Interaction of endophytic fungus *Piriformospora indica* with plant and its role in regulation of plant defense response

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CERTIFICATE

The research work embodied in this thesis entitled **"Interaction of endophytic fungus *Piriformospora indica* with plant and its role in regulation of plant defense response"** has been carried out by **Manoj Kumar** for the degree of Master of Philosophy (M.Phil.). It is certified that the work presented is original and has not been submitted in part or full for any degree or diploma of any other university or institute.

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ABBREVIATIONS

AM	:	Arbuscular mycorrhiza
AMF	:	Arbuscular mycorrhizal fungi
ATP	:	Adenosine triphosphate
°C	:	Degree Celsius
CDNB	:	1-chloro-2,4-dinitrobenzene
DNA	:	Deoxyribonucleic acid
d	:	Day
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
g	:	Gram
h	:	Hour
HC	:	Hyphal Coil
kD	:	kilo Dalton
K_m	:	Michaelis constant
m	:	Minute (s)
mm	:	Millimeter (10^{-3} meter)
M	:	Molarity (moles per liter)
mM	:	Millimolar (10^{-3} molar)
mg	:	Milligram (10^{-3} gram)
ml	:	Milliliter (10^{-3} liter)
µg	:	Microgram (10^{-6} gram)
µl	:	Microliter (10^{-6} litre)
mRNA	:	Messenger RNA
µm	:	Micrometer
µM	:	Micromolar
NaCl	:	Sodium chloride
nm	:	Nanometer

PAGE	:	Polyacrylamide gel electrophoresis
PGPRs	:	Plant growth promoting rhizobacterias
pH	:	-ve log of hydrogen ion concentration
RNA	:	Ribonucleic acid
rpm	:	Rotations per minute
sec	:	Seconds
sp	:	Species
TEMED	:	N,N,N',N', tetra methylethylenediamine
TE	:	Tris-EDTA
VAM	:	Vesicular arbuscular mycorrhiza
V_{max}	:	Maximum velocity
U	:	Unit
UV	:	Ultraviolet
v/v	:	Volume/Volume
w/v	:	Weight/Volume
w/w	:	Weight/Weight

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1. Introduction

Plants interact with microorganisms in a number of ways ranging from beneficial or symbiotic interactions and pathogenic or non-symbiotic interactions. Beneficial relations may be caused by the symbiotic or non symbiotic associations between the plants and the host microorganism. Some of the beneficial relations are mycorrhizal symbiosis between the plant and the fungus residing in the roots of the plants and actinorhizal symbiosis between the plant and the nitrogen fixing gram positive bacteria. Mycorrhizas are among the most universal, intimate and important symbioses in terrestrial ecosystem. Among these, Arbuscular Mycorrhiza (AM) is the most ancient and widespread mycorrhizal symbiosis with a wide range of plant species, and effectively reduce root diseases caused by a number of soil borne pathogens (Linderman, 1994; Bodker et al., 2002; Li et al., 2006). *Piriformospora indica* is an endophytic fungus (Verma et al., 1999) showing a great deal of similarity with AM fungi, has growth promoting effects on plant and confers resistance against biotic and abiotic stress (Waller et al., 2005). However the role of *P. indica* in defense response of plant during biotic and abiotic stress has not been well studied. *Fusarium moniliforme* is a parasitic fungus to several crops and is commonly seed born and has been isolated from both symptom less and rotted maize kernels (Koehler, 1942) however reports suggest that this seed born inoculum is less important than the inoculum in the air and soil (The American Phytopathological society, 1973). *F. moniliforme* produces mycotoxins called fumonisins and moniliformin. Moniliformin was found highly lethal to animals and experiments show the growth inhibition effect on plant (Cole et al., 1973).

Plant defense mechanisms are characterized by a combination of two responses constitutive and inducible. Constitutive responses consist of general barriers or preexisting biochemical defenses. Inducible responses can be localized or systemic and are more sophisticated because they involve the recognition of the pathogen by the host plant, signal transduction, and the expression of several genes. We are more concerned with the antioxidative defense system (against oxidative stress), which involves several antioxidants and antioxidative enzymes in balancing redox level of cell and defense mechanism mediated by Pathogenesis Related Proteins (PR Proteins) that are antimicrobial in nature. These small PR Proteins (e.g., thionins, defensins, heveinlike proteins, and knottin-like peptides) have been classically divided into five groups, PR-1, -2, -3, -4, and -5, based on serological

and amino acid sequence analyses. These are the mainly co-ordinated, often complex and multifaceted defense mechanism against the fungal pathogen (Pozo et al., 2002; Pozo et al., 1998; Pozo et al., 1999).

The work presented here is mainly focused on effects of *P. indica* on plant growth, morphology, antioxidative defense system and PR proteins during the colonization. We have studied regulation of antioxidants and antioxidant enzymes (e.g. SOD, GR, CAT and GST) levels by *P. indica* during colonization, its compatibility with maize plant and expression of PR proteins. Finally our work focuses the role of *P. indica* in bioprotection against *F. moniliforme*, effect of state of affair of inoculum of *P. indica* and suspected role of these defense systems in bioprotection.

2. Review of Literature

Micro-organisms are crucial to the functioning of terrestrial ecosystems, and soils contain not only a vast amount of microbial biomass, but also the world's largest reservoir of biological diversity. Soil provides a spatially and temporally heterogeneous environment, especially at the scale encountered by microbes. Factors influencing the dispersion and growth of microbial populations include the presence of solid surfaces, strongly fluctuating moisture conditions, variable and often limiting levels of organic and inorganic substrates, and interactions with other soil-borne organisms. However, the most critical selective force for microorganisms in soil is influence of plant roots. The soil zone influenced by plant roots, the rhizosphere, differs markedly from the root-free soil environment. Not only are physical and chemical conditions different in the rhizosphere, but plant roots also provide the majority of substrate for energy and biosynthesis of microorganisms in soil. Plants can therefore have a profound effect on soil-borne microbial communities. However, a strong feedback also exists between the microbial communities in the rhizosphere and vegetation dynamics, as micro-organisms can significantly influence plant establishment, growth, and succession.

Microorganisms affect individual plants and plant communities both beneficially, e.g. by the provision of nutrients and direct hormonal stimulation of growth, as well as negatively, e.g. as the causal agents of disease. Taken together, plant-microbe interactions in the rhizosphere are of prime importance to the functioning of terrestrial ecosystems.

PLANT MICROBE INTERACTIONS

Plants constitute an excellent ecosystem for microorganisms. The environmental conditions offered differ considerably between the highly variable aerial plant part and the more stable root system. Microbes interact with plant tissues and cells with different degrees of dependence (Montesinos et al., 2002). Interactions between microorganisms and plants have major effects on the development of civilization since humans began to rely extensively on cultivated crops for food.

Microorganisms have developed several strategies to adapt themselves to the plant environment, including beneficial interactions and harmful or pathogenic interactions. Beneficial interactions are caused by symbiotic (*e.g., Rhizobium*) or non-symbiotic bacteria and by a highly specialized type of fungi, the mycorrhizae. The pathogenic or detrimental interactions of microbes with plants involve viroids, viruses, bacteria and fungi, and lead to infectious diseases affecting the host plant (Montesinos et al., 2002).

In symbiotic relationships, the microorganism assists the plant with nutrient absorption or contributes biochemical activities that the plant lacks. The plant, in turn, contributes photosynthate, to the competitive advantage of the corresponding microbial symbiont in the rhizosphere. By altering the balance of microflora in the rhizosphere, symbiotic associations may also help to protect plants from disease causing microorganisms.

To successfully colonize a particular host, a microorganism must develop the ability to pass defensive barriers of the plant to prevent infection. Once these barriers are breached, the newly susceptible host then faces selection pressure to develop counter measures that block invasion by the pathogen. After a novel resistance response has evolved in the plant, the pathogen must again respond with an alternative mechanism that restores virulence. These dynamic battles have resulted in the utilization of highly specific and extremely sophisticated attack strategies by the pathogen and equally elaborate defense responses by the host (Jackson et al., 1996). In this kind of relationship, the appearance of disease symptoms, *i.e.,* "compatibility," is the outcome in most cases and results from the ability of the pathogens to overcome the complexities of the host defense responses. However, "incompatibility," a resistance reaction that prevents or severely retards pathogen growth, may be conditioned by a single interacting gene pair-a host resistance (R) gene and a pathogen avirulence *(Avr)* gene (Flor, 1971).

NON-SYMBIOTIC INTERACTIONS

There are the three major categories of disease-causing organisms, viruses and viroids, bacteria and mycoplasma-like organisms, and fungi (Jones et al., 1991).

Viruses

Viruses were first recognized as pathogenic entities about a century ago. Despite differences in the properties of their genomes, all plant viruses face the same two fundamental challenges during the establishment of systemic infections in their plant hosts. The first necessity is to replicate in the initially infected cells. This is achieved in a wide variety of ways, all relying to some extent on the utilization of host components to complement replication determinants encoded by the virus. The second requirement is for the viruses to move through adjacent plant cells to the vascular system, before spreading throughout the plant. This process also depends on highly specific interactions with host proteins (Shaw, 1996).

Prokaryotes

Many important plant diseases exhibiting a wide range of symptom phenotypes are caused by bacteria and mycoplasma-like organisms. These organisms have diverse life styles, ranging from facultative pathogens to more fastidious obligate pathogens (Agrios, 1988). Members of these groups have two modes of pathogenesis: biotrophic pathogens kill their host plants relatively slowly, thus allowing maximal opportunity for pathogen replication, whereas necrotrophic pathogens use a brute force strategy that results in rapid tissue death. Biotrophic bacteria elicit fire blights, wildfire, halo blights, leaf and fruit spots, scalds and yellowing diseases, vascular wilts, scabs, cankers, and galls. In contrast, necrotrophic pathogens generally have broader host ranges than biotrophic bacteria and their pathology is more dependent on environmental conditions that stress the host.

In an interesting parallel with population monitoring by some rhizosphere inhabitants, *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas, Xonthomonos, Streptomyces* that the necrotrophic bacterial pathogens engage in a stealth mode of pathogenesis by lying in wait to express virulence factors until a population of bacteria that is sufficient to overcome the host response, has accumulated. This mob attack response is proposed to be mediated by "quorum

sensing" mechanisms in which quantitative autoinduced signals monitor bacterial populations and regulate the release of antibiotic or virulence factors (Mok et al., 1996).

In contrast to the diverse lethal syndromes caused by biotrophic and necrotrophic bacteria, the tumor-forming Agrobacteria do not kill cells directly. Instead, characteristic tumors often appear just below the soil level at the crown of the plant, hence the name crown-gall disease.

Fungi

Numerous species of fungi in each of the major phylogenetic groups cause serious plant diseases. These include lower fungi, such as Plasmodiophoromycetes, Chitridomycetes, and Oomycetes, and higher fungi, such as Ascomycetes, Basidiomycetes and Deuteromycetes (Webster, 1980). Fungal pathogens produce many different fruiting body and spore forms, and their life styles range from obligate parasites, such as the chitrids, downy mildews, and rust fungi, to facultative parasites that are capable of attacking plants only under special circumstances. Obligate fungal parasites have established intimate and highly evolved relationships with their plant hosts. During the course of infection, these pathogens engage in many sophisticated but poorly understood activities that redirect nutrient flow in plant tissues and alter the growth and morphology of the plant. Changes in the morphology of the pathogen are also evident during pathogenesis.

