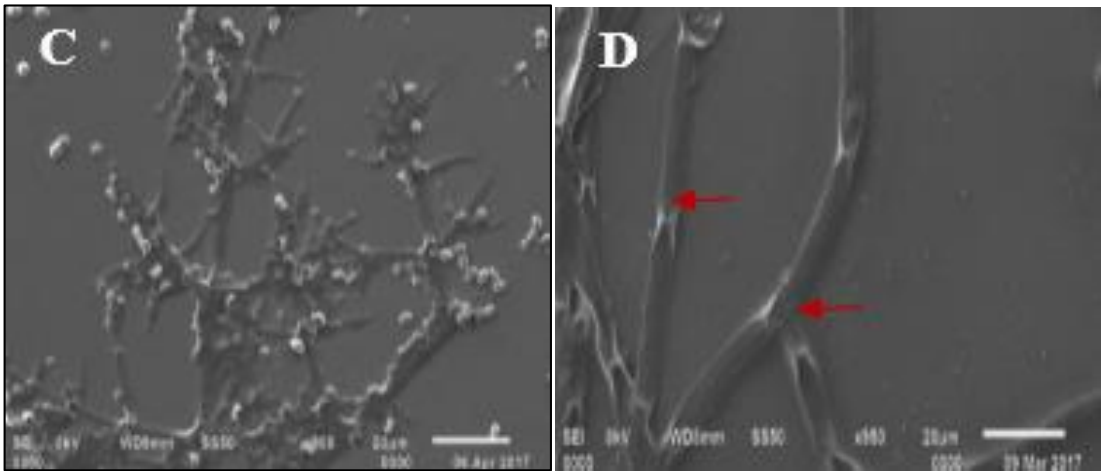
Electron microscopy

Electronic microscopic studies (SEM and TEM) revealed that the *T. asperellum* (MH593785) adsorbed/accumulated copper on the call wall, while the sensitive isolate (TCMS 5) absorbed the copper ions inside the cell. Hence ultra-structural changes such as shrinking of cytoplasmic material, destruction of mycelia due to penetration of copper into the cell wall and increase in the vacuole size and hyphal swelling were also observed. SEM pictures depicted surface view of mycelia (Fig 1). Whereas, TEM photographs depicted cross section of the mycelia (Fig 2). Probably, binding of copper on the cell surface of (MH593785) immobilized the metal to cross the cell wall. These findings are also supported by the findings of Eryya and Kumar (2014) [1].

Further, Energy dispersive X-ray spectrometery (EDS) quantified copper accumulation by *T. asperellum* (MH593785). Two peaks of copper element at 0.9 keV and 8.1 keV were found in copper hydroxide 500 ppm amended *T. asperellum* (MH593785). Results indicated that 1.78% copper peak area percent was present in *T. asperellum* (MH593785) when cultured in CuOH 500 ppm amended PDB medium (Fig 3). Any peak of copper element was not observed in untreated *T. asperellum* (MH593785) (Fig 4). These studies further support the presence of copper tolerance in *T. asperellum* (MH593785).

