Journal of Biotechnology and Crop Science
1(1): 60-65, 2012

Molecular characterization of antagonistic bacteria against pathogen of green mold disease of oyster mushroom

Priyanka Pandey, RC Ram, Saket Kumar

Received: 15 April 2012 Revised Accepted: 17 May 2012

ABSTRACT

In the present investigation, the root-colonizing bacteria were isolated on different media like Pikovskaya's Agar media for Phospahte solublizer, King's B media for Fluoresceent *Pseudomonas* and Actinomycetes Agar media for Actinomycetes and screened using Dual culture analysis on N.A. medium to select antagonistic bacteria from a random group of bacterial isolates and to compare with selected strains for their fungal growth inhibition capabilities. In this way 15 bacterial strains displaying a wide antifungal spectrum were selected as the most interesting bacteria to test for biological control of green mold disease caused by *Trichoderma harzianum.* A total of 17 strains of *Actionmomycetes* were isolated from different soil samples on the basis of different parameters of colony characteristic like colony shape, size, surface, margin and color on the actinomycetes agar medium. Most of these isolates were from Rose and Wheat soil. Biochemical characterization including Gram-staining test, IAA test, Indole-production test, MRVP test and Antibiotic assay of different bacterial strains were performed successfully. Molecular characterization followed by PCR amplification based 16S rDNA of antagonistic bacterial strains was done. The bacterial strains such as PKB-1, PPB-4, PAM-5, PPB-3, PKB-19, PAM-11, PAM-12, PKB-9, and PKB-18 showed clear DNA bands. The bacterial strains which were successfully amplified by PCR were PKB-1, PKB-18, PPB-3, PPB-4, and PAM-11 along with the ladder.

Key Words: *Actionmomycetes, Antagonistic bacteria, Bacterial strains, Molecular characterization*

INTRODUCTION

Mushroom is a fungus belongs to *Basidiomycota* division. It does not contain chlorophyll and therefore it is known as eukaryotic heterotrophs which obtain food from decaying organic matter (Allen and Allen 1999). Mushroom cultivation is dominated by the production of *Agaricus bisporus* , which is followed by *Lentinula edodes* (shiitake) and *Pleurotus ostreatus* (oyster mushroom) (Chang *et al* 1997). Infections in mushroom cultivation due *Trichoderma* have come to be known as weed mould, or more commonly as the "green mould disease". Historically, *Trichoderma* viride and *Trichoderma Koningi* were reported to cause losses in cultivation (Sinden *et al* 1953). Sinden considered the genus *Trichoderma* as species competing with the mushroom or indicator of poor compost, associating their presence to situations with acidic pH or soluble sugar residues. Pathogens of the green mould disease of *Pleurotus ostreatus* (Kredics 2009).

Natural substrates and the surface of wild-growing *Agaricus species* are possible reservoirs of *T. aggressivum*. Antagonistic bacteria from fifty samples of wild-growing *Agaricus species* were isolated. From the investigation, it was expected to minimize the possibility of infection of the freshly

Priyanka Pandey (✉)
Department of genetics and Plant Breeding, Institute of Agicultural Sciences BHU Varanasi
Email: prppandey25@gmail.com

RC Ram
Department of Mycology and Plant Pathology, Institute of Agicultural Sciences BHU Varanasi

Saket Kumar
Department of Genetics and Plant Breeding, Institute of Agricultural Sciences BHU Varanasi

