Jurnal Penelitian Kelapa Sawit, 2006, 14(2): 93-102

ISOLATION OF PARTIAL LACCASE GENE OF *GANODERMA* IN OIL PALM USING PRIMER DESIGNED FROM COPPER-BINDING REGION

Condro Utomo

ABSTRACT

A primer pair designed from the consensus sequences of the copper-binding regions in the N-terminal domain of known basidiomycete laccases were used to isolate laccase-gene from Ganoderma *isolated from oil palm. Primer Lac 2a-Lac 2r gave the PCR product of about 1617 bp. Computer searches of the databases identified the sequence of the PCR product analyzed as laccase gene sequence, suggesting the specificity of the primers. PCR product of* Ganoderma *showed 44.5 to 76.7 % nucleotide sequence similarity to other basiodiomycete fungi and cluster to* Tremetes villosa *laccase gene.*

Keywords: *partial laccase gene, oil palm*, Ganoderma

ABSTRAK

Sepasang primer yang dirancang dari sekuen konsensus yang berasal dari daerah "copper-binding" pada domain N-terminal dari gen-gen laccase jamur dari klas basiodiomycet digunakan untuk mengisolasi gen laccase yang berasal dari Ganoderma *pada kelapa sawit. Pasangan primer Lac 2a-Lac 2r menghasilkan produk PCR berukuran kira-kira 1617 bp. Pencarian identitas berdasarkan komputer pada bankdata dari produk PCR menunjukkan bahwa produk PCR tersebut adalah benar gen laccase. Sekuen dari gen laccase dari* Ganoderma *asal kelapa sawit menujukkanhomologi sekuen sebesar 44,5-76,7% dengan gen laccase dari jamur basidiomycet lain dan mengelompok pada jamur* Tremetes villosa *pada pohon philogenetik.*

Kata kunci : *gen laccase partial, kelapa sawit*, Ganoderma

1. INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is one of the most important estate crops in Indonesia. Onwards from the second and subsequent planting cycles, oil palm is threatened by fungal pathogens, especially by *Ganoderma* spp. (Aphyllophorales, Basidiomycota), the causal agent of basal stem rot (BSR) disease that attacks the root system of oil palm. In basidiomycete fungi, the gene encoding the laccase enzyme (benzenediol: O_2 oxidoreductase; E C 1.10.3.2) has been widely studied, apart from its role in delignification of plant

material. Laccase appears to be involved also in different cellular processes such as sporulation, pigment production, fruiting body formation, rhizomorph induction and also in plant pathogenesis.

An intensive research concerning the role of laccase enzyme involved in plant pathogenesis for various fungi such as *Botrytis cinerea* and *Cryphoneria parasitica* have been conducted. *B. cinerea* causes soft rot infections in many horticultural crops and this fungus produces extracellular laccases that involved in the pathogenic process because cucurbitacins, tetracyclic triterpinoids produced by the cucumber, protect the plant from infection (3,4) and this protection is mediated by specific repression of laccase synthesis by the fungus (19). Evidence of a role for laccase in pathogenesis has also been obtained in the chestnut blight fungus *C. parasitica* (17). There are hipovirulent strains of this fungus in which the diminution of virulence is associated with the presence of a double-stranded RNA of viral origin. These strains are repressed for laccase synthesis by prevention of accumulation of laccase mRNA.

The aim of the future research is to study the role of the laccase gene of *Ganoderma* in oil palm pathogenesis.

2. MATERIALS AND METHODS

2.1. Fungal isolates and DNA extraction

Ganoderma isolates from oil palm (from Bukit Sentang estate/BS) were grown in malt-yeast medium (6) for a month at 30 °C. Mycelia were harvested from liquid cultures by filtration onto Whatman No.1 filter paper and rinsed two times with double distilled water. Mycelia were freeze-dried for two days, ground to a fine powder in a pestle and mortar and then stored at -20 °C until use. Total genomic DNA of each *Ganoderma* isolate was extracted according to the method of Möller *et al.* (16) with an additional phenol/chloroform extraction. The extracted DNA was quantified by UV spectrophotometry (Beckman DU-50 Spectrophotometer, Germany) and checked by agarose gel electrophoresis.

