African Journal of Biotechnology Vol. 9(12), pp. 1758-1763, 22 March, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1770
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Assessment of genetic and biochemical diversity of ecologically variant ectomycorrhizal *Russula* sp. from India

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Accepted 1 March, 2010

The aim of this study was to develop the phylogenetic relationship amongst the ecologically variant *Russula* species by using polymerase chain reaction (PCR) based technique, random amplified polymorphic DNA (RAPD) and isozyme analyses. Two groups could be characterized amongst the total isolates by cluster analyses. Protease, cellulase, glutamate dehydrogenase, pectinase and acid phosphatase designated band $P2_{20.16}$, $C4_{72.18}$, $GLD1_{30.21}$, $Pe5_{69.12}$ and $AP4_{72.12}$, respectively, were common in all the isolates and four monomorphic RAPD bands viz; 818, 512, 298 and 201 bp were also diversified in the isolates. This common band reveals that diversity of these alleles or loci in all ecologically variant isolates. Thus, the present studies discuss the genetic diversity of ecologically variant *Russula* species on the basis of RAPD and isozyme analysis.

Key words: Genetic diversity, isozyme, random amplified polymorphic DNA, *Russula* sp., zymography.

INTRODUCTION

Most of the species of family Rassulaceae are economically important, they inhabit temperate zone and are considered as ectotrophic mycorrhizae associated with forest trees of various families. Most of the members of this family live normally under the conditions of symbiosis (Singer, 1986). The diversity of ecologically variant isolates of *Russula* species was previously determined on the basis of morphological characteristics, which sometime fails to recognize ecologically variant isolates. Therefore some para-morphological criteria especially RAPD and isozyme analysis are used for differentiation of morphologically similar or ecologically variant isolates or both (Micales et al., 1986; Cruickshank and Pitt, 1987; Huhtala et al., 1999; Stanosz et al., 1999; Upadhyay et al., 2003). Some of the enzyme (protein) bands which are obtained by electrophoretic separation may be common in all the isolates. The common band (enzyme band) reveals that this biochemical (protein) is diversified in all the isolates or in other words the loci or alleles which code to these enzyme bands are diversified in all the isolates (Banke et al., 1997). This concept gives a novel idea for phylogenetic analysis of not only the *Russula* species but also for other fungi. The basic concept of diversity of alleles or loci in electrogram of RAPD is same as in zymography (Williams et al., 1990; Upadhyay et al., 2003.).

The aim of the present study is to characterize the diversity of *Russula* species using RAPD and isozyme analysis. The resulting binary matrices are analyzed by computer based programme and to find out whether isolates are evolutionarily similar or not.

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Abbreviations: PCR, polymerase chain reaction; **DNA,** deoxyribonucleic acid; **RAPD,** random amplified polymorphic DNA; **EtBr,** ethidium bromide; **UPGMA,** unweighted pair group method with arithmetic mean.

MATERIALS AND METHODS

Fungal isolates

A total of eight isolates of ectomycorrhizal *Russula* species were isolated from different forests (Mandala, Balaghat, Jabalpur, Rewa, Sahdol, Bllaspur, Dantewara and Kanker) of Madhya Pradesh and

Chhattisgarh, India during rainy season by employing standard techniques and identified on the basis of morphological characteristics (Rinaldi and Tyndalo, 1985; Singer, 1986; Jordan, 1995). Detailed taxonomic studies were done in the laboratory. Pure culture of *Russula* sp. were maintained on malt extract slant and stored at 4°C in refrigerator for further use. These cultures have been deposited in Fungal Germplasm Collection Center, R.D. University, Jabalpur, India for further references.

Extraction of extra-cellular isozyme

Enzyme extraction was done (Upadhyay et al., 2005). 500 mg of solid substrate (starch for amylases, protein source for proteases, cellulose for cellulases and pectin for pectinases) were autoclaved with 10 ml of basal salt solution (1.5 g KH_2PO_4; 0.025 g $MgSO_4.7H_2O$; 0.025 g $CaCl_2$; 0.015 g $FeSO_4.7H_2O$; 0.005 g $ZnSO_4$; 1000 ml D/W) in 100 ml of Erlenmeyer flasks which were seeded with 5 mm disc separated from seven days old culture mycelium and incubated at 28°C for 8 days. Before harvesting, the flasks were kept at 4°C for 15 min, then 2 ml of chilled 50 mM Tris buffer (pH 7.4) was added and vortexed properly. Crude enzyme was pipette out and centrifuged at 10,000 rpm at 4°C for 1 min. Supernatant was taken as enzyme extract and stored at 20°C until used for electrophoresis.

