Nepal Journal of Science and Technology 9 (2008) 65-71

Antifungal Factor Produced by *Pseudomonas fluorescens* against an Endophytic Fungus

Rajani Malla[1] and Suprada Pokharel[2]
[1]*Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal*
e-mail : rajanimalla2000@yahoo.com

Abstract
Growth characteristics of *Piriformospora indica* an endophytic fungus with plant growth promoting rhizobacteria in an aspergillus medium were studied. *P. indica* showed promotional effects with species of *Azotobacter chroococcum*, *Azospirillum brasilensis*, *Bradyrhizobium* spp., and inhibitory effect with *Pseudomonas fluorescens.* The biochemical and molecular level of inhibitory factor produced by *P. fluorescence* suggests that the antifungal substance was thermolabile and the protease test showed it to be protease resistant in nature. Dialysis test indicated that this substance had a molecular weight of more than 12,000 kDa. The inhibitory substance turns out to be 'fungistatic' as well as 'fungicidal' in nature. Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) studies showed the cell wall in damaged form suggesting the high potency of this antifungal substance. The inhibitory compound produced by *P. fluorescence* was suspected to be pyoverdine, a kind of siderophore, an iron containing substance.

Key words: *Pseudomonas fluorescens, Piriformospora indica*, siderophore, rhizobacteria, TEM, SEM

Introduction
Exploiting the potential of fluorescent pseudomonads to act as crop protectants has become the focus of many research groups. *Pseudomonas* spp. have been investigated as potential biological control agents due to their ability to colonize in the rhizosphere and protect plants against a range of agronomically important fungal diseases (Stutz *et al.* 1986). Their biocontrol capabilities result largely from their ability to produce antifungal metabolites. *Piriformospora indica,* a novel fungus, is a good representative of Basidiomycetes- the highest evolved group of fungi, in the sense that its interaction with *Pseudomonas* can turn out to be a role model in understanding the inhibitory factors secreted from the bacteria (Malla *et al*. 2002).

P. indica was isolated from the rhizosphere of the woody shrubs *Prosopsis julifera* and *Zizyphus nummularia* growing in the western part of Rajasthan (Verma *et al.* 1998). The characteristic features of *P. indica* are: axenically cultivable on synthetic media, simple septum with dolipore and continuous straight parenthosomes, spore 8-45 µm in diameter and 8-25 nuclei per spore. The fungus promises to serve as the substitute of arbuscular mycorrhizal (AM) fungi to overcome the long-standing enigma of science. The functional similarities with AM fungi are: broad and diverse host spectrum, positive phyto-promotional effects on test host, hyphae never invade the endodermis, sexual stages not seen, phosphorus mobilizer and transporter (Malla *et al.* 2004, 2007a), tool for biological hardening of micropropagated plantlets and a potent biological control agent against root pathogens. Based on the 18S and 28S rDNA analysis and the ultrastructure of the septal pore, its phylogenetic relationship is within the Hymenomycetes. Proteomics and genomics data about this fungus have recently been described (Kaldorf *et al.* 2005, Peškan-Berghöfer *et al.* 2004, Shahollari *et al.* 2005, Malla *et al*. 2005, 2006, Malla & Varma 2007c). *Sebacina vermifera* sensu consists of a broad complex of species possibly including mycobionts of jungermannioid and ericoid mycorrhizas. Extrapolating from the known rDNA sequences in *Sebacinaceae*, it is evident that there is a cosm of mycorrhizal biodiversity yet to be discovered in this group (Weiß *et al.* 2004). The fungus was found close relationships with *Sebacina vermifera* sensu according to morphological immunological and phosphatase isozymes characteristics (Malla *et al.* 2007b & Malla 2008). The properties of *P. indica* have been patented in Muenchen, Germany (Patent No. 97121440.8-2104, Nov. 1998). The culture has been deposited at Braunsweich, Germany (DMS No. 11827) and 18S rDNA fragment deposited with GenBank, Bethesda, USA, AF 014929.

Pseudomonas fluorescens encompasses a group of common, non-pathogenic saprophytes that colonize soil, water and plant surfaces. It produces a soluble, greenish fluorescent pigment, particularly under conditions of low iron availability. It is an obligate aerobe, except for some strains that can utilize NO_3 as an electron acceptor in place of O_2. On the basis of rRNA/DNA-homology studies, *Pseudomonas* has been divided into five groups. However, recent polyphasic and genomic taxonomic studies indicated that it is a multigeneric entity and that the five rRNA groups are distantly related to each other.