2.2. Primer design and polymerase chain reaction (PCR) conditions

Primers were designed based on the conserved amino acid sequence in copper-binding region I and copper-binding region IV of six laccase genes of published basidiomycete fungi. Six amino acid sequences of the laccase genes were derived from National Centre for Biotechnology Information/NCBI. The following basidiomycete fungi were used: *Trametes villosa* (accession number AAC41686), *T. villosa* (L49377), *T. villosa* (L78077), Basidiomycete CECT 20197 (AAB63443), Basidiomycete PM 1 (CAA78144), *Trametes versicolor* (CAA59161). Two 17- or 18-base sequences designed from DNA sequences of the conserved amino acid sequences of copper-binding region are primers Lac 2a (5`TGGCACGGCTTCTTCCAG 3`) and Lac 2r (5`CACTGCCACATCGACTTC

3'). For PCR amplification, 5 µl of the extracted DNA (100 ng) was added to 20 µl reaction mix. The thermocycler was programmed, as followed: after 5 min heating at 95 °C, the DNA amplification was carried out in 40 cycles of 35 sec denaturation at 94 °C, 45 sec annealing at 65 °C and 60 sec extension at 72 °C. The 40 cycles were ended after 10 min extension at 72 °C and cooled to 4 °C. The PCR products were either analysed immediately or stored at -20 °C. The PCR products were analyzed by electrophoresis on a 1.5 % agarose gel and stained with ethidium bromide to visualize the amplicons under UV light.

2.3. Cloning and Sequencing

PCR products derived from PCR amplification of primer Lac 2a and Lac 2r were purified using QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. After purification, PCR products were cloned in plasmid vector of pCR^R 2.1-TOPO from TOPO TA cloning kit (Invitrogen, Netherlands) according to the manufacturer's instructions. The cloned DNA fragments were sequenced on both strands using forward and reverse universal primers M13. Ready Reaction BigDye Terminator Cycle Sequencing kit (Perkin Elmer Corp., USA) was used to sequence laccase gene. The sequence was determined using an ABI prism 310 DNA sequencer (Applied Biosystem Inc., USA).

2.4. Data analysis and µhylogeny

For the laccase gene, computer-assisted comparisons of the nucleotide sequences were made to find the similarities of nucleotide sequences in

Table 1. Laccase amino acid sequences of various basidiomycete fungi used for comparison with the laccase gene from oil palm *Ganoderma* BS

Fungi and gene	Laccase amino acid sequence GenBank accession numbers
1. *Agaricus bisporus* Lac 1	AAC18877
2. Basidiomycete CECT 20197 Lac pox 1	AAB63443
3. Basidiomycete PM 1 Lac	CAA78144
4. *Coprinus cinereus* Lac 1	AAD30964
5. *Ceriporiopsis subvermispora* Lac	AAC97074
6. *Lentinula edodes* Lac 1	AAF13037
7. *Marasmius quercophilus* Lac 1	AAF06967
8. *Pycnoporus cinnabarinus* Lac 1	AAF13052
9. *Pleurotus ostreatus* Lac	CAA06291
10. *Phlebia radiata* Lac	CAA36379
11. *Schizophylum commune* Lac (mRNA)	BAA31217
12. *Trametes versicolor* Lac 1	CAA59161
13. *Trametes villosa* Lac 1	AAC41686

NCBI/GenBank, using BLASTN program (1). Nucleotide sequences that encoded amino acid (exons) for the laccase gene were translated to the (deduced) amino acid sequences by using EditSeq (DNAstar, Madison, USA). To compare the laccase gene of oil palm *Ganoderma* with other laccase genes, several laccase genes of basidiomycete fungi were used as described in Table 1. Alignments of sequences were done using Clustal V algorithm method (MegAlign; DNAstar, Madison, USA). Phylogenetic tree of the laccase gene was calculated and constructed using MegAlign program (DNAstar, Madison, USA).

