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Cloning and characterization of a laccase gene from *Ganoderma* spp. causing basal stem rot disease in coconut

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Summary. Basal stem rot disease in coconut is caused by the white-rot fungus *Ganoderma lucidum,* which is soil-borne in nature. Its degree of virulence is governed by the activity of the laccase enzyme. Of twenty-five isolates belonging to the genus *Ganoderma* obtained from different host species, the isolate from Silent Valley (SV) showed the greatest laccase activity *in vitro,* followed by the isolate from Veppankulam (CRS-1). These two isolates also reacted positively in the laccase assay *in vitro*. The laccase-positive SV and CRS-1 isolates were further amplified by polymerase chain reaction (PCR) using degenerate primers for the partial sequence, which showed the fragment size of 200 bp. The highly virulent SV isolate was cloned in a plasmid vector and sequenced. It was confirmed as a partial-length laccase gene and submitted to the GenBank database. The nucleotide sequence of the DNA of this isolate showed high homology with those of the laccase genes of other basidiomycetes.

Key words: lignolytic enzymes, cloning, degenerate primers.

Introduction

Basal stem rot (BSR) disease caused by *Ganoderma lucidum* (Leys). Karst. is the most destructive disease in coconut production, and a major limiting factor especially in Tamil Nadu, Andhra Pradesh and other coconut growing states of India. The pathogen has an ecological role in the delignification of woody plants (Peries, 1974; Bhaskaran *et al*., 1989; Lattiffah *et al*., 2002) by means of lignolytic enzymes. Among these enzymes, laccase (EC 1.10.3.2) is important. It belongs to a family of multi-copper oxidases that are widespread in fungi, bacteria (Faure *et al*., 1994), actinomycetes (Kalaichelvan and Ramasamy, 1989), as well as in insects (Thomas *et al*., 1989) and plant species (Mayer, 1987). The laccase enzyme has various functions, including participation in lignin biosynthesis (O'Malley *et al*., 1993), plant pathogenicity (Sbaghi *et al*., 1996), the degradation of plant cell walls (Machuca and Duran, 1993), insect sclerotization (Anderson, 1985) and bacterial melanization (Faure *et al*., 1994). The laccases from plants oxidize monolignols to form polymeric lignins, whereas the laccases from the white-rot fungi degrade and depolymerize the lignins. The fungal laccases (benzenediol:oxygen oxido-reductase, EC 1.10.3.2) probably have more functions, including morphogenesis, fungus/plant interaction, stress defence and lignin degradation (Thurston, 1994). Chemically, all these functions of the laccases are related to the oxidation of a range of aromatic substances. The net effects of such oxidation can be very different and even work in opposite directions. Laccase is involved in the transformation of polyphenolic compounds, such as lignin, into for-

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est litter (Criquet *et al*., 1999). Several novel laccase genes have been obtained from fungi isolated from soils (Farnet *et al*., 2002). Fungal laccases usually contain four relatively conserved regions that bind the copper atoms directly involved to their active site and are good candidates for a molecular approach in soil fungal ecology (Thurston, 1994).

Laccase is particularly abundant in many lignin-degrading white-rot fungi and this has led to speculation that it has a role in wood and pulp delignification (Dominguez *et al*., 2007). Low concentrations of several laccases are produced constitutively on wood and in submerged fungal cultures, while higher concentrations are induced by the addition of aromatic compounds (Eggert *et al*., 1996). The most extensively investigated laccase mediator is ABTS (2,2'-azinobis-3-ethylbenzthiazoline-6-sulfonate) a synthetic nitrogen-substituted aromatic compound that oxidizes non-phenolic lignin model compounds (Bourbonnais and Paice, 1990). DNA amplification from soil samples detects a higher diversity of laccase genes (Luis *et al*., 2004). Laccase genes have been isolated and cloned from fungi isolated from different sources *viz*., soils (Farnet *et al*., 2002), forest soils (Luis *et al*., 2004), *Pleurotus ostreatus* (Giardina *et al*., 1995), *Pleurotus sajor-caju* (Soden and Dobson, 2001), *Gaeumannomyces graminis* var. *tritici*, the take-all fungus (Litvintseva and Henson, 2002), *Pleurotus sajor-caju* (Soden *et al*., 2002) *Melanocarpus albomyces* (Kiiskinen and Saloheimo, 2004), and *Trametes* sp. strain I-62 (Gonzalez *et al*., 2003). Against this background, the present study was carried out to identify the wood degrading enzyme, the laccase, produced by *Ganoderma* isolates, and to carry out amplification by polymerase chain reaction, to clone and sequence the laccase gene and to compare the sequences of this gene with sequences of other fungal laccase genes.