The presence of pseudomonads leads to the suppression of plant deleterious microorganisms both by the production of secondary metabolites (Leisinger 1979) antibiotically active substances (Budzikiewicz 1993) e.g. phenanines, pyrroles, acetylphloroglucinols and cyanides (Voisard, 1989) and by the excretion of effective siderophores, with high iron binding constants that cannot be utilized by pathogenic fungi or bacteria (Kloepper 1980). Among better known siderophores of pseudomonads are high-affinity iron chelators such as pyoverdins (Leong 1986) that help compete out harmful microorganisms by reducing the levels of Fe (III) (Davison 1986).

Materials and Methods

Interaction with rhizobacteria

P. indica was grown in aspergillus medium (Hill & Kaefer 2001, Pham *et al.* 2004) along with rhizobacteria at corners (Malla *et al.* 2002) and incubated at 28 ± 2°C. The growth characteristics were recorded at 2days intervals.

Dialysis tube test

The fungus was routinely cultivated on aspergillus agar and incubated at 28 ± 2°C in dark. The activated tube was cut and the monolayer was made with the help of a sterile surgical knife in the laminar flow bench. It was gently placed in between growth of *P. fluorescens* and *P. indica*. The tubing retained most proteins with molecular weight of 12,000kDa or greater.

Protease test

The *P. fluorescens* culture broth was dissolved in 100mM ammonium bicarbonate. The enzyme (trypsin-pancreatic trypsin) was added at 1:50-100 and incubated for 1-4 hours at 30°C. optimum pH was 7-9.

Absorption spectroscopy

The zone between *P. fluorescens* and *P. indica* (4 days and 22 days old) was cut out in the laminar flow bench, with the help of a sterile surgical knife. It was put into 3ml of methanol overnight. The recordings were taken from 200 to 900nm in the Shimadzu UV-visible recording spectrophotometer (UV-260) using Sigma cuvettes.

Spectrofluorimetry

The zone of growth between *P. fluorescens* and *P. indica* (4 days and 22 days old) was cut out. It was put into 3ml of methanol overnight. Here the instrument used was Carry Eclipse (EL01035456). The excitation and emission slit width was set at 5nm. Each spectrum was an average of 5 scans. The excitation wavelength was 250nm. The sample was scanned from 270nm to 400nm.

Similarly, a second set of spectra were recorded. The excitation wavelength was 400nm. It was scanned from 415nm to 600nm.

Scanning electron microscopy (SEM)

The hyphae and bacterial cells growth mixture were fixed in 2.5% glutaraldehyde (70%, Research industries, USA) buffered with 0.1M Sodium Phosphate Buffer, pH 7.4 for one hour. The fixed samples were washed in distilled water three times at 15 minutes intervals. Cleaned samples were dehydrated through a graded series of ethanol. The specimens were mounted on aluminium SEM stubs. The samples were transferred to Edwards Vacuum Evaporation unit. The samples were coated at a pressure of 10^{-5} torr. with approximately 100Å thick layer of carbon followed by about 200Å thick coating of silver. For the uniformity of coating, the samples were made to rotate at 45° from the vapour source. The coated specimens were scanned in scanning electron microscope (Philips), at an anode potential of 15.00 KV. For studying the interspecific differences, the pictures were taken at different magnifications for providing more details.

Transmission electron microscopy (TEM)

The samples were fixed in modified Karnovsky's fluid (David *et al*, 1973) buffered with 0.1M sodium phosphate buffer at pH 7.4 post fixeded for two hours in 1% osmium tetroxide (Palade 1952) in the same buffer at 4°C. After fixation, samples were dehydrated and embedded.in CY 212 araldite. Ultrathin sections

were cut on ultramicrotome using the diamond knife on copper grid. The sections were then stained in 0.5% aqueous uranyl acetate for 15 minutes and lead citrate (Reynolds 1963) for 3-4 minutes. The stained sections were observed with Philips, CM – 10 electron microscope. The microscope was operated at 60 – 80 KV.