spawned compost with green mould (Rinker 1997). According to old records, levels of green mould contamination were 60% higher in weeks with dry, windy conditions during compost bagging, furthermore, peak heat tunnels most exposed to windborne contamination (Seaby 1996). *Trichoderma* green mould disease of *A. bisporus* also appeared in Hungary (Szczech *et al* 2008), Poland (Sobieralski *et al* 2009). Pathogenic green moulds may colonize the substrate or grow on the surface of the emerging mushrooms. These symptoms are not appear in the bags until 10-35 days following the apparently normal spawn run of *A. Bisporus*. *Trichoderma spp.* produce whitish mycelia indistinguishable from those of the mushrooms during spawn run, therefore it is difficult to recognize the infection at this stage (Rinker 1996). Subsequently, large patches of compost turn green rapidly as spore production begins on the Trichoderma mycelia (Seaby 1996) which had run through the compost with Agaricus. Morris *et al* (1995) described the symptoms of green mould disease as the presence of green fungal sporulation in the mushroom compost or casing layer between 2-5 weeks of the production cycle in the mushroom growing unit. The crop loss is proportional to the area infected, where generally no mushrooms are produced in contaminated bags in the case of serious out breaks. Mushrooms are unsaleable as they become severely spotted, distorted (Seaby. 1989) and infested with red pepper mites (Pygmephorus mesembrinae), which feed on the Trichoderma and gather on the mushrooms (Morris 1995).

MATERIALS AND METHODS

Collection of Rhizospheric bacteria: 50 strains of bacteria collected from the soil samples of Varanasi, Mirzapur, Allahabad, Bareilly, Lucknow region. Strains of bacteria characterized by growing on the selective media as actinomycetes for actinomycetes bacterial, Pikovaskya for Phosphate solublizing bacteria, and king's B media for fluorescence bacteria After growing on selective media 12 strains were found actinomycetes, 25 strains fluorescence, and the rest were phosphate solublizing bacteria. Antagonistic test- During antagonistic test 3 bacteria K1, K8, K14 were found antagonistic

Identification and isolation of pathogenic fungus Trichoderma harzianum: An extensive survey of different regions of estern Uttar Pradesh was done for soil sample collection from rhizopheric soil of particular plants. Fifty soil samples were collected from different part of Uttar Pradesh. These samples were taken from Maize, Rose, Parthenium, Brassica, Wheat, Rice, Guava, Pea and Chenopodium. A small infected portion of dark green patches of *Trichoderma harzianum* was taken from mushroom beds. The fungus was isolated in petriplate containing PDA medium under aseptic condition in laminar flow. The inoculated petriplates were incubated at 28^{o}C in BOD incubator for mycelia growth.

Microbial analysis of soil sample: The soil samples were microbial analysis by serial dilution. Taken 90 ml sterile water and labeled as blanks 1,2,3,4,5,6 and 7 and autoclave along with petriplates. 10 ml suspension was transferred from flask number 1 into blank number 2 with a sterile pipette under aseptic conditions to make 1:100 dilution (10^{-2}) and shake well about 5 minutes to make 1:100 (10^{-1}) dilution. Another dilution 1:1000(10^{-3}) was prepared by pippeting 10 ml of the suspension into water blank number 3, using a fresh sterile pipette and was shake well. Further dilution was prepared as above mentioned method similarly upto10^{-7} dilutions. 15 ml of cooled nutrient agar medium (45^{0}C) was taken and added to each petriplate and mixed the inoculum with gentle rotation of petriplates by pour method. The three selective media viz. Pikovaskya's agar medium, King;s B agar medium and Actinomycetes agar medium were added to various dilutions. Upper solidification of media, all plates was incubated in BOD incubator in a inverted position at 28^{o}C for 2-7 days.

Culture preparation of bacteria in nutrient broth: 100- 300 µl of Nutrient broth culture poured in the sterilized petri plate. Add nutrient agar medium in petri plate and spread properly, make 3 well one in the center and another two at the periphery, poured the antibiotic stock solution of different conc. Incubate for 24 hrs. After incubation period observe MIC (Minimum Inhibition Concentration).

Screening of antagonistic bacteria against Trichoderma harzinum: The study of colony interaction between soil bacteria and individual species of test pathogen studied by Dual Culture Experiment. Dual culture assay was done by placing the inoculom of pathogenic fungus in the centre of petriplate containing PDA medium. The bacterial inoculants were streaked parallel on opposite sides of fungal inoculam. After inoculation, the petriplate was stored at ambient temperature in the incubatore for 4-7 days. After few days, the cleared zone was observed and diameter was observed and diameter was observed. The radial growth of pathogenic fungus and bacteria was measured at interval of 24 hrs upto 7 days after inocubation.Inhibition of mycelia growth of pathogenic fungus by each bacterial strain was recorded. Percentage growth was calculated as per formula by Dennis and Water (1971).

