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Molecular Characterization of Selected Hybrid Fusants of the Parent Strains of *L. edodes*

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Abstract: ***Five hybrid fusants viz., L4, L13, L31, L58, and L76 were selected on the basis of horizontal radial growth after protoplast fusion carried out between LeS and LeC strains of Lentinus edodes. A total of 4 malate dehydrogenase isozymes loci were observed all together. Hybrid strains (L4 and L58) showed two bands at the Rm values of 0.33 and 0.50. Hybrid L31 strain exhibited two bands at the Rm value of 0.43 and 0.50, while L76 showed two bands at the Rm value of 0.33 and 0.40. Hybrid L13 produced no band on gel. It was observed that hybrid L4, hybrid L31, and hybrid L58 shared Rm value with LeS while hybrid L13 and hybrid L76 strains shared Rm value with LeC in case of peroxidase. Only one isoform of isozyme was observed for superoxide dismutase which was common in all the strains including one parental strain viz, LeC and 5 hybrid fusants viz, L4, L13, L31, L58, L76 except LeS. The combined effect of all the primers on all the genotypes indicated that there was no 100% similarity between any 2 genotypes. Maximum similarity coefficient of value 0.950 was obtained between hybrid fusants L58 and L13. A combined dendrogram was obtained from the RAPD data of all the four primers together and it was found that three hybrid fusants i.e. L58, L13 and L31 were closer to LeS parent strain.***

Key words: Hybrid fusants, Isozyme, *Lentinus edodes*, RAPD.

1. Introduction

Lentinus edodes called as Xiangu in China and Shiitake in Japan. It is cultivated on large scale because of its taste and nutritional values in addition to its medicinal values which is due to lentinan, a ß -(1→3)-D-glucan compound having demonstrated anti-tumor activity. Till date, 40 species of *Lentinus* has been reported throughout the world, out of which 37 species have been described from India. Some of the well-known species of this genus are *Lentinus squarrosulus* (Mont.) Singer, *L. cladopus* Lev., *L. lepideus* (Buxb.) Fr., *L. tuber-regium* (Fr.) Fr., *L. sajor-caju* (Fr.) Fr., *L. crinitus* (L.) Fr., *L. tigrinus* (Bull.) Fr., *L. polychorus* Lev., *L. conatus* Berk., *L. strigosus* Fr., and *L. kauffmanii* A. H. Smith, etc. Most of the physiological, biological and cultivation work has been done on *Lentinus edodes* (Berk.) Sing. because of medicinal property [1-7].

Molecular characterization at protein level by PAGE (Native/SDS), isozymic analysis and many other techniques has been used by various scientists. Molecular markers of RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), microsatellites or SSR(simple sequence repeat) and many other markers were used to discriminate inter or intraspecific identification of mushrooms like of *Agaricus, Auricularia, Ganoderma, Lentinula, Stropharia rugoso-annulata* and *Volvariella* . These technologies provide easily obtain reliable data for mushroom strain identification [8].

Among molecular markers, RAPD analysis was first developed to detect polymorphism between organisms, to produce genetic markers, and to construct genetic maps in the absence of prior sequence information [9-11]. It has permitted the study of the population structure of many fungi that were difficult to characterize with other markers. This technique allows rapid generation of reliable and reproducible DNA fingerprints and has been used to investigate the genetic variation within several fungal groups [12].

In this work, two molecular techniques (Isozyme patterning and RAPD-PCR) have been used to establish a relationship between the hybrid fusants (Viz., L4, L13, L31, L58, and L76) and the parent strains of *L. edodes* (LeS and LeC).

2. Material and Methods:

2.1 Fungal Culture

Two parent strains (LeS and LeC) of *L. edodes* obtained from Department of Microbiology, Punjab Agricultural University, Ludhiana, were fused through PEG-mediated protoplast fusion technique and five fusants viz., L4, L13, L31, L58 and L76 were selected on the basis of horizontal radial growth and were used for further characterization through molecular techniques (PAGE and RAPD-PCR).