The penetration processes through which fungi gain entry into plant tissue are clearly complex events that in many cases involve the attachment of fungus to plant surface (Nicholson and Epstein, 1991) and secretion of a cocktail of hydrolytic and proteolytic enzymes (Kolattukudy,1985; Dickman et al., 1989), the composition of which varies depending on the particular fungus-plant interaction, however In case of mechanically penetrating fungi, penetration is characterized with formation of specialized penetration organs, called appressoria, at the tip of their germ tubes. Now appressorium attached to plant surface by extracellular adhesives and it form a penetration pore by exposing enormous turgor pressure (>8 megapascals; Howard et al., 1991). From this penetration pore, an infection peg develops and pierces through

the cuticle and cell wall, possibly assisted by hydrolytic enzymes (reviewed in Mendgen and Deising, 1993). During penetration, the hydrolytic enzymes and/or plant defense responses generate fragments of fungal and/or plant cell walls. These compounds, which are often oligosaccharides, can elicit broad host range defense responses that slow pathogen ingress. Symptoms often result from the effects of fungal toxins, the genetics, biosynthesis, and modes of action of which are steadily being resolved. These low molecular weight molecules appear to target critical biochemical pathways, and their action can have pleiotropic effects on plant metabolism. For example, fusicoccin blocks the function of plasma membrane ATPases, thus perturbing the energy status of cells, and tentoxin affects energy transfer in chloroplasts. Fusicoccin and tentoxin affect a broad range of plant species. However, other fungal toxins, the so-called host-selective toxins (HSTs), appear to be specific for individual plant species, and in some cases their effects are mediated by gene-for-gene interactions.

The interactions of *Avr* and *R* genes during pathogenesis are also studied. The *Avr* gene products are virulence components that have strategic roles in pathogenicity or in pathogen fitness. In fact, any gene expressed by a pathogen could become an *Avr* gene if an *R* gene capable of recognizing its product evolved in the plant host virulence may be restored after the evolution of successful resistance strategies. Among the possibilities are the deletions of *Avr* genes, frame shifts that lead to the production of truncated (and unrecognizable) *Avr* proteins, and ectopic expression from altered promoters. The time taken for evolution of these modified *Avr* determinants likely depends on the nature of the originally targeted specificities. If resistance is targeted against indispensable pathogen products, such as those involved in replication, it is likely that the effectiveness of the corresponding *R* gene (its "durability") may be extended. HSTs were the first compounds shown to confer plant disease specificity. Single host genes condition sensitivity to HSTs, and the ability of HST-producing fungi to cause disease is strictly correlated with synthesis of the toxin. Thus, HSTs are potent weapons in the arsenals of otherwise relatively weak pathogens.

Fusarium species are common plant pathogens and are causative agents of superficial and systemic infections in humans are facultative fungal parasites.

Infections caused by *Fusarium* sp. are collectively referred to as fusariosis. This fungus is the most common cause of mycotic keratitis. The most virulent *Fusarium* sp. is *Fusarium solani*. Fusarium produces very harmful toxins, especially in storage of infected crops. Filamentous fungus (*F. moniliforme*) is the hyphae producing and asexual reproductive stage of the genus *Fusarium*. Specialized spores are produced at the ends of filamentous hyphae branches. *F. moniliforme* is a major parasite of members of Gramineae. It causes stalk rot in maize (Ayers et al., 1970; Christensen and Wilcoxson, 1966; Hooker, 1972) and poor stand, reduced root and shoot weight and reduced plant growth and emergence in maize seedlings (Futrell and Kilgore, 1969; Van wyk et al., 1988; Palmer and MacDonald, 1974; Scott and Futrell, 1970). *F. moniliforme* is commonly seed born and has been isolated from both symptomless and rotted maize kernels (Koehler, 1942) however reports suggest that this seed born inoculum is less important than the inoculum in the air and soil (The American Phytopathological society, 1973). *F. moniliforme* produces mycotoxins called fumonisins and moniliformin. Moniliformin was found highly lethal to animals as single dose of 500 μg cause death of cockerels within two hours. A study with plant shows growth inhibitor and phytotoxic effects of the toxin. Experiments show the growth inhibition was 24 and 57% at 20 and 200 ppm of toxin respectively (Cole et al., 1973).

The fungus exists as an intercellular infection within the plant, although under certain circumstances it may become intracellular. The fungus endophytically infects seedling from its systemic infection of the seed, usually by the second day following germination, or within 10 days. The systemic infection of maize seeds produces maternal line vertical transmission from generation to generation. The endophytic hyphae are neither latent nor dormant but instead physiologically active (Bacon et al., 2001).

SYMBIOTIC INTERACTIONS

Among the favorable plant-microbe interactions that have been studied in the greatest detail are those in which bacteria or fungi enter into mutually beneficial symbioses with higher plants (Stacey et al., 1992). As is the case for the majority of

plant-pathogen interactions, symbioses are characterized both by their complexity and by their specificity, they are also of enormous importance for global agricultural productivity.

In most nitrogen-fixing symbioses, soil bacteria of the unrelated genera *Rhizobium* and *Frankia* induce cell divisions in fully differentiated (and quiescent) cells in the root cortex or pericycle of plants in the families Rosaceae and Leguminosae. Bacteria enter the root and migrate, intercellularly or intracellularly, toward these foci of dividing plant cells. As cell division continues and the nascent structures mature into nodules, the bacteria differentiate into forms that are capable of fixing nitrogen. The fixed nitrogen is transported throughout the plant and, in return, the bacteria are supplied with photosynthate and a protected environment in which to divide. In contrast to the restricted phylogenetic distribution of nitrogen-fixing symbioses, mycorrhizal associations are almost ubiquitous, and the effects of these associations on plant fitness and health and on the ability of plants to grow productively in suboptimal environments are profound. Mycorrhizas are intimate associations between plant roots and certain soil fungi, and are typified by outgrowths of fungal hyphae from mycorrhizal roots. These hyphae serve to increase the absorptive surface area of the root, thus facilitating the uptake of nutrients and minerals, particularly phosphorus (which is limiting for plant growth in many soil types). Mycorrhizal associations may also help to protect roots from infection by pathogenic organisms in the soil.

These symbioses share a number of similar features. For example, infection by both kinds of nitrogen-fixing bacteria, and by mycorrhizal fungi, triggers similar, but limited subsets of the plant defense responses. This implies that, between them, the symbionts must be capable of modulating these responses. Furthermore, there is evidence that Nod factors, which mediate the specificity of legume-*Rhizobium* interactions, can also stimulate mycorrhizal formation (Ane et al., 2004). After infection, the developmental program of nodule formation is controlled by the plant in both kinds of symbioses.

Mycorrhizal symbiosis

The importance of mycorrhizal associations was recognized when difficulties in transplanting forest trees to new soils were encountered. The new environments lacked the appropriate fungal species, and the trees were not able to thrive without them. Mycorrhizal associations are now known to be important for a wide variety of cultivated and native plants and form an essential component of their ecology. These associations can take on a number of different morphologies but they fall into two broad categories (Smith and Read, 1997). In endomycorrhizal associations, such as arbuscular mycorrhizas (AM), the mycorrhizal fungus penetrates root cells in response to specific signals from the plant. In the cortical cells the fungi differentiate nutrient exchange structures, termed arbuscules. These are anatomically similar to the haustoria (feeding structures) formed by pathogenic fungi, although their function is very different (Gianinazzi et al. 1983). Gross changes in root morphology are not generally seen in these symbioses, although subcellular modifications are extensive. By contrast, in ectomycorrhizal symbioses fungi grow within the cortical cell walls and their hyphae form a sheath around the root. Exchange of nutrients at the interface between the plant and fungal symbionts, occur through a plant hexose transporter gene whose expression increases markedly in cortical cells containing arbuscles (Harrison, 1996).

AM is characterized by highly branched haustorium–like fungal structures within root cortical cells. The symbiotic association formed between a wide range of plant species including angiosperms, gymnosperms, pteridophytes, and some bryophytes. Only a few plant species, *e.g.,* members of Brassicaceae, caryophyllaceae, Chenopodiaceae, do not engage in AM interactions (Vierheilig et al., 1996). The fungi involved in AM symbiosis are obligate biotrophs *i.e.,* they have not been cultured in the absence of a plant host. They reproduce asexually, forming multinucleate spores (Trouvelot et al., 1999). Development of the symbiosis is initiated when a fungal hypha contacts the root of the host plant where it differentiates to form an appressorium. Although the components of root exudates are capable of stimulating hyphal growth and branching, they are unable to elicit the formation of appressoria, which were initially on intact plant roots (Giovannettim et al., 1993). Appressorium formation is followed by the development of a penetration

hypha and penetration of the root. It can occur in two different ways, in some cases the hypha enters by forcing its way between two epidermal cells, whereas in other cases, the hypha penetrates an epidermal or root hair cell wall and grows through the cell (Bonfante-fasolo, 1984). The localized production of cell wall–degrading enzymes, in combination with mechanical force, may facilitate entry of the hypha without inducing defense responses (Bonfante and Perotto, 1995). AM fungi produce exo- and endoglucanases, cellulases, xyloglucanases, and pectolytic enzymes including polygalacturonases (Garcia-Romera et al., 1991; Garcia-Garrido et al., 1992; Garcia-Garrido et al., 1996; Rejon-Palomares et al., 1996) all of which would expedite their passage through a cell wall.

After the fungus colonizes the intercellular space of the root cortex the pant mediates two steps allowing the fungus to penetrate the rhizodermis. (1) anticlinal cell walls of two adjacent epidermal cells separate from each other in the vicinity of fungal hyphae allowing the intercellular passage of the hyphae, and (2) fungal hyphae are allowed to pass intracellularly through an exodermal cell and an adjacent cell from the outermost cortical layer. After the subsequent penetration of the innermost cortical layers, tree-like fungal structures (arbuscules) are formed within individual root cortical cells by repeated dichotomous branching of fungal hyphae (Smith et al., 1997).

The arbuscules are responsible for nutrient exchange. They represent a dead end in the growth of AM fungi (Bonfante and Perotto, 1995), because they finally senesce and collapse after 4–10 days of symbiosis (Sanders et al., 1977). The fungal structures are then degraded completely by the plant cell and the plant cell recovers its original morphology (Jacquelinet-Jeanmougin et al., 1987). This way, cortical cells are able to allow a second fungal penetration and arbuscule formation. The life cycle of AM fungi is completed by the formation of extraradical spores, which may enter another colonization process. The fungal cell wall becomes progressively thinner as the arbuscules develops and consequently in these cells, there is an extensive intracellular interface in which the two symbionts are in extremely close contact, separated only by their membranes and a narrow plant- derived apoplast (Bonfante-Fasolo, 1984; Bonfante and Perotto,1995). This interface is thought to be the site at which phosphate and carbon are transferred between symbionts (Smith

and Gianinazzi-Pearson, 1988; Smith, 1993). The life span of an arbuscule is only a few days, after which it collapses and decays leaving the cell undamaged and capable of hosting another arbuscule (Alexander et al., 1988). Following formation of arbuscules, some species of AM fungi also form lipid-filled vesicles within the roots, which are presumed to act as a storage reserve for the fungus (Smith and Gianinazzi-Pearson, 1988).

Despite of several beneficial features, AM symbiosis is not much applicable in sustainable agriculture because it is unable to make symbiosis with several group of plants and is can't cultured as axenically due to which the exploitation of AM symbiosis restricted to *in vivo*.

