Melanin Production and Laccase Mediated Oxidative Stress Alleviation During Fungal-fungal Interaction Among Basidiomycetes Fungi

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Research

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Abstract

Fungal-fungal interaction often leads to the change in metabolite profile of both the interacting fungus which may have potential implication in industry or agriculture. In the present study, we performed two sets of fungal-fungal interaction - *Trametes coccinea* (F3) with *Leiotrametes lactinea* (F9) and *Trametes coccinea* (F3) with *Trametes versicolor* (F1) to understand the changes in the metabolite profile during the interaction process and how this process impacts the hyphal/mycelial morphology of the participating fungi. The metabolites produced during interaction of *Trametes coccinea* (F3) with *Leiotrametes lactinea* (F9) and *Trametes coccinea* (F3) with *Trametes versicolor* (F1) was analysed through Liquid Chromatography coupled to Mass Spectroscopy (LC-MS). Most of the metabolites secreted or produced during interaction are associated with defensive response. Further, visualization with scanning electron microscopy revealed that interaction between the tested fungi led to the change in the hyphal morphology of the one participating fungus. The bipartite fungal interaction resulted in the production of a dark brown colour pigment – melanin as confirmed by the LC-MS, FTIR and NMR analysis. Moreover, the fungal-fungal interaction also led to increase in the production of laccase, a group of multicopper oxidases involved in detoxification of toxic compounds. Further increased activity of superoxide dismutase, an enzyme that catalyzes the dismutation of the superoxide anion to hydrogen peroxide was also recorded during fungal– fungal interaction. Quantitative real-time PCR revealed upregulation of *lcc1* (encoding a laccase enzyme) and few other stress related genes of *T. versicolor* during its hyphal interaction with *T. coccinea*, suggesting a direct correlation between laccase production and melanin production.

Introduction

Fungal-Fungal interactions are highly dynamic phenomenon which occurs in nature, whereby the interacting fungi compete for available nutritional source and territory. These interactions lead to the induction of an array of bioactive products by stimulating the complex metabolic pathways (Bertrand et al. 2014). Co-culturing fungal species results in the production of some novel metabolites as a response to antagonistic interaction (Chatterjee et al. 2016). Few studies have been carried out to unravel the morphological and enzymatic changes during fungal interaction. As it has been reported, during interaction of two different fungal species, various pathways get induced in the barrage zones. The induction of certain metabolic pathways are due to the production of toxins, growth inhibitors and their by-products (Rodriguez et al. 2011). Phelligridin C, phelligridin H, methyl inoscavin A, inoscavin C, methyl davallialactone and foscoparianol D are some compounds observed during the co-culture of *Inonotus obliquus* and *Phellinus punctatus* (Zheng et al. 2011). During interaction, alcohols, aldehydes, ketones, terpenes, aromatic compounds and reactive oxygen species (ROS) are produced as a result of antagonism (Evans et al. 2008), which leads to the up-regulation of many oxidative enzymes like laccase, manganese peroxidase, lignin peroxidase (Gregorio et al. 2006). Tamayo et al. (2016) reported that the increase in ROS activates enzymes like superoxide dismutases (SODs) which act as first line of defense.

Interspecific interaction among wood rotting basidiomycetes is a natural phenomenon of the ecosystem dynamics. For example, *Gloeophyllum trabeum*, *Irpex lacteus*, *Trametes coccinea*, *Trametes versicolor*, *Pleurotus ostreatus*, *Ganoderma applanatum* are some of the common wood inhabiting basidiomycetes that occupy the same microhabitat (Song et al. 2012). The outcome of such interactions can be either replacement, where one fungus gains the territory of the other; or deadlock where neither of the two interacting fungi gains the territory of one another. Interactions among these basidiomycetes in the environment may help decompose and cycling

of nutrients like carbon and nitrogen, thus adding environmental benefit. Besides environmental utility, the interactions also have numerous industrial application such as induction of industrially important enzymes like laccase, xylanase, cellulase; production of important novel bioactive compounds. Therefore, understanding the basic metabolism and molecular mechanism is essential. In order to study the metabolic response between the confronting mycelia of the two different fungal species, metabolomics analysis of interaction zone of *T. coccinea* with *T. versicolor* and *T. coccinea* with *L. lactinea* was performed by liquid chromatography coupled to mass spectrometry (LC–MS). The interaction of *T. coccinea* with *T. versicolor* and *T. coccinea* with *L. lactinea* was chosen based on their morphological studies as well as their promising laccase and superoxide dismutase activity observed during interaction. The study also focuses on analyzing an important natural product-melanin produced during the interaction.

Materials And Methods

Strains and culture condition

Three previously isolated fungal isolates *viz*., *Trametes versicolor* F1 (MK370665), *Trametes coccinea* F3 (MK168589) and *Leiotrametes lactinea* F9 (MK168586) were obtained from the Microbial Biotechnology laboratory, Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat for the interaction studies. The cultures were grown on potato dextrose agar (PDA) (Himedia, India) and incubated at 28 °C.

Interaction study between the fungal isolates

For mono-cultures, a 7 mm agar plug of the fungal culture was inoculated in petri dish containing potato dextrose agar (PDA). *T. coccinea* vs *L. lactinea* and *T. coccinea* vs *T. versicolor* were dual cultured by inoculating 7 mm agar plugs of each isolate onto the opposite sides of a PDA plate and incubated at 28 °C for 14 days.

Visualization of interacting fungi under Scanning electron microscope (SEM)

The hyphae from both the interacting cultures i.e., *T. coccinea* vs. *T. versicolor* and *T. coccinea* vs. *L. lactinea* and the monocultures on the 9th, 12th and 15th day of incubation were taken and fixed in 2.5% glutaraldehyde (prepared in 0.1 M phosphate buffer), following the protocol of Kathuria et al. (2014). Scanning electron microscopic analysis was done using FEI Quanta 250 SEM at an accelerating voltage of 10 kV with a scanning electron detector for taking micrographs at different magnifications.

Metabolite extraction for LC-MS

The two fungal dual cultures (*T. coccinea* with *L. lactinea* and *T. coccinea* with *T. versicolor*) and mono-cultures of *T. coccinea*, *T. versicolor*, *L. lactinea* at their 8th day of interaction were considered for metabolite extraction for LC-MS (Fig. 1). The excised mycelia were mixed in a solvent containing Methanol: Dichloromethane: Ethyl acetate in the ratio of 1: 2: 3. It was placed in a rotary shaker at 120 rpm for 12 hrs and sonicated. The filtrate was dried in a rotary evaporator system (IKA®, Germany). The powdered extract of each interaction and monoculture obtained was re-dissolved in methanol. The extract was filtered through 0.22 µ syringe filter and LC-MS was carried out in AB Sciex 4000 Q – Trap – Mass Spectrometer coupled to Waters Acquity UPLC™ system. ESI positive spectra were obtained in an ODS-2 column with mobile phase: (a) acetonitrile: (b) 0.1 % formic acid in water (gradient mode) with an injection volume of 20 µL. The scan range was from 80-1000 m/z.

Data extraction and Compound Identification

The peaks from ESI positive containing the m/z ratio in one of the axes and the relative abundance (%) in the other axis for different retention time were analyzed for the presence of different compounds based on the adduct ions formed. The molecular mass for each retention time was predicted by identifying the different adducts like M+H, M+NH_4, M+Na, M+Na+2H, M+ACN+H, 2M+ACN+H, M+ACN+Na, M+CH_3OH+H, M+2Na-H, 2M+H, M+K, 2M+K, M+2K+H, 2M+Na. The molecular mass thus obtained were compared with the molecular weight of the reference compounds from different sources including Yeast Metabolome Database, PubChem, NIST molecular database. Some references were specific for a particular species whereas some were based on common metabolites secreted by fungal species.

Production and extraction of brown pigment

For brown pigment production during interaction, 7 mm mycelial plug from *T. coccinea* vs. *L. lactinea* and *T. coccinea* vs. *T. versicolor* were inoculated in 100 mL of Sabouraud Broth (*SB) (Himedia). Monocultures of *T. coccinea*, *L. lactinea* and *T. versicolor* were also inoculated and incubated at 28 ˚C for 21 days. A flask containing media without inoculum was taken as control. When the colour of the broth changed, the mycelial biomass was removed and 1 M NaOH was added to the filtrate making the solution alkaline with pH 10 and autoclaved at 120 ˚C. The solution was centrifuged at 8000 rpm for 20 min. The filtrate was acidified to pH 2 by adding HCl and centrifuged at 8000 rpm for 15 min. The precipitate was washed with HPLC grade water to remove any acid traces and dried at room temperature. The dried pigment was collected and weighed (Arun et al. 2015).

Characterization of brown pigment

The dried pigment was characterized for its solubility in water, 1M KOH, 1M NaOH and organic solvents like ethanol, hexane, and acetone. Reactivity of the pigment with hydrogen peroxide was tested by reacting 50 mg melanin with 30% H_2O_2, followed by precipitation with 1 mol/L HCl.