Extraction of intra-cellular isozyme

Biomass of the fungus was harvested from the cultures grown as in modified complete medium (15 g sucrose; 5.0 g dextrose; 0.5 g $MgSO_4.7H_2O$; 1.0 g K_2HPO_4; 0.46 g KH_2PO_4; 5.0 g yeast extract; 4.0 g bacto-peptone; 1000 ml D/W) (Choi et al., 1987). Crude intra-cellular enzyme was extracted from harvested biomass by centrifugation, which had been washed 4 times in 5 mM acetate buffer to remove extra-cellular enzyme. The mycelia were grinded in precooled mortar and pestle with Tris-HCl grinding buffer (20 mM Tris-HCl; 0.1 mM $Na_2EDTA.2H_2O$; 10 mM β-mercaptoethanol; 10.0 g sucrose; 1 ml triton-X-100 and pH 8.0 was adjusted with conc. HCl) (Skovgaard and Rosendahl, 1998). The extract was centrifuged at 20,000 rpm at 4°C, supernatant was collected and transferred to new 1.5 ml eppendorf tube and stored at 40°C until used for electrophoresis.

Zymography

The enzymes were separated by SDS polyacrylamide gel electro-phoresis in a discontinuous buffer system. The resolving gel contained 10% acrylamide with substrate and stacking gel was made 5% acrylamide (Sambrook et al., 1989). A Tris base with pH 8.8 having concentration 375 mM in resolving gel and 126 mM in stacking gel with pH 6.8 were used. The reservoir buffer contained 25 mM Tris base and 192 mM glycine with pH 8.3 (Hames, 1990). 15 µl enzyme samples were loaded in the wells along with gel loading dye. The gel was run at 80 V and 15 mA current at 4°C for 4 h or until the dye front reaches the bottom. The gel was washed for 10 min. each with 1st washing buffer (50 mM Tris buffer, 49 ml; Triton-x-100, 1 ml) and 2nd washing buffer (50 mM Tris buffer) in shaking condition, and then it was incubated in recommended substrate buffer of individual enzyme (Upadhyay et al., 2005). The zymograms were prepared and relative position of bands was calculated (Upadhyay et al., 2003).

Random amplified polymorphic DNA (RAPD)

Genomic DNA from fungal mycelium was extracted (Williams et al.,1990) and polymerase chain reaction (PCR) was carried out in 25 µl reaction mixture containing 2.5 µl of 5× PCR buffer, 100 µl each of dATP, dGTP, dTTP and dCTP, Taq DNA polymerase decamer random primer and 50 ng of genomic DNA. Amplification reaction performed in DNA Mini Thermal Cycler and the sequential steps were: 1 cycle 3 min at 94°C, 2 min at 52°C and 3 min at 72°C followed by 38 cycle of 1 min at 94°C, 2 min at 40°C and 2min at 72°C. The last 10 min, extension at 72° C was carried out. 10 µl of amplification products along with PCR loading buffer were loaded in 1.0% agarose gel containing 1 µg/ ml EtBr. Separation was carried out in 0.5× TBE buffer at 90 V (Sambrook et al., 1989). Electrophoretic separation was stopped when dye reaches at bottom. 2 µl of 1000 bp Banglore Genei molecular markers was added as a standard. The gel was observed under gel documentation and photograph was taken.

RESULTS AND DISCUSSION

Eight isolates of *Russula* species belonging to *Russula rosea*, *Russula cyanoxantha* and *Russula solaris* were collected from various forests of Central India. The biochemical and molecular biodiversities amongst all 8 isolates of *Russula* species were analyzed for their electrophoretic profile of extra-cellular and intra-cellular isozyme pattern (for Biochemical diversity) and RAPD (for molecular diversity).