O_2, ROS, AND OXYGEN TOXICITY

In its ground state (its normal configuration, O_2) molecular oxygen is relatively unreactive. However, during normal metabolic activity, and as a consequence of various environmental perturbations (e.g., extreme temperatures, radiation, xenobiotics, toxins, air pollutants, various biotic and abiotic stresses, and diseases) O_2 is capable of giving rise to frightfully reactive excited states such as free radicals and derivatives (Scandalios, 1997; Fridovich, 1995) The oxidation powers of O_2 are restricted because electrons can only be absorbed from another species whose electron spin is antiparallel to the two unpaired, parallel-spin electrons in diatomic oxygen. This spin restriction renders ground state molecular oxygen sufficiently unreactive, so that it cannot abstract electrons from other species. However, removal of the spin restriction by adding a single e-, or upon transfer of energy to oxygen from a photosensitizer (e.g., chlorophyll, flavin-containing compounds), increases the reactivity of oxygen. Photosensitizers can harvest light and energize O_2 to form singlet oxygen ($^1\Delta_gO_2$), which can interact directly with another molecule, transferring the additional energy to the target molecule. The complete reduction of O_2 to water requires four electrons. O_2 has a preference for a stepwise univalent pathway of reduction resulting in partially reduced intermediates. The reactive species of reduced dioxygen 'Reactive Oxygen Species (ROS)' include the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$). The latter can also be generated by the interaction

of $O_2^{\bullet-}$ and H_2O_2 in the presence of metal ions. Both $O_2^{\bullet-}$ and $OH^{\bullet}$ are extremely reactive and can cause molecular damage, leading to cell death. The hydroxyl radical reacts with virtually anything, inflicting indiscriminate and extensive intracellular damage. The $O_2^{\bullet-}$ is the conjugate base of a weak acid, the perhydroxyl radical ($^{\bullet}HO_2$), whose p*K*a is 4.69 ± 0.08. Thus, under acidic conditions, the very reactive perhydroxyl radical may predominate following a one electron reduction of dioxygen, while at higher pH values the $O_2^{\bullet-}$ is predominant. These and the physically energized form of dioxygen, singlet oxygen ($^1O_2$), are the biologically most important ROS (**Table 1**). An activation energy of ~22 kcal/mol is required to raise molecular oxygen (O_2) from its ground state to its first singlet state. In higher plants, this energy is readily obtained from light quanta via such transfer molecules as chlorophyll. Unless abated, all of these intermediate oxygen species are extremely reactive and cytotoxic in all organisms (Scandalios, 1997; Halliwell, 1996). ROS can interact with proteins, lipids, and nucleic acids to cause severe molecular damage (**Table 2**). Thus, oxygen provides a paradox, in that it is essential for aerobic life, yet in its reduced forms is one of the most toxic substances with which life on Earth must cope. ROS are found in virtually all intracellular organelles or compartments as a consequence of normal metabolic activity. Each organelle or compartment has potential targets for oxidative damage, as well as mechanisms for the elimination of excess ROS accumulation (**Figure 1**).

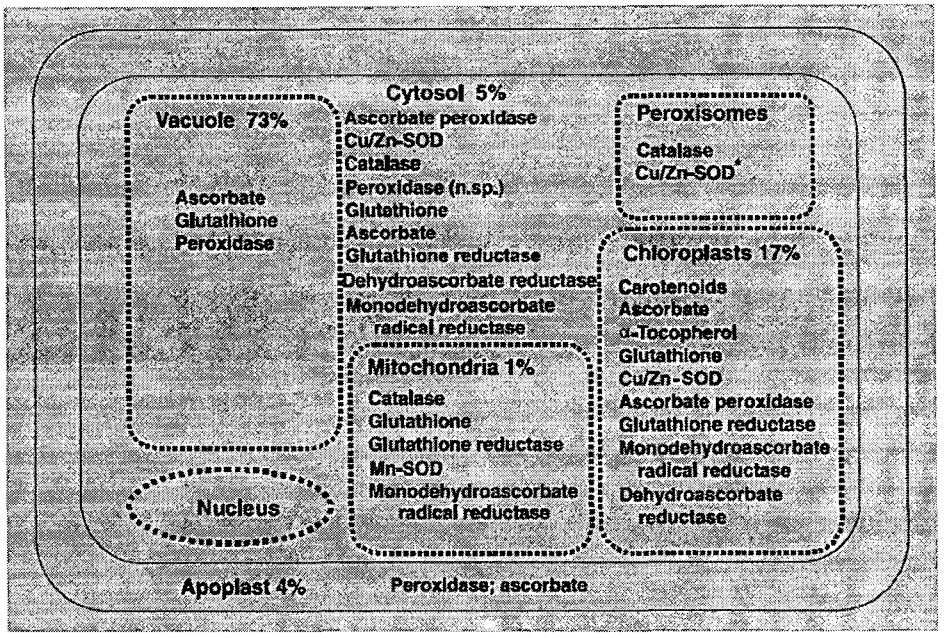

Figure 1. Intracellular antioxidant resources in plant cells. SOD = superoxide dismutase (Adopted from Scandalios, 2005).

Table 1. Reactive oxygen species (ROS) of interest in oxidative stress.

Name	*Notation*	*Some comments and basic sources*
Molecular oxygen (triplet ground state)	O_2; $^3\Sigma$	*Common form of dioxygen gas*
Singlet oxygen (1st excited singlet state)	$^1O^2$; $^1\Delta$	*Photoinhibition; UV irradiation; PS II e- transfer reactions (chloroplasts)*
Superoxide anion	$O_2\bullet-$	*Formed in many photooxidation reactions (flavoprotein, redox cycling); Mehler reaction in chloroplasts; mitochondrial e- transfer reactions; glyoxysomal photorespiration; peroxisomal activity; nitrogen fixation; reactions of O_3 and $OH\bullet$ in apoplastic space; defense against pathogens; oxidation of xenobiotics*
Hydrogen peroxide	H_2O_2	*Formed from $O_2\bullet-$ by dismutation; photorespiration; ß-oxidation; proton-induced decomposition of $O_2\bullet-$; defense against pathogens*
Hydroxyl radical	$OH\bullet$	*Decomposition of O_3 in apoplastic space; defense against pathogens; reactions of H_2O_2 with $O_2\bullet-$ (Haber-Weiss); reactions of H_2O_2 with Fe^{2+} (Fenton); highly reactive with all macromolecules*
Perhydroxyl radical	$O_2H\bullet$	*Protonated form of $O_2\bullet-$; reactions of O_3 and $OH\bullet$ in apoplastic space*
Ozone	O_3	*UV radiation or electrical discharge in stratosphere; reactions involving combustion products of fossil fuels and UV radiation in troposphere*

Table 2. Examples of reactive oxygen species (ROS) damage to lipids, proteins and DNA.

Oxidative damage to lipids
• *Occurs via several mechanisms of ROS reacting with fatty acids in the membrane lipid bilayer, leading to membrane leakage and cell death.* • *In foods, lipid peroxidation causes rancidity and development of undesirable odors and flavors.*
Oxidative damage to proteins
• *Site-specific amino acid modifications (specific amino acids differ in their susceptibility to ROS attack)* • *Fragmentation of the peptide chain* • *Aggregation of cross-linked reaction products* • *Altered electrical charge* • *Increased susceptibility to proteolysis* • *Oxidation of Fe-S centers by $O_2\bullet-$ destroys enzymatic function* • *Oxidation of specific amino acids "marks" proteins for degradation by specific proteases* • *Oxidation of specific amino acids (e.g., Try) leads to cross-linking*
Oxidative damage to DNA
• *DNA deletions, mutations, translocations* • *Base degradation, single-strand breakage* • *Cross-linking of DNA to proteins*

DEFENSE RESPONSE IN PLANTS

Each plant, either at the species or subspecies level, has a range of pathogens to which it is susceptible, and another to which it is resistant. Non-host plants of a given pathogen group develop a type of blocking necrosis against these pathogens when their tissues are invaded, which is called the hypersensitivity reaction (HR). The resultant HR contrasts with the compatible reaction of a pathogen on its host plant, which consists of infection and invasion of plant tissues or organs, and often plant death (Montesinos et al., 2002).

The defense mechanisms utilized by plants can take many different forms, ranging from passive mechanical or preformed chemical barriers, which provide nonspecific protection against a wide range of organisms, to more active host-specific responses that provide host- or varietal-specific resistance (Jackson and Taylorbi, 1996)

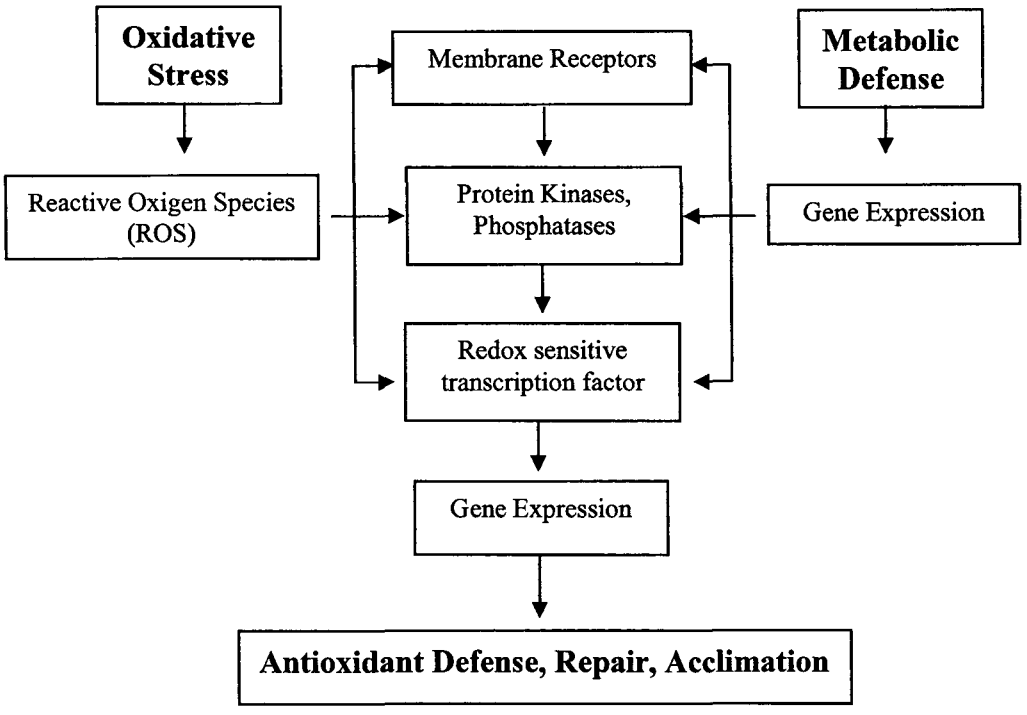

Figure 2: Redox Regulation of cellular systems (Adopted from https:// bioinformatics.cs.vt.edu/~expresso/awards/proposals/ITR_2002)

Plant defense mechanisms are characterized by a combination of two responses constitutive and inducible. Constitutive responses consist of general barriers or preexisting biochemical defenses. Inducible responses can be localized or systemic and are more sophisticated because they involve the recognition of the pathogen by the host plant, signal transduction, and the expression of several genes. In a localized response, plant tissues react against pathogens by a type of programmed cell death consisting essentially of electrolyte leakage from the cytoplasm and an oxidative burst. In systemic defense, a signal spreads from the site of interaction as a response to chemicals, microorganisms, insects, mechanical damage or stress. The signal is mediated by several molecules which function as messengers in plants, for example, salicylic and jasmonic acid, or even volatiles such as nitric oxide and ethylene (Baker et al., 1997). These messengers interact with specific binding proteins that are implicated in the transcriptional activation of PR genes in response to pathogen aggression. The products of several of these genes are enzymes, *e.g.* peroxidases, lipo-oxygenases, superoxide dismutases, and phenylalanine-ammonia-lyase (PAL), involved in the secondary metabolism of plants and specifically in the synthesis of phenolic compounds, or are phytoalexins, glucanases and chitinases, with antimicrobial activity. The inducible defense

response in plants produces a limited variety of biochemical compounds with low specificity for the target pathogen. However, the specificity of the response in plants lies in the fact that they detect intracellularly specific types of proteins secreted by the pathogen (elicitors), using an interaction recognition system which triggers the defense response. There is now experimental evidence that avr genes are present in plant viruses, plant pathogenic bacteria, and plant pathogenic fungi.

The genetic basis of this strong specificity in plant-bacteria interactions is explained by the gene-for-gene elicitor-receptor model. This model takes into account *avr* genes in the pathogen which are homologous to the *R* genes in the host plant. A complementary combination of *avr* and *R* genes results in an incompatible plant pathogen interaction (rejection) and triggers defense mechanisms in the host cells. By contrast, a non-complementary combination of *avr* and *R* genes (compatible) results in infection. According to this elicitor receptor model in plant-bacteria interactions the virulence genes and factors in the pathogen are confronted with the defense genes and factors in the plant host cell. Incompatibility genes in the bacterial pathogen encode avirulence factors (avrX) which are secreted by a type III secretion system controlled by the *hrp* genes (hrpX) . The avirulence factors are detected by the resistance genes (*resX*) or factors in the plant cell which trigger the hypersensitive reaction and defense (Flor et al., 1955, Baker et al., 1997).