FTIR and NMR analysis

For fourier-transform infrared spectroscopy (FTIR) analysis, two milligrams of the dried pigment were ground properly with infra-red grade KBr (1:10) and pressed into a disc under vacuum (Khatib et al. 2018). The spectrum from 4500 – 400 cm^{-1} was recorded in a Perkin Elmer Inc. spectrophotometer. The pigment composition and structure were predicted by searching the spectrum against a database of reference spectra.

For proton nuclear magnetic resonance spectroscopy (NMR) analysis, the method of Kurian and Bhat (2017) was followed. The pigment was dissolved in deuterated DMSO and the solution was subjected to ^1H NMR analysis using a Bruker 300 MHz instrument with a magnetic field of 11.4 T.

Laccase Assay

Qualitative screening was done by inoculating a 5 mm diameter mycelial disc of 5-day old culture onto PDA plates containing 4 mM guaiacol Bodke et al. (2012). Intense brown coloration around the fungal colony was considered positive for laccase production.

For laccase quantification, a mycelial disc from each of the interacting fungi was inoculated into a 250 mL Erlenmeyer flask containing yeast extract powder (1 g/L), D-glucose (10 g/L), KH_2PO_4 (1 g/L), $MgSO_4.7H_2O$ (5 g/L), NaCl (5 g/L), lignin (0.1 g/L) and incubated at 28 °C with 150 rpm for 14 days (Vantamuri and Kaliwal 2016). A flask containing the media without inoculum was taken as control. A reaction mixture containing 10 mM sodium acetate buffer (pH 4.5), 0.15 mM Diammonium 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), and 0.1 mL of extract incubated at 30 ºC for 10 min. Oxidation of ABTS was monitored periodically for 14 days by measuring the increase in absorbance at 420 nm in an Evolution 202UV vis spectrophotometer (Thermo Scientific, USA). One enzyme unit is defined as the amount of enzyme that oxidizes 1 µmol of ABTS per min.

Superoxide dismutase (SOD) Assay

Superoxide dismutase activity was monitored by following the protocol of Debona et al. (2014). Mycelia from the interacting zone was weighed and crushed in phosphate buffer (pH 7.8). SOD activity was assayed on the basis of its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT). A reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA, and 100 µL of extract incubated in light for 30 min was used to perform the assay. Controls which were incubated in dark were maintained for each set. Spectrophotometric readings were taken at 460 nm periodically for 14 days post inoculation.

Quantitative Real-Time PCR (qRT-PCR) analysis of associated genes

A total of 14 test genes and one housekeeping gene were selected for analyzing their expression at transcript levels by qRT-PCR during the interaction of *T. coccinea* vs. *T. versicolor*, *T. coccinea* and *T. versicolor* alone. Primers used in this study along with their target genes are listed in Table 1. On the 8th day of interaction, total RNA of *T. coccinea* vs. *T. versicolor* and their monocultures were isolated using TRI Reagent® (Sigma-Aldrich, MO, USA). The isolated RNA samples were treated with DNase I (Thermo Fisher Scientific, USA) and purified using silica column (Ambion, Life technology, USA). First strand cDNA was prepared from the isolated RNA using GoScript™ Reverse Transcription kit (Promega, Madison, USA). Quantitative real-time PCR was performed on the first strand cDNA using GoTaq qPCR Master Mix (Promega, Madison, USA) in a total reaction volume of 20 µL containing 10 nM of each primer and 50 ng cDNA template according to the manufacturer's protocol. Real-time PCR was performed using three biological replicates on the Quant studio 5 Real-Time PCR System (Applied Biosystems, USA) with alpha-tubulin (*atn*) gene as the reference gene. The relative gene expression of each gene was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 1
List of oligonucleotide primers used for quantitative real time PCR analysis

Gene/Locus	Product	Primer	Sequence (5'->3')	BP
TRAVEDRAFT_29635	Alcohol dehydrogenase	AlcDH F	GGGCAAACGGTCCTCATCTT	20
		AlcDH R	GTAGTCCGCCCCAAGAGAAC	20
TRAVEDRAFT_144610	Alcohol Oxidase	AlcO F	CGTGGTGCTTGGTCCTACAT	20
		AlcO R	GCAGGTCTTTGACCACCGTA	20
TRAVEDRAFT_128490	Amine Oxidase	AmnO F	TTACGGAGGACCTTTTCGGC	20
		AmnO R	AAGAAGTTGACGGGGTGCAA	20
TRAVEDRAFT_73942	Glutathione-S-transferase	GST F	ATCGAGACGGCCAACTTTGA	20
		GST R	TCATCGCCAGCAAGGAACTT	20
lcc1	Laccase	LCC F	GGTGGGATTAACTCCGCCAT	20
		LCC R	AAGACCATGTTCAGCGGTGT	20
VP3at	Manganese Peroxidase	MPX F	TGGCTTTCAAAACTCTCGCC	20
		MPX R	CGAACAGGTTCTGCTGGATG	20
TRAVEDRAFT_158338	3-Ketoacyl-CoA thiolase	KCT F	ATGGCACGACGAAGGAGAAC	20
		KCT R	AGTCGTGACATGCTTTGCCA	20
TRAVEDRAFT_31517	ABC-transporter	ABCT F	TTGGGCTTCATTTTCGCCAC	20
		ABCT R	CGAGTGGACCCAACGAAGAA	20
TRAVEDRAFT_45420	Copper Radical Oxidase	CRO F	GATGAGTGCTGGTGTGGTGA	20
		CRO R	ATAGAGCTGCAACCGTGCTT	20
TRAVEDRAFT_75578	Terpenoid Synthase	TPS F	GCTCCACATAAACCGCCTCT	20
		TPS R	CAGCGACGCTACATGGATCA	20
TRAVEDRAFT_139368	CDF Metal transporter	CMT F	TTCTCATGGGCTCGGTCAAC	20
		CMT R	AACTTGACACCTAGAGCCGC	20
TRAVEDRAFT_73497	Glutamate decarboxylase	GDC F	AGCGGGATGTTCACAGTGTT	20
		GDC R	TTTGAACGCCACTACGGGAA	20
TRAVEDRAFT_26075	FAD – linked oxidoreductase	FDO F	AGGGGTGCGGCTTATATTCG	20
		FDO R	GGCCTCCTTGTACCAAACCA	20

Gene/Locus	Product	Primer	Sequence (5'->3')	BP
TRAVEDRAFT_28066	Aromatic compound dioxygenase	ACDO F	GGCAAAATTCTCGCGAACCC	20
		ACDO R	TATCCAGGGGCCTTGACTGT	20
TRAVEDRAFT_69843	α-Tubulin	ATN F	AGAACAGGCTCAACGTCTCG	20
		ATN R	ACGGGGAAATGAATACGGGG	20

Statistical analysis

The data obtained from laccase and SOD assay were analyzed in SPSS 25.0 software by one-way Analysis of Variance (ANOVA). Duncan test was performed to study the level of significance ($p \leq 0.05$). The results of quantitative Real-time PCR were analyzed using student *t*-test in Origin Pro 6.0 and $p \leq 0.05$ was considered as significant.

Results

SEM Analysis during *in vitro* fungal-fungal interaction

The SEM analysis for the interaction between *T. coccinea* and *L. lactinea* as well as *T. coccinea* and *T. versicolor* on the late stages of interaction, i.e., on the 9th, 12th and 15th days of interaction showed formation of pores on the hyphae (Fig. 2). The number of pores increased with the increase in number of days of interaction, which ultimately led to the death of the hyphae in the interaction zone.

Metabolites of the mono and dual cultures by LC-MS

The metabolic profiling of the different metabolites during the interaction of *T. coccinea* with *T. versicolor* along with that of the monocultures, i.e., *T. coccinea* and *T. versicolor* (Table 2) resulted in identification of 39 compounds. 18 of these compounds were produced exclusively during the interaction, 9 compounds were produced exclusively by *T. coccinea* monoculture and 13 were produced exclusively by *T. versicolor* monoculture. Similarly, during the interaction of *T. coccinea* with *L. lactinea* and their growth as monocultures produced 41 compounds (Table 3) of which 20 were produced exclusively during the interaction, 9 compounds were produced by *T. coccinea* and 12 compounds were produced by *L. lactinea*. The result showed that more numbers of compounds were obtained from the barrage zone as compared to the monocultures indicating the induction of metabolite production during the interaction. One of the significant results obtained from the LC-MS analysis is the detection of compounds like tyrosine, L-DOPA and melanin in the barrage zone (Fig. 3). Tyrosine and L-DOPA are involved in melanin synthesis pathway (Blagoeva 1984; Rzepka et al. 2016).