2.2 Culture media and crude enzyme extract preparation

Mycelium of parent strains and the hybrid fusants were grown for 7 days at 25±2^{o}C in 250ml Erlenmeyer flasks containing 25ml YPG broth having composition (g/l) of glucose 10, yeast extract 5, peptone 5, pH 6.0. The mycelial mass of each strain (1g each) was washed with ddH_2O twice and drained off, crushed over ice cubes using mortar and pestle in 0.1M phosphate buffer containing 0.1% Triton X-100, then transferred into 2ml eppendorf tube containing

600µl extraction buffer, incubated at 4°C overnight after which it was centrifuged at 10,000 rpm for 30 minutes at 4°C. Supernatant collected out and used as crude enzyme extract for isozyme pattern analysis.

2.3 Isozyme Analysis

Three isozymes viz., Malate dehydrogenase (MDH), peroxidase (PO) and superoxide dismutase (SOD) were taken for isozyme pattern analysis to ascertain relationship between the parent strains and hybrid fusants. Total protein content present in crude enzyme extract of parent strains and hybrid fusants was also estimated by standard methodology [13].

2.4 Polyacrylamide gel electrophoresis (PAGE)

A discontinuous buffer native-PAGE with a stacking gel system was used [14-15], with little modification where the SDS component was omitted.

2.5 Staining of gels for Isozymes

Malate dehydrogenase isozyme activity was done using tetrazolium as staining agent. Peroxidase activity was detected using 3, 3', 5, 5'-Tetramethylbenzidine (TMBZ) as staining dye and Superoxide dismutase activity was detected using tetrazolium system [16-18].

2.6 Molecular characterization of hybrid fusants and the parent strains using RAPD-PCR

Pure mycelial cultures of hybrid fusants and parent strains of *L. edodes* were maintained on PDA media in petri plates and raised on MYG broth for 4-5 weeks at 25±2°C.

2.7 DNA Extraction and Purification

DNA of the hybrid fusants and parent strains of *L. edodes* (150-200mg) was extracted using CTAB extraction method [19] in 1.5mL microfuge. The pellets were resuspended in 20µl 1X TE buffer (stored at -20^{o}C until use) and 5µl of the above preparation was used for PCR amplification.

2.8 CR Amplification

10 random primers were used for amplification of hybrid fusants and parents strains of *L. edodes*. The reaction mix for PCR amplification and PCR conditions were maintained [20]. Primers used were OPN 08 (5´-AATCGGGCTG-3´), OPN 09 (5´-CACCG TATCC-3´), OPN 10 (5´-GTGGGCTGAC-3´), OPN 11(5´-AAGGGCGAGT-3´), OPA 02 (5´-GGAGT GCCTC-3´), OPA 04 (5´-GGTGTGCCTC-3´), OPA 06 (5´-GGTCCTTGAC-3´), OPAA 03 (5´-TTAGCGCC CC-3´), OPAA 07 (5´-CTACGCTCAC-3´) and OPAA 10 (5´-TTCCCTCCCA-3´). After amplification, 3µl of 6X loading dye was added to each of the amplified product. This mixture of each sample was loaded in 1.5 percent agarose gel prepared in 0.5X TBE buffer. PCR product was resolved by running gel at 5V/cm for 2-3 hr. The gels were visualized under UV light and photographed using UV gel documentation system (BIORAD). The RAPD band size was determined on the position of bands relative to the ladder (1Kb). The amplified bands were recorded as 1 (band present) or 0 (band absent) in a binary matrix.

2.9 Estimation of genetic similarity and cluster analysis

Computer software programme Numerical Taxonomic and Multivariate Analysis System (NTSYS-PC) VERSION 2.02E [21] was used for estimation of genetic similarities among the lines using SIMQUAL mode of NTSYS and the similarity matrix value based on [22] coefficient of similarity was used to generate dendrogram. Clustering was done by (Unweighted Pair Group Method with Mathematical Average) UPGMA using SAHN module of NTSYS version 2.02e.

3. Results and Discussion

Five fusants (L4, L13, L31, L56 and L76) and two parents (LeS and LeC) were characterized on the basis of Isozyme pattern analysis and RAPD analysis. Results obtained are given below.

3.1 Isozyme analysis of hybrid fusants and parents

Isozymes have been widely used in many researches to screen variability in fungal populations, to identify sexual and somatic hybrids, in linkage studies. They have been also intensively utilized in the identification, phylogenetic classification, species and strain discrimination of many fungal groups.