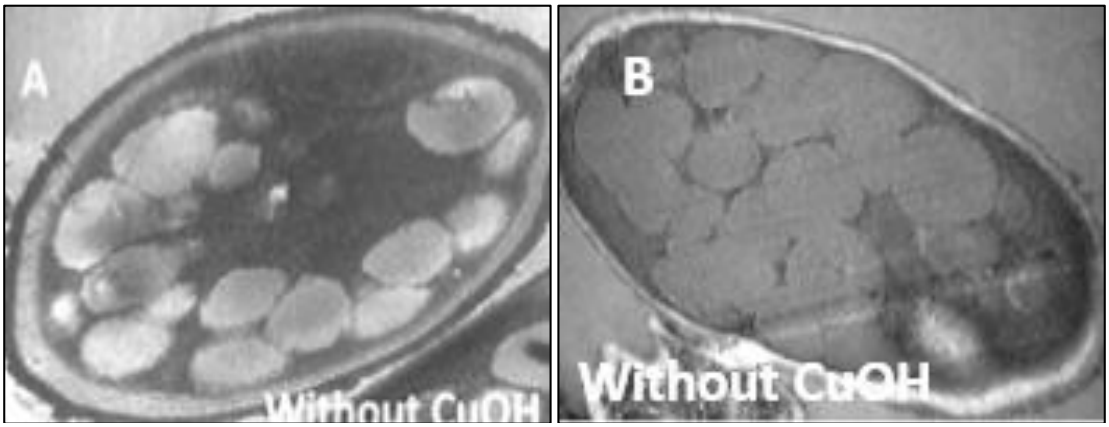


A. *T. asperellum* (MH593785) (CuOH untreated) **B.** *T. asperellum* (MH593785) (CuOH-500ppm treated)

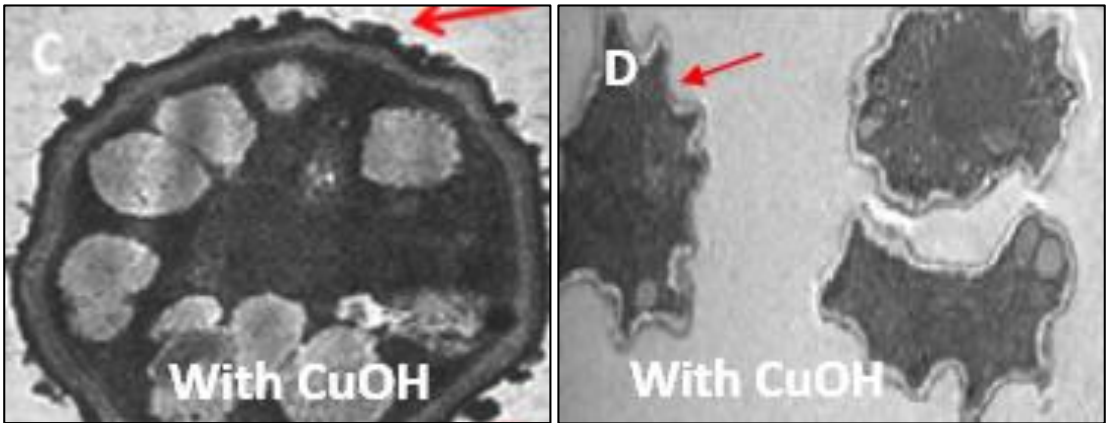


C. *Trichoderma* strain TCMS5 (CuOH untreated). **D.** *Trichoderma* strain TCMS5 (CuOH 500ppm treated)

Fig 1: Scanning electron microscopic view of *Trichoderma asperellum* (MH593785) and TCMS 5 showing (arrows) effect of copper after exposure to copper compounds.



A. *T. asperellum* (MH593785) (CuOH untreated) **B.** *Trichoderma* strain TCMS5 (CuOH untreated)



C. *T. asperellum* (MH593785) (CuOH-500ppm treated). **D.** *Trichoderma* strain TCMS5 (CuOH 500ppm treated).

Fig 2: Transmission electron microscopic view of cross section of *Trichoderma asperellum* (MH593785) showing and TCMS 5 showing (arrows) effect of copper after exposure to copper compounds

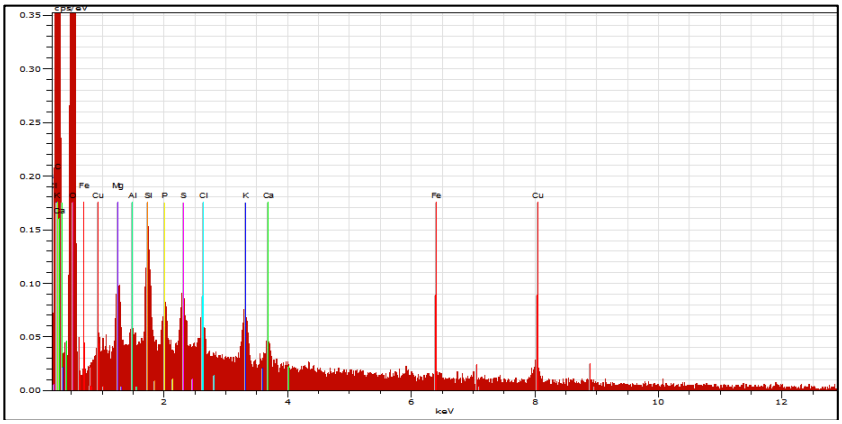


Fig 3: CuOH 500ppm treated *Trichoderma asperellum (MH593785) peak showing copper peak area percent 1.78% at 8Kev*