HR that is elaborated in response to invasion by all classes of pathogens is the most common feature associated with active host resistance. In most cases, activation of the HR leads to the death of cells at the infection site, which results in the restriction of the pathogen to small areas immediately surrounding the initially infected cells. At the whole plant level, the HR is manifested as small necrotic lesions. The number of cells affected by the HR is only a small fraction of the total in the plant, so this response obviously contributes to the survival of plants undergoing pathogen attack.

Although the biochemical basis for hypersensitivity is unknown, during the HR, controls regulating ion flux are compromised, damaging concentrations of ROS accumulate, and marked changes occur in normal metabolic processes, including the synthesis of salicylic acid (SA). The ROS may also function either to cross-link cell

wall components or as toxic substances that attack the pathogen, or both. Both ROS and SA appears to have roles in signal transduction cascades that coordinate various defense responses in the plant. HR induces the synthesis of pathogenesis related (PR) proteins and the accumulation of hydroxyproline-rich glycoproteins, which may function in the strengthening of cell walls, and of biosynthetic precursors of callose and suberin, which may be involved in sealing off the infection site. Systemic Acquired resistance (SAR) occurs during necrotic resistance responses against viruses, bacteria, and fungi, and it culminates in the activation of broad spectrum resistance against a large number of biotrophic pathogens. During necrosis, or upon application of SA, normally susceptible tissues develop highly resistant responses during which expression of at least nine families of so-called SAR proteins are elicited.

Most plants have a battery of preformed secondary metabolites that are directly antimicrobial. These secondary compounds were among the first components to be implicated in resistance to plant pathogens. Some of the compounds are biologically active, whereas others are converted to active forms by host enzymes released during infection or injury. There are three classes of preformed compounds: saponins, cyanogenic glycosides, and glucosinolates as inhibitors of plant disease-causing organisms (Jackson et al., 1996).

ANTIOXIDANTS IN PLANT DEFENSE

Certain Reactive oxygen intermediates (ROI) such as hydrogen peroxide and the superoxide anion radical accumulate in many plants during attack by microbial pathogens. These are formed during certain redox reactions and incomplete reduction of oxygen or oxidation of water by mitochondrial or chloroplast electron transfer chains. The mechanism of producing superoxide from molecular oxygen involves a plasma membrane associated NADPH oxidase. The superoxide anions produced outside the plant cells are rapidly converted to (H_2O_2), a molecule that crosses the plasma membrane and enters the plant cells. This H_2O_2 is eventually removed from cells by conversion to water by certain antioxidant enzymes. Family of ROIs consists of the superoxide radical anion $O_2^{\bullet}$, the hydroperoxyl radical $HO_2^{\bullet}$, hydrogen peroxide H_2O_2, the hydroxyl radical $HO^{\bullet}$, Superoxide, its protonated form

$HO_2^{\bullet}$ and $HO^{\bullet}$ are relatively short-lived whereas H_2O_2 is comparatively stable and can cross membranes. In particular, the hydroxyl radicals among the ROIs are toxic due to their extraordinary ability to react spontaneously with organic molecules such as phenols, fatty acids, proteins and nucleic acids (Huckelhoven and Kogel, 2003)

The term ''oxidative defense'' has been used to characterize ROI-dependent plant defiance reactions. ROI accumulation is closely associated with the induction of plant defense reactions against viral, bacterial, and fungal pathogens, HR, defense gene expression, and cell wall strengthening via cross-linking reactions of phenylpropanoids and proteins (Bradley et al., 1992, Levine et al., 1994; Jabs et al., 1997; Lamb and Dixon, 1997; Grant and Loake, 2000). The ascorbate-glutathione cycle is the major antioxidant pathway in plastids, where ROI are generated during normal biochemical processes. The increased production of toxic oxygen species is a feature commonly observed under certain stress conditions (Foyer et al., 1994), when the equilibrium of formation and detoxification of active oxygen species can no longer be maintained. Both enzymic and nonenzymic mechanisms have evolved to overcome oxygen toxicity. Plants induce 'antioxidant defense system' in response to ROS generation to diminish cytotoxic functions such as lipid peroxidation, protein modification and DNA damage (Mittler, 2002). Detoxification of ROS may reduce cell death and allow plants to eliminate microbial pathogens by producing antimicrobial compounds and cell wall stiffening. The primary components of this system include carotenoids, ascorbate, glutathione, tocopherols and enzymes such as superoxide dismutase, catalase, glutathione peroxidase, peroxidases and the enzymes involved in the ascorbate–glutathione cycle, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase (Noctor and Foyer, 1998).

To minimize the damaging effects of ROS, aerobic organisms evolved both nonenzymatic and enzymatic antioxidant defenses (**Table 3 and 4**). Non-enzymatic defenses include compounds of intrinsic antioxidant properties, such as vitamins C and E, glutathione, and β-carotene. Purely enzymatic defenses, such as superoxide dismutases (SOD), catalases (CAT) and peroxidases, protect by directly scavenging

superoxide radicals and hydrogen peroxide, converting them to less reactive species. SODs catalyze the dismutation of O2 •− to H_2O_2, and CAT and peroxidases reduce H_2O_2 to $2H_2O$. The similarity between the SOD and CAT reactions is that each is an oxidation-reduction in which the substrate, $O_2^{\bullet-}$ for SOD and H_2O_2 for CAT, is both reductant and oxidant, whereas different reductants are required for the peroxidases, depending upon their specificities. Under some conditions CAT can act as an efficient peroxidase. SODs deal with the first product of the univalent reduction of O_2, converting it to H_2O_2, which must then be destroyed by CAT and/or peroxidases. Thus, the SOD and CAT serve, in tandem, as front-line antioxidant defenses:

$$O_2\bullet- + O_2\bullet- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \ (K2 = 2.4 \times 109 \text{ M-1 s-1})$$

$$H_2O_2 + H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2 \ (K1 = 1.7 \times 107 \text{ M-1 s-1})$$

$$H_2O_2 + R(OH)_2 \xrightarrow{Px} 2\,H_2O + R(O)_2 \quad (K4 = 0.2\text{-}1 \times 103 \text{ M-1 s-1})$$

Table 3. Non enzymatic Antioxidant Molecule

Antioxidant molecule	*Sub-cellular location*
Ascorbate (vitamin C)	*Plastid; apoplast; cytosol; vacuole*
Proline	*Cytoplasm, vacuole*
β-Carotene	*Plastid*
Glutathione, reduced (GSH)	*Plastid; mitochondrion; cytosol*
Polyamines (e.g., putrescine, spermine)	*Nucleus; plastid; mitochondrion; cytosol*
α-Tocopherol (vitamin E)	*Cell and plastid membranes*
Zeaxanthin	*Chloroplast*

Table: 4. Antioxidant enzymes.

Enzyme	*EC number*	*Subcellular location*
Ascorbate peroxidase	*1.11.1.11*	*Plastid stroma and membranes*
Peroxidases (non-specific)	*1.11.1.7*	*Cytosol; cell wall-bound*
Catalase	*1.11.1.6*	*Glyoxysome; peroxisome; cytosol;*
Superoxide dismutase (SOD)	*1.15.1.1*	*mitochondria*
		Cytosol (Cu/ZnSOD); plastid (Cu/ZnSOD; FeSOD); mitochondrion (MnSOD);
Dehydroascorbate reductase	*1.8.5.1*	*peroxisome*
Glutathione reductase	*1.6.4.2*	
Monodehydroascorbate reductase	*1.6.5.4*	*Cytosol; plastid*
Glutathione S-transferases	*2.5.1.18*	*Mitochondrion; cytosol; plastid*
		Plastid stroma
		Cytosol; microsomal

Glutathione-S-Transferase (GST)

Glutathione-S-transferases are ubiquitous enzymes catalysing the addition of reduced glutathione to electrophilic substrates, to form a polar δ-glutathionylated reaction product which tags them for vacuolar sequestration (Edwards et al., 2000). GSTs were first discovered in animals in the 1960s as a result of their importance in the metabolism and detoxification of drugs (Wilce and Parker, 1994). Their presence in plants was first recognized shortly afterwards in 1970, when a GST activity from maize was shown to be responsible for conjugating the chloro-δ-triazine atrazine with GSH, thereby protecting the crop from injury by this herbicide (Edwards et al., 2000).

GSTs are soluble proteins with typical molecular masses of around 50 kDa, and each is composed of two polypeptide subunits. The native enzyme appeared to be a monomer with a molecular mass of approximately 30 kD and an apparent isoelectric point at pH 5.2. The enzyme had a pH optimum between 7.5 and 8.0 and apparent Km values of 4.4 and 1.9 mM for reduced glutathione and p-coumaric acid, respectively (Dean et al., 1995).

The enzyme displayed activity towards 1-chloro-2, 4-dinitrobenzene, a general GST substrate and high activities towards ethacrynic acid. It also exhibited glutathione peroxidase activity toward cumene hydroperoxide. It detoxify endobiotic and xenobiotic compounds by covalent linking of glutathione to hydrophobic substrate GSTs have direct cytoprotective activities and they might be essential for the preservation of plants during environmental stress and disease, as well as for the support of normal development (Marrs, 1996). GSTs are predominantly expressed in the cytosol, where their GSH-dependent catalytic functions include the conjugation and resulting detoxification of herbicides, the reduction of organic hydroperoxides formed during oxidative stress and the isomerization of maleylacetoacetate to fumarylacetoacetate, a key step in the catabolism of tyrosine. GSTs also have non-catalytic roles, binding flavonoid natural products in the cytosol prior to their deposition in the vacuole. Recent studies have also implicated GSTs as components of ultraviolet-inducible cell signaling pathways and as potential regulators of apoptosis.

GSH a tripeptide, L-glutamyl- L-cisteinyl-glycine, first reported in 1988 as a philothion, distributed in the intracellular space of plants, animals, and microorganisms has two general functions: to remove toxic metabolites from the cell and to maintain cellular sulfhydril groups in their reduced form. It contains glutamic acid, cysteine, and glycine. The carboxyl acid "side chain" is part of the backbone peptide structure. The normal carboxyl group is the so called side chain in this case. The function of glutathione is to protect cells from oxidizing agents which might otherwise damage them. The oxidizing agents react with the -SH group of cysteine of the glutathione instead of doing damage elsewhere. Many foreign chemicals get attached to glutathione, which is really acting as a detoxifying agent (Boyland and Chasseaud, 1969, Meister, 1989).

Glutathione metabolism involves many reactions where glutathione is synthesized, degraded, conjugated or oxidized. In one of these pathways, the GSTs, a family of multifunctional isozymes in vertebrates, plants, insects and aerobic microorganisms (Armstrong, 1993), catalyzes both GSH- dependent conjugation and reduction (Ketterer et al., 1993).

Catalase (CAT)

Catalase is an enzyme present in the cells of plants, animals and aerobic bacteria. It promotes the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen and is one of the major antioxidant enzymes (Scandalios et al. 1997). Catalase also uses hydrogen peroxide to oxidize toxins including phenols, formic acid, formaldehyde and alcohols. Catalase is located in a cell organelle called the peroxisome. It is a tetrameric heme containing enzyme. Plant catalases are generally localized in microbodies (Huang et al., 1983, Scandalios, 1994, Willekens et al., 1995). It is one of the first enzymes to be purified and crystallized and has gained a lot of attention in recent years because of its link to cancer, diabetes, and aging in humans and animals. In plants, catalase scavenges H_2O_2 generated during mitochondrial electron transport, β-oxidation of the fatty acids, and most importantly photorespiratory oxidation (Scandalios et al. 1997). Accumulating evidence indicates that catalase plays an important role in plant defense, aging, and senescence and it is indispensable for stress defense in plants (Willekens et al., 1997).