Table 2

Comparative analysis of metabolite profiles during interspecific interactions between *T. coccinea* - *T. versicolor*. '+' indicates presence and '-' indicates absence of a particular compound

Retention Time	Predicted Molecular mass	Adduct	*Trametes coccinea*	*Trametes versicolor*	*Trametes coccinea* – *Trametes versicolor*	Compound
1.09	157.1	158.1 (M + H)	+	-	-	N-acetyl-D-proline
1.13	117.1	118.1 (M + H), 136.1 (M + NH_4)	-	+	-	L-aspartic 4-semialdehyde
1.44	286.2	287.2 (M + H)	+	-	-	Cinnabarin
1.59	300.1	301.1 (M + H); M + ACN + H)	+	-	+	Cinnabarinic acid
2.29	147.9	148.9 (M + H), 171.1 (M + Na)	-	-	+	3,5-Dibromosalicylaldehyde
5.67	147.9	148.9 (M + H), 211.2 (M + ACN + Na)	-	+	-	Unknown
7.72(PDA)			-	-	+	
7.56	278.1	279.1 (M + H), 301.0 (M + Na)	-	-	+	Unknown
7.74	240.2	241.2 (M + H), 481.4 (2M + H)	-	+	-	Anserine
8.74	285.3	286.3 (M + H), 318.3 (M + CH_3OH + H)	-	+	-	Unknown
9.92	421.5	422.5 (M + H)	-	-	+	Unknown

Retention Time	Predicted Molecular mass	Adduct	*Trametes coccinea*	*Trametes versicolor*	*Trametes coccinea* – *Trametes versicolor*	Compound
10.31	313.3	314.3 (M + H)	-	+	-	Unknown
11.19	278.1	279.1 (M + H), 301.3 (M + Na)	-	-	+	Unknown
11.21	278.1	279.1 (M + H), 342.4 (M + ACN + Na)	-	+	-	Unknown
11.37(PDA)			-	-	+	
11.77	229.3	230.3 (M + H)	-	+	-	Unknown
12.14	273.3	274.4 (M + H)	-	+	-	Unknown
12.18			-	-	+	Unknown
12.84	299.3	300.3 (M + H)	-	+	-	4-amino-2-methyl-5-diphosphopyrimidine
13.96	285.3	286.3 (M + H)	-	+	-	β-Alanine, N-isobutyryl-, decyl ester
14.19	583.2	584.2 (M + H)	-	-	+	Unknown
14.71	197.2	436.1 (2M + ACN + H)	-	-	+	L-DOPA
15.60	183.2	184.2 (M + H)	+	-	-	Phenoxazine
15.73	313.3	314.3 (M + H)	-	+	-	Unknown
16.39	240.3	241.3 (M + H), 279.2 (M + K)	-	-	+	D-Cystine
16.56	170.1	171.1 (M + H), 203.1 (M + Na)	-	-	+	D-Glyceraldehyde 3-phosphate

Retention Time	Predicted Molecular mass	Adduct	*Trametes coccinea*	*Trametes versicolor*	*Trametes coccinea* – *Trametes versicolor*	Compound
17.05	181.2	401.3 (2M + K)	-	-	+	Tyrosine
18.48	300.9	377.9 (M + 2K + H)	-	-	+	Unknown
18.73	633.3	634.3 (M + H), 678.5 (M + 2Na-H)	-	-	+	Unknown
19.44	338.3	339.3 (M + H), 356.4 (M + NH_4)	+	-	-	Oleic acid, butyl ester
19.87	355.4	356.4 (M + H)	+	-	-	S-Adenosylmethioninamine
20.00	506.2	507.2 (M + H)	-	-	+	Fumaric acid, 3,4-dimethoxyphenyl undecyl ester
20.81	318.3	357.3 (M + K)	-	-	+	Melanin
21.76	427.4	428.4 (M + H)	+	-	-	Unknown
21.97	474.2	475.2 (M + H), 507.2 (M + Na)	-	-	+	Unknown
24.25	337.3	338.3 (M + H)	-	+	-	S-(Hydroxymethyl)glutathione
24.52	351.3	352.3 (M + H), 384.4 (M + Na)	-	-	+	Unknown
24.70	355.4	356.4 (M + H), 733.7 (2M + Na)	+	-	-	S-Adenosylmethioninamine
24.87	385.3	386.3 (M + H)	-	+	-	Unknown
25.89	240.1		-	-	+	Unknown

Retention Time	Predicted Molecular mass	Adduct	*Trametes coccinea*	*Trametes versicolor*	*Trametes coccinea* – *Trametes versicolor*	Compound
26.22	455.4	456.4 (M + H)	+	-	-	Unknown

Table 3

Comparative analysis of metabolite profiles during interspecific interactions between *T. coccinea - L. lactinea*. '+' indicates presence and '-' indicates absence of a particular compound

Retention time	Predicted Molecular mass	Adduct	*Trametes coccinea*	*Leiotrametes lactinea*	*Trametes coccinea – Leiotrametes lactinea*	Compound
1.09	157.1	158.1 (M + H)	+	-	-	Unknown
1.13	181.2	365.1 (M + H)	-	-	+	Tyrosine
1.44	286.2	287.2 (M + H)	+	-	-	Cinnabarin
1.48	143.0	144.0 (M + H)	-	-	+	Unknown
1.59	300.1	301.1 (M + H); M + ACN + H)	+	-	-	Cinnabarinic acid
2.12	292.1	293.1 (M + H)	-	-	+	Unknown
7.70	240.2	241.3 (M + H), 481.5 (2M + H)	+	-	+	Anserine
7.79	286.1	287.1 (M + H)	-	-	+	N(1)-(5-phospho-D-ribosyl)glycinamide
8.95	326.2	327.2 (M + H)	-	-	+	Fertaric acid
10.77	148.1	171.1 (M + Na)	-	+	-	2-hydroxyglutaric acid
11.16 (PDA)			-	+	-	
11.19	278.1	279.1 (M + H)	-	-	+	Unknown

Retention time	Predicted Molecular mass	Adduct	*Trametes coccinea*	*Leiotrametes lactinea*	*Trametes coccinea* – *Leiotrametes lactinea*	Compound
11.35	278.1	279.1 (M + H)	-	+	-	Unknown
11.64	229.2	230.2 (M + H)	-	-	+	Unknown
13.32	414.2	415.2 (M + H), 437.2 (M + Na)	-	-	+	Unknown
14.17 (PDA)			-	+	-	
14.69 (PDA)			-	+	-	
14.71	476.1	477.1 (M + H)	-	-	+	Unknown
14.88	197.2	395.4 (2M + H)	-	-	+	L-DOPA
15.58	226.1	227.1 (M + H)	-	+	-	Chorismate
15.60	183.2	184.2 (M + H)	+	-	-	Phenoxazine
16.58	575.3	576.3 (M + H)	-	-	+	Unknown
17.01	399.1	401.1 (M + H)	-	+	-	Unknown
18.44	382.2	446.0 (M + ACN + Na), 767.6 (2M + 1)	-	+	-	Unknown
18.48	383.0	766.5 (2M + 1)	-	-	+	Unknown

Retention time	Predicted Molecular mass	Adduct	*Trametes coccinea*	*Leiotrametes lactinea*	*Trametes coccinea* – *Leiotrametes lactinea*	Compound
18.73	339.0	678.3 (M + H)	-	-	+	5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxylate
18.71	476.0	477.0 (M + H)	-	+	-	Unknown
19.44	338.3	339.3 (M + H), 356.4 (M + NH_4)	+	-	-	Unknown
19.87	355.4	356.4 (M + H)	+	-	-	S-Adenosylmethioninamine
19.98	324.3	325.3 (M + H)	-	+	-	Uridine 5'-monophosphate
20.74	318.3	357.3 (M + K)	-	-	+	Melanin
21.76	427.4	428.4 (M + H)	+	-	-	Adenosine 3',5'-diphosphate
21.97	484.3	485.3 (M + H), 502.3 (M + NH_4)	-	-	+	Unknown
22.51	397.4	398.4 (M + H), 420.3 (M + Na)	-	-	+	Phytosphingosine 1-phosphate
22.86	383.4	384.4 (M + H), 406.3 (M + Na)	-	-	+	Unknown

Retention time	Predicted Molecular mass	Adduct	*Trametes coccinea*	*Leiotrametes lactinea*	*Trametes coccinea* – *Leiotrametes lactinea*	Compound
24.70	355.4	356.4 (M + H), 733.7 (2M + Na)	+	-	-	S-Adenosylmethioninamine
24.75	382.5	788.6 (2M + Na)	-	+	-	Unknown
24.48	281.3	282.3 (M + H)	-	-	+	Unknown
25.45	390.3	391.3 (M + H), 413.2 (M + Na)	-	+	-	Unknown
25.70	390.3	391.3 (M + H), 413.3 (M + Na)	-	-	+	Unknown
26.22	455.4	456.4 (M + H)	+	-	-	Unknown
26.28	535.3	536.3 (M + H), 599.1 (M + ACN + Na	-	+	-	Unknown

Table 4

Biochemical Characterization of melanin

Sl. No	Assay	Result
1	Solubility in Water	Insoluble
2	Solubility in Ethanol	Insoluble
3	Solubility in Hexane	Insoluble
4	Solubility in Acetone	Insoluble
5	Solubility in 1M KOH	Soluble
6	Solubility in 1M NaOH	Soluble
7	Reaction with Hydrogen peroxide	Decolorization of the pigment
8	Reaction with Hydrochloric acid	Precipitated readily

Extraction of extracellular melanin pigment

The SB broth where the two different fungi were dual cultured (i.e., *T. coccinea* and *T. versicolor* (Fig. 4A); *T. coccinea* and *L. lactinea* (Fig. 4B) showed dark brown coloration. On the other hand, no dark brown coloration was observed in the control and the monocultures of *T. coccinea*, *T. versicolor* and *L. lactinea*. As the color of the control did not change, it means that the difference in coloration is due to the compound secreted by the fungi and not due to the result of the oxidation of the media. An amount of 37 mg/L and 31 mg/L of extracellular pigment were obtained as a product from the culture broth, where *T. coccinea* – *T. versicolor* and *T. coccinea* and *L. lactinea* were allowed to interact respectively.