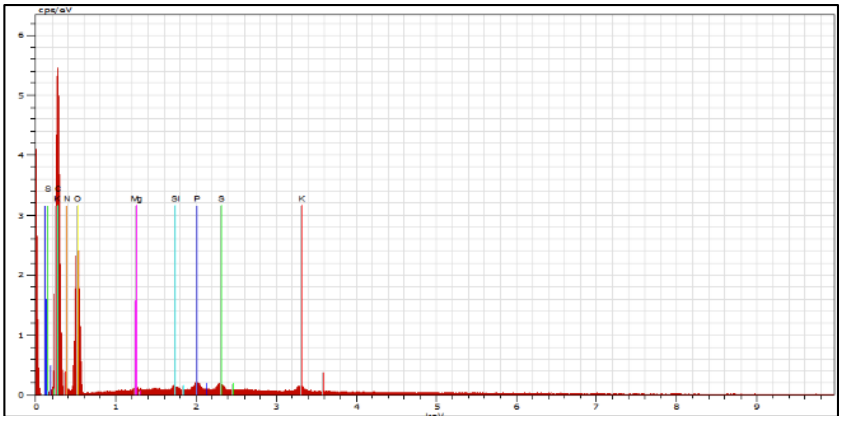


Fig 4: *Trichoderma asperellum* (MH593785) (Control) Cu peak not detected

Plasma membrane is made up of lipid and proteins. The physical properties of a membrane are largely determined by its lipid composition and degree of unsaturated fatty acid (Simon *et al*., 1996) [7]. Fatty acids (lipid) are hydrophobic in nature and play an important role in membrane fluidity and stability (Avery *et al*., 1996) [6]. Its high concentration makes it more compact and prevents the entry of free Cu ions inside the plasma membrane by regulating the membrane fluidity and ultimately membrane stability (Avery *et al*., 1993) [4].

Fatty acid profiling of CuOH tolerant *Trichoderma asperellum* (MH593785) indicated that an increase in total fatty acid content in presence of CuOH 500ppm made the plasma membrane stable in copper amended PDB media (Fig 5, Table 3.1). Total fatty acid in CuOH treated *T. asperellum* was observed as 55.82% while in control it was 40.46%. Specifically octadecenoic acid derivatives (9.67%), octadecadienoic acid derivatives (17.46%), hexadecanoic acid (4.37%) and petroselinic acid (2.51%) were observed at high concentration in *T. asperellum* (MH593785) cultured in CuOH 500 amended PDB as compare to untreated control in which octadecenoic acid/ oleic acid derivatives (1.15%), octadecadienoic acid/linoleic acid derivatives (11.86%) and hexadecenoic acid/palmitic acid (2.18%) were observed (Fig 6, Table 3.2). However, petroselinic acid peak were found in CuOH amended *T. asperellum* culture only. Phospholipids play an important role in metal ions movement across the plasma membrane. Its hydrophilic motif facilitate the free movement of copper ions inside the cell (Vossen, *et al*., 1995) [19] In our study high amount of phospholipid was observed in control (3.73%) while in in CuOH-500 ppm, treated *Trichoderma asperellum* (MH593785) showed less amount (1%) of phospholipid. Hence, absorption of copper ions inside the cell was reduced by reducing the hydrophilic phospholipid composition of cell membrane (Vossen *et al*., 1995) [19]. Nischwitz and coworkers (2007) [14] reported as 4 (cis-9-hexadecenoic acid/ Palmitic acid, 2-hydroxy-13-methyltetradecanoic acid/myristic acid) and 7 (cis-9/trans-12/cis-7-octadecenoic acid/oleic acid) were the highest or second highest amount in copper tolerant *Pantoea ananatis*. Two unsaturated fatty acids linoleic acid and oleic acid were markedly increased at high copper concentration in copper tolerant fungi (Abboud and Alawlaqi 2011) [2].