Three enzymes, namely malate dehydrogenase (MDH), peroxidase (PO) and superoxide dismutase (SOD) gave different staining reaction to show different banding patterns. The protein content of all hybrids along with their parents was estimated before to start native-PAGE electrophoresis for isozymes (Table 1). Maximum total protein content was observed in hybrid fusant L58 (182.5µg/mL) while minimum protein content was found in parent LeC (58.60 µg/mL). Hybrid fusant L58 and L4 showed almost same protein content with the parent LeS. It was reported to be 18±2.0 mg protein content in crude extract of *Lentinus edodes* IBB 363[23].

Table 1: Protein content of parent and hybrid fusants of *L.edodes*

Sr. No.	Strains	Protein content (µg/mL)
1	LeS	161.45
2	LeC	58.60
3	L4	175. 2
4	L13	149.1
5	L31	109.5
6	L58	182.5
7	L76	96.9

3.1.1. Malate dehydrogenase (MDH)

Bands were obtained at Rm values corresponding to two isozymes (Table 4.41). Out of parental strains, one band appeared on the gel in LeS while zero band appeared in LeC strains. Hybrid strains (L4 and L58) showed two bands at the Rm values of 0.33 and 0.50. Hybrid L31 strain exhibited two

bands at the Rm value of 0.43 and 0.50, while L76 shown two bands at the Rm value of 0.33 and 0.40. Hybrid L13 produced no band on gel. A total of 4 malate dehydrogenase isozymes loci were observed all together (Figure 1).

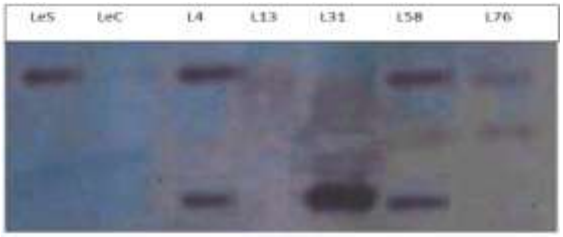

Figure 1: Native-PAGE of malate dehydrogenase isozyme of parent and hybrid fusants of *L.edodes*

Distinct banding patterns of malate dehydrogense and esterase were observed in mycelium of 40 different strains of *L. edodes* [24]. It was analyzed that 93 strains of *L. edodes* taken from many countries to find the genetic variability in geographical distributed population of the fungus by studying electrophoresis patterns which indicate degree of variation in isozymes [25]. Six types of malate dehydrogenase enzymes was observed in *L. edodes* while observed 3 malate deydrogenase isozymes in fusants between *P. florida* and *P. cystidiosus* [26, 27]. The present study also showed a high variation in malate dehydrogenase systems of *L. edodes*.

3.1.2. Peroxidase

Two isozymes loci were observed for peroxidase at an Rm value of 0.70 and 0.62 by LeS and LeC (Figure 2). It was observed that hybrid L4, hybrid L31, and hybrid L58 shared Rm value with LeS while hybrid L13 and hybrid L76 strains shared Rm value with LeC. No additional bands were observed at Rm value other than parental band position. Esterase and peroxidase isozyme pattern showed that fusant strains of *P. sapidus* and *P. ostreatus* had bands from both the parents [28]. Isozyme patterning of esterase, peroxidase and acid phosphatase of sixty three isolates of *L. edodes* was studied to find genetic similarity [29].

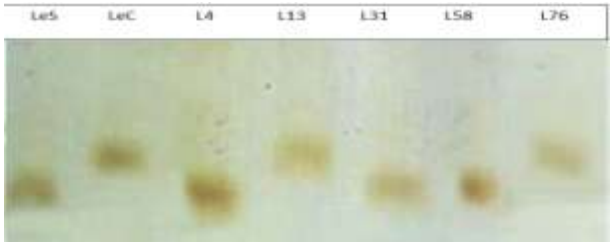

Figure 2: Native-PAGE of Peroxidase isozyme of parent and hybrid fusants of *L.edodes*

3.1.3. Superoxide dismutase

Only one isoform of isozyme at Rm value of 0.22 was observed for superoxide dismutase which was common in all the strains including one parental strain viz, LeC and 5 hybrid fusants viz, L4, L13, L31, L58, L76 except one parental strain viz, LeS (Figure 3). A total of 11 different bands of four different enzymes (alcohol dehydrogenase (ADH), malate dehydrogenase (MDH), peroxidase (Po) and superoxide dismutase (SDH))were recorded in the intergeneric fusants of *P. florida* and *V. volvacea* [30].