Materials and methods

Fungal material

Ganoderma lucidum was isolated from the fruiting body (bract/basidiocarp) or the infected roots of diseased palms and forest trees on potato dextrose agar (Aneja, 1993). The fungus was purified using the single hyphal tip technique (Rangaswami, 1972) and used for further study. Cultures were maintained on potato dextrose agar slants throughout the period of study. Twenty-five isolates of *Ganoderma* were obtained from various hosts, including *Cocos nucifera*, *Areca catechu*, *Pinus* spp., *Tamarindus indica*, *Borassus* sp. and *Prosopis julifera* (Sw.) DC., in Tamil Nadu. The *Ganoderma* isolates used in the study and their sources are listed in Table 1.

Cultural characteristics of the pathogen

The fungus was grown on potato dextrose agar for seven days at room temperature (30–35°C). The colour of the mycelium, growth characters, appearance etc., was recorded for all isolates.

Assay of laccase activity in liquid culture

One or two discs of mycelium from each isolate were placed in 250 ml conical flasks containing potato dextrose broth (100 ml). The discs were incubated for sufficient growth at room temperature (30–35°C) and the laccase enzyme was assayed at three-day intervals. The assay buffer used was a mixture of 0.1 M acetic acid and 0.1 M phosphoric acid adjusted to pH 5.0 with NaOH. A 200 μl sample of culture was mixed with 750 μl of assay buffer in a 1.6 ml semimicrocuvette. To this, 50 μl of a 20 mM aqueous solution of ABTS (extinction coefficient of 35 mM^{-1} cm^{-1} at 405 nm) was added. The increase in absorbance at 405 nm was recorded over one min on a Cary Spectrophotometer (Varian instruments, Melbourne, Australia) (Wahleithner *et al*., 1996).

In vitro assay of laccase activity

For the laccase *in vitro* assay, 15 ml of 2% sterile agarose medium containing 500 μM of ABTS per ml in 50 mM glycine-HCl buffer (pH 5.0) was placed in sterile Petri dishes. Wells were formed in solidified agarose dishes with a sterile cork borer and filled with 40 μl of fungal culture filtrate (2–4 days old) (Srinivasan *et al*., 1995). Boiled culture filtrate was used as a control. The test for laccase activity was positive when the medium around the wells turned an intense bluish-green.

DNA extraction

Template DNA was extracted from pure cultures, obtained from the field as described by Moller *et al*. (1992) with slight modifications. The fungus was grown in potato dextrose broth at room temperature and the mycelial mat was dried in a conical flask when it was fully grown. A dry weight of 30–60 mg, or corresponding amounts of fresh weight,

Table 1. Isolates of *Ganoderma* spp. used in this study and their source.