Amylases

A total of 26 bands were obtained in amylase isozyme pattern. The fractions with $A1_{18.76}$ and $A2_{22.80}$ were present in most of the isolates except R-49 and R-29. Fraction $A4_{36.31}$ was present only in R-63, R-64 whereas band $A3_{30.78}$ was present only in R-49, R-63 and R-64. The band $A5_{38.12}$ was recorded in all the isolates except in R-38 and R-29 (Figure 1A).

Proteases

Five polymorphic enzymes containing a total of 30 bands of protease isozymes were observed which clearly divided these in two zymogram groups that is, Zg1 and Zg2. Two fractions with R_f $P1_{16.66}$ and $P2_{20.16}$ were diversified in all the isolate of *Russula* species. $P3_{24.93}$ and $P5_{36.61}$ were restricted only in the Zg1 whereas $P4_{30.81}$ was common in all the isolates of Zg1 and two isolates viz., R-49 and R-61 of Zg2 (Figure 1B).

Pectinases

A total of 31 pectinase bands which contained 5 polymorphic isozymes were observed. Two fractions with relative mobility $Pe1_{12.62}$ and $Pe5_{69.12}$ were common in all of the isolates whereas band $Pe2_{18.18}$ was absent only in R-25, Fraction $Pe3_{29.22}$ was present in 5 isolates viz., R-29, R-49, R-61, R-63 and R-64 (Figure 1C).