Biochemical analysis of the melanin pigment

The solubility test of the pigment showed that it was insoluble in water and organic solvents like ethanol, hexane, and acetone but soluble in 1M KOH and 1M NaOH. Also, the brown coloration of the pigment faded away when it was treated with an oxidizing agent like hydrogen peroxide. Treatment of melanin with hydrochloric acid resulted in precipitate formation (Table 3).

Characterization of the melanin pigment from FTIR spectrum

In FTIR spectrum, peaks were obtained at 3399 cm^{-1}, 2,926 cm^{-1}, 2,851 cm^{-1}, 1,586 cm^{-1}, 1385 cm^{-1}, 1030 cm^{-1} and 618 cm^{-1}. Absorption at 3399 cm^{-1} attributes to the polymeric OH groups. The stretching vibrations for aliphatic CH bonding appear at 2,926 cm^{-1} and 2,851 cm^{-1}. At 1,586 cm^{-1}, the symmetric carboxylate stretching vibrations (COO) are detectable. The indole ring vibration / CNC stretching was observed at 1385 cm^{-1}. CH in-plane / CH out-of plane deformation are attributed at 1030 cm^{-1}. 618 cm^{-1} indicates to the out-of-plane bending of the aromatic carbon-hydrogen bond (Fig. 5A). The presence of these functional groups indicates the presence of melanin.

Characterization of the melanin pigment from NMR spectrum

In NMR spectrum, peaks were obtained at 0.8 ppm, 1 ppm, 1.2 ppm, 1.3 ppm, 1.043 ppm, 2.341 ppm, 2.5 ppm, 3 ppm, 7.2 ppm. 0.8 to 1 ppm can be attributed to the CH_3 groups of alkyl fragments. Peaks from 1.2 to 1.3 ppm can be attributed to the long chain methylenes. Peaks at 1.043 and 2.341 ppm are associated with the CH at aliphatic region. The signals at 2.5 ppm are associated with DMSO, which comes from the solvent deuterated DMSO or sulfonate groups bound to the pyrrole nitrogen which is relative to the occurrence of N-sulfonation. Peak at 7.22 ppm is related to the pyrrole –CH group of a carboxyl substituted indole. A peak ranging from 3 to 4 ppm is also observed tentatively near the signal from residual water in the DMSO. Peaks from 2.2 to 2.3 ppm indicates CH3 group and 2.5 to 3 ppm indicates NH groups which are connected to the indole groups. Also peaks from 7 to 7.5 ppm indicate the presence of aromatic heterocyclic ring of the pigment (Fig. 5B). The presence of these functional groups infers the compound to be melanin.

Assessment of Laccase Activity during *in vitro* fungal-fungal interaction

Qualitative analysis of laccase activity through plate assay indicated *T. coccinea*, *T. versicolor* and *L. lactinea* to be laccase positive (Fig. 6). Quantification of laccase activity revealed that the interactions increased laccase activities as compared to the enzyme activity in pure cultures. The laccase activity increased from 2nd day post inoculation up to the 8th day, and then slightly decreased on the succeeding days until the 14th day post inoculation for both the interactions (Fig. 7A, Fig. 7B). The laccase activity in case of the pure cultures also increased till the 8th days post inoculation but was less (significantly low), compared to the interaction ones. The laccase activity for *T. coccinea* with *T. versicolor* (F3-F1) and *T. coccinea* with *L. lactinea* (F3-F9) in the interaction zone was found to be 253.86 U/L and 272.25 U/L respectively. This was more as compared to the laccase activity of the monocultures of *T. coccinea*, *T. versicolor* and *L. lactinea* was found to be 70.64 U/L, 115.03 U/L and 134.42 U/L ($p \leq 0.05$).

Assessment of Superoxide Dismutase (SOD) activity during *in vitro* fungal-fungal interaction

The superoxide dismutase assay showed that during the initial days of interaction (4th, 6th and 8th days) the superoxide dismutase activity was higher for the interacting fungi. But their activity gradually decreased from the 10th day post inoculation. Superoxide dismutase activity was very negligible as observed for the monocultures *i.e.*, 1.5 U/mgFW in *T. coccinea*, 0.57 U/mgFW in *T. versicolor* and 0.79 U/mgFW in *L. lactinea* (Fig. 7C, D). The SOD activity for *T. coccinea* with *T. versicolor* (F3-F1) and *T. coccinea* with *L. lactinea* (F3-F9) in the interaction zone was found to be highest, *i.e.*, 207.81 U/mgFW and 246.74 U/mgFW respectively at $p \leq 0.05$ (Fig. 7C and 7D).

Gene expression profile during interaction of *T. coccinea* and *T. versicolor*

Quantitative Real time PCR (qRT-PCR) analysis of the 12 genes during interaction of *T. coccinea* and *T. versicolor* was performed and compared to the expression profile of *T. versicolor* control culture to understand how the interaction of the two fungal isolates affect the expression of these genes. The results of qRT-PCR analysis are shown in Fig. 8. Among the genes that showed upregulation during interaction, the *lcc1* gene that encodes laccase showed 11.1-fold increased expression during interaction followed by *tps* (6.2-fold), *cro* (2.8 folds), *amnO* (1.7-fold) and *alcDH* (1.6-fold). Among the genes tested, *fdo* (gene encoding FAD-linked oxidoreductase), *gdc* (gene encoding glutamate decarboxylase), *cmt* (gene encoding CDF metal transporter), *gst* (gene encoding glutathione S transferase), *alcO* gene encoding alcohol oxidase) and *kct* (gene encoding 3-

ketoacyl-CoA thiolase) showed significantly decrease in expression during interaction. The rest of the genes *viz*., *abcT* (gene encoding an ABC transporter), *acdo* (gene encoding aromatic compound dioxygenase), and *mpx* (gene encoding manganese peroxidase) did not reveal any significant difference in their expression during interaction of *T. coccinea* and *T. versicolor*.

Discussion

Fungal melanins are secondary metabolite made up of complex heterogeneous polymers of phenolic and/or indole monomers. They are reported to play a myriad of biological roles such as in morphogenesis, virulence, energy transduction including acting as scavengers of stresses, and in turn protect cell viability (Toledo et al. 2017). Brown pigmentation occurs in the barrage zone due to deposition of certain quinone compounds assumed to be melanin or melanin like compounds and is associated with morphological changes at interacting hyphal fronts (Peiris et al. 2008). However, an interesting change was observed in the present study in response to deadlock interaction. To explore these interactions fully, the changes in hyphal morphology, metabolite production and gene expression were studied. The occurrence of pigmentation in the barrage zone could be correlated to the deposition of melanin pigments which got induced during stress condition due to the physical contact between the competing hyphae. Biophysical analysis using FTIR and NMR further confirmed that the brown pigment is melanin. The amount of melanin produced during fungal-fungal interaction in the present study was higher (31–37 mg/L) when compared to the melanin produced by monocultures of different species as observed in the basidiomycetes *Schizophyllum commune* which produced only 0.25 mg/L of melanin from the culture broth (Arun et al. 2015).

Morphological changes were also observed in the zone of interaction which is often related to altered metabolism conferred by various metabolic and oxidative enzymes, increase or decrease of which could lead to a series of other changes. Scanning electron microscopic analysis of fungal hyphae from interaction zone revealed formation of pores on the hyphae of the interacting isolates leading to distortion of the hyphae and culminated in death of hyphae which can be attributed as a characteristic feature of deadlock interaction. Microscopic observations of this type of deadlock interaction have not been visualized earlier. Though in many studies, various authors have mentioned that in case of deadlock interaction, both the interacting fungi compete strongly so that neither of the fungi can acquire the territory of the other (Boddy 2000). One or both of the interacting fungi secrete some important metabolites which results in the death of the interacting hyphae in the interaction zone (Peiris et al. 2008). Therefore, the changes in metabolite profile during the interaction of *T. coccinea* with *T. versicolor* and *T. coccinea* with *L. lactinea* was analyzed. The metabolic profile revealed that during the interaction process many compounds were synthesized or induced which were otherwise not produced by the mono cultures. The presence of compounds like tyrosine and L-DOPA could be co-related to the production of melanin – an important bioactive molecule (Slominski et al. 2012; Luo et al. 2017; Almeida-Paes et al. 2018).

Traditionally, there is also a possibility of competition between fungal species for nutritional and habitat resources resulting in stress and laccase enzyme induction (Gregorio et al. 2006). The enzyme is also reported to play a crucial role in detoxification. The increase in laccase activity in the interaction zone may be due to the defense reaction resulting from mycelial confrontations (Baldrian 2004). Eyre et al. (2010) reported that enzymes involved in reactive oxygen species (ROS) generation, like the NADPH oxidases, laccase and

peroxidases are occasionally upregulated during interaction. Accumulation of ROS in barrage zones causes oxidative damage to competitor mycelia, particularly in the deadlock ones. Laccase not only could function as antioxidants to remove ROS for fungal survival, but also showed a strong ability of xenobiotic detoxification as reported earlier (Zhong et al. 2019). In the present study, the increase in superoxide dismutase in the early days of post inoculation indicates the increase production of ROS. Reports suggested that ROS increases in the interaction zone due to oxidative stress during the interaction of *Dichomitus squalens* and *P. ostreatus*. This increase in ROS results in the activation of first line of defense by enhancing the activity of superoxide dismutase which helps in scavenging the ROS compounds (Tamayo et al. 2016).