Super-Oxide Dismutase (SOD)

These are metalloproteins that catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen. It is ubiquitous in aerobic organisms where it plays a major role in defense against oxygen radical-mediated toxicity (Tsang et al., 1991). Superoxide radicals (O^{2-}) are toxic by-products of oxidative metabolism (Fridovich, 1986). Their toxicity has been attributed to their interaction with hydrogen peroxide to form highly reactive hydroxyl radicals ($OH^{\cdot}$) through the reaction:

$$H_2O_2 + O_2^{\cdot} \xrightarrow{Fe2+,Fe3+} OH^{-} + O_2 + OH^{\cdot}$$

These hydroxyl radicals are thought to be largely responsible for mediating oxygen toxicity *in-vivo*, they can react indiscriminately with DNA,

proteins, lipids, and almost any constituent of cells (Halliwell, 1987; Cadenas, 1989; Fridovich, 1989). The oxygen generated in the chloroplasts during photosynthesis can accept electrons passing through the photosystems, form the superoxide radicals (Asada and Takahashi, 1987). SOD converts these highly harmful superoxide radicals to hydrogen peroxide and oxygen.

SOD provide protection against oxygen toxicity, against compounds that cause exacerbation of oxygen toxicity, against ionizing radiation, and also against the damaging sequelae of prolonged inflammation (Hasan and Fridovich, 1981). The reaction catalysed by SOD in plants is as follows:

$$2H^{+} + O_2^{\bullet} \longrightarrow H_2O_2 + O_2$$

SOD activity is also induced by diverse stresses (Bowler et al., 1992), presumably because of the increase in the concentration of superoxide radical in cells under those conditions. Therefore, SOD is an important enzyme family in living cells for maintaining normal physiological conditions and coping with stress (Chen and Pan, 1996).

Glutathione reductase (GR)

GR (EC 1.6.4.2) is a ubiquitous flavoenzyme that converts oxidized glutathione (GSSG) to the reduced form (GSH), with concomitant oxidation of NADPH (Halliwell and Gutteridge, 2000). The tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is the majorfree intracellular thiol compound in plants, where it functions as a powerful reductant that maintains protein thiols in their reduced state and protects membranes against peroxidation by ROS. The thiol group of two glutathione molecules can be oxidized, yielding the dimeric oxidized glutathione form, which is unable to protect against oxidation (Halliwell & Gutteridge, 2000; Foyer and Noctor, 2001; Noctor et al., 2002). Reduced glutathione participates in signalling processes by itself, and also as nitrosoglutathione (GSNO) after its reaction with nitric oxide (Noctor et al., 2002; del Rio et al., 2003; Barroso et al., 2006). In plants grown under abiotic stress in the form of heavy metals and xenobiotics, GSH is an essential molecule. It is the

repetitive piece from which phytochelatins, Small peptides involved in zinc (Zn) and cadmium (Cd) detoxification, are synthesized (Cobbett and Goldsbrough, 2002). It is also used by glutathione-S-transferases to bind and detoxify xenobiotic compounds (Marrs, 1996; Edwards et al., 2000). GR is the GSH-regenerating enzyme of the ascorbate-glutathione cycle, which removes hydrogen peroxide (H_2O_2) with the concerted action of three other enzymes–ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DAR) plus GSH and ascorbate (Noctor and Foyer, 1998; Asada, 2000; Halliwell and Gutteridge, 2000). Glutathione reductases have been linked to many situations where oxidative stress occurs, such as salinity drought, UV radiation, high light intensity, chilling and contamination by ozone, heavy metals and herbicides, among others (Foyer et al., 1991; Mullineaux and Creissen, 1997).This has also been shown that GR was found upregulated and are specific for the state of cold hardiness of red spruce (Hausladen and Alscher, 1994) with increased antioxidant capacity and more resistanse to photooxidation. Recently Waller et al. (2005) have described the role of GR in antioxidative defense system in plants colonized with an endophytic fungus *P. indica*.

PATHOGENESIS RELATED PROTEINS (PR PROTEINS)

Plants when exposed to pathogens such as fungi and viruses produce low-molecular-weight antimicrobial compounds called phytoalexins, antimicrobial peptides, and small proteins (Bloch et al., 1988; Broekaert et al. 1995; Segura et al. 1993). The mechanism of action of these PR proteins is not known well except PR-2 and PR-3.

PR-2 proteins (β-glucanses). PR-2 proteins have β-1, 3- endoglucanase activity in vitro and have been grouped into three classes on the basis of amino acid sequence analysis (Beffa and Meins, 1996; Cote et al., 1991). Class I glucanases are basic proteins of 33 kDa and are found in the plant vacuole. Classes II and III include acidic, extracellular proteins of about 36 kDa. The major structural difference between class I proteins and the other two classes is that class I proteins are synthesized as pre-proteins that are processed prior to being enzymatically active.

PR-2 proteins have been found in a wide variety of plants, including tobacco, *A. thaliana*, peas, grains, and fruits (Cote et al., 1991; Kim and Hwang, 1997), the proteins are active in vitro at micromolar levels against a wide number of fungi, including human and plant pathogens (e.g., *Rhizoctonia solani, C. albicans*, and *Aspergillus fumigatus*). The antifungal activity of PR-2 proteins has been convincingly demonstrated by a number of in vitro enzyme and whole-cell assays (Stintzi et al., 1993) as well as *in planta* using transgenic plants over-expressing a PR-2 protein (Jach et al., 1995). The antifungal activity of plant β-1, 3-glucanases is thought to occur by PR-2 proteins hydrolyzing the structural β-1, 3-glucan present in the fungal cell wall, particularly at the hyphal apex of filamentous molds where glucan is most exposed, resulting in a cell wall that is weak. This weakened cell wall results in cell lysis and cell death.

PR-3 proteins (chitinases). A number of enzymatic assays have shown PR-3 proteins to have in vitro chitinase activity. Most PR-3 proteins have molecular masses of between 26 and 43 kDa (Nielsen et al., 1997; Watanabe et al., 1999). Chitinases (both plant PR-3 chitinases and chitinases from other sources) have been divided into five groups. class I chitinases contain an N-terminal cysteine-rich domain of 40 amino acids (also known as the wheat germ agglutinin domain), a chitin-binding hevein-like domain, a highly conserved central portion, and a hinge region; most class I proteins have molecular masses of 32 kDa. Class II proteins are similar in amino acid sequence to class I proteins, but they lack the N-terminal cysteine-rich domain and have molecular masses of 27 to 28 kDa. Class IV proteins resemble class I chitinases but are significantly smaller due to four major deletions. Class III proteins do not share amino acid sequence homology to any other class and have molecular masses of 28 to 30 kDa. Class V chitinases show sequence similarities to bacterial exochitinases and have molecular masses of 41 to 43 kDa. In addition to chitinases, a chitosanase (chitosan is deacetylated chitin) from *Streptomyces* strain N174 with antifungal activity has been isolated (Money and Harold, 1992), and its X-ray structure has been determined. Chitinases have been isolated from fungi, plants, cucumber, beans, peas, grains and many others and bacteria and have potent antifungal activity against a wide variety of human and plant pathogens.

PR-5 (TL) proteins. PR-5 proteins share significant amino acid homology to thaumatin (a sweet-tasting (to humans) protein from the South African ketemfe berry bush) and are known as TL proteins. TL proteins have been isolated from *A. thaliana*, corn (Huynh et al., 1992), soybeans, rice, wheat, tobacco, tomato, pumpkin, beans, barley, fla and many other plants. The majority of PR-5 proteins have molecular masses of 22 kDa and are stabilized by eight disulfide bonds. This highly stabilized structure allows PR-5 proteins to be very resistant to protease degradation (Roberts and Selitrennikoff, 1990). Although the precise mechanism of action of PR-5 proteins is not completely understood, there are a number of interesting observations that may eventually lead to a unified hypothesis for how these proteins function to kill fungi (Ibeas et al., 2000) First, several TL proteins cause cell permeability changes in fungal cells with a cell wall but have no or little effect on protoplasts (Roberts and Selitrennikoff, 1990). For example, zeamatin (a TL protein from corn) caused very rapid cell lysis of *N. crassa*, even at 4°C; lysis occurred primarily at subapical regions (Roberts and Selitrennikoff, 1990). Second, a number of PR-5 proteins bind (1,3) β-glucan and have detectable in vitro (1,3) β-glucanase activity (Trudel et al., 1998).

Proline: An osmolyte cum non-enzymatic antioxidant

The class of small molecules known as "compatible osmolytes" includes certain amino acids (notably proline), quaternary ammonium compounds (e.g. glycinebetaine, prolinebetaine, B-alaninebetaine, and choline-*o*-sulfate), and the tertiary sulfonium compound 3-dimethylsulfoniopropionate (DMSP). The quaternary ammonium compounds and DMSP are derived from amino acid precursors. These compounds share the property of being uncharged at neutral pH, and are of high solubility in water (Ballantyne and Chamberlin, 1994). Moreover, at high concentrations they have little or no perturbing effect on macromolecule-solvent interactions (Yancey et al, 1982; Low, 1985; Somero, 1986; Timasheff, 1993; Yancey, 1994). Unlike perturbing solutes (such as inorganic ions) which readily enter the hydration sphere of proteins, favoring unfolding, compatible osmolytes tend to be excluded from the hydration sphere of proteins and stabilize

folded protein structures (Low, 1985). These compounds are thought to play a pivotal role in plant cytoplasmic osmotic adjustment in response to osmotic stresses (Wyn Jones et al, 1977).

Proline accumulation is a common metabolic responses of higher plants to water deficits, and salinity stress (Stewart and Larher, 1980; Thompson, 1980; Stewart, 1981; Hanson and Hitz, 1982; Rhodes, 1987; Delauney and Verma, 1993; Samaras et al, 1995; Taylor, 1996; Rhodes et al, 1999) and is primarily localized in the cytosol (Leigh et al, 1981; Ketchum et al, 1991; Pahlich et al, 1983). Proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions and temperature extremes (Pollard and Wyn Jones, 1979; Paleg et al, 1981; Nash et al, 1982; Paleg et al, 1984; Brady et al, 1984; Gibson et al, 1984; Rudolph et al, 1986; Santarius, 1992; Santoro et al, 1992). Proline may also function as a protein-compatible hydrotrope (Srinivas and Balasubramanian, 1995), and as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989).

Exogenously supplied proline can also be osmoprotective (Tal and Katz, 1980; Handa et al, 1986; Lone et al, 1987) and cryoprotective (Withers and King, 1979; van Swaaji et al, 1985; Duncan and Widholm, 1987; Songstad et al, 1990; Santarius, 1992) to higher plant cells. Proline synthesis is implicated as a mechanism of alleviating cytoplasmic acidosis, and may maintain $NADP^+$/NADPH ratios at values compatible with metabolism (Hare and Cress, 1997). Rapid catabolism of proline upon relief of stress may provide reducing equivalents that support mitochondrial oxidative phosphorylation and the generation of ATP for recovery from stress and repair of stress-induced damage (Hare and Cress, 1997; Hare et al, 1998). Antioxidative nature proline was shown in a study by Chen and Dickman (2005) in which they demonstrate that Proline is able to protect the yeast cells from lethal level of ROS generated by paraquat and inhibit the stress induced apoptotic response. This work open the possibility that proline may reveal an elusive mechanism responsible for the ability of symbiotic fungi to protect plants against abiotic and biotic stress (Rodriguez and Redman, 2005).

ANTIOXIDANTS AND ARBUSCULAR MYCORRHIZAL SYMBIOSIS

During interaction, first, plant recognizes the invader through some signal molecules secreted by the invader known as elicitors. Elicitors can be secreted from the microbe (exogenous elicitors) or generated as a result of physical and/or chemical cleavage of the plant cell wall (endogenous elicitors). After perception of an elicitor, a number of biochemical changes contribute to the early response in host cells. These processes include changes in the ion permeability of the plasma membrane, the activation of plasma membrane-bound enzymes, the activation of kinases, phosphatases, phospholipases, and the production of signal molecules, including active oxygen species. The result of these processes is the transcriptional activation of defence-related genes. A hypersensitive response and some elements of signal transduction pathways are activated after pathogen recognition by the plant during the early stages of AM formation.