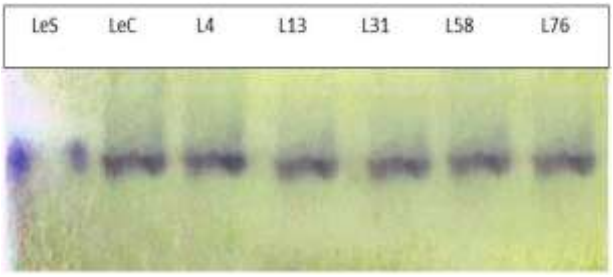

Figure 3: Native-PAGE of Superoxide dismutase isozyme parent and hybrid fusants of *L.edodes*

Fusants of *P. ostreatus* and *P. djamor* possessed bands common to their parents when esterase was used for isozyme studies. Possession of common bands directly indicate relationship of parents with hybrids while appearance of new non-parental bands indicate occurrence of new interaction between the two parental genomes [31, 32].

3.2 Molecular characterization of hybrid fusants and parents

Molecular markers are used to characterize the genotypes among different species of fungi on the basis of their sequence similarity. RAPD-PCR technique became very popular for molecular characterization of fungus genome.

3.2.1. RAPD analysis

Eighteen polymorphic amplified bands were obtained with primer OPN-8 with similarity coefficient values ranged from 0.388 to 0.833(Figure 4-5). Highest similarity coefficient was 0.833 between hybrid fusants L58 and L13. Lowest similarity coefficient was 0.388 in hybrid fusant L31 and parent LeS.