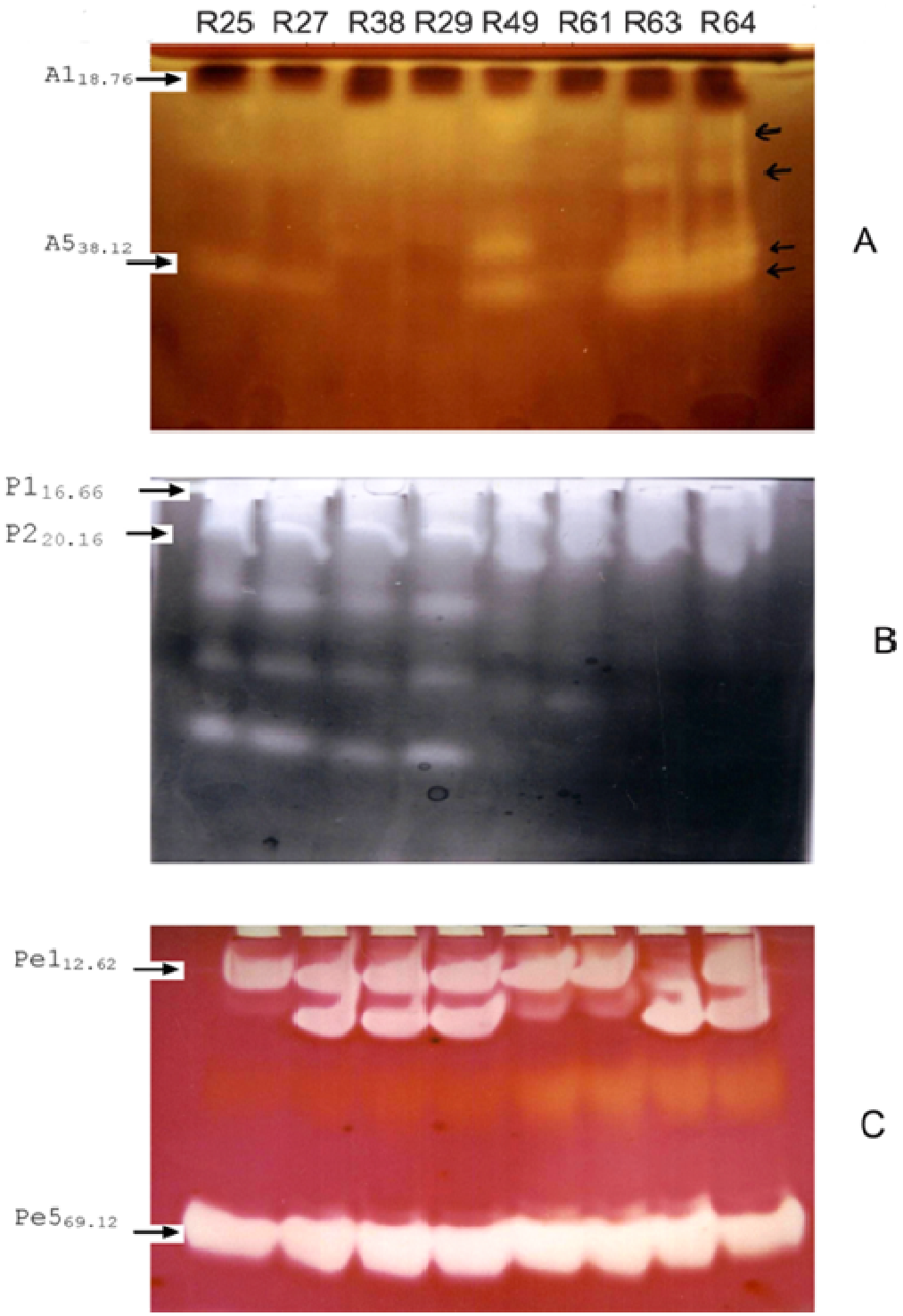

Figure 1. Polyacrylamide gel stained for (A) amylases, (B) proteases and (C) pectinases. Lanes 1 to 8 are different species of *Russula*.

Cellulases

Four polymorphic bands containing a total of 27 bands of cellulase isozymes patterns were recorded. Band $C1_{12.82}$ which diversified in most of the isolates except R-25 and R-27, $C2_{26.16}$ was absent only in R-25 and R-49 whereas $C3_{32.12}$ was absent in R-64. Fraction with R_f $C4_{72.18}$ was common in all the isolates of *Russula* sp. (Figure 2A).