An oxidative burst could be detected at sites where hyphal tips of AM fungi attempted to penetrate a cortical root cell of the plant. Accumulation of SA during the early stages of infection also has been observed in the interaction. In the early stages of root colonization by AMF, the plant defense response is characterized by a weak and transient activation, but at later stages, for example, the arbuscule formation, this activation appears to become stronger, although only in those cells which contain fungal structures (Garcia-Garrido and Ocampo, 2002).

The levels of H_2O_2 in mycorrhizal associations are increased (Salzer et al., 1999). Transient increases of catalase and peroxidase activity were observed to coincide with appressoria formation and fungal penetration into the root. Catalase and peroxidase play a role in the catabolism of hydrogen peroxide and/or in cross-linking reactions between proteins and polysaccharides in the interface between the arbuscule and the plant cell plasma membrane (Blee and Anderson, 2000). Glutathione-S-transferase accumulates only in certain cells containing arbuscules. Alterations in the pattern of anti-oxidative enzymes, such as catalase and peroxidase in mycorrhizal roots may indicate that oxidative compounds are produced during the colonization process. The increase in catalase and peroxidase activity could be due to their function as antioxidants for any active oxygen molecules generated during

the initial stages of fungal penetration (Garcia-Garrido and Ocampo, 2002). Since H_2O_2 and other reactive oxygen species are involved in signal transduction cascades in plant–pathogen interactions, it is possible that degradation of H_2O_2 by catalase in AM could be a possible mechanism for avoiding the activation of defense response genes (Garcia-Garrido and Ocampo, 2002)

PIRIFORMOSPORA INDICA –A SYMBIOTIC FUNGUS

Piriforomospora indica is a recently discovered fungus which is an axenically cultivable endophyte that colonizes roots (Varma et al., 1999). It can be easily grown on various complex and minimal substrates, where it asexually forms chlamydospores containing 8 to 25 nuclei. It has been related to the Sebacinales (form genus *Rhizoctonia*, Hymenomycetes, Basidiomycota) on the basis of an alignment of nuclear DNA sequences from the D1-D2 region of the large ribosomal subunit. It has the added trait of being able to be grown in axenic cultures. Colonization was observed under a light microscope. When hyphae of *P. indica* contacted roots, they developed appressoria and roots were colonized intercellularly. The fungus also colonized cortex cells intracellularly, where it formed coils and branches or round bodies, but a typical arbuscule, as developed by the *Glomales*, not observed (Varma et al., 1999). Fungal colonization commenced at 4 to 7 days after treatment. Appressorium-like structures were observed at the point of contact of hyphae with root epidermis, whereas a broken epidermal layer was observed near the point of contact. Recently it has been shown that this fungus also colonizes the root through root hair (Waller et al., 2005). The round bodies, however, might function as storage organs. *P. indica* requires host cell death for its colonization (Deshmukh et al., 2006) and never invaded the stellar tissue nor traversed upwards into the shoot (Varma et al., 1999).

Hosts include the cereal crops rice, wheat, and barley as well as many dicotyledonous including the crucifers that is not in case of mycorrhizal fungus. The fungus has the potential to render protection to the micropropagated plantlets and help them escape the transient transplant shock (Sahay et al., 1999). Interaction of this endophytic fungus with plant roots, results in better nutritional status. In interaction with *Arabidopsis* and tobacco, fungus stimulated nitrate reduction and

acquisition (Sherameti et al., 2005), in contrast to the activity of arbuscular mycorrhiza fungi. This has also been proved that this fungus encodes a high affinity Phosphate transporter that which transport phosphorus from media to plant (Yadav et al., 2007).

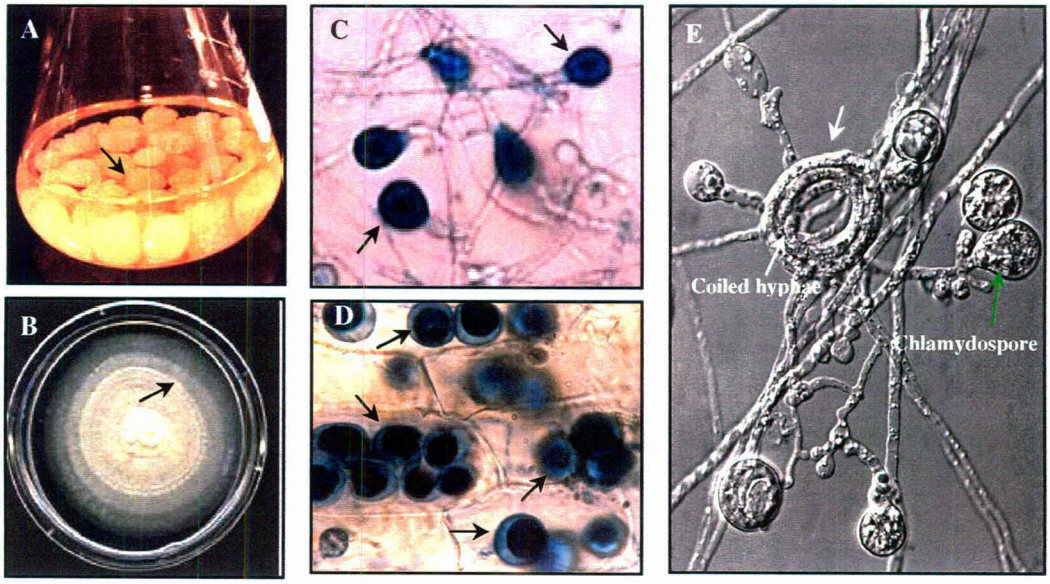

Figure 3: Growth and Morphological Characteristics of *P. indica*

(A) Axenic growth of *P. indica* in aspergillus medium at 28±2°C. The fungus shows coral like growth (black arrow)

(B) *P. indica* growth on solidified aspergillus medium. Black arrow showing concentric ring indication the rhythmic growth of the fungus.

(C) The characteristic pear shaped chlamydospore of *P. indica* stained with trypan blue.

(D) Chlamydospore of *P. indica* in root cortical cells of colonized maize roots stained by trypan blue observed under Leica microscope 10X magnification.

(E) Morphology of *P. indica* (Green arrow showing chlamydospore and white arrow showing coiled hyphae)

Table: 5. Complete Phylogenetic Classification of *P. indica*.

Kingdom	Fungi
Division	Basidiomycota
Class	Hymenomycetes
Sub class	Heterobasidiomycetes
Order	Sebacinales
Family	Sebacinaceae
Genus	*Piriformospora*

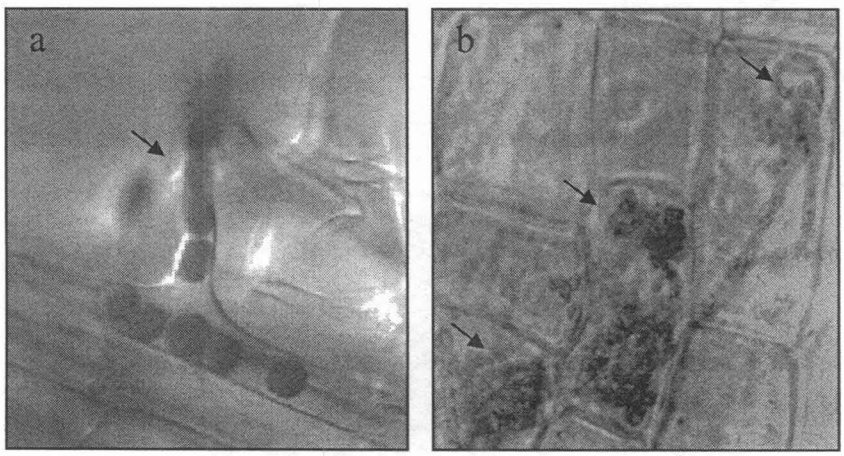

Figure 4: Colonization pattern of *P. indica* in plant root. Fungal hyphae enters root through root hair and forming round bodies (chlamydospores) in cell (a). In later stage it colonizes the cortical cell which contains coiled hyphae (black arrows) (b). (Adopted from Waller et al., 2005 and Varma et al., 1999.)

Interaction of fungus leads to several beneficial effects on plant, Firstly, the growth-promoting activity of the fungus resulted in enhanced barley grain yield. Recently it was found that a leucine-rich repeat protein is required for growth promotion and enhanced seed production and its mutation in plant (*ppi-2*) dose not respond to fungus (Shahollari et al., 2007). Second, *P. indica* amended tolerance to mild salt stress, and third, *P. indica* conferred resistance in barley against root (Waller et al., 2005) and leaf pathogens and having an antifungal activity (Rai et al., 2004). The interaction of barley with *P. indica* constitutes a model system for systemic disease resistance in cereals (Waller et al., 2005). It is evident from a

recent study that the interaction of this fungus with the plants (barley plant) in the presence of a pathogen (*Fusarium culmorum)* is helpful in defense mechanism. Therefore the present study is taken up to study the mechanism of defense response mediated by *P. indica* in the presence of a plant parasite like *Fusarium moniliforme.*

The present study includes the antioxidant property of maize plant (*Zea mays*) in the presence of *P. indica* along with PR protein and plant with parasite *F. moniliforme* using roots as well as shoots and role of *P. indica* in bioprotection mediated either through antioxidant enzyme and/or PR proteins.

3. Materials and Methods

Plant Material

Maize (*Zea mays*) : Bio 9681 variety (Provided from IARI, New Delhi)

Fungal Material

Piriformospora indica : A newly discovered root symbiotic fungus

Fusarium moniliforme : A parasitic fungus

Chemicals and Enzymes

The commercially available chemicals and enzymes used are mentioned in **Table 6.**

Table 6: List of chemicals and enzymes those were used.

Type	*Material*	*Sources*
Dyes	*Coomassie Brilliant Blue, Coomassie blue G 250*	*Sigma*
Locally available chemicals	*Isopropanol, iso-amyl alcohol, $CaCl_2$, NaCl, NaOH, Glucose, Methanol, $MgCl_2$, KOH, Potassium acetate, Chloroform, Glycerol, Acetic acid, NaH_2PO_4, Na_2HPO_4, $MgSO_4$, HCl, H_2SO_4, Glycine, KCl, Sucrose, Sodium hypochlorite, Mercuric chloride, tri-Sodium citrate, Formaldehyde, Triton-X-100, Polyvinyl Polypyrollidine. NADPH, Pyrogallol, β-Glucan, Aniline Blue, CDNB,*	*Qualigens, HiMedia, SRL and Merck.*
Chemicals from abroad	*Acrylamide, Bis-Acrylamide, TEMED, Spermine, Spermidine,*	*Sigma*

General Sterilization Procedures Used

All the glasswares, tissue culture tools and culture media were sterilized by autoclaving at 121.6°C under 15 lb psi pressure for 15 min. The antibiotics and other heat labile components were filter sterilized with dispensable syringe driven PVDF filter unit of 0.22µm pore size (Millex^{TM}, Millipore, USA).

Maintenance of Fungal Culture

P. indica and *F. moniliforme* were maintained routinely on solidified Aspergillus medium (Hill and Kaefer, 2001). The inoculated petri plates were incubated for 7-10 days at 30±2 °C.

Seed Surface Sterilization and Germination

Maize seeds (*Zea mays* L.) were surface sterilized for 2 min. in 0.1 % mercuric chloride solution, 4% NaOCl solution for 2 min. and 70 % ethanol for 5 min. followed by six times washing with sterile water and germinated under sterile condition on 0.8% water agar plate at 30±2 °C for 4 days. Seedlings were transferred to pots containing sterile soil.