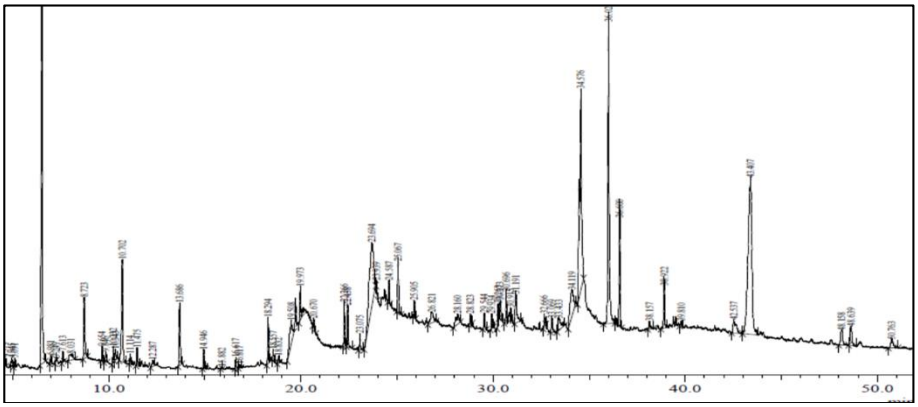


Fig 5: Chromatogram showing fatty acid profiling of *T. asperellum* (MH593785) (CuOH500ppm treatment)

Table 2: Peak area percent of different compounds identified in fatty acid profiling of *T. asperellum* (MH593785) (CuOH 500 ppm treated

Sl. No.	R. Time	Area%	Compound Name
1	4.579	0.16	3-Decen-2-one
2	4.945	0.18	3-Hexadecene, (Z)-
3	5.091	0.21	Tridecane
4	6.526	14.52	6 Amyl. Alpha. Pyrone
5	6.981	0.21	Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(6
6	7.249	0.08	3-Oxabicyclo[3.3.0]Octan-2-ON, 7-Methylen-6,6-Dimethyl-
7	7.613	0.25	Phenol, 3,5-bis(1,1-dimethylethyl)-
8	8.031	0.56	.Beta.-Sesquiphellandrene
9	8.723	1.85	1-Hydroxy-6-methoxyisoquinoline-4-carbaldehyde
10	9.654	0.33	1-Pentadecene
11	9.846	0.27	2-Bromo dodecane
12	10.232	0.33	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol
13	10.343	0.31	(1R,4R)-1-methyl-4-(6-Methylhept-5-en-2-yl)cyclohex-2-enol
14	10.702	1.72	(-)-GUAIOL
15	11.114	0.23	5-Azulenemethanol, 1,2,3,4,5,6,7,8-octahydro-.alpha.,.alpha.,3,8-tetramethyl-
16	11.475	0.49	3-Buten-2-one, 4-(3-hydroxy-6,6-dimethyl-2-methylenecyclohexyl)-
17	12.287	0.29	2H-Pyran-2-ONE, 5,6-Dihydro-6-Pentyl-, (R)-
18	13.686	1.74	2(1H)-Naphthalenone, 4a,5,6,7,8,8a-hexahydro-8a-methyl-, trans-
19	14.946	0.44	1-Heptadecene
20	15.882	0.04	4,4-DIMETHYL-2-ADAMANTANOL
21	16.617	0.26	1,2-Benzenedicarboxylic Acid, Bis(2-Methylpropyl) Ester
22	16.817	0.09	Limonene Dioxide 2
23	18.294	0.99	Hexadecanoic acid, methyl ester
24	18.557	0.26	Isopimara-9(11),15-diene
25	18.832	0.17	Phenanthrene, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydro-1,1,4a,7-tetramethyl-,
26	19.508	1.80	9-Octadecenoic Acid (Z)-
27	19.973	0.84	Heptadecanoic Acid, Ethyl Ester