3. RESULTS

By using the primer pair Lac 2a-Lac 2r, *Ganoderma* isolated from oil palm produced a single PCR product of about 1,650 bp as shown in Figure 1. To confirm the identity of nucleotide sequences of oil palm *Ganoderma* amplified by the primer pair Lac2a-Lac 2r, a computer-assisted comparison of the nucleotide sequences with the existing nucleotide sequences in gene databases was performed by using the BLASTN program (1). By sending a partial nucleotide sequence of 300-500-bp to the gene databases, the identity of the sequenced DNA fragment could be determined. Similarity report of the nucleotide sequence amplified by primer pair Lac 2a-Lac 2r with nucleotide sequence in GenBank (here only one similarity report is presented as an example):

```
L49376.1 TMTLCCA Trametes villosa (clone LCC1) laccase gene, exons 1-
9, complete cds.Length = 2417

 Score = 111 bits (56), Expect = 3e-22Identities = 193/239 (80%)
 Strand = Plus / Minus

Query: 236  ccgtgcaagtgnaacgggtgcggggtgccgggggcgctggcggtcgcgggaaggtgagc   295
            ||||||||||| || ||||| || |  |||||||||  ||||||||||||||| || |
Sbjct: 1925 ccgtgcaagtggaaggggtggggcgcaccgggggcggcggcggtggcgggaaggagatc  1866

Query: 296  tcgatggaggagttcattgggagctcgtagacgctgccggaggggaggagctcctgcgcg   355
            ||||||  || ||||   || |||  ||||||||| |||||||| ||||| |||||||||
Sbjct: 1865 tcgatgtcggcgttcgagggaagcgagtagacgctaccggagggcaggaggtcctgcgcg  1806

Query: 356  gtctgtgcgccgctgaggatctgcaggagcacgggcacggtgggcgggacgaaggtgtcg   415
             |||| |||||||||| |||||| || || || |||||||| || ||  ||| |  |
Sbjct: 1805 ttctgcgcgccgctgatgatctggagcaggacaggcacggtcgggggcgtgaaagacgtg  1746

Query: 416  ccgttgatgaagaagcgggagccgttgaagttgaacgctaagttgatcgccaggtcgac   474
            ||||||||||||||  || ||||||||||||||||| | |||||| ||||||||||
Sbjct: 1745 ccgttgatgaagaagttggtgccgttgaagttgaacgccatgttgatggccaggtcgac  1687
```

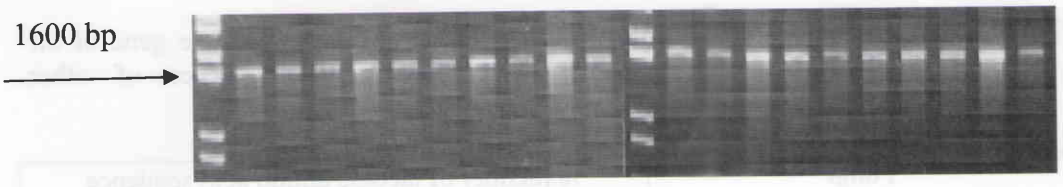

Figure 1: Results of PCR amplification of *Ganoderma* isolated from oil palm using primer pair Lac 2a-Lac 2r

Based on nucleotide comparison with the published nucleotide sequence in GenBank, it could be shown that the sequenced DNA fragment of the *Ganoderma* isolated from oil palm (isolate BS) had similarity with the laccase genes of other basidiomycete fungi. Therefore, the PCR product amplified by these primers was confirmed as DNA fragments of the laccase gene.