Morphological changes are associated with changes in gene expression compared to non-interacting mycelia during interactions. For example, during antagonistic interaction, downregulation of chitin synthase in *S. gausapatum*; there is decrease in growth with its possible replacement by *T. versicolor* (Eyre et al. 2010). Quantitative real-time PCR suggested that several stress related genes from *T. versicolor* were differentially expressed during the interaction process. We could not study the differential gene expression of *T. coccinea* during interaction due to the unavailability of whole genome sequence of *T. coccinea*. The *lcc1* gene of *T. versicolor*, which encodes the laccase enzyme showed significant upregulation ($p \leq 0.05$). Laccases have been reported earlier to be involved in melanin production (Boddy and Hiscox 2016). Although many studies have revealed that interspecific fungal interactions contribute to the increase in laccase activity, (Baldrian 2004; Chi et al. 2007; Gregorio et al. 2006; Hiscox et al. 2010; Kuhar et al. 2015; Wei et al. 2010) the mechanism of laccase production caused by mycelial interactions sill remains elusive. Here, the production of melanin in the interaction culture (as revealed by LC-MS, FTIR and NMR analysis) could be correlated to the high laccase activity, which was due to the upregulation of *lcc1* gene. Other oxidative stress related genes, such as *tps* (gene encoding terpenoid synthase), *cro* (gene encoding copper radical oxidase), *amnO* (gene encoding amine oxidase) and *alcDH* (gene encoding alcohol dehydrogenase) were also induced during interaction, which was at par the earlier report (Zhong et al. 2019). Differential expression of these stress related genes indicated the occurrence of stress signals during the interspecific mycelial interaction of the two mushroom species (Silar 2005).

Conclusion

The present study revealed that the changes in the hyphal pattern during the fungal interaction among the three white rot fungi are mostly related to the metabolites produced during interaction. An overproduction of the extracellular enzyme laccase during the fungal interaction was also marked to have a role in the detoxification process. This may be due to the production of some toxic compounds or may be due to the production of ROS during interaction by one or both of the interacting fungi. As a result of this, the production of laccase in the interaction zone increases by either or both the fungi. This study is further supported by the increase in the activity of superoxide dismutase which acts as a scavenging radical. The present study analysed differential genes expression during interspecific fungal interactions, revealing that defense-related responses and a myriad of signaling pathways might be associated with the upregulation of oxidative stress-resistant genes, along with the production of an industrially important bioactive compound (melanin), and thereby competing for both nutrient and territory.

Declarations

Ethics approval consent to participate -

No animal or human volunteers were used in this study

Consent to participate –

Not Applicable

Adherence to national and international regulations –

Not Applicable

Consent for publication –

Not Applicable

Availability of data and material –

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests –

The authors declare that they have no competing interests

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Authors' contributions –

RCB conceived the idea and acquired funding; SD and RCB designed the study; SD performed enzyme assay, scanning electron microscopy, liquid chromatography-mass spectrometry, analyzed the findings and prepared the draft manuscript; DJH, GG and SD designed the primers, interpreted the results and prepared the figures; AG, DJH, GG and TB performed the gene-expression studies; AB, MB and RCB provided the technical support during the study; All authors read the manuscript, critically revised and agreed to the content of the manuscript.

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Figures

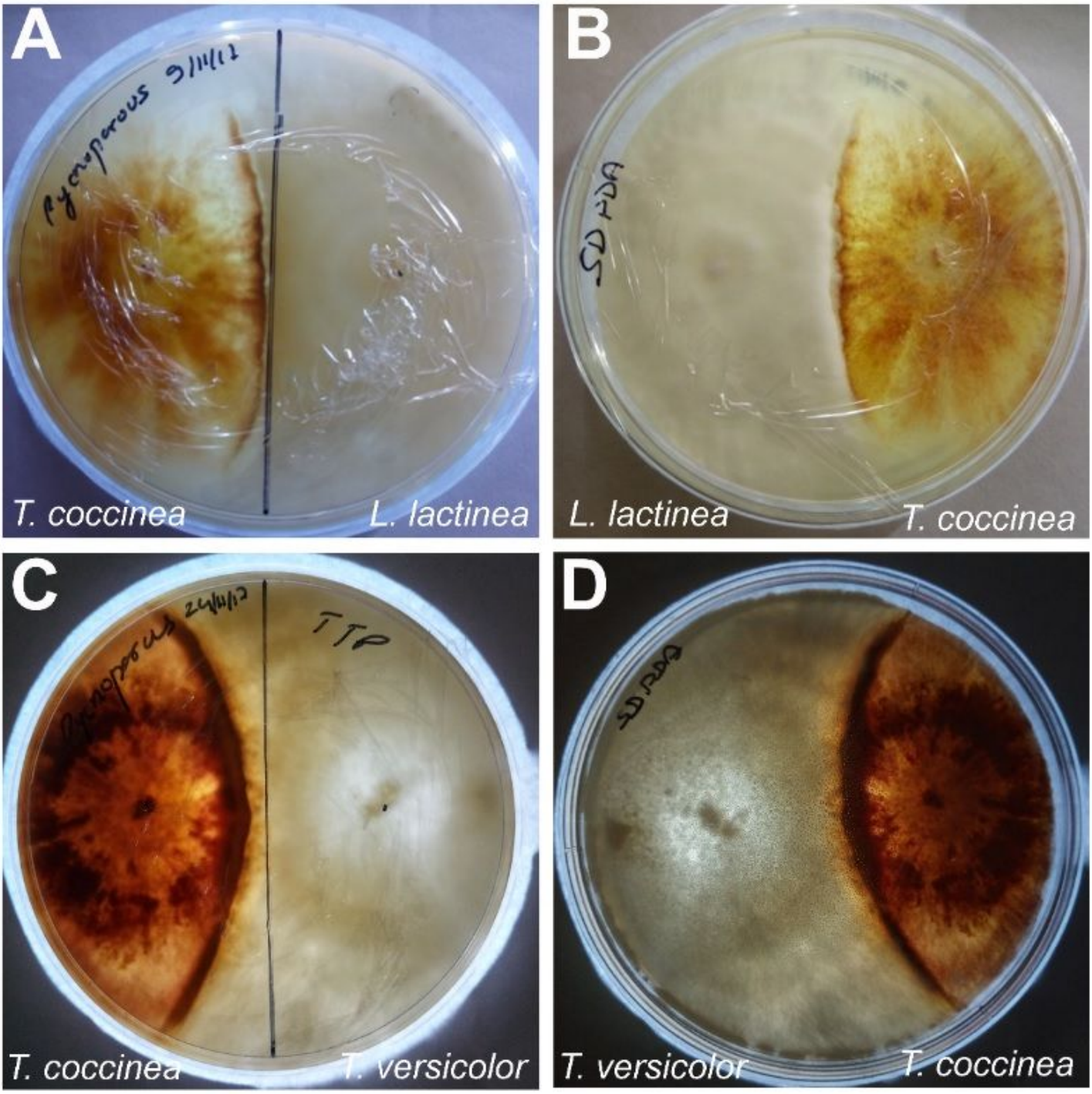

Figure 1

Interspecific interactions of T. coccinea with L. lactinea and T. coccinea with T. versicolor A and B are the front and back view of the interaction between T. coccinea and L. lactinea, C and D are the front and back view of the interaction between T. coccinea and T. versicolor.