Percentage growth inhibition = R-**r x 100 f**
Where, r = Colony growth in control plate
R = Colony growth in intersecting plate

Isolation of Bacterial Genomic DNA

Bacterial genomic DNA isolated by Minikit method. Took, 1 ml of bacterial culture were transferred to a eppendorf tube and centrifuged for 10 minutes and suspension was discord. 200µl of GT buffer was added to the tube and the cell pallet resuspended by vertex or by pippetting and then incubated at room temperature for 5 minutes. 200µl of GB buffer was added to the sample and vortexed for 5 seconds and incubated at 70^{o}C for 10 minutes. The sample lysate was clear. During incubation, tube were inverted every 3minutes. After incubation added 200µl elution buffer in a 70^{o}C. After incubation 5 µl of RNase-A added (10mg/ml) to the sample lysate, mixed by vortexing and incubated at room temperature for 5 minutes. 200 µl of absolute ethanol was added to the sample lysate and vertex immediately for 10 seconds, if precipitate appears, break it by pipeting. Now GD column was placed in a 2ml collection tube. All the mixture was transferred to to the GD column andplaced in a new 2 ml collection tube. 400 µl and centrifuged to full speed for 30 seconds and wash with flow and discord. 600 µl of wash buffer was added to the GD column and centrifuged at full speed. Through the flow discard and GD column was placed back in the 2ml collection tube and centrifuged again for 3minutes to full speed and dry column matrix. Slandered elution volume was 100 µl. If less sample is to be used, reduce the elution volume (30-50 µl) to increase DNA concentration. If higher DNA required, repeat the DNA elution step and increase DNA recovery. The dried GD volume was transferred to a clean 1.5 ml eppendorf tube. 100 µl of preheated elution buffer of TE was added to the centre of column matrix. Now, waited for 3-5 minutes elution buffer and absorbed by matrix and centrifuged at full speed for 1 minute to elute the purified DNA (Vogelstein *et al* 1979).

Molecular characterization of antagonistic bacteria: PCR Amplification Primer Sequences- 5' AGAGTTGATCCTGGCTCAG3' 16S 27F 5' AGGGTTGCGCTCGTTG3' 16 S 1115 R PCR mix (50 µl) was prepared by adding Taq DNA polymerase 1.25U, dNTPs mixture 1 µl, Template DNA (60ng/µl) 1µl, Forward Primer (10pmol/µl) 1 µl, reverse primer (10 pmol/µl) 1 µl, Buffer (10X) 5µl. Amplification was carried out as following reaction condition for 30 cycles. Initial temperature provides 94^{0}C for 120 seconds, denaturation 94^{0}C for 30 second, annealing 48^{0}C for 30 second, Extension 72^{0}C for 60 second, Final extension 72^{0}C for 120 seconds. After amplification runs PCR

product in 1% agrose gel for detection. 16 S r-DNA sequencing to amplified DNA. After sequencing performed NCBI BLAST, and compared homology with other strains of bacteria. DNA of antagonistic bacteria were isolated and amplified by PCR with the help of primers (16S 27F and 1115F). The bacterial strains which were successfully amplified by PCR were PKB-1, PKB2, PPB3, PPB-4 and PAM-11 along with leader. The 16Sr DNA sequence has hyper variable regions, where sequence have diverged over evolution time. These are often flanked by strongly conversion regions. Primers are design to bind to conversion region and amplify variable reasons. The DNA sequences of 16S are DNA gene has been determine for an extremely large number of species (Pasquale *et al* 2006). Factually, there is no other gene that has been as well as characterize as in many species. Thus, result obtained for present investigation was conformed with the results (Reinhard *et al* 2008).