Results

Inhibition of growth

P. indica showed promotional growth effect with *Azotobacter chroococcum, Azospirillum brasilensis, Bradyrhizobium* spp. and inhibitory effect with *P. fluorescens.* In the presence of *Pseudomonas* the growth of *P. indica* in agar plate was suppressed from the day one (Fig.1). When the culture filtrate was taken, the inhibition continued. Treatment of this culture filtrate with trypsin did not affect the inhibition, Boiling the *Ps. fluorescens* filtrate stoped the inhibitory action and the fungus grew normally. Filtering the culture filtrate by 0.2µ Minisart filter also rendered the property of inhibitory factor secreted by *P. fluorescens.* When an activated dialysis tube was introduced between bacterial and fungal growth, areas the inhibition was put at halt and the hyphae grew normally indicating the molecular size of inhibitory substance to be bigger than 12,000kDa. In normal medium initially up to 20 days the usual inhibition of fungus was shown, but by the 22nd day fluorescence pigments were observed in the petri plate (Fig. 2).

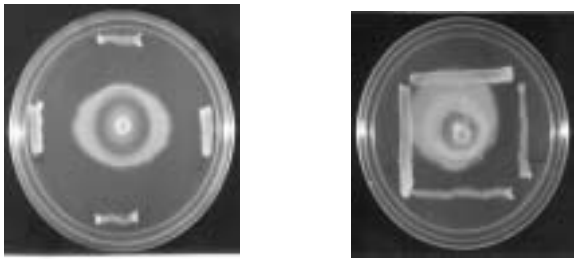


Fig.1. Interaction of *P. indica* with rhizobacteria *P. indica* showed promotional effects with species of *Azotobacter* and inhibitory effect with *Pseudomonas fluorescens.*
Ps: *Pseudomonas fluorescens*
Az: *Azotobacter chroococcum*
Pi*: Piriformospora indica*

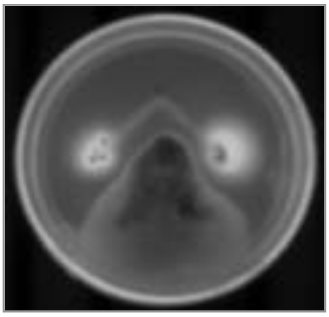


Fig.2. Growth of *P. indica* (←) with *Pseudomonas fluorescens* (←) in normal aspergillus medium showing release of fluorescent compound after 20 days of interaction. Note the inhibition of fungus by bacteria.

Spectrophotometric analysis

When the four days old, non-fluorescent zone (boiled and unboiled) was dissolved in methanol and its absorbance spectra was recorded, it showed a peak at 300nm. The sample was scanned from 220nm to 700nm. Similarly, a 25-day- old non-fluorescent sample showed a peak at 250nm. The 25 -days -old fluorescent sample also showed two peaks, at 240nm and 410nm(Fig. 3).

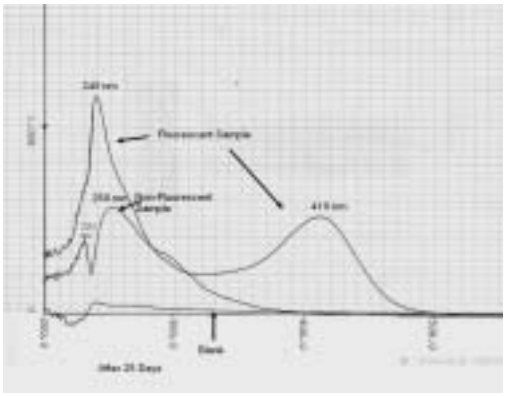


Fig.3. UV Spectrophotometric analysis showing the non-fluorescent sample's (25 days) absorption maximum which undergoes a blue shift from 300nm to 250nm

Spectrofluorimetric analysis

When the fluorescence spectrum of fluorescent sample was excited at 250nm; a peak was observed at 385nm (Fig. 4). Similarly, when excited at 400nm and the sample was scanned from 400nm to 600nm a peak was observed at 488nm (Fig.5).