The total length of nucleotide sequence of oil palm *Ganoderma* amplified by the primer pair Lac 2a-Lac 2r is (about 1650 bp). To completely sequence this fragment, internal sequencing primer P1: 5` TTGGGA AAACGCAGGCTT 3`, P2: 5` GGGCTTGTTA TCCGAAGG 3` and P3: 5` GGGAGGG GTGTGGTCAGC 3` were used. Deduced intron sequences were based on the comparison with the published laccase gene sequences from other basidiomycete fungi such as *Trametes villosa* (20), Basidiomycete CECT 20197 (12) and *Pycnoporus cinnabarinus* (8) and consensus sequence for 5` splicing GT(AG)(AT)GT and 3` splicing (CT)AG junctions present in filamentous fungi (2,10). For better visualisation, the deduced exon and intron sequences are presented as capital and lowercase characters, respectively as shown in Figure 2. The deduced exon sequences were translated to the predicted amino acid sequences by using EditSeq program (DNAstar, Madison, USA). Based on the consensus introns, oil palm *Ganoderma* has seven putative introns ranging from 55 to 71 bp in size.

The deduced amino acid sequences of the laccase gene of *Ganoderma* (BS) had identities to laccase genes of other basidiomycete fungi ranging from 44.5 to 76.7 % (Table 2). Amino acid sequence of the laccase gene of oil palm *Ganoderma* was aligned with those of published sequences of other basidio-mycete fungi to infer phylogenetic trees. The phylogenetic trees are shown in Figure 3. The laccase gene of oil palm *Ganoderma* clustered to *T. villosa* Lac1.

Table 2: Percent amino acid identities of the partial sequenced laccase gene of oil palm *Ganoderma* compared to published laccase genes of other basidiomycete fungi

Fungi	% Identity of laccase amino acid sequence
	Ganoderma isolated from oil palm
A. bisporus Lac 1	44.5
Basidiomycete CECT 20197	71.4
Basidiomycete PM1	72.8
C. cinereus Lac 1	57.8
C. subvermispora Lac	63.4
L. edodes Lac 1	54.4
M. quercophilus Lac 1	72.8
P. cinnabarinus Lac 1	73.7
P. ostreatus Lac	56.5
Ph. radiata Lac	66.2
Sh. commune Lac (mRNA)	55.8
T. versicolor Lac 1	65.0
T. villosa Lac 1	76.7
Ganoderma Lac 1.8	63.9

Percent identities of amino acid sequences of the laccase genes among other basidiomycete fungi are not shown.

```
TGGCACGGCTTCTTCCAGAAGGGCACGAACTGGGCGGACGGCGTTGCCTTCGTCAACCAGTGCCCGATC   69
W H  G  F  F  Q  K  G  T  N  W  A  D  G  V  A  F  V  N  Q  C  P  I
   I
TCCAGTGGCAACTCCTTCCTGTACGACTTCCAAGTCCCTGGCCAGGCCGgtaagcatcgccgcccccttcggcctgac   149
S  S  G  N  S  F  L  Y  D  F  Q  V  P  G  Q  A

atcagatgatgctcatggtagttgcgcagGCACCTATTGGTATCACAGCCATCTGTCCACTCAGTACTGCGATGGTCTC   228
                              G  T  Y  W  Y  H  S  H  L  S  T  Q  Y  C  D  G  L
                                          II
AGGGGCCCGTTCGTCGTATACGACCCTGAAGACCCGCTGTTGTCCATGTATGACGTCGATGATG gtgagat   298
R  G  P  F  V  V  Y  D  P  E  D  P  L  L  S  M  Y  D  V  D  D  D

tttccccgacgttcctccactgacacatgagtgaactttgtgcttattcgccctatacagACTCTACGGTGATCACCCT GACCGACTGGT   386
                                                        S  T  V  I  T  L  T  D  W  Y

ACCACACTGCCGCTAAACTTGGGCCGGCCTTCCCgtgagtcttcgcgtgcctcttttcaagggtccaggtacagcagccgctgac   471
  H  T  A  A  K  L  G  P  A  F  P

gcattgggaaaacgcagGCTTGGCGCGGACGCGACCCTTATCAACGGGCTGGGGCGGAGCCCCGCTACGTCC   543
                 L  G  A  D  A  T  L  I  N  G  L  G  R  S  P  A  T  S

ACGGCTGAGCTCGCTGTCATCAACGTCACGCAGGGCAAGCGgtacgcacacgtgcgaaggcctccaagacaagcggta   621
T  A  E  L  A  V  I  N  V  T  Q  G  K  R
```