28	20.670	0.26	(S,E)-8,12,15,15-Tetramethyl-4-methylenebicyclo[9.3.1]pentadeca-7,11-diene
29	22.266	2.02	9,12-Octadecadienoic acid, methyl ester
30	22.438	2.04	9-Octadecenoic Acid (Z)-, Methyl Ester
31	23.075	0.39	Methyl stearate
32	23.694	7.65	9,12-Octadecadienoic Acid (Z,Z)-
33	23.939	0.20	(E)-9-Octadecenoic acid ethyl ester
34	24.587	0.54	Heptadecanoic Acid, Ethyl Ester
35	25.905	0.41	Quinolin-2(1H)-one, 4-hydroxy-1,3-diethyl-7-methyl-
36	26.821	0.86	1-Tridecanol
37	28.160	0.80	9-Octadecenamide
38	28.823	0.17	1-Heneicosanol
39	29.544	0.47	Octadecanal
40	29.934	0.32	3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester
41	30.263	0.51	9,12-Octadecadienoyl chloride, (Z,Z)-
42	30.373	0.69	cis-9-Hexadecenal
43	30.696	0.83	(1R,4aR,4bS,7S,10aR)-1,4a,7-Trimethyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,10,10a
44	30.915	0.22	2-Methyltetracosane
45	31.191	3.38	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
46	32.666	0.33	2H-2,4A-Methanophenanthren-3(4H)-ONE-4-D, Decahydro-2,4B,
47	33.069	0.49	Cyclohexane, 1,1'-tetradecylidenebis-
48	33.433	0.35	4-Cyano-4-Hydroxy-2,2,6,6-Tetramethyl-Piperidine
49	34.119	2.51	Petroselinic acid, TMS derivative
50	34.576	12.82	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester
51	36.023	10.26	8-Pentadecanone
52	36.600	1.25	Squalene
53	38.157	0.29	2-methyloctacosane
54	38.922	1.36	9(11)-Dehydroergosteryl benzoate
55	39.810	0.20	2-Methylhexacosane
56	42.537	1.07	Ergosta-5,7,9(11),22-tetraen-3-ol, (3.beta.,22E)-
57	43.407	16.21	Ergosterol
58	48.158	0.86	9,19-Cyclolanost-25-en-3-ol, 24-methyl-, (3.beta.,24S)-
59	48.639	1.00	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite ester (3:1)
60	50.763	0.62	Phenylalanine, N-isovaleryl-, methyl ester
		100.00	

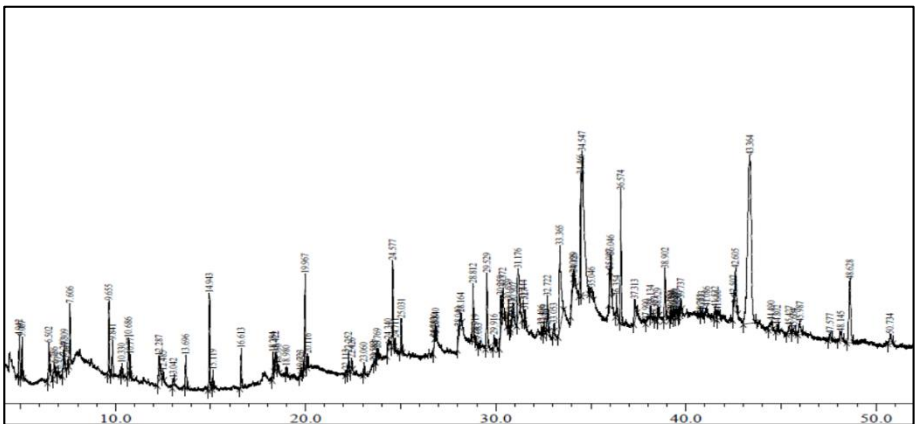


Fig 6: Chromatogram showing fatty acid profiling of *T. asperellum* (MH593785) in control

Table 3: Peak area percent of different compounds identified in fatty acid profiling of *T. asperellum* (MH593785) in control

Sl. No.	R. Time	Area%	Name
1	4.942	1.2	1-Tridecene
2	5.087	1.16	Hexadecane
3	6.502	0.96	6 Amyl. Alpha. Pyrone
4	6.786	0.49	N,N-Dimethyl-3-Buten-1-Amine #
5	6.975	0.11	Copaene
6	7.260	0.15	1,1'-Biphenyl, 4-methyl-
7	7.309	0.31	Tetradecane
8	7.497	0.23	1,1'-Biphenyl, 2-methyl-
9	7.606	1.56	Phenol, 3,5-bis(1,1-dimethylethyl)-
10	9.655	1.91	1-Pentadecene
11	9.841	0.93	Tridecane
12	10.330	0.31	Oxiranemethanol, 3-methyl-3-(4-methyl-3-pentenyl)-
13	10.686	1.09	5-Azulenemethanol, 1,2,3,4,5,6,7,8-octahydro-.alpha.,.alpha.,3,8-tetramethyl-
14	10.771	0.38	Dehydrolinalool