RESULTS AND DISCUSSION

PCR Amplification Primer by following sequences: 5' AGAGTTGATCCTGGCTCAG3' 16S27 F 5' AGGGTTGCGCTCGTTG3' 16 S 1115 R PCR mix (50 µl) was prepared by adding Taq DNA polymerase 1.25U, dNTPs mixture 1 µl, Template DNA (60ng/µl) 1µl, Forward Primer (10pmol/µl) 1 µl, reverse primer (10 pmol/µl) 1 µl, Buffer (10X) 5µl. Amplification was carried out as following reaction condition for 30 cycles. Initial temperature 94^0C for 120 seconds, denaturation 94^0C for 30 second, annealing 48^0C for 30 second, Extension 72^0C for 60 second, final extension 72^0C for 120 seconds after amplification runs the PCR product in 1% agrose gel for detection. 16 S r-DNA sequencing – sequenced the amplified DNA (Anand et al., 2011). After sequencing performed NCBI BLAST, and compared homology with other strains of bacteria.

Molecular characterization of selected bacterial isolates showing antagonism against green mold disease The isolated bacterial antagonistic DNA were isolated and amplified by PCR with the help of universal primers (16S 27F and 16 S). The progressive research is fully concentrated on development, such type of tools which are pollution free and hazardous chemical less. So the most of the scientist are aware for biocontroling agent for treating most of the disease in plants. These are important contributions because biocontrol agents offer disease management alternatives with different mechanisms of action than chemical pesticides. Recently a trend develop in research as increase use of bio-rational screening processes to identify microorganisms with potential for biocontrol. This increased testing under semi commercial and commercial production conditions and increase emphasis on combining biocontrol strains with each other. With the above controling methods, integrating biocontroling system on overall system.

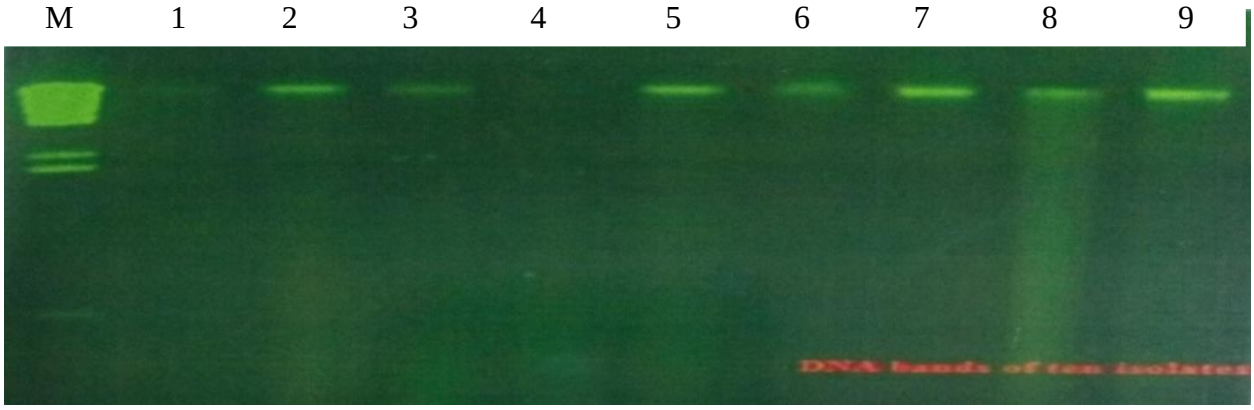

Figure 1 Analysis of DNA molecule after PCR amplification in variable temperature

Green moulds develop billions of spores that are easily carried by workers, insects and contaminated equipment. Therefore, infestations are quickly spread and the control of the disease is difficult. Failure to treat the disease outbreaks in the early stages can be very costly as untreated areas of disease produce the spores and propagules that will spread the disease throughout the rest of the crop and the farm. Poor hygiene either through ineffective disinfection of equipment or through the ingress of contaminated air into spawning halls is the likely route of the entry of the pathogen. Stringent sanitation standards coupled with the use of the proper fungicide will be required to prevent a buildup of pathogen propagules around the farm. It used to be a common practice for a long time to sprinkle salt over the green mould specks or to irrigate them with a binomial containing solution.