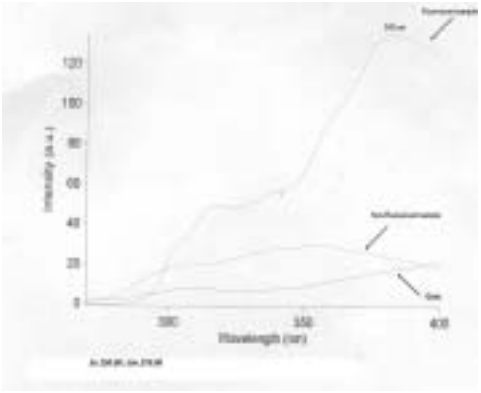


Fig.4. Spectrofluorimetric analysis of the 25 days old fluorescent sample showing an emission maximum at 385nm when excited at 250nm and scanned from270nm to 400nm

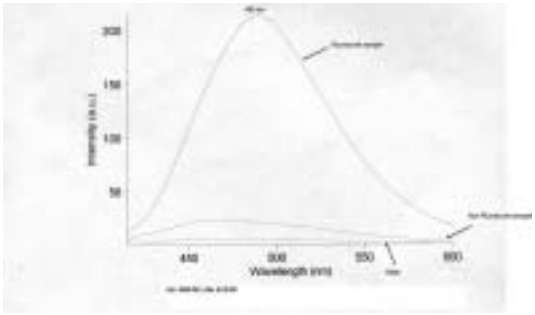


Fig. 5. Spectrofluorimetric analysis of the fluorescent sample showing an emission at ~ 488nm, when excited at 400nm. The sample was scanned from 415nm to 600nm

Scanning electron micrograph

Scanning electron micrograph of the control (only *P. indica*) at 8000X showed healthy fungal hypha with smooth chitinous cell wall. Damaged fungal structure was observed (at 8000 X) when the bacteria was in direct physical contact with it. Certain dotted ooze like substances were released which were clearly visible (Fig.6) were suspectede to be â-Proteobacteria which inhabit the fungus as symbionts.

β-proteobacteria

Fig. 6. Scanning electron micrograph of interaction between *Piriformospora indica* and *Pseudomonas fluorescens* showing damaged hyphae (Left) Control hyphae of *P. indica* (Right) note β-protobacteria within the hyphae.

Transmission electron micrograph

Transmission electron micrograph further unraveled the morphology of the dotted substance released during *P. fluorescens* interaction. At 1100X, the control sample showed smooth surface while pseudomonad treated sample showed dotted structure oozing out into the medium as well as the tuberculated surface without chitinous cell wall layer. Pseudomonas treated fungal hyphae showed highly damaged surface, with blown away cell-wall and loosen up cytosolic mass (Fig.7).

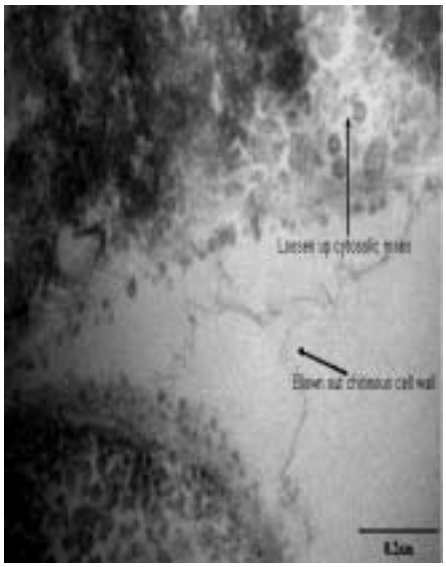
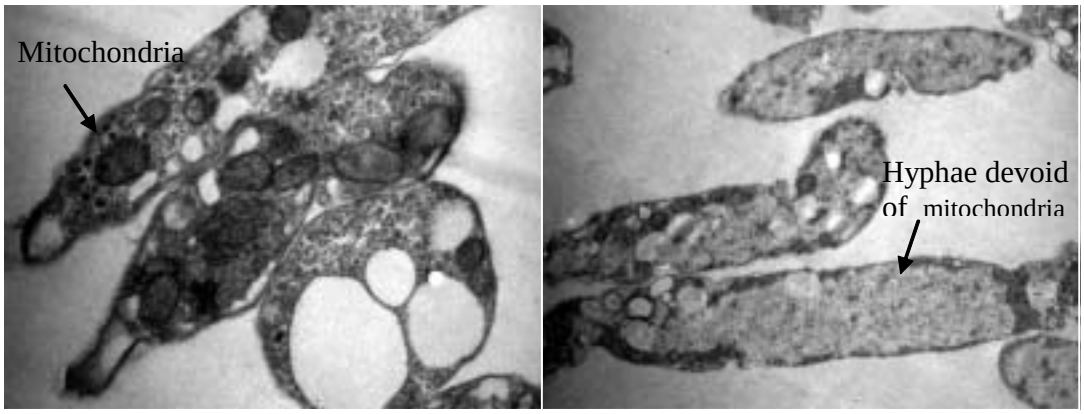