15	12.287	0.95	2H-Pyran-2-one, 5,6-dihydro-6-pentyl-
16	12.485	0.16	1-Heptanol, 2-propyl-
17	13.042	0.17	7-Amino-3-Methyl-4-Oxo-1,4-Dihydropyrazolo[5,1-C][1,2,4]TRIAZINE-
18	13.696	0.96	(6R,7R)-Bisabolone
19	14.943	2.43	1-Heptadecene
20	15.119	0.45	Sulfurous acid, 2-ethylhexyl isohexyl ester
21	18.284	0.59	Tridecanoic Acid, Methyl Ester
22	18.422	0.5	2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-
23	18.550	0.1	9,12-Octadecadienoic Acid (Z,Z)-, 2-[(Trimethylsilyl)Oxy]-1-[[(25
24	18.980	0.16	1,2-Benzenedicarboxylic acid, diheptyl ester
25	19.723	0.13	1,4-Methanocycloocta[d]pyridazine, 1,4,4a,5,6,9,10,10a-octahydro-11,11-dimethyl-
26	19.857	0.01	Hexane, 1,1-diethoxy-
27	19.967	2.41	1-Heptadecene
28	20.116	0.33	Decane, 2,3,5,8-tetramethyl-
29	22.113	0.1	Acetic Acid, Decyl Ester
30	22.252	0.42	11,14-Eicosadienoic acid, methyl ester
31	22.426	0.3	6-Octadecenoic acid, methyl ester, (Z)-
32	23.060	0.28	Octadecanoic Acid, Methyl Ester
33	23.583	0.09	3,5-Dimethyl-2-furyl methyl ketone
34	23.687	0.01	1,2,4-Thiadiazole, 5-amino-
35	23.769	0.19	(R)-(-)-14-Methyl-8-hexadecyn-1-ol
36	24.340	0.31	2-Methyl-4-(2,6,6-Trimethyl-1-Cyclohexen-1-YL)-2-Buten-1-
37	24.577	2.29	9-Tricosene, (Z)-
38	24.711	0.23	2-Methyltetracosane
39	25.031	0.9	1,1,4,7-Tetramethyldecahydro-1H-Cyclopropa[E]Azulen-4-
40	26.753	0.12	1-Decalincarboxamide, 5-hydroxy-
41	26.800	0.03	2-ethoxyethyl ester
42	26.840	0.05	15-Hydroxypentadecanoic acid
43	28.040	0.36	Cyclooctene, 5-chloro-
44	28.164	0.36	Cyclohexane propionic Acid, 2-Propenyl Ester
45	28.812	1.34	1-Heneicosanol
46	28.919	0.15	Nonadecane
47	29.083	0.13	trans-2,7-Dimethyl-3,6-octadien-2-ol
48	29.529	2.31	Octadecanal
49	29.916	0.37	3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester
50	30.259	1.34	1,E-8,Z-10-Hexadecatriene
51	30.372	1.11	cis-9-Hexadecenal
52	30.659	0.8	1S,2S,5R-1,4,4-Trimethyltricyclo[6.3.1.0(2,5)]dodec-8(9)-ene
53	30.760	0.02	Naphthalene, Decahydro-1-Methyl-2-(Methylene-D2)-
54	30.813	0.11	Dimethyl-n-propylphosphonate
55	30.907	0.49	octyl ester
56	31.176	2.18	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
57	31.444	0.48	1,2-Benzenedicarboxylic Acid
58	31.527	0.41	13-Octadecenal
59	32.406	0.14	1-(1-Heptadecynyl)Cyclopentanol
60	32.519	0.08	03027205002 Flavone 4'-OH,5-OH,7-DI-O-Glucoside
61	32.650	0.03	3-[(3,3-Dimethyl-1-Piperidinyl)Methyl]-5A,9-Dimethyl-3A,5,5A,63
62	32.722	1.81	n-Tetracosanol-1
63	33.053	0.23	4-Hexyl-2,2,6,6-Tetramethyl-1,4-Piperidinediol
64	33.365	4.75	4-Cyano-4-Hydroxy-2,2,6,6-Tetramethyl-Piperidine
65	34.129	0.28	(9Z,12Z)-1-Hydroxy-3-methoxypropan-2-yl octadeca-9,12-dienoate
66	34.466	3.99	Heptanoic acid, propyl ester
67	34.547	10.76	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester
68	35.046	0.52	1-Hydroxy-Cyclododecanecarbonitrile
69	35.987	0.24	8-Pentadecanone
70	36.046	0.57	9-Octadecenamide, (Z)-
71	36.354	0.5	1-Nonadecene
72	36.574	3.57	Squalene
73	37.313	0.97	Cyclooctacosane
74	38.134	0.36	Eicosane, 7-hexyl-
75	38.353	0.19	1,3-Cyclohexanedicarboxamide, CIS-
76	38.529	0.32	Solanid-5-EN-3-OL #
77	38.902	1.67	Solanidane (Z)-
78	39.234	0.12	Decanoic Acid
79	39.333	0.02	Bis(cyclohex-3-enylmethyl)amine
80	39.380	0.08	Bornylamine, N-cyclopropylcarbonyl
81	39.493	0.27	3,5-Cyclo-6,8(14),22-ergostatriene
82	39.737	0.3	Behenic alcohol
83	40.753	0.01	5-Methyl-2-methylamino-2-thiazoline