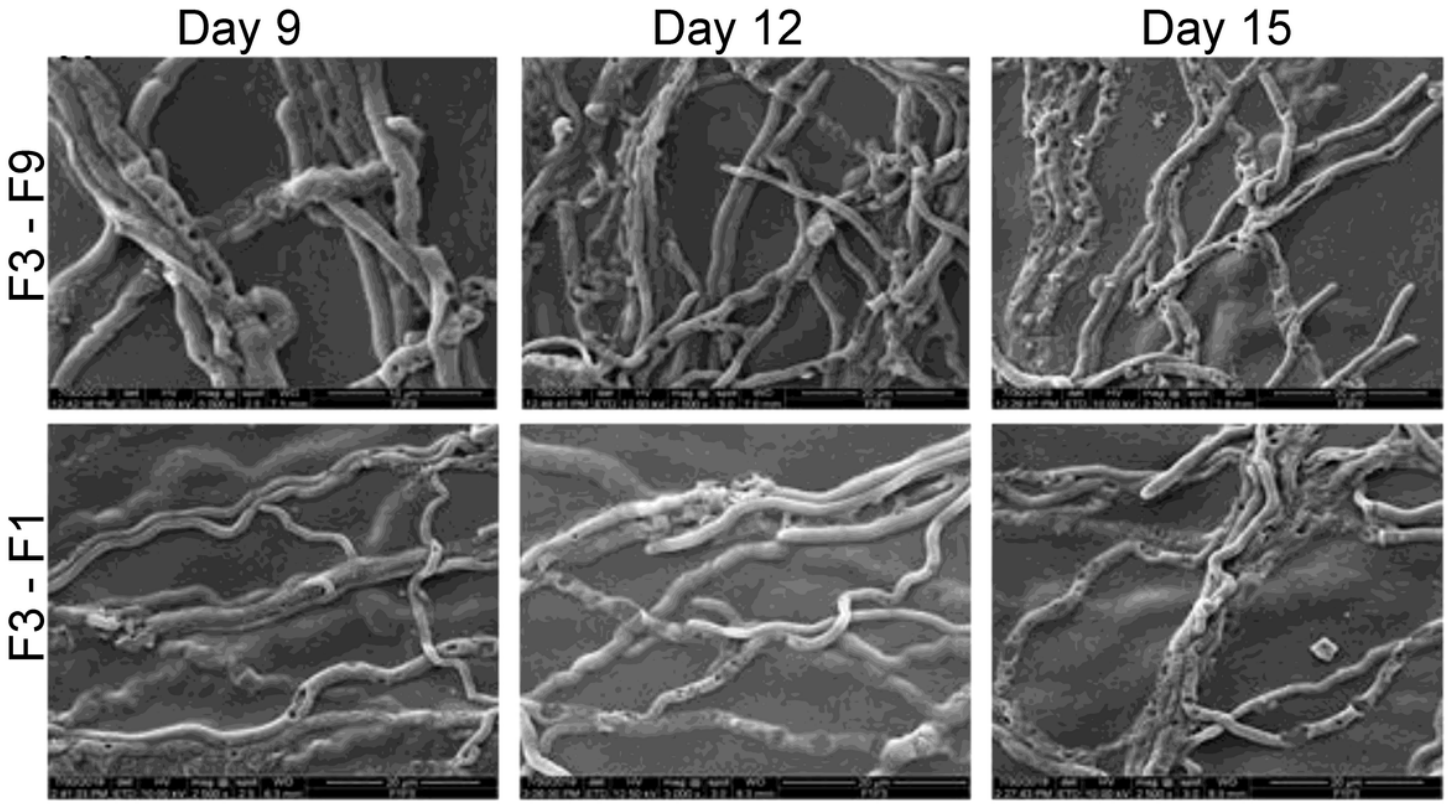

Figure 2

Scanning electron microscopic observation during interaction A and B depicts the SEM images during the interaction of T. coccinea with T. versicolor and T. coccinea with L. lactinea respectively on 9th, 12th and 15th days after inoculation.

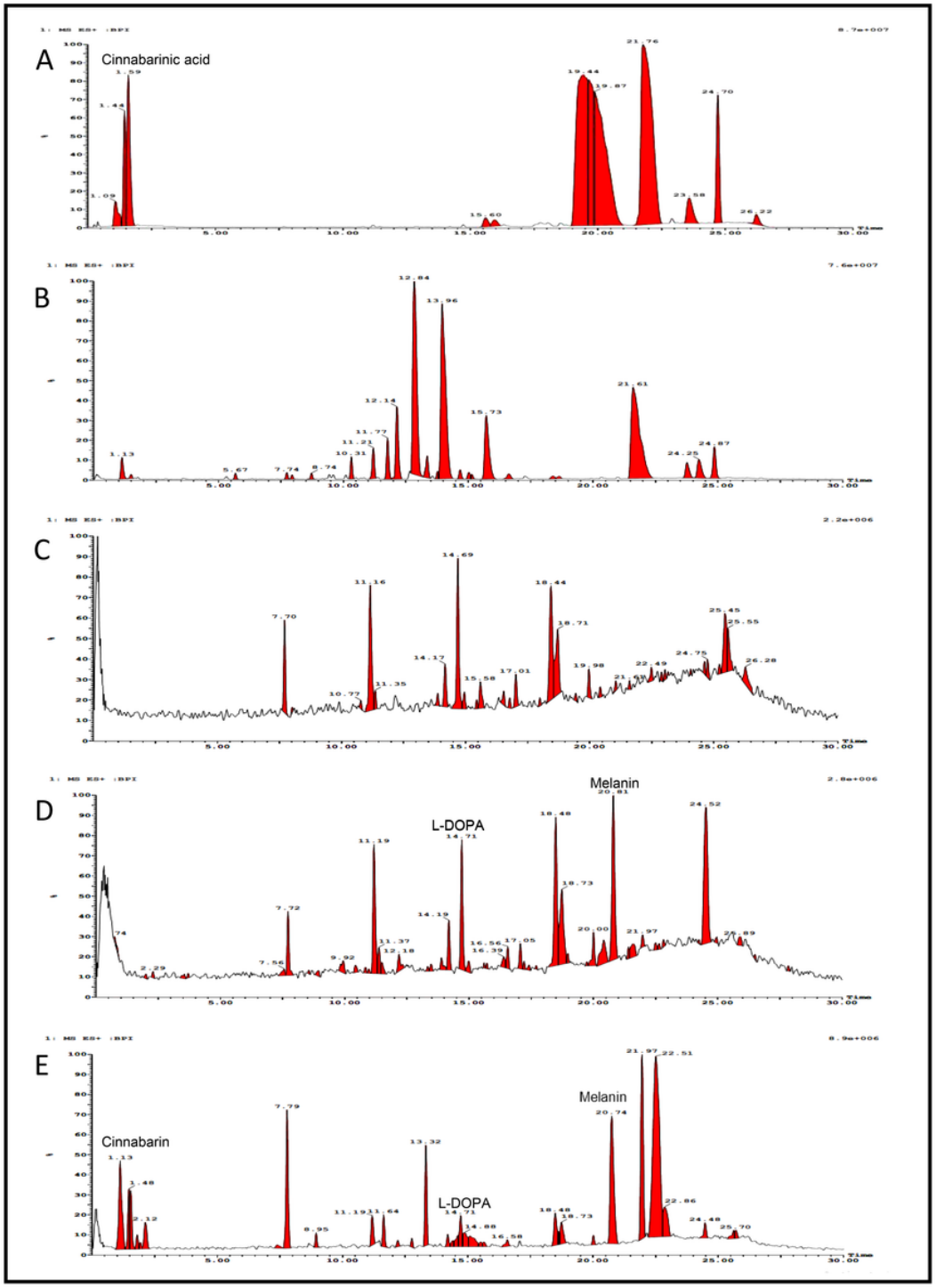

Figure 3

LC-MS chromatogram of the extracts A Trametes coccinea B Trametes versicolor C Leiotrametes lactinea D T. coccinea-T. Versicolor E T. coccinea- L. lactinea.