Fig. 7. TEM Showing the Surface Anatomy of Fungal Hypha Under the Influence of *Pseudomonas fluorescens* Fungicidal Factors. Pseudomonas treated fungal hyphae showed highly damaged surface, with blown away cell-wall and loosen up cytosolic mass (14000X) mitochondria

Mitochondrial density

At 3500X, the control showed 2.26µm wide hypha. The hyphal radius of the fungus was calculated to be 1.13µm with help of bar. A 5µm length of hypha was considered to be one unit length. It was observed in several micrographs that the number of mitochondria varied from 2 to 7 (considering longitudinal as well as transverse sections).

At 3500X, the *P.* treated fungus showed 1.74µm wide hypha, which was less than control. Number of mitochondria were found less than in control (Fig.8).



Control, 3500X

Pseudomonas treated fungal hyphae, 3500X

Fig. 8. TEM (Magnification 3500X) showing number of mitochondria in control and *Pseudomonas* treated fungal hypha

Discussion

Knowledge concerning plant-growth-promoting cultivable root endophytes is low and most studies have been conducted with mycorrhizal fungi. Arbuscular mycorrhizal fungi occur on the roots of 80% of vascular flowering plant species but they are obligate photobiotrophs and cannot be cultured without the plant. *P. indica*, a plant root-interacting fungus, can be easily grown on various complex and minimal substrates. *P. indica* has growth-promoting effects on a broad range of plants, as do the AM fungi, but has the added trait of being able to be grown in axenic cultures. *P. indica* has the potential to markedly promote plant growth, while exhibiting some similarities to mycorrhizal fungi.

The hunt for newer antifungal compounds or biocontrol is still on for obvious reasons. In recent years, the interest in the use of bacteria for biological control of plant-pathogenic fungi has increased. The fluorescent pseudomonads produce a variety of biologically active natural products (Budzikiewicz 1993, Leisinger 1979) many of which have an ecological function in these gram-negative bacteria. Some of these natural products contribute to the suppression of plant-pathogenic fungi (Dowling 1994, Thomashow 1996) whereas others are important virulence factors of certain plant-pathogenic *P. fluorescens* (Bender 1999). *P. fluorescens* is important as a biological control organism due to its rhizospheric competence, the large spectrum of antimicrobial substance and other secondary metabolites that it produces, and its status as model environmental strain for studies of gene regulation (Howell 1980).

Siderophores are extracellular, low molecular weight substances which are selectively complex iron with high affinity. Fluorescent pseudomonads produce siderophores such as pseudobactin and pyoverdine which chelate the iron available in the soil and make it unavailable to pathogens thus the pathogen dies for want of iron. *In vitro* experiments have shown that cyclic lipopeptides secreted by *P. fluorescens* having antibiotic and surfactant properties cause morphological changes as well as alternations in intracellular pH and mitochondrial organization in pathogenic fungi like *Pythium ultimum* and *Rhizoctonia solani* (Thrane *et al*. 1999).

P. fluorescens promotes mycorrhization *in-vivo* increasing root colonization by the fungus. Few strains of *P. fluorescens* have been characterized as helper bacteria, as, they increase mycorrhization and/or growth of the fungus. Around the roots of the non-mycorrhizal host plants they have a saprophytic behaviour. In the mycorrhizas, they are organized in small colonies inside cavities dug in the polysaccharidic matrix of the interhyphal-cement (Collignon & Dexheimer 1994).