Isolate code	Geographic origin[a]	Host	Source of material	External symptoms of host
CRS-1	Veppankulum, TN	*Cocos nucifera*	Sporophore	BSR lesion
SV	Silent Valley, KL	Unknown (Forest tree)	Sporophore	Dead
SLP	Sultan pet, TN	Unknown (Forest tree)	Sporophore	Dead
PR	Paiyur, TN	*Cocos nucifera*	Infected root	BSR lesion
PY	Pondicherry, TN	*Tamarindus indica*	Sporophore	Dead
OTY-1	Ooty, TN	Silver oak	Sporophore	Dead
CRS-2	Veppankulum, TN	*Cocos nucifera*	Infected root	Dead
TKT- 5	Thambikottai, TN	*Tamarindus indica*	Sporophore	Dead
TKT-2	Thambikottai, TN	*Cocos nucifera*	Infected root	BSR lesion
UDP	Udumalpet, TN	*Cocos nucifera*	Sporophore	Dead
OTY-2	Ooty, TN	*Pinus* spp.	Sporophore	Dead
SM	Salem, TN	*Cocos nucifera*	Sporophore	Dead, BSR lesion
MDU	Madurai, TN	*Cocos nucifera*	Sporophore	Dead, BSR lesion
CUD	Cuddalore, TN	*Cocos nucifera*	Infected root	BSR lesion
CRS-3	Veppankulum, TN	*Borassus* sp.	Sporophore	BSR lesion
CBE-1	Coimbatore, TN	*Cocos nucifera*	Sporophore	BSR lesion
CBE-2	Coimbatore, TN	*Cocos nucifera*	Infected root	Dead
CBE-3	Coimbatore, TN	*Cocos nucifera*	Sporophore	Dead
PK	Pudukottai, TN	*Cocos nucifera*	Infected root	Foliar, BSR lesion
VPM-1	Veppankulum, TN	*Cocos nucifera*	Infected root	Foliar, BSR lesion
TKT-1	Thambikottai, TN	*Cocos nucifera*	Infected root	BSR lesion
TKT-4	Thambikottai, TN	*Prosopis julifera*	Sporophore	No discolouration
TKT-3	Thambikottai, TN	*Cocos nucifera*	Infected root	Foliar, BSR lesion
MTP	Mettupalayam, TN	*Areca catechu*	Sporophore	BSR lesion
CBE-4	Coimbatore, TN	Red gloom over	Sporophore	Lesion

[a] TN, Tamil Nadu; KL, Kerala.

were sufficient to permit microextraction in 500 μl buffer. Lyophilized mycelium was ground with fine sand in a mortar and the powdered mycelium was transferred to a microtube containing 500 μl TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS). Proteinase K (50–100 μg) from a stock solution was added and incubated for 30 to 60 min at 55–60°C with occasional gentle stirring. The salt concentration was adjusted to 1.4 M with 5 M NaCl, and 1/10 vol 10% CTAB (cetyltrimethylammoniumbromide) was added. It was incubated for 10 min at 65°C and 1 vol SEVAG (chloroform: isoamylalcohol, 24:1, v:v) mix was added gently. The mixture was incubated for 30 min at 0°C and then centrifuged for 10 min at 4°C. The supernatant was transferred to a fresh tube, 0.55 vol isopropanol was added to the precipitate DNA, and this was centrifuged immediately for 5 min at 5000 g. The supernatant was discarded and the pellet was washed twice with cold 70% ethanol, dried and dissolved in 50 μl TE buffer.

Cloning and sequencing of the laccase gene

The DNA of the Silent Valley (SV) isolate, selected on the base of high laccase activity, was

amplified with degenerate primers specific to the basidiomycetes Cu1F (5'-CAT(C) TGG CAT(C) GGN TTT(C) TTT(C) CA-3') and Cu2R (5'-G G(A)CT GTG GTA CCA GAA NGT NCC-3') (D'Souza *et al*., 1996). PCRs were run on a Master cycler gradient system (Eppendorf, Hamburg, Germany) with an initial cycle of denaturation (3 min at 94ºC) followed by 35 cycles with denaturation (30 s at 94ºC), annealing (30 s at 50ºC) and elongation (2 min at 72ºC), and by a final elongation (10 min at 72ºC). Amplified DNA was purified from each reaction mixture by agarose (1.2%, w:v) gel electrophoresis in TBE buffer containing 0.5 μg of ethidium bromide per ml. A small agarose slice containing the band of interest (observed under long-wavelength [312-nm] UV light) was excised from the gel and purified using a QIAquick gel extraction kit (Qiagen, Inc., Chatsworth, CA, USA) following manufacturer's instructions. Purification was performed to remove primer dimers and other residues from the PCR amplification. Fragments were cloned into the T/A vector pCR2.1 (Fermentas, St. Leon-Rot, Germany) and transformed into *Escherichia coli* strain DH5α according to the procedure recommended by the manufacturer. Transformants were selected on lima bean (LB) agar amended with ampicillin (75 mg ml^{-1}) and X-Gal (20 mg ml^{-1}). Clones were randomly selected and used as templates in PCR to produce products of the required size, 203 bp, in agarose gel. Clones producing PCR fragments of the appropriate size were subjected to DNA sequencing. DNA sequencing was performed at Genei Pvt Ltd, Bangalore, India. For sequence determination of the cloned PCR products, a generally applicable sequencing strategy was developed. The sequences for entire cloned PCR products, approximately 203 bp in length, were determined using vector-encoded M13 sequencing primer sites, forward primer (5'-CACGACGTTGTAAAACGAC-3') and reverse primer (5'-GGATAACAATTTCACACAGG-3').