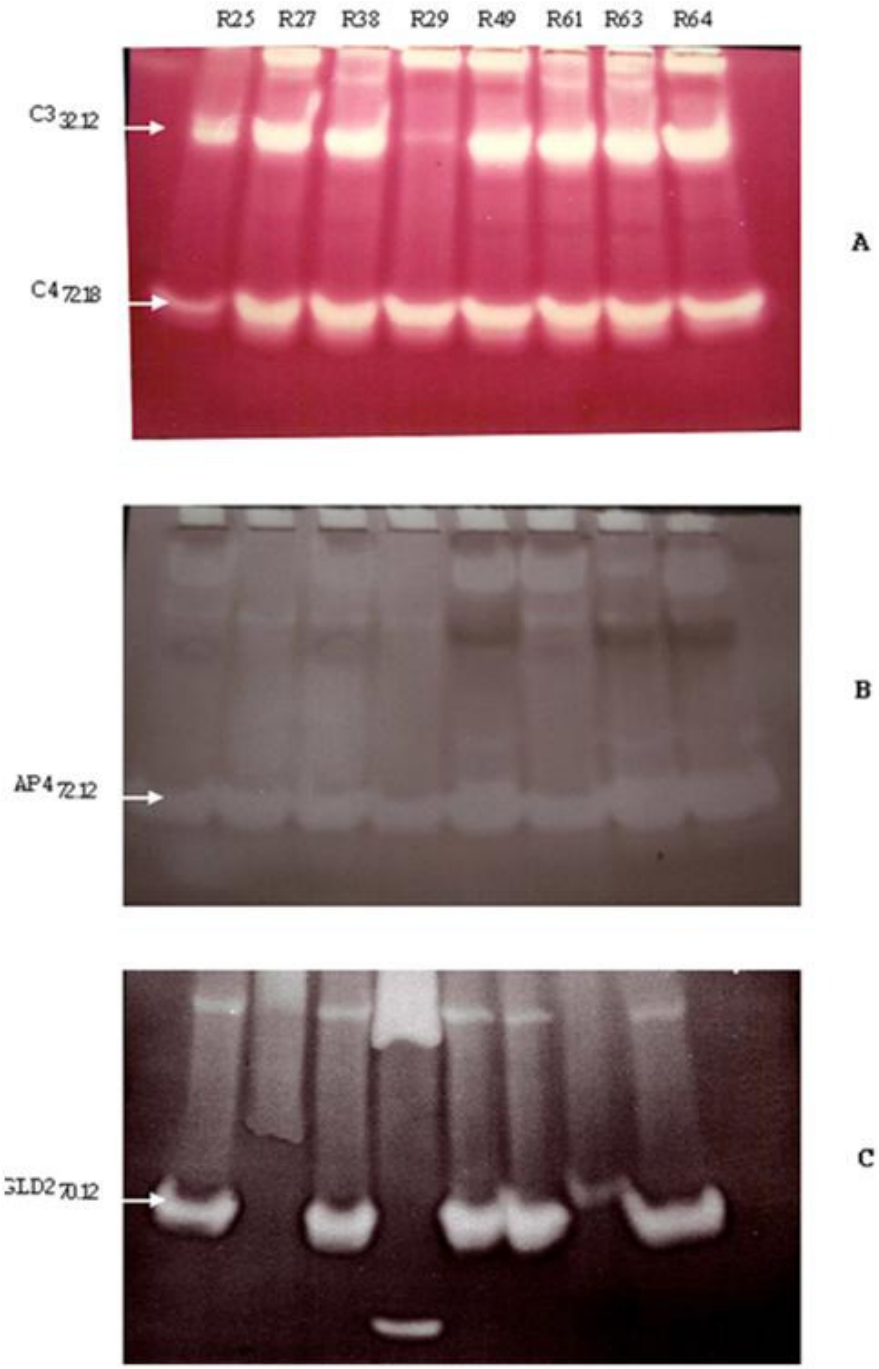

Figure 2. Polyacrylamide gel stained for (A) cellulases, (B) acid phosphatases (negative mode) and (C) glutamate dehydrogenases (negative mode). Lanes 1 to 8 are different species of *Russula*.

Glutamate dehydrogenases

Only three polymorphic bands of glutamate dehydrogenase isozymes were obtained. Fraction with R_f $GLD1_{26.66}$ and $GLD2_{70.12}$ was common in most of isolates except R-63 and R-29, respectively. Fraction $GLD3_{78.12}$ was restricted only in R-29. No common band was observed (Figure 2B).

Acid phosphatases

Two fractions of acid phosphatase, that is $AP1_{21.8}$ and $AP4_{72.18}$, were present in all the isolates whereas $AP2_{31.6}$ was absent in R-61. Fraction with R_f $AP3_{66.66}$ was present only in three isolate that is, R-49, R-68, and R-64 (Figure 2C).

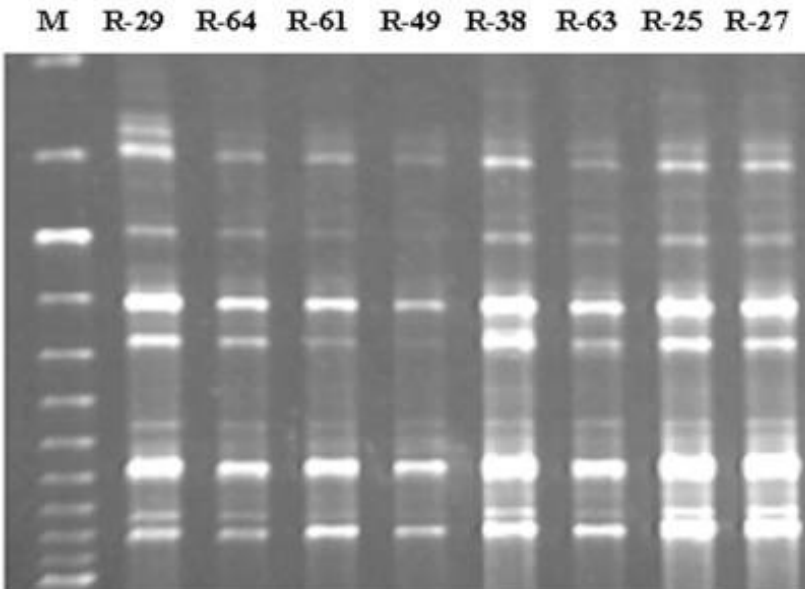

Figure 3. RAPD of ecologically variant isolates of *Russula* sp.