P. indica showed positive growth effects with species of *Azotobacter chroococcum, Azospirillum brasilensis, Bradyrhizobium* spp. and inhibitory effect with *P. fluorescens.* The anti-fungal factor produced by the bacteria is potent enough to degrade the chitinous cell wall of the fungus and bring down the mitochondrial count within the hyphae which is one step beyond the finding of Thrane *et al.* (1999) who showed the alteration in the mitochondrial organization. When the fungus is growing normally, it requires energy for its growth and development. It colonizes the media surface on the petri plate – showing it's healthy growth. The lesser diameter of hypha in the *Pseudomonas* treated sample suggests the fungistatic nature of the 'inhibitory compound', while the damaged chitin cell wall layer in the bacterial treated fungal hypha shows the fungicidal nature of the antifungal substance. Its hyphal movement is supported by the cytoskeleton network in its cytosol. The cytoskeleton gets the energy from mitochondria in the form of ATP. So, the number of mitochondria observed in the control sample is much higher than the *Pseudomonas* treated fungal sample, which supports this hypothesis. On the basis of absorption spectra under spectrophotometer the antifungal agent was suspected to be siderophore The result of gas chromatography/mass spectrometry is awaited and the chemical nature of this anti-fungal substance will be unraveled.

Microbial products are so complex in their chemical structures that one compound may have two or more totally different chemical moieties which can interact with different receptors. However, the use of microbial products as fungicides has limitations. One disadvantage is that they may suffer from the emergence of natural resistance in plant pathogens due to their highly specific mechanisms of action. In our *in-vitro* study *P. fluorescence* inhibited the growth of *P. indica*, in *in-vivo* condition the bacteria may interact as growth promoting organism or mycorrhization helper bacteria by the way of signal transduction since the fungus is not pathogenic and its interaction with other PGPRs were positive. Research on the cellular and molecular basis of the interaction should contribute to the understanding of the beneficial associations of *P. indica* and PGPRs. A panoramic effort is required to unravel the nature of this high quality antifungal factor.

References

Bender, C.L., F. Alarcon-Chaidez and D. C. Gross. 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.* **63**: 266-292.

Budzikiewicz, H. 1993. Secondary metabolites from fluorescent pseudomonads. *FEMS Microbiol. Rev.* **104**: 209-228.

Collignon, A.M., J. Dexheimer. 1994. Parietal interactions between *Pseudomonas fluorescens* strain BBC6 and the ectomicorrhizal fungus *Laccaria laccata* S238. *Journal of Trace and Microprobe Techniques* **12**: 201-208.

David, G.F.X., J. Herbert and C.D.S. Wright. 1973. The ultrastructure of the pineal ganglion in the ferret. *J. Anat.* **115**: 79.

Davison, J. 1986. Plant beneficial bacteria *Biotechnology* **6**: 282-286.

Dowling, D.N. and F. O'Gara. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *Trends Biotechnol.* **12**:133-144.

Hill, T.W. and E. Kafer. 2001. Improved protocols for aspergillus medium: trace elements and minimum medium salt stock solutions. *Fungal Genetics News Letter* **48**: 20-21.

Howell, C. R. and R.D. Stipanovic. 1980. Suppression of *Pythium ultimum* induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic pyoluteorin. *Phytopathology* ***70***:712-715.

Kaldorf, M., B. Koch, K. H. Rexer, G. Kost, A. Varma. 2005. Patterns of interaction between *Populus* Esch5 and *Piriformospora indica*: A transition from mutualism to antagonism. *Plant Biology* **7**: 210-218.

Kloepper, J.W., J. Leong, M. Teintze and M.N. Schroth. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* ***286***: 885-886.

Leisinger, T. and R. Margraff. 1979. Secondary metabolites of fluorescent pseudomonads. *Microbiol. Rev.* **43**: 422-442.

Leong, J. 1986. Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. *Annu. Rev. Phytopathol*. ***24***: 187-209.

Malla, R. and A. Varma. 2005. Phosphatase (s) in microorganisms. In: Biotechnological applications of microbes. IK International- India. 125-50.

Malla, R., R. Prasad, P.H. Giang, U. Pokharel, R. Oelmueller and A. Varma. 2004. Characteristic features of symbiotic fungus *Piriformospora indica*. *Endocytobiosis Cell Res.* **15:** 579-600.

Malla, R., Z. M, V. Yadav, Suniti, A. Verma, M. Rai, and A. Varma A. 2002. *Piriformospora indica* and plant growth promoting Rhizobacteria: An Appraisal. In*: Frontiers*

of fungal diversity in India. (GP Rao, DJ Bhat, Lakhanpal TN and C Manoharichari, Eds.). IBDC. pp. 401-419.