[illegible]CTACCGCTTCCGTCTGATCTCCATGTCTTGCGATCCGAA CTACACCTTCAGTGTGG 697
Y R F R L I S M S C D P N Y T F S V D

ACGGCCACGACATGA CTGTCATTGAGGCGGA CGGTATTGAGACGCAGCCC GTCACGGTGAACGCCATC 765
G H D M T V I E A D G I E T Q P V T V N A I

CAGATCTTCGCCGCTCAACGTTACTCCTTTGTG gtgagtccctgtagtgtgactgttctgtgtcctagaagctaaaaccccttcaca 853
Q I F A A Q R Y S F V

gCTCACCGCTGACCAGGACGTCGATAACTACTGGGTCCGCGCCAA CCCCAACTTCGGTAACGTCGGCTT 923
L T A D Q D V D N Y W V R A N P N F G N V G F

CACGGACGGCATCAACTCTGCCATCCTGCGC TATGACGGCGCGGACCCCGTCG AGCCCACGACCTCG 989
T D G I N S A I L R Y D G A D P V E P T T S

CAGCAGACGACGCAGAACCTCCTGAACGAGGTCGATCTCCACCCAT ACGTCGCAAT CCCCACGGTACg 1057
Q Q T T Q N L L N E V D L H P Y V A I P T V P

tcgctcgtcctatctctccgagtcgccattgaatgctcactgcctgtccccttcggataacagCCGGCAGCCCGACCCCCGGAGGCGTC 1146
G S P T P G G V

GACCTGGCGATCAACTTCGCGTTCAACTTCAACGGCTCCCGCTTCTTCATCAACGGCGACACCTTCGTC 1215
D L A I N F A F N F N G S R F F I N G D T F V

CCGCCCACCGTGCCCGTGCTCCTGCAGATCCTCA GCGGCGCACAGACCGCGCAGGAGCTCCTCCCCT 1282
P P T V P V L L Q I L S G A Q T A Q E L L P S

CCGGCAGCGTCTACGAGCTCCCAATGAACTCCTCCATCGAGCTCACCTTCCCCGCGACCGCCAGCGC 1349
G S V Y E L P M N S S I E L T F P A T A S A

CCCCGGCACCCCGCACCCGTTCCACTTGCACGGTgtaagtctccccttattccctcctcccctgtccgatgccgacgctgacca 1433
P G T P H P F H L H G
III

caccctcccgtcgcgtgcagCACGAGTTCGCGGTGATCCGCAGCGCGGG CTCGACCGAGTACAACTACGA C 1505
H E F A V I R S A G S T E Y N Y D

AACCTCCGTGTGCGCGACGTCGTGTCGACGGGCGTGGCGGGCGACAACGTGACGATCCGGTTCCAG 1571
N L R V R D V V S T G V A G D N V T I R F Q

ACGAACAACCCGGGGCCGTGGATCCTCCA CTGCCACATCGACTTC 1617
T N N P G P W I L H C H I D F
IV

Figure 2: Partial nucleotide sequence of the laccase gene of oil palm *Ganoderma* BS. Copper-binding regions are indicated by roman numerals (I to IV).