Plant Fungus Interaction

Surface sterilized pre-germinated maize seedlings were placed in pots filled with a mixture of sterile sand and soil (3:1; garden soil from Jawaharlal Nehru University campus and acid-washed riverbed sand). *P. indica* inoculation was performed as described by Varma and Schuepp (1994), whereas in control plants, autoclaved dH_2O was used. Initially the plants were inoculated with *P. indica* or *F. moniliforme* by direct mixing of culture in sterile soil and allow one set of plants to grow without any fungus. In order to study the bioprotection and protective potential, the colonized plants with *P. indica* were reinoculated with *F. moniliforme*; plants infected with *F. moniliforme* were reinoculated with *P. indica*. While the last set of pots (without any fungus) were inoculated with *P. indica* and *F. moniliforme* simultaneously. The controlled experiment was run for 35 days. Three replicates were used for each experiment. Plants roots were harvested at different time intervals after inoculation, and a random sample of the root system was assessed for colonization.

The delayed and alternate inoculation of both fungus have chosen to see the effect of colonization of *P. indica* in bioprotection, whether it is effective in recovery of biomass, phenotypic appearances and decrease in colonization of *F. moniliforme* to infected plants with *F. moniliforme*. In other controlled experiment, the plants

inoculated with *P. indica* were used to study the growth promoting effect of fungus by measuring the fresh and dry weight.

Growth Condition and Sampling

Maize plants were grown in a controlled environment in green house at 30±2°C temperature with 16 h light/8 h dark and relative humidity 60-70%, with light intensity of 1000 Lux. Plants were watered twice and fertilized with Hoagland's solution (Arnon and Hoagland, 1940) once in week. Plant sample were harvested in different time periods after re-inoculation of fungus, carefully washed under running tap water, rinsed in deionised autoclaved water and weighed. Samples were freezed (in liquid Nitrogen) for enzyme assay and to study colonization in plant root tissue.

Histochemical analysis

To check colonization, root segments (1.0 cm approximately) were heated in 10% KOH for 15 min. followed by treating with 1N HCl and were stained with 0.05% trypan blue overnight or 60°C for 1 h and mounted on lactophenol (Dickson et al., 1998; Phillips and Hayman, 1970). Observation was done under light microscope (Leica Microscope, Type 020-518.500, Germany). Per cent colonization was calculated for the inoculated plants using the following formula (McGonigle et al., 1990).

$$\textbf{Percent colonization} = \frac{\textbf{No. of colonized root segments}}{\textbf{Total number of segments}} \textbf{ X 100}$$

Antibiosis assay of *P. indica* and *F. moniliforme*

To demonstrate the antibiotic activity *P. indica* and *F. moniliforme* to each other, both the fungus are inoculated on the same plate leaving some distance between two inoculums to grow without interrupting each other. The growth pattern

of margins of hyphal ferry of individual fungus approaching to each other that is due to inhibition is the sign of antibiotic activity of particular fungus.

Protein Extraction and Quantification

Frozen root and shoot tissue were homogenized at 4°C in an ice chilled mortar with liquid Nitrogen in QB buffer contained no DTT (1,4-Dithiothreitol) {for Superoxide-dis-mutase (SOD) assay, Catalase (CAT) assay and Glutathione-S-transferase (GST) assay} with 50 mg polyvinyl pyrrolidone (PVP) per gram tissue {for Glutathione reductase (GR) assay}. Extraction of protein for Native-PAGE was done in Mcilavaine buffer (citrate-phosphate buffer, pH 6.8). Crude homogenates were centrifuged at 15000X *g* for 15 min at 4°C and the supernatant fractions were kept frozen at -20°C. Protein contents were determined by the method of Bradford (Bradford, 1976) using BSA as standard.

SOD assay:

SOD activity was monitored as described by Roth and Gilbert, (1984). One milliliter of reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 100 µM EDTA, with an appropriate aliquot of enzyme extract and 10 mM of Pyrogallol. The enzyme activity was calculated by scanning the reaction mixture for 120 seconds (60 seconds interval) at 420 nm in presence of blank containing no protein sample. Due to auo-oxidation of Pyrogallol, blank containing no containing no protein sample is also scanned at same parameter. The enzyme activity (U/mg Protein) can be calculated by formulae bellow-

Specific Activity = V. $\Delta OD_{420.}$ Dilution / $(0.5\Delta OD_{blank})$ .v. protein concentration

Where as v = Sample volume V = Reaction Mixture Volume

NOTE: Due to auto-oxidation of Pyrogallol ΔOD_{420} is represented as

$$\Delta OD_{420} = \Delta OD_{420\ sample} - \Delta OD_{420\ blank}$$

CAT assay:

Catalase activity was assayed by measuring the initial rate of hydrogen peroxide disappearance using the method of Beers and Sizer (1952). One milliliter of catalase assay reaction mixture contained 0.05 mM sodium phosphate buffer, pH 7.0 with an appropriate aliquot of enzyme extract and 1 mM of hydrogen peroxide. The decrease in hydrogen peroxide concentration due to catalase activity was followed as a decline in optical density at 240 nm, and the activity was calculated using the extinction coefficient of 40 mM cm^{-1} for hydrogen peroxide. The enzyme activity (U/mg Protein) can be calculated by formulae bellow-

Specific Activity = V. $\Delta OD_{240.}$ / E. v. Protein concentration

Where as E= 40 $mM^{-1}cm^{-1}$ v = Sample volume V = Reaction Mixture Volume

GST assay:

GST activity was monitored according to the method given by Habig et al. (1974). One milliliter of reaction mixture contained 0.1M sodium phosphate buffer (pH 6.5) with an appropriate aliquot of enzyme extract, 2% CDNB (1-chloro-2,4-dinitrobenzene) and 1 mM glutathione reduced. The enzyme activity was calculated by adding the sample and scanning the reaction mixture for 180 seconds (60 seconds interval) at 340 nm in presence of blank containing no protein sample. The enzyme activity (U/mg Protein) can be calculated by formulae bellow-

Specific Activity = V. $\Delta OD_{340.}$ / E. v. protein concentration

Where as E= 9.6$mM^{-1}cm^{-1}$ v = Sample volume V = Reaction Mixture Volume

GR assay:

Glutathione reductase activity was determined by the oxidation of NADPH (reduced nicotinamide adenine dinucleotide phosphate) at 340 nm with the extinction coefficient of 6.2 mM cm^{-1} as described by Nordhoff et al. (1993). The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.8), 2 mM

EDTA, 0.2 mM NADPH and 0.5 mM glutathione (oxidized form, GSSG) with an appropriate volume of enzyme extract in a 1 ml volume. The reaction was initiated by the addition of NADPH at 25°C. The enzyme activity (U/mg Protein) can be calculated by formulae bellow-

$$\textbf{Specific Activity} = \textbf{V. } \Delta OD_{340.} \textbf{/ E. v. protein concentration}$$

Where as E= 6.22 $mM^{-1}cm^{-1}$ v = Sample volume V = Reaction Mixture Volume

Estimation of proline content

Approximately 0.5 gm of plant material was homogenized in 10 ml of 3 % aqueous sulphosalicylic acid (solvent) and the homogenate centrifuged at 10000 rpm for 8 min. 2 ml of collected supernatant was incubated with 2 ml acid ninhydrin (2.5 gm ninhydrin in 60 ml glacial acetic acid and 40 ml of 6 M orthophosphoric acid) and 2 ml glacial acetic acid in capped tube for 1 hour at 100°C. Reaction was terminated by keeping tube in ice, the reaction product (chromophore) was extracted with 2 ml ice cold Toluene, mixed vigorously with stirrer for 15-20 sec. The toluene containing chromophore was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm. using toluene as a blank. The proline concentration was determine from a standard curve and calculated on fresh weight basis as follows:

$$\textbf{Concentration of proline (µg)/ml of solvent} = \textbf{K. OD} + \textbf{B}$$

$$\textbf{Proline content (µg/gm tissue)} = \frac{\textbf{\{Proline conc. (µg/ml) x Volume of sulphosalicylic acid\}}}{\textbf{Weight of sample in gm.}}$$

Where the value of K and B are taken from standard curve which was drawn between the concentration of proline (10, 20, 30.....80, 90, 100 µg) and relative absorbanse.

Gel electrophoresis and In-gel enzymatic assays for Pathogenesis Related Protein-2 (PR-2, β-1,3-Glucanase)

All Protein extracts were analyzed by 10% or 15% (w/v) polyacrylamide gel electrophoresis (PAGE) under native conditions, at pH 8.9 (Davis, 1964). For all treatments 15 µg and 20 µg of proteins were loaded for analyses. A soluble fraction of purified laminarin from *Laminaria digitata*, was used as substrate for β-1,3-glucanase activity (Grenier and Asselin, 1993). laminarin were incorporated at a final concentration of 0.6 mg/ml directly in the separation gels for the Davis system. After electrophoresis, gels were incubated in 50 mM sodium acetate buffer, pH 5.0, for 3 h at 37°C. β-1, 3-Glucanase activities on gels were revealed by staining the gels for 15 min with 0.025% (w/v) Aniline blue in 150 mM K_2HPO_4, pH 8.6, and visualized under long wave UV transilluminator (Grenier and Asselin, 1993).

BIOINFORMATICS AND CALCULATIONS

Prediction of theoretical isoelectric point (*pI*) and molecular weight (*Mw*) of β-1,3-Glucanase of *Zea mays*

The theoretical Isoelectric Point (*pI*) and molecular weight was calculated using "Compute *pI*/Mw" on ExPASy Proteomics tools (http://scansite.mit.edu/calc_mw_pi.html).

Statistical Methods

All graphs and statistical calculations were performed on "Microsoft excel" from Microsoft corporation and unpaired student *t*- test and ANOVA was performed using program "SigmaStat 2.0" from Jandel Corporation.

4. Results

Colonization of *P. indica* and Plant Growth

The fungal inoculation treatments showed different levels of effectiveness in improving the growth of the maize plant. Initially it was observed that the fungal spore germinates and enter root through root hair or other root surface within 3 to 5 days. After 5 days period it was observed that fungal mycelium grow intercellular and intracellular in the root cortex and forms a network of hypha. Colonization of fungus depends on the size of inoculum and time. Using one gram of *P. indica* fresh culture per pot, we have found 15%-35% colonization for 10 days, and at the end of 20 day 70% or more colonization was observed (**Table 7**). Colonization of fungus characterized with intracellular densely packed pear shaped chlamydospores.

Note: The distribution of chlamydospore within the root was taken as an index for colonization.

We have observed dramatic morphological alteration in root initially and later in shoot also. Colonization results in an increase in the length of primary, seminal, crown and lateral roots of maize root system and increase in number of seminal and crown roots. In later stages fine secondary and tertiary roots were also observed which was found as interwoven network (**Fig. 7**). The shoot length and leaves broadness were significantly increases in response to *P. indica* colonization (**Fig. 7**). Effect of colonization *P. indica* stimulate the total fresh and dry weight of plant, which shows 157 % and 148 % increase respectively as compare to control plant (**Fig. 6 a and b**).

Table 7. Percent Colonization of *P. indica* in maize root. Plants were inoculated with *P. indica* culture (1 gm/pot) and roots were harvested at 5, 10, 20 days after inoculation. Roots were chopped into ~2 mm pieces and observed for fungal structure (chlamydospores) under light microscope.