Malla, R., U. Pokharel, and A. Varma. 2005. Random amplified polymorphic DNA of the two fungi from *Sebacinales*. *Journal of Institute of Science and Technology* **14**: 34-43.

Malla, R., M. Zeaullah, U. Pokharel and A. Varma. 2006. Immunological characterization of acid phosphatase in an endophytic fungus. *Nepal Journal of Science and Technology* **7**: 77-84.

Malla, R., U. Pokharel, R. Prasad and A. Varma. 2007a. Proteomics and genomics approach to study plant-microbe cross communication. In: *Microbes for human life* (A.K. Chauhan, H. Kharkwal and A. Varma, Eds.) Anshan Pub, Uk. pp. 609-636.

Malla, R., U. Pokharel, R. Prasad, R. Oelmüller and A. Varma. 2007b. Immuno-technology for the localization of acid phosphatase using native gel bands in *Piriformospora indica* and other soil microorganisms. In: *Modern tools and techniques.* Springer-Verlag, Germany **11**: pp. 211-234

Malla, R. and A. Varma. 2007c. Use of short oligonucleotide primers in random amplified polymorphic DNA techniques for species identification. (Oelmüller R and Varma A., Eds.) Springer-Verlag, Germany **11**:235-244.

Malla, R. 2008. Isozymes involved in phosphate metabolism in *Piriformospora indica*. *Journal of Institute of Science and Technology* **15:** 1-13.

Palade, G.E. 1952. A study of fixation for electron microscopy. *J. Exp. Med*. **95**: 285-97.

Peškan-Berghöfer, T., B. Shahollari, H. G. Pham, S. Hehl, C. Markent, V. Blank, G. Kost, A. Varma and R. Oelmueller. 2004. Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represent a novel system to study beneficial plant-microbe interactions and involve in early plant protein modifications in the endocytoplasmic reticulum and in the plasma membrane. *Physiology of Plant* **122**:465-471.

Pham, G. H., R. Kumari , A. Singh, R. Malla, R. Prasad, M. Sachdev, M. Kaldorf, F. Buscot, R. Oelmüller, R. Hampp, A.K. Saxena, K.H. Rexer, G. Kost and A. Varma. 2004 Axenic cultures of *Piriformospora indica*. (Eds. A. Varma, L. Abbott, D. Werner and R. Hampp). In: *Plant Surface Microbiology. Springer-Verlag*. pp. 593-613.

Reynolds, E.S. 1963. The use of lead citrate at high pH on an electron opaque stain in electron microscopy. *J. Cell Biol.* **17**: 208-212.

Shahollari, B., A. Varma and R. Oelmüller. 2005. Expression of a receptor kinase in *Arabidopsis* roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. *Journal of Plant Physiology* **162**: 945-958.

Stutz, E.W., G. Defago and H. Kern. 1986. Naturally occurring fluorescent pseudomonads involved in the suppression of black root rot of tobacco. *Phytopathology* **76**: 181-185.

Thrane, C., S. Olsson, T.H. Nielsen and J. Sørensen. 1999. Vital fluorescent strains for detection of stress in *Pythium ultimum* and *Rhizoctonia solani* challenged with viscosinamide from *Pseudomonas fluorescens* DR54. *FEMS Microbiol. Ecol.* **30**: 11-23.

Thomashow, L. S. 1996. Biological control of plant root pathogens. *Curr. Opin. Biotechnol.* **7**:343-347.

Verma S., A. Varma, K-H. Rexer, A. Hassel, G. Kost, A. Sarbhoy, P. Bisen, P. Buetehorn and P. Franken. 1998. *Piriformospora indica* , a new root-colonizing fungus. *Mycologia,* **90**: 895-909.

Voisard, C., C. Keel, D. Hass and G. Defago. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black rot of tobacco under gnotobiotic conditions. *EMBO J.* **8**: 351-358.

Weiß, M., M.A. Selosse, K.H. Rexer, A. Urban and F. Oberwinkler. 2004. *Sebacinales*: a hitherto overlooked cosm of heterobasidiomycetes with abroad mycorrhizal potential. *Mycol. Res.***108**:1-8.