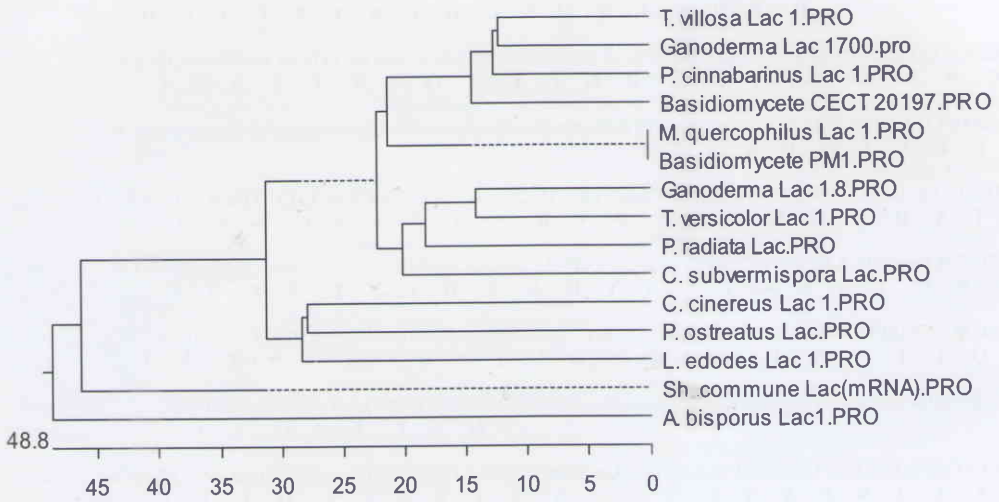

Figure 3: A phylogenetic tree of the partial amino acid sequences of laccases was constructed using the Clustal method with the PAM 250 residue weight table. Multiple alignment parameters used were: gap penalty = 10 and gap length penalty = 10. Pairwise alignment parameters were: ktuple = 2, gap penalty = 5, window = 4 and diagonals = 4. The length of each pair of branches represents the distance betw een sequence pairs. The units at the bottom of the tree indicate the number of substitution events.

4. DISCUSSION

During recent years, laccase genes have been isolated from several basidiomycete fungi. The sequences of these genes display a common pattern in that they encode polypeptides of approximately 520-550 amino acids residues or 2100-2500 nucleotide sequences including introns (8). It is in the copper-binding amino acid residues and their general distribution in the polypeptide chain the laccases are all similar (7), this region is suitable to design primer in order to amplify laccase gene. By using primer pair Lac 2a-Lac 2r designed from the copper-binding region this primer pair amplified a single PCR product of 1617 bp or the primer could amplify approximately 77 % of the complete laccase gene of *Ganoderma* from oil palm. This *Ganoderma* laccase gene has similarity 44.5 to 76.7 % compare to other basidiomycete fungi and cluster to *T. villosa* Lac1.

In fungi, besides lignin degradation (5), laccase has been involved in different biological process such as sporulation (12), pigment production during fruit body development (18), and plant pathogenesis (9,15) in which laccase could potentially contribute to pathogen-mediated degradation of lignified zones (13). With regard to

lignification as a defense reaction of chestnut bark attacked by *C. parasitica* (11), laccase perhaps interferes with this process or participates in the penetration of mycelial fan through lignified zones. It also was suggested that laccase plays a role during the infection process by detoxifying host phenolics, therefore studies in the future for *Ganoderma* isolated from oil palm are focusing on the structure and regulation of laccase-coding genes may help in the elucidation of the role and enzymatic mechanism in the pathogenesis process in oil palm.