rDNA ITS sequencing matching

Comparison of the sequence obtained from Genei Pvt Ltd, Bangalore, India was performed, using BLAST analysis, with the database already available in the National Centre for Biotechnological Information (NCBI) (Altschul *et al.,* 1990). Average linkage cluster analysis of aligned sequences for the construction of the phylogenetic trees was performed with Treecon version 1.15. Clustering was determined by UPGMA analysis of pairwise genetic distance values. Amino acid and nucleotide sequences were aligned using CLUSTAL X 1.81. The newly obtained sequence was deposited with the NCBI database, GenBank, New York (NY), USA.

Statistical analysis

The data were statistically analyzed (Rangasamy, 1995) using IRRISTAT version 92 developed by the International Rice Research Institute Biometrics Unit, the Philippines (Gomez and Gomez, 1984). The percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two levels of significance (P<0.05 and P<0.01) and means were compared by Duncan's Multiple Range Test (DMRT).

Results

Cultural characteristics of the pathogen

Most of the isolates produced a dense mycelial growth on PDA, while a few isolates showed sparse mycelial growth. Most isolates were white in the initial stage of growth and later the colony colour changed to pale yellow or light brown. Fast growing isolates produced a dense mycelial growth on PDA. The aerial mycelium mostly consisted of thin-walled, branched hyphae with clamp connections. Basidiocarp primordia and basidiospores did not form in culture. Isolates SV and CRS-1, which were fast growing, overgrew the entire Petri dish in five to seven days and produced a dense mycelial growth on PDA.

All isolates were identified as belonging to the genus *Ganoderma*, while only isolate SV was fully identified as *G. lucidum*.

Growth of *Ganoderma* spp. isolates in liquid broth

The isolates took nine to fifteen days to overgrow the entire surface of the potato dextrose broth in the Petri plate and conical flask. The isolates SV and CRS-1 produced greater mycelial growth than the other isolates.

Colorimetric assay for laccase

Laccase activity of *Ganoderma* isolates was studied in stationary culture. The highest laccase activity on the ninth day after inoculation occurred in the fast growing isolates (double that of the slow growing ones). Activity was less in

the medium and in slow growing isolates (Table 2). Laccase activity gradually increased from the third to the ninth day of inoculation, then gradually decreased. A greater laccase activity was observed on the ninth day after inoculation on all the isolates tested. Isolate SV produced the greatest laccase activity (66.00) followed by CRS-1 (65.27). The laccase activity of isolate SM was the lowest at 27.04.

***In vitro* assay for laccase**

The extracellular laccase produced by the isolates was assayed on agarose Petri dishes containing ABTS. The development of an intense bluish-green colour indicated the presence of laccase . There was no colour development in the fluid when it was heated or boiled. The CRS-1 isolate showed laccase activity *in vitro*. An intense bluish green colour was seen after three to five days.

PCR amplification of the laccase gene

PCR was performed to detect the laccase gene using degenerate primers. The degenerate primers amplified a fragment size of 200 bp, corresponding to the region of the 16s-23s rRNA intervening sequence for the laccase gene. The DNA region most commonly used for molecular determination of filamentous fungi is the gene cluster that codes for ribosomal RNA (rRNA). This cluster consists of the genes for the 5.8S, the small and the large ribosomal subunits, which are separated by internal transcribed spacers (ITS). The isolates CRS-1, SV and SLP showed amplification of 200 bp which indicated the presence of the laccase gene (Fig. 1).