84	40.833	0.14	1-Heptadec-1-ynyl-cyclopentanol
85	41.086	0.34	1,4-Methanoazulen-7-ol, decahydro-1,5,5,8a-tetramethyl-, [1s-(1.alpha.,3a.beta.,
86	41.532	0.33	2-Methyltetracosane
87	41.666	0.52	Uvidin B
88	42.507	0.29	Ergosta-5,7,9(11),22-tetraen-3-ol, (3.beta.,22E)-
89	42.605	2.35	Solanidan-3-one
90	43.364	19.57	Ergosta-5,7,22-TRIEN-3-OL, (3.BETA.,22E)-
91	44.490	0.54	Diethylmalonic acid, 2-isopropylphenyl 2-methylhex-3-yl ester
92	45.427	0.37	Isosolanidan-3-ol
93	45.664	0.19	Carbonic acid, but-3-yn-1-yl heptadecyl ester
94	45.987	0.68	D:A-Friedooleanan-28-al, 3-oxo-
95	47.577	0.35	Glutaric acid, 8-chlorooctyl 4-acetylphenyl ester
96	48.145	0.6	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol
97	48.628	3.73	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite ester (3:1)
98	50.734	0.8	4,4-Dimethyl-2-Adamantanol
		100.00	

Hence, the study proved that copper tolenant *T. asperellum* (MH593785) isolate had ability to modulate the fatty acid composition of plasma membrane which prevented the penetration/ absorption of coppers ions inside the cell and copper are accumulated only outside of the cell membrane. Although Cu toxicity towards microorganisms is well documented, the precise molecular mechanism(s) of toxicity has yet to be clearly ascertained. Similarly, while a number of characteristics which enhance resistance or tolerance to copper have been described (e.g., Cu^{2+} efflux activity, production of extracellular ligands, cell surface precipitation, metallothionein synthesis (Nischwitz and Gitaitis, 2007) [14] which depends on cell membrane integrity, microbial survival in copper toxic habitats can rely on intrinsic properties rather than adaptive changes, and in many cases these mechanisms are unknown. We have shown that copper tolerance towards *T. asperellum* (MH593785) increases considerably with increased plasma membrane fatty acid. In view of the large inherent differences in membrane lipid composition between (and within) the major microbial taxonomic groups (Tang, 2013) [13], as well as the variation attributable to environmental acclimation, it seems reasonable to speculate in light of our results that membrane fatty acid composition may be one important intrinsic characteristic that determines the differential tolerance of individual microorganisms to copper toxicity.

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