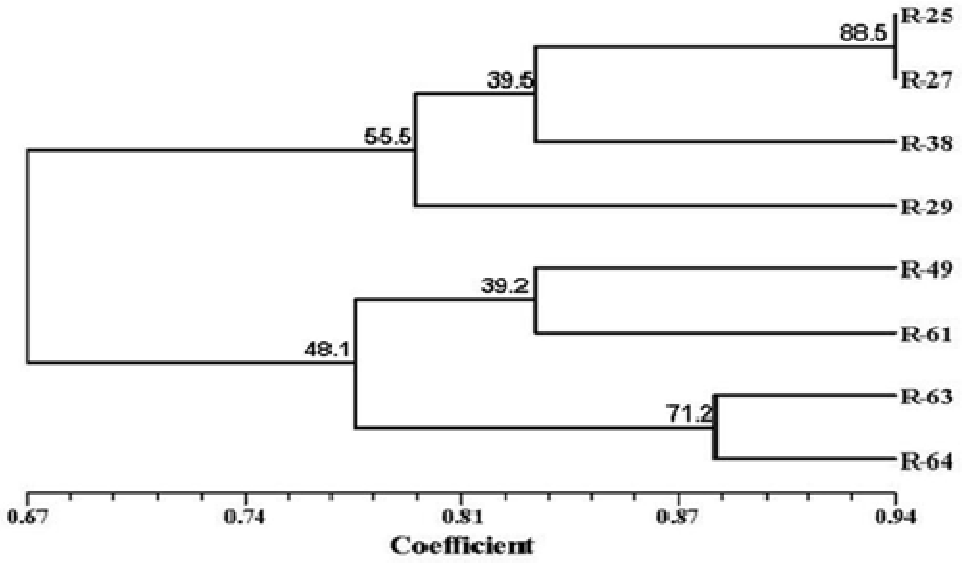

Figure 4. Dendogram based on eucladian distance analysis of isozyme and RAPD of indigenous *Russula* sp.

Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) was detected by use of polymerase chain reaction with arbitrary 10-mer primers for molecular characterization of ecologically variant isolates of *Russula* sp. For this analysis, OPA-14, OPA-17, OPA-19, OPF-8 and OPF-10 primers (Operon Technologies. Inc. USA) were used.These primers were selected from preliminary screening and on the basis of their ability to generate highly reproducible RAPD fingerprints that could be used to differentiate ecologically variant isolates. Out of these primers, OPA-19 gave a maximum of 10 polymorphic bands whereas 4 bands were gotten from OPA-14 and OPA-17 and only two bands from OPF-8. OPF 10 was not amplified with the genomic DNA of tested mushroom isolates. From the amplification of genomic DNA of all eight isolates of *Russula* sp. by OPA-19 primers, 10 polymorphic bands (loci) were produced. Four bands viz, 818, 512, 298 and 201 bp were obtained in all the isolates whereas loci 916 bp was present in R-29 only (Figure 3).

The dendrogram (Figure 4) was constructed by using

eucladian distance and UPGMA based on simple matching coefficient of 8 isolates of *Russula* sp. based on the presence or absence of RAPD bands and isozyme analysis. The significant feature of the dendrogram was the hierachial order of clustering and division of the various species into two different groups and both groups have 4 isolates. The isolates R-25, R-27, R-38 and R-29 were on one branch of clad whereas the rest 4 isolates were on same clad of other group. From Figure 4, it is clear that these were genetically near to each other because the similarity value of all isolates of group 1 and group 2 is > 0.75. Two isolates of *R. rosea* (R-25 and R-27) and one isolate of *R. cyanoxantha* (R-38) showed resemblance with each other (similarity value > 0.94). Two morphologically similar isolates of *R. solaris* (R-63 and R-64) were also on same cluster but at different branches (similarity value > 0.90). It might be clear that these are ecological variants. Similarly, on the basis of pectic isozyme analysis Cruickshank and Pitt, (1987) and Cruickshank (1990) differentiated various species of *Penicillium* and *Rhizoctonia,* respectively. The result of RAPD and isozyme analysis showed that there was diversity within the genus as well as in species, which other wise could not be detected by morphological studies. The methods used in this work have permitted recognition of remarkable differences, which are not evident at morphological level between ecological variant isolates of *Russula* sp. Similar observations were also recorded by Correll et al. (1993), Vicente et al. (1999) and Alymanesh et al. (2009) while studying the vegetative compatibility group variation of isolates of *Colletotrichum dematium*, *Acremonium cucurbitacearum* and *Fusarium solani,* respectively.

ACKNOWLEDGEMENTS

We wish to thank the Head, Department of Biological Sciences, R.D. University, Jabalpur (M.P.) for providing the laboratory facilities. We also thankfully acknowledge DBT, New Delhi for financial assistance.

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