Cloning and sequencing of the laccase gene

The 16s-23s rDNA fragments of isolate SV, the one showing the highest laccase activity, were cloned into the T/A vector pTZ57R/T and transformed into *Escherichia coli* strain DH5α. Transformants on LB agar amended with ampicillin were randomly selected and used as templates in PCR to produce the products of the required size of 200 bp in agarose gel (Fig. 1). The partial sequence of the laccase gene of isolate SV was determined and submitted to the NCBI. The accession number was DQ333343 (laccase gene, partial sequence). They were compared with the nucleotide and aminoacid sequences of different laccases from various countries in the GenBank database (Table 3). The unweighted pair group method with arithmetic means (UPGMA) tree resulting from the analysis of nucleotide and aminoacid sequences of the DNA gene using the sequences from the GenBank is shown in Fig. 2. The resulting SV 200 bp partial length sequence showed a greater similarity with the laccase gene of *Panus rudis* than with the sequences of other laccase genes. The genetic distances between sequences from the GenBank and from the cloned sequences of DNA based on UPGMA are shown in Table 4.

Table 2. Laccase activity of *Ganoderma* spp. isolates.

Isolates	Laccase activity nine days after inoculation
CRS-1	65.27 ab
SV	66.00 a
SLP	41.13 i-j
PR	40.07 kl
PY	41.03 i-j
OTY-1	38.93 l
CRS-2	37.10 l
TKT-5	45.23 g-k
TKT-2	46.53 fgh
UDP	46.27 f-i
OTY-2	27.07 m
SM	27.04 m
MDU	42.17 h-l
CUD	46.60 fgh
CRS-3	51.17 def
CBE-1	52.10 de
CBE-2	60.50 bc
CBE-3	40.70 jkl
PK	45.47 g-j
VPM-1	59.53 c
TKT-1	48.12 efg
TKT-4	54.93 d
TKT-3	52.97 de
MTP	62.47 abc
CBE-4	44.70 g-k

Values are the means of two replications One unit (U) of an oxidase oxidises 1 μmol substrate/min. Means in a column followed by a common letter are not significantly different by DMRT at P=0.05.

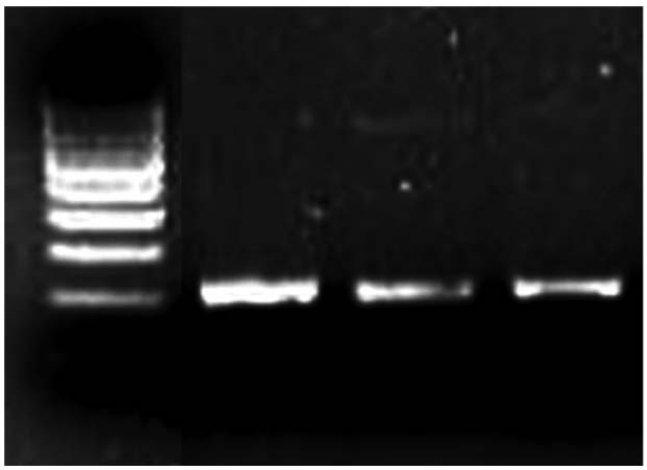

Fig. 1. Laccase gene amplification. Lane 1, 100 bp ladder; lane 2, SV; lane 3, CRS-1; lane 4, SLP.