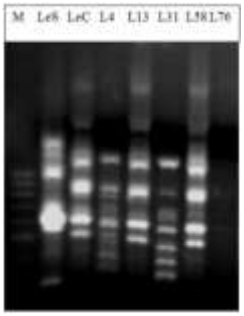

Figure 4: RAPD amplification on gel using primer OPN 8

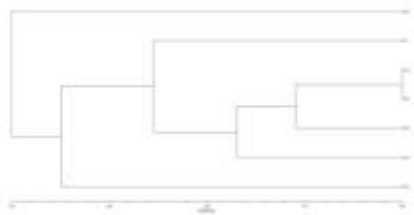

Figure 5: Dendrogram obtained using OPN-8 RAPDs marker

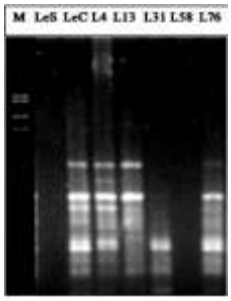

Figure 6: RAPD amplification on gel using primer OPN9

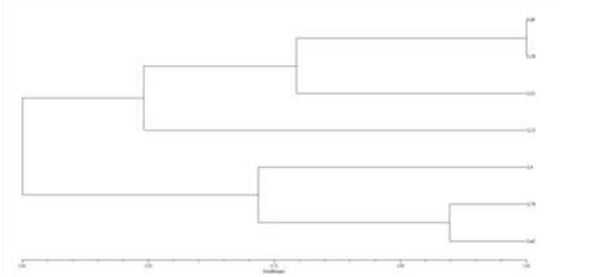

Figure 7: Dendrogram obtained by using OPN-09 RAPDs marker

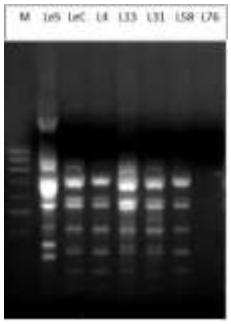

Figure 8: RAPD amplification on gel using primer OPN 10

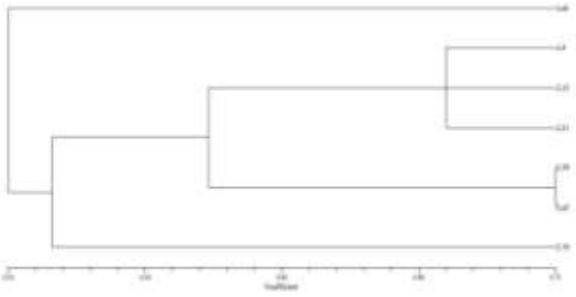

Figure 9: Dendrogram obtained by using OPN-10 RAPDs marker

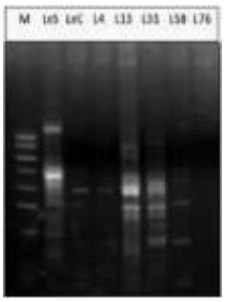

Figure 10: RAPD amplification on gel using primer OPN 11

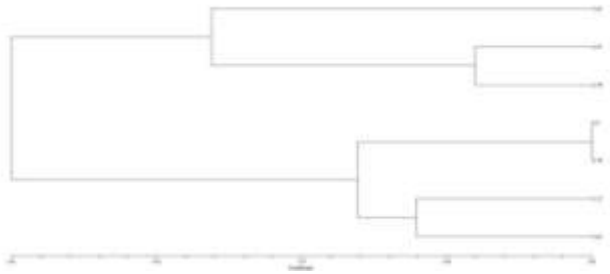

Figure 11: Dendrogram obtained by using OPN-11 RAPDs marker

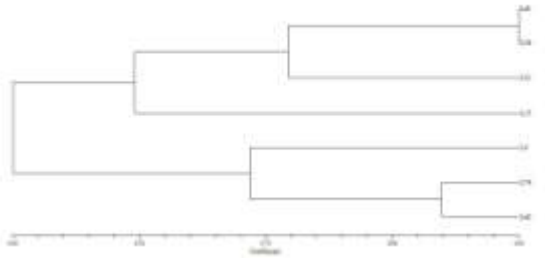

Figure 12: Combined dendrogram of all the four RAPD primers

The primer OPN 9 produced a total of 12 (Figure 6-7).The highest similarity coefficient was 0.916 between hybrid fusants L76 and LeC. Lowest similarity coefficient was 0.416 between hybrid fusants L4 and parent LeS.

Primer OPN 10 produced 18 bands (Figure 8-9).Highest similarity coefficient was 0.750 between L58 and parent LeC. Least similarity was 0.400 between hybrid fusant L13 and parent LeS. Amplification of DNA using primer OPN11 showed 21 bands (Figure10-11). Highest similarity coefficient was 0.950 for hybrid fusants L4 and L76. Lowest similarity coefficient was 0.300 for hybrid fusants L4 and L58.

The combined effect of all the primers on all the genotypes indicated that there was no 100% similarity between any 2 genotypes. Maximum similarity coefficient of value 0.952 was obtained between hybrid fusants L58 and L13. Similarity coefficient of parent LeC with parent LeS was 0.416 which could be compared with hybrid fusants L13 and L58. A combined dendrogram was obtained from the RAPD data of all the four primers together and it was found that three hybrid fusants i.e. L58, L13 and L31 were closer to LeS parent strain (Figure 12).

Several workers also used RAPD analysis for studying the genetic variability among *L. edodes* strains obtained from diverse regions and have reported that RAPD assay can be used to differentiate the strains of *L. edodes*. There was degree of genetic homogeneity between *Agaricus bisporus* and *Volvariella volvacea* on the basis of AP-PCR, RFLP and RAPD marker profile profiles [33]. Twenty RAPD molecular markers were used, 17 showed good polymorphism (OPA01 to OPA05, OPA07 to OPA14, OPA17 to OPA20) to assess strain variability of 34 *Lentinula edodes* strains thereby clustering and similarity coefficients made strains in two odds with different geographic origin [34]. It was concluded

from the observation that *Pleurotus smithii* was actually a synonym of *Pleurotus cystidiosus* when analysed through RAPD markers profile [35]. Similarly, Nineteen different arbitrary RAPD primers were used to study *Pleurotus ostreatus* and *Pleurotus sajor-caju*, and reported that only 6 primers (AC-04, AD-08, AD-09, B-14, D-20, G-02) produced amplifications for both species, 5 (S-03, D-06, G-07, G-08, B-15) produced amplifications only for *P. ostreatus*, the primer S-17 produced amplifications only for *P. sajor-caju* and 7 primers (AC-09, B-02, B-05, B-07, D-05, G-06, G-09) did not amplify any fragment from both species [36].

4. Conclusion

Molecular characterization using Isozyme pattern analysis and RAPDs-PCR showed positive relationship between hybrid fusants and one parent strain (LeS) of *L. edodes*. So the present work revealed that genetic discrimination between the strains or genus can be worked out easily through molecular characterization which is at par with older genetic manipulation methods to study genetic similarity or dissimilarity.

5. Acknowledgement

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