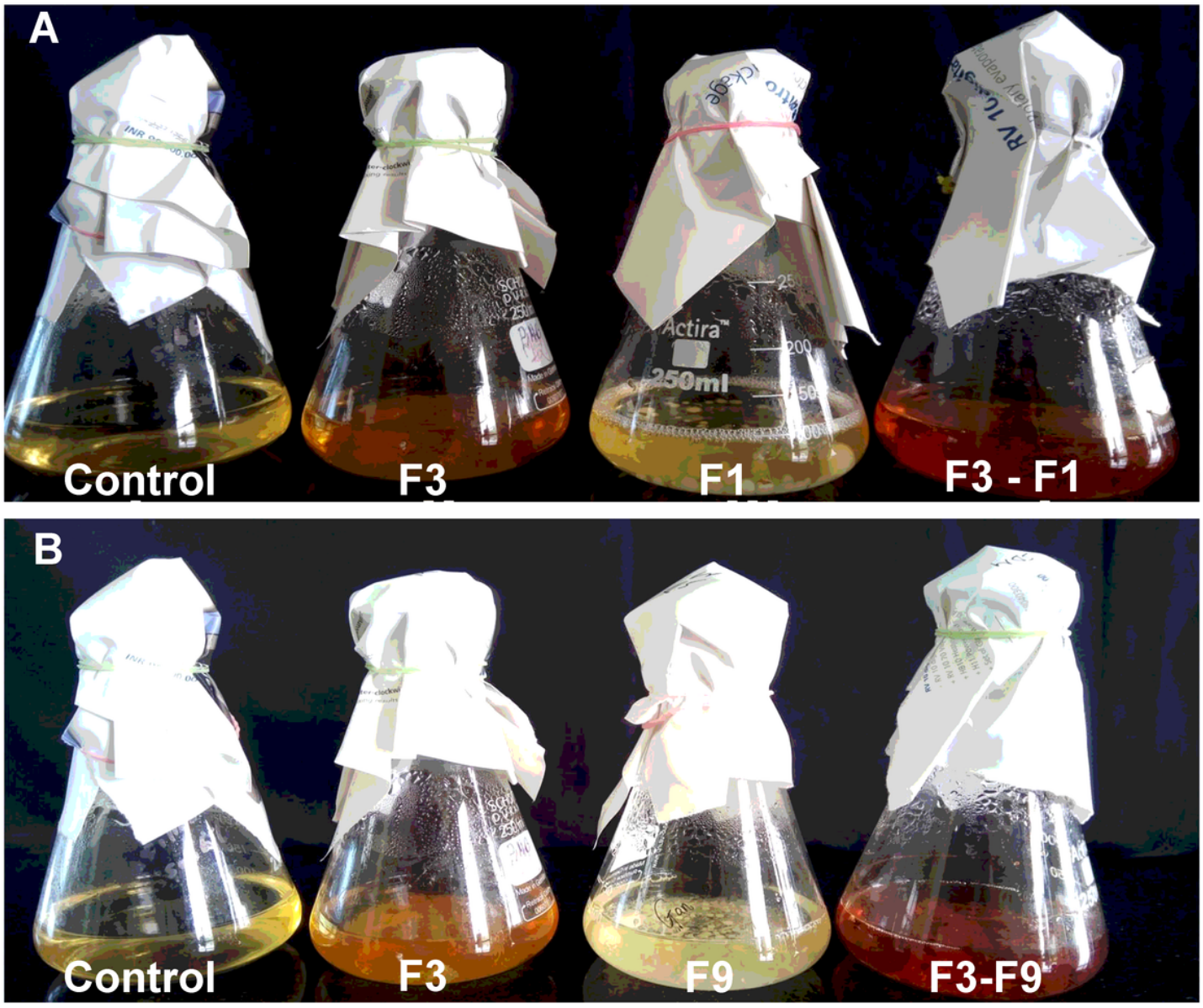

Figure 4

Culture broth showing melanin production A and B depicts dark brown color formation in the flask containing the interacting mycelia for T. coccinea - T. versicolor and T. coccinea - L. lactinea respectively.

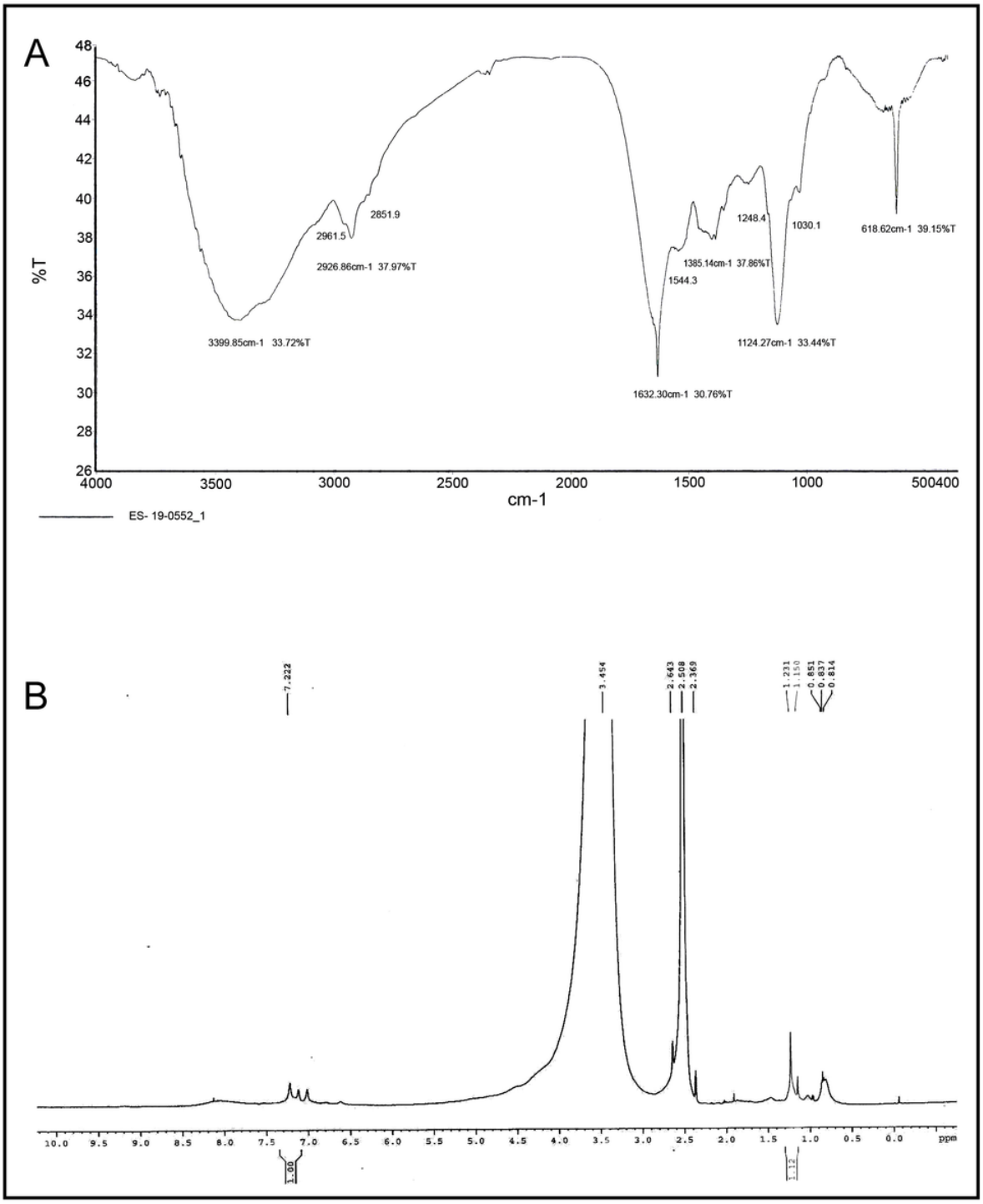

Figure 5

Spectroscopic characterization of melanin pigment purified from T. coccinea – T. versicolor A FTIR spectrum B NMR spectrum.

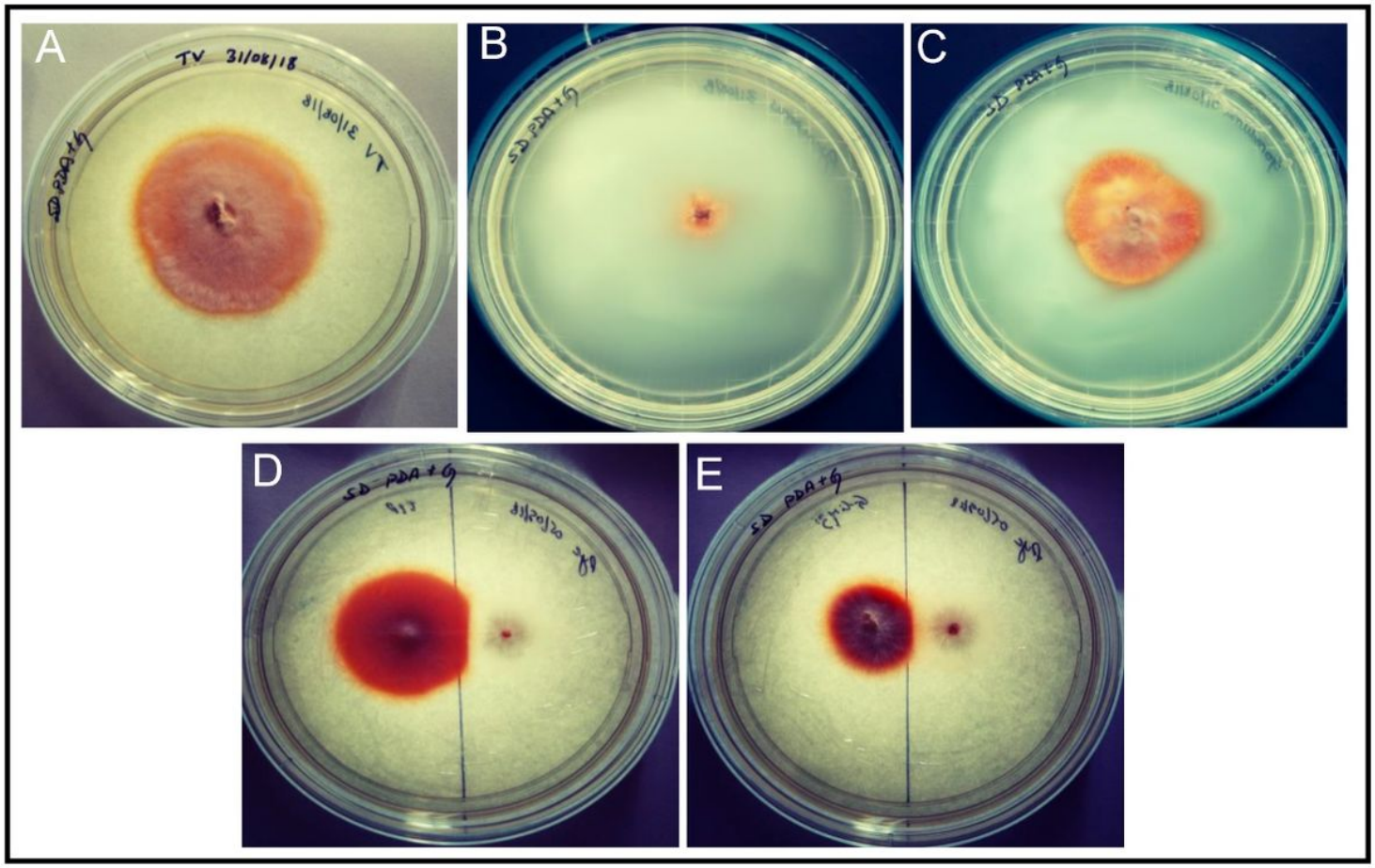

Figure 6

Qualitative laccase activity for plate cultures A T. versicolor (F1), B T. coccinea (F3), C L. lactinea (F9) D T. coccinea – T. versicolor and E T. coccinea – L. lactinea