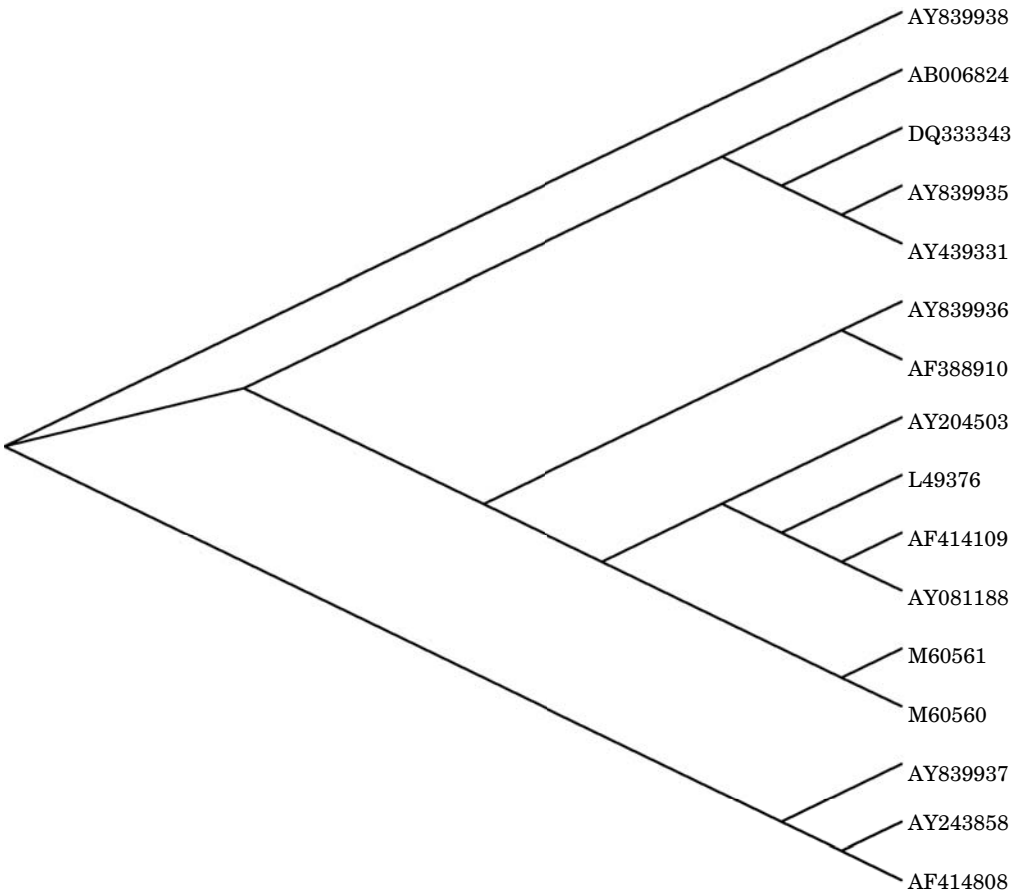

Fig. 2. UPGMA tree of the laccase gene of *Ganoderma* sp. isolate SV with other nucleotide sequences from GenBank.

Table 3. Nucleotide sequences of laccase genes used in this study.

Fungal species and laccase gene	Accession number
Crilipha hirsutus ligninolytic phenol oxidase gene	M60561
C. hirsutus ligninolytic phenoloxidase gene	M60560
Trametes sp. AH28-2 laccase C	AY839937
Trametes sp. AH28-2 laccase D	AY839938
Trametes sp. AH28-2 laccase A	AY839936
White-rot fungus AH28-2 laccase gene	AF388910
Trametes versicolor putative laccase gene	AY204503
Ganoderma lucidum isolate Lcc9 laccase gene	AY243858
Panus rudis laccase A (lacA) gene	AY839935
Trametes versicolor laccase B precursor (lac1)	AF414109
Panus rudis laccase mRNA	AY439331
Trametes villosa (clone LCC1) laccase gene	L49376
Ganoderma tsunodae mRNA for bilirubin oxidase	AB006824
Trametes pubescens laccase 1A (lap1A) gene	AF414808
Trametes versicolor laccase III gene	AY081188
Coriolus versicolor CVL3 gene for laccase	D13372

Table 4. Sequence identity similarity matrix for the laccase gene of the *Ganoderma* sp. isolate SV based on sequence data with of the GenBank database reference isolates.

Isolates	DQ333343	M6056	M6056	AY839	AY839	AY839	AF388	AY204	AY243	AY839938	AF414	AY439	L4937	AB006	AF414	AY081
DQ333343	1.000	0.133	0.133	0.28	0.251	0.236	0.236	0.128	0.182	0.256	0.162	0.206	0.182	0.192	0.088	0.182
M6056		1.000	1.000	0.111	0.142	0.110	0.110	0.858	0.206	0.155	0.568	0.193	0.578	0.164	0.176	0.572
M6056			1.000	0.111	0.142	0.110	0.110	0.858	0.206	0.155	0.568	0.193	0.578	0.164	0.176	0.572
AY839				1.000	0.229	0.720	0.720	0.134	0.608	0.182	0.233	0.187	0.204	0.192	0.134	0.204
AY839					1.000	0.229	0.229	0.147	0.125	0.215	0.224	0.224	0.202	0.311	0.098	0.196
AY839						1.000	1.000	0.139	0.569	0.215	0.220	0.215	0.220	0.181	0.122	0.215
AF388							1.000	0.139	0.569	0.215	0.220	0.215	0.220	0.181	0.122	0.215
AY204								1.000	0.267	0.161	0.621	0.193	0.632	0.153	0.169	0.62
AY243									1.000	0.123	0.159	0.149	0.168	0.137	0.181	0.156
AY839938										1.000	0.247	0.236	0.241	0.161	0.112	0.236
AF414											1.000	0.298	0.786	0.230	0.112	0.775
AY439												1.000	0.281	0.489	0.121	0.27
L4937													1.000	0.203	0.114	0.981
AB006														1.000	0.098	0.197
AF414															1.000	0.12
AY081																1.000