REFERENCES

1. ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHÄFFER, J. ZHANG, Z. ZHANG, W. MILLER and D.J. LIPMAN, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

2. BALLANCE, D. J. (1986). Sequences important for gene expression in filamentous fungi. Yeast 2, 229-236.

3. BAR-NUN, N and A. M. MAYER. 1989. Cucurbitacins-repressors of induction of laccase formation. Phytochemistry 28, 1369-1371

4. BAR-NUN, N and A. M. MAYER. 1990. Cucurbitacins protect cucumber tissue against infection by *Botrytis cinerea*. Phytochemistry 29, 67-71

5. BOURBONNAIS, R. and M. G. PAICE. 1992. Demethylation and delignification of kraft pulp by *Tremetes versicolor* laccase in the presence of 2,2 azinobis-(3-ethylbenzthiazoline-6-sulphonate). Appl. Microbiol. Biotechnol. 36, 823-827

6. BURGER, M. N., J. C. MSUYA, M. CAMERON and W. H. STIMSON. 1994. A monoclonal antibody for the detection of *Serpula lacrymans*. Mycol. Res. 98, 356-362.

7. COLL, P. M., C. TABERNERO, R. SANTAMARIA and P. PERES. 1993. Characterisation and structural analysis of the laccase I gene from the newly isolated lignino-lytic basidiomycete PM 1 (CECT 2971). Appl. Environ. Microbiol. 59, 4129-4135.

8. EGGERT, C., P. R. LAFAYETTE, U. TEMP, K. E. L. ERIKSSON and J. F. D. DEAN. 1998. Molecular analysis of a laccase gene from the white rot fungus *Pycnoporus cinnabarinus*. Appl. Environ. Microbiol. 64, 1766-1772.

9. GEIGER, J. P., M. NICOLE, D. NANDRIS and B. RIO. 1986. Root rot diseases of *Hevea brasiliensis*. I. Physiological and biochemical aspects of root aggression. Eur. J. For. Pathol. 16, 22-37.

10. HAHN, M., U. NEEF, C. STRUCK, M. GÖTTFERT and K. MENDGEN. 1997. A putative amino acid transporter is specifically expressed in haustoria of the rust fungus *Uromyces fabae*. Mol. Plant-Microbe Inter. 10, 438-445.

11. HEBARD, F. V., G. J. GRIFFIN, and J. R. ELKINS. 1984. Developmental histopathology of cancers incited by hypovirulent and virulent isolates of *Endothia parasitica* on susceptible and resistent chestnut trees. Phytopathology 74, 140-149.

12. LEATHAM, G. and M. A. STAHMANN. 1981. Studies on the laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. J. Gen. Microbiol. 125, 147-157.

13. LEWIS, N.G. and E. YAMAMOTO. 1990. Lignin: occurrence, biogenesis and biodegradation. Annu. Rev. Plant Physiol. Plant. Mol. Biol. 41, 455-496.

14. MANSUR, M., T. SUAREZ, J. B. FERNANDEZ-LARREA, M. A. BRIZUELA, and A. E. GONZALES. 1997. Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT 20197. Appl. Environ. Microbiol. 63, 2637-2646.

15. MARBACH, I., E. HAREL and A. M. MAYER. 1985. Pectin, a second inducer for laccase production by *Botrytis cinerea*. Phytochemistry 24, 2559-2561.

16. MÖLLER, E. M., G. BAHNWEG, H. SANDERMANN and H. H. GEIGER 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected plant tissues. Nucleic Acids Res. 20, 6115-6116.

17. RIGLING, D. and N. K. VAN ALFEN. 1991. Regulation of laccase biosynthesis in the plant-pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. Journal of Bacteriology 173, 8000-8003.

18. SMITH, J. F., N. CLAYDON, M. E. LOVE, M. ALLAN and D. A. WOOD. 1989. Effect of substrate depth on extracelluler endocellulase and laccase production of *Agaricus bisporus*. Mycol. Res. 93, 292-296.

19. VITERBO, A., B. YAGEN and A. M. MAYER. 1993. Cucurbitacins, attack enzymes and laccase in *Botrytis cinerea*. Phytochemistry 32, 61-65

20. YAVER, D. S. and E. J. GOLIGHTLY. 1996. Cloning and characterization of three laccase genes from the white-rot basidiomycete *Trametes villosa*: genomic organization of the laccase gene family. Gene 181, 95-102.