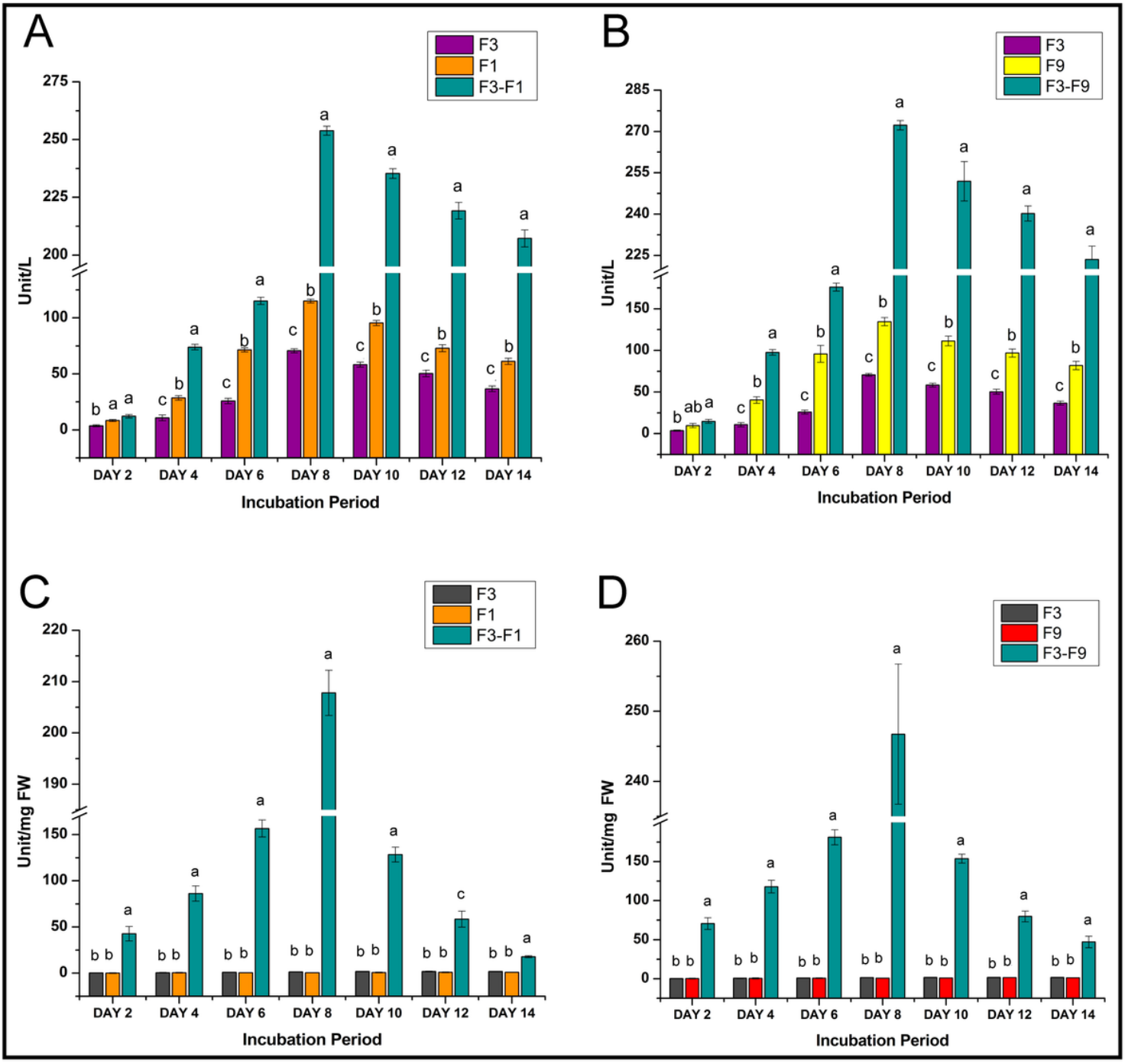

Figure 7

Quantitative analysis for enzyme production A Laccase production by T. coccinea (F3), T. versicolor (F1) and their interaction (F3-F1) B Laccase production by T. coccinea (F3), L. lactinea (F9) and their interaction (F3-F9) C SOD production by T. coccinea (F3), T. versicolor (F1) and their interaction (F3-F1) D SOD production by T. coccinea (F3), L. lactinea (F9) and their interaction (F3-F9) (level of significance with $p \leq 0.05$)

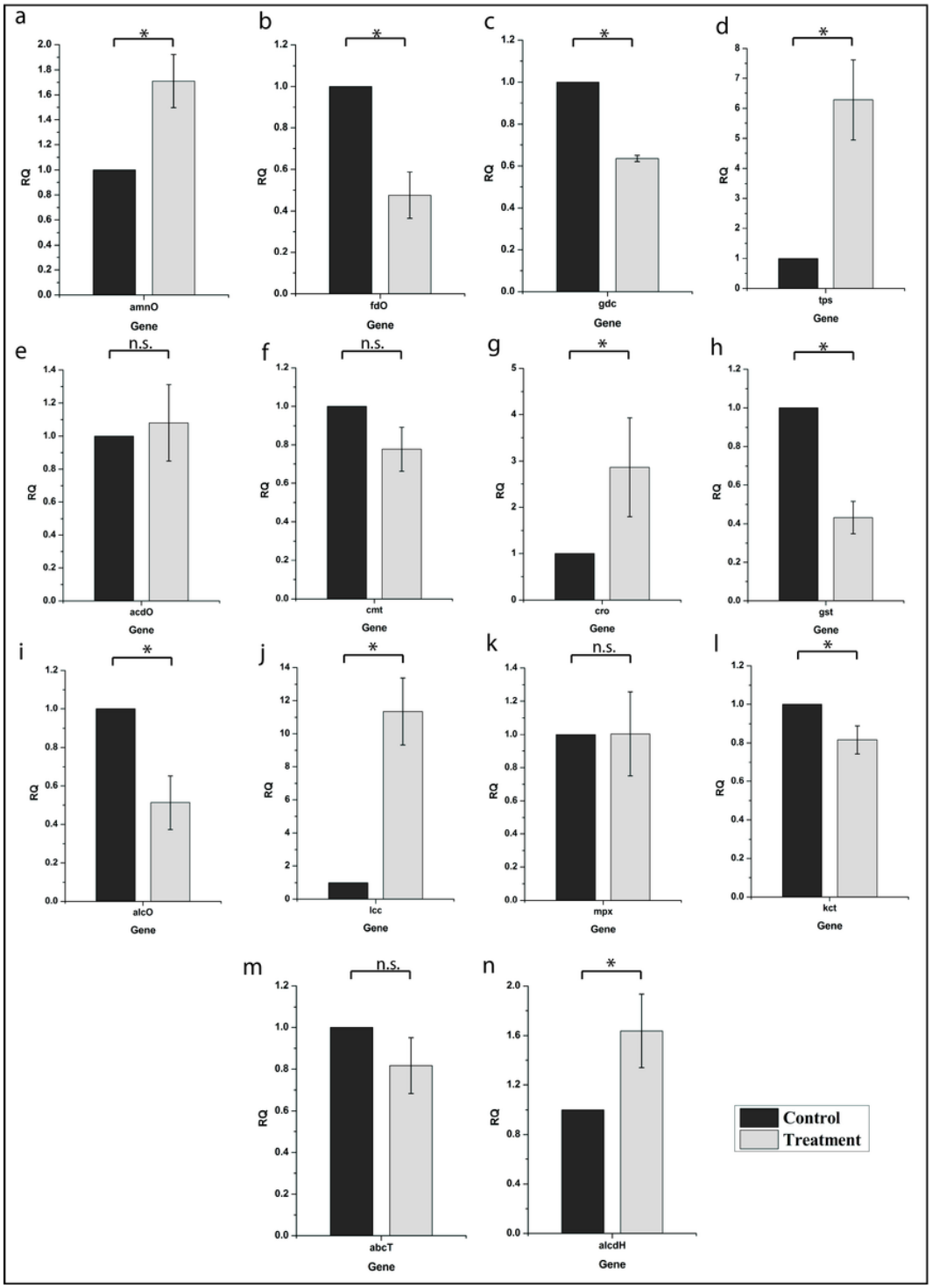

Figure 8

qRT-PCR analysis of T. versicolor (control) and T. coccinea-T. versicolor (treatment) A Amine Oxidase B FAD – linked oxidoreductase C Glutamate decarboxylase D Terpenoid Synthase E Alcohol dehydrogenase F CDF Metal transporter G Copper Radical Oxidase H Glutathione-S-transferase I Alcohol Oxidase J Laccase K Manganese Peroxidase L 3-Ketoacyl-CoA thiolase M ABC-transporter N Alcohol dehydrogenase. Significance was calculated using student's t test with $p \leq 0.05$

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1.xlsx
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