Discussion and conclusions

Ganoderma species are believed to be highly effective degraders of lignin polymers by producing lignolytic enzymes. Of these enzymes, laccase is the most important, and it oxidizes nonphenolic aromatic compounds with relatively low redox potentials (Youn *et al*., 1995; Dominguez *et al*., 2007). Decaying wood is the most typical environment in which laccase is produced. The mechanisms that are involved in lignocellulose degradation by laccases have been studied in detail by many authors (Baldrian, 2006). In the present study, laccase activity of 25 *Ganoderma* spp. isolates was measured. Measurements showed a gradual increase in enzyme activity over 9 days, followed by a slow decrease. Differences in laccase production by *Ganoderma* isolates were reflected by differences in the growth habit (fast, moderate or slow) of the isolates under laboratory conditions: enzyme activity was very low in slow growing isolates and high in fast growing isolates. The reduction in enzyme activity after nine days was very great in slow growing isolates compared to that in moderate and fast growing isolates. This may be correlated with the virulence factor of the pathogen and is in agreement with Johansson *et al*. (1999), who documented the virulence factor of extracellular laccase production by *Heterobasidion annosum* on many forest trees. Low concentrations of several laccases are produced constitutively in wood and in submerged fungal cultures, while higher concentrations are produced when aromatic compounds such as 2,5-xylidine or ferulic acid (Eggert *et al*., 1996) were added. Apart from this, the isolates SV and CRS-1 turned an intense bluish-green on an ABTS-amended medium. These results corroborate Srinivasan *et al*. (1995). These authors also stated that the appearance of an intense bluish-green colour on ABTS amended medium indicated that laccase was produced by the micro-organism. According to the available nucleotide sequences of laccase genes from basidiomycetes, the PCR performed with this primer pair was expected to produce fragments of approximately 200 bp (Luis *et al.,* 2004). The DNA region most commonly used for the molecular determination of filamentous fungi is the gene cluster that codes for ribosomal RNA (rRNA). This cluster consists of the genes for the 5.8S, the small and the large ribosomal subunits, which are separated by internal transcribed spacers (ITS). It supported the present study, in that the virulent isolates amplified the 200 bp partial length fragment size of the laccase gene. Laccase enzymes in ectomycorrhizal (ECM) fungi are directly involved in degrading organic polymers in which nitrogen and phosphate resources are sequestered. In the present study, the partial length laccase gene 200 bp was cloned and sequenced from the isolate SV and was similar to *Panus rudis* and other laccase-producing organisms. In other studies with similar findings, four laccase isozymes (LCC1, LCC2, LCC3 and LCC4), synthesized by *Pleurotus ostreatus* strain V-184, were purified and characterized (Gonzalez *et al.,* 2003); the laccase gene was cloned and sequenced from the lignin-degrading basidiomycete *Pleurotus ostreatus* (Giardina *et al.,* 1995), and when four laccase isozyme genes, Psc *lac1*, *2*, *3* and *4* were cloned from the edible mushroom, *Pleurotus sajor-caju* (Soden and Dobson, 2001) the genes displayed a high degree of homology with other basidiomycete laccases (55–99%).

The *Ganoderma* sp. isolate SV and the *G. Lucidum* isolate CRS-1 showed the greatest laccase activity and the gene sequence reported here will be useful in the mass production of fungal laccase, which is expected to be applicable in various methods. The laccase enzyme can also be exploited for the degradation of polycyclic aromatic hydrocarbons (PAHs) in the pulp industry.

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