In certain cases, the whole of the infected casing material was removed and a new casing was applied. In the commercial production of *A. bisporus*, disinfectants are frequently used as an aid to general hygiene procedure and as inhibitor of the activity of many undesirable microorganisms . They are used to clean shelves, growing containers, machinery and working surface as well as floors and walls and in foot dips. Pasteurization of compost or wood materials used in the construction of mushroom growing rooms resulted in low green mould infection as well as high yield and high number of flushes (Catlin *et al* 2004).

CONCLUSION

Prevention has to play a central role in green mould management, however, if the infection already occurred at a mushroom producing facility, it has to be controlled. Chemical treatments are often the most effective means of managing green mould. The minimal inhibitory concentrations of a series of fungicides towards *T. aggressive umisolates* were determined and compared with other *Trichoderma* species occurring in mushroom cultivation as well as Agaricus bisporus the results are presented. Despite the expansion in the commercial both the bands were eluted by cutting the gel and partially sequenced. Finally, the 16S rDNA sequences were compared with those available in the NCBI database combining a BLAST search and analysis, which allowed the identification of BMBK1 as *Pseudomonas entomophila* L-48 and BMBK6 as *Pseudomonas putida* GB-1. These species are also reported earlier to have antagonistic activity and growth-promoting effects (Gardner *et al* 1984). Analysis of variation of the ribosomal 16S gene allows the inference of the phylogenetic relationships among taxonomically relatively close and distant organisms (Ludwig *et al* 1998).

ACKNOWLEDGMENT

This study is supported by Banaras Hindu University (BHU) through M. Sc. thesis program (ID. No./Enrolment No. PB-08202/309275) for financial support. Special thanks are conveyed Dr. Rajesh Singh, (Professor), Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University (BHU), Varanasi for their constant incouragement & providing the resources to carry out the research work.

REFRENCES

Allen Z, Allen R, (1999) The nut gourmet: nourishing for every occasion. Vegetarians in Paradise. Vol 12(7).

Anand K, Shiv KV, Ashis Dhiraj KC, (2011) Biochemical and molecular characterization of antagonistic bacteria against yellow blotch of oyster. International Journal of Research in Engineering and Technology. eISSN 2319-1163.

Chang ST, Miles PG (2004) Mushrooms Cultivation nutritional value medicinal effect and environmental intact. Second Edition CRC Press.

Denis M, Proinflammatory cytokines in hypersensitivity pneumonitis. Am J Respir Crit Care Med 1995 151: 164-9

Kredics L, Cseh T, Körmöczi P, Hatvani L, Manczinger L, Vágvölgyi C (2008) Extracellular enzyme production of the two causative agents of oyster mushroom green mould under inductive and non-inductive conditions. Abstracts of the 6th International Conference on Mushroom Biology and Mushroom Products. Bonn Germany 29: 8-3.

Mori S, Nakagawa-Yoshida K, Tsuchihashi H (1995) Mushroom worker's lung resulting from indoor cultivation of Pleurotus osteatus. Occup Med 48: 465-8.

Rinker DL (1996) Biological Control. Pennsylvania Mushroom Integrated Pest Management Handbook. The Pennsylvania State University University Park PA 16802.

Rinker DL (1997) Response of the oyster mushroom to supplementation prior to pasteurization. Mush Sci 13 (II): 189-198.

Seaby DA, (1996) Differentiaation of T.taxa associated with mushroom cultivation associated with mushroom production. Plant Pathology 45: 905-912.

Sinden JE, Hauser (1953) Nature and control of three mildew diseases of mushrooms in America. Mushroom Sci 2: 177–180.