Time After Inoculation (Days)	% Colonization
5	9 ± 6.35
10	26 ± 9.10
20	70 ±10.54

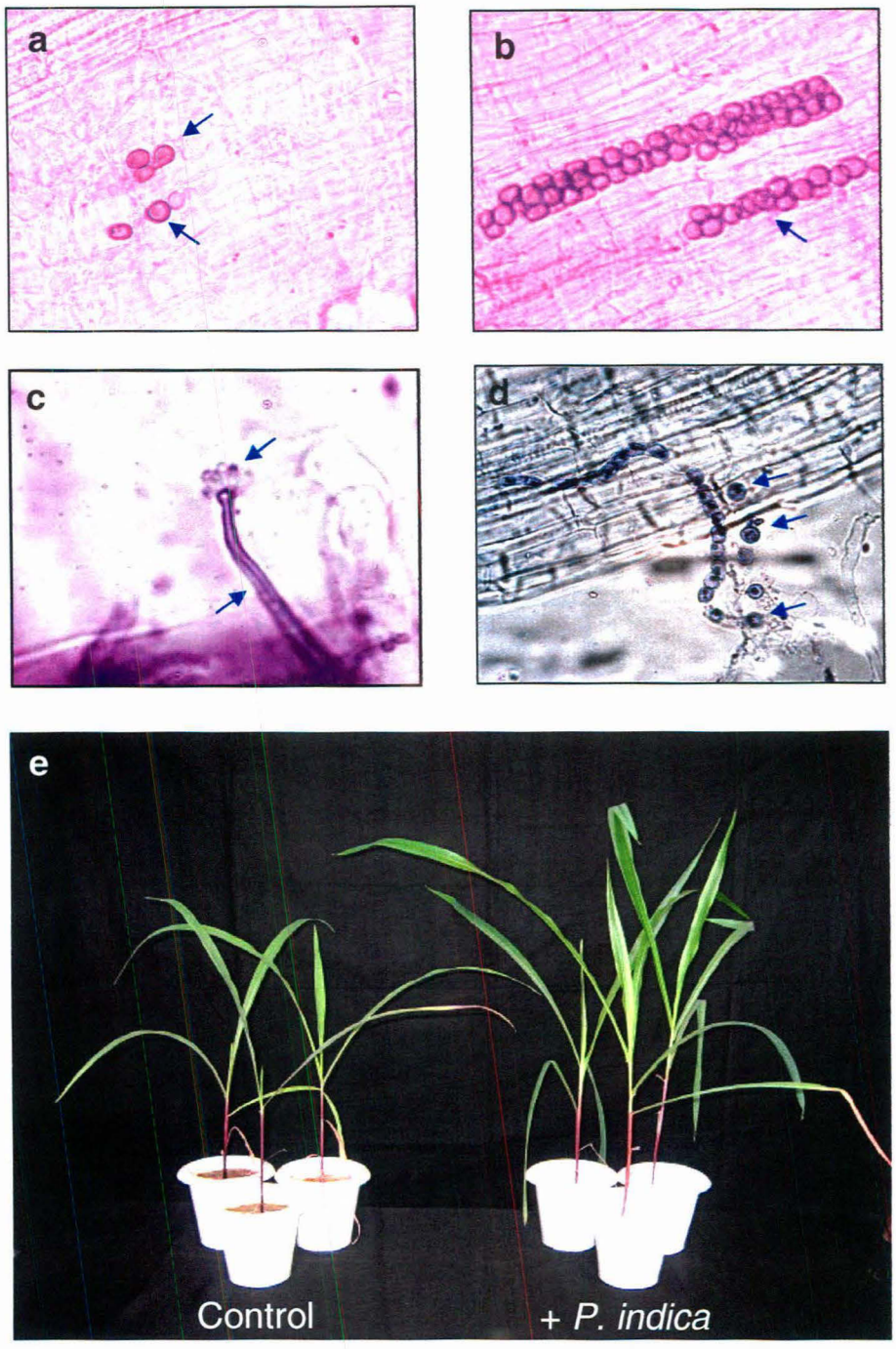

Figure 5. Colonization of *P. indica* in maize root. Fungus first establishes it self in root tissue a; arrow indicating a few chlamydospore in plant root after 5 days of inoculation and the colonization of fungus increases by time b; arrow indicating the densely packed chlamydospore in cortical cells of root tissue after 15 days of inoculation. Fungus also showing some basidium likes structure observed during colonization process c; arrow indicating basidiophore-like structure bearing 8 basidiospore-like structures). d; Fungus show a unique way of colonization in which it forms chlamydospores in chain and released from end of chain, released spores are get attached on root surface. e; Effect of colonization of *P. indica* on maize plants, after inoculation, maize plants were grown for 35 days, along with control plants (non inoculated with *P. indica*).

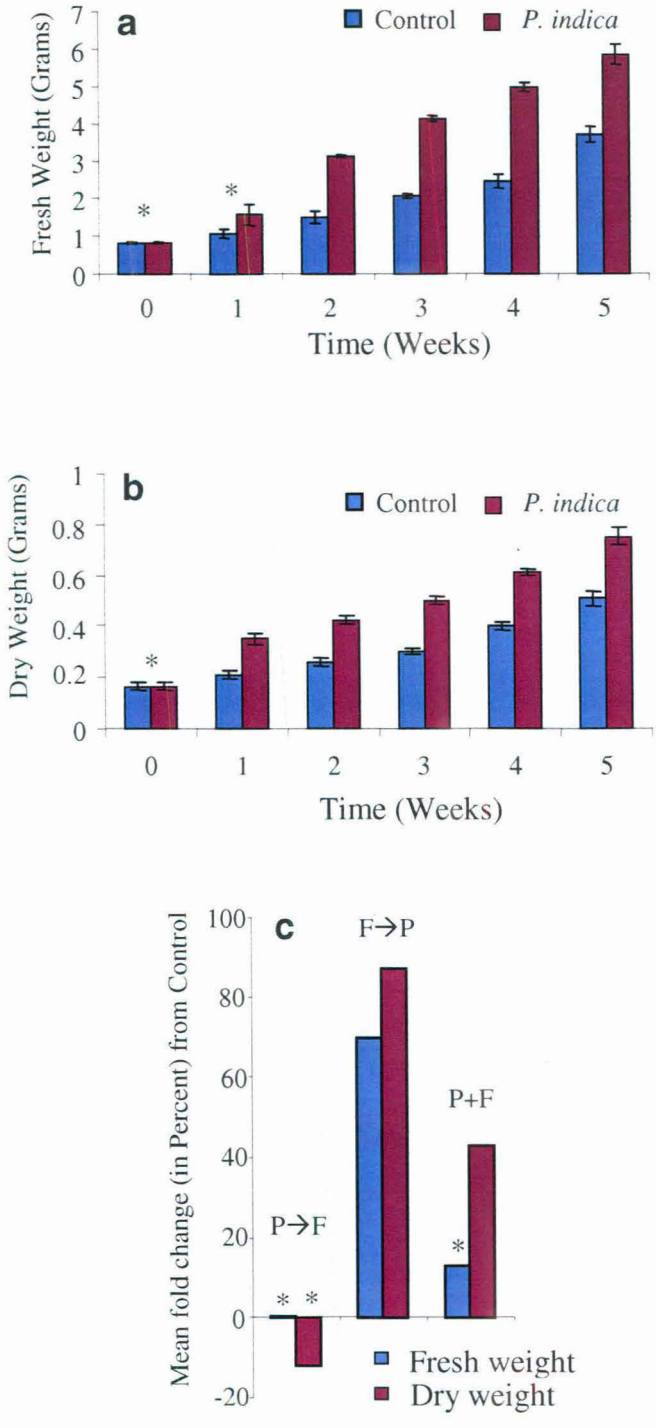

Figure 6. Impact of simultaneous and alternate inoculation of *P. indica* and *F. moniliforme* on biomass yield. Growth promoting effect of *P. indica* on Fresh weight (a) and Dry weight (b) of maize plant. Weight of *P. indica* colonized and control plants were determined every week. Mean fold change in biomass yield (c) relative to *P. indica*, *F. moniliforme* and non-inoiculated control six week old plants respectively. P→F, inoculation of *F. moniliforme* in 10 days old plant precolonized with *P. indica*; F→P, inoculation of *P. indica* in 10 days old plant precolonized with *F. moniliforme* and inoculation of both fungus (P+F) in 10 days old plant alone. Bars labeled with the (*) represents non-significantly different means, according to unpaired student *t*-tests ($P < 0.05$), after ANOVA.

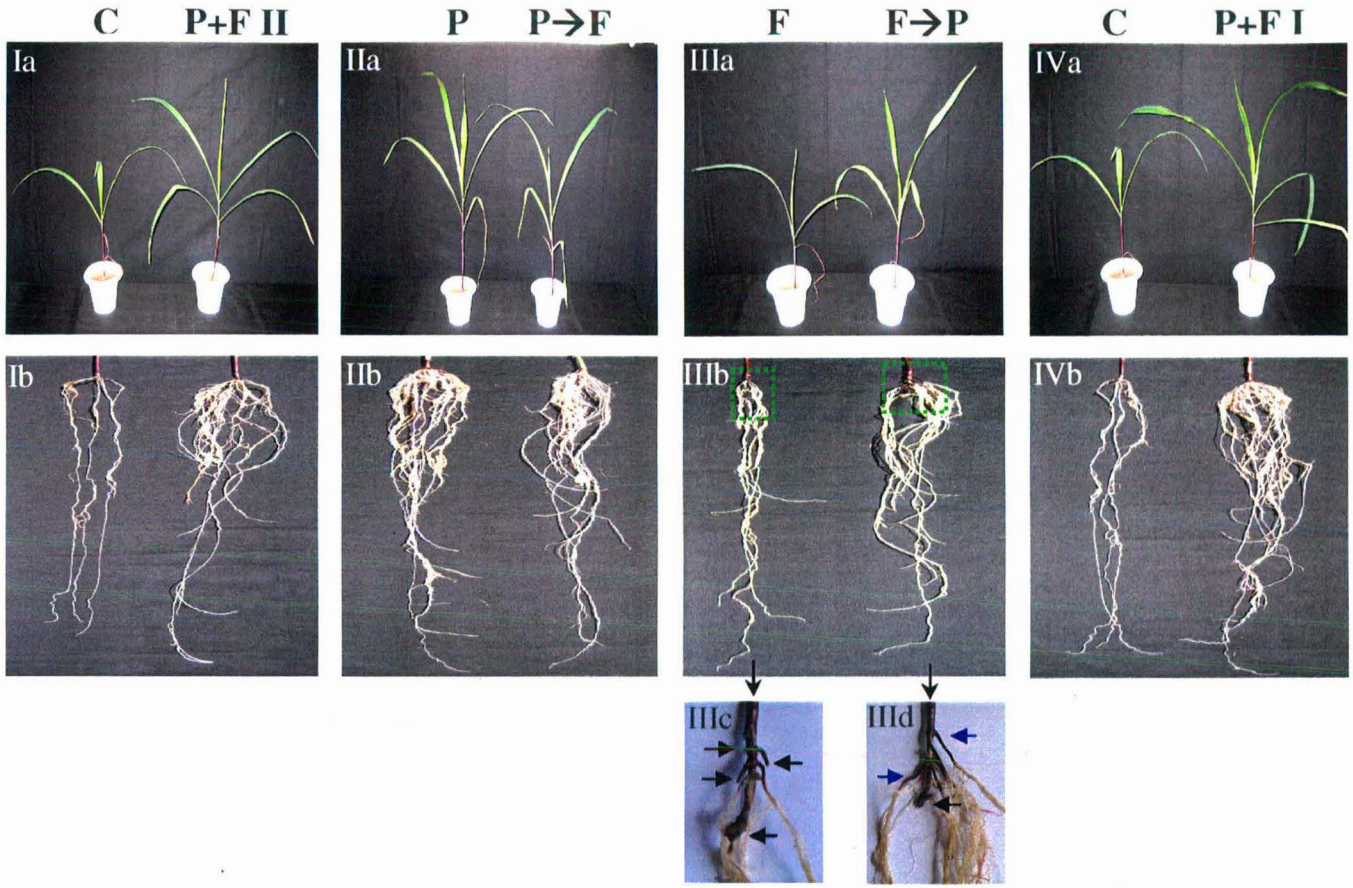

Figure 7. Plant shoot and root phenotype demonstrating the protective potential of *P. indica* against *F. moniliforme*. Plants were allowed to colonize either with *P. indica* (Pi) or *F. moniliforme* (Fm) or both (P+F-I), whereas control plants were allowed to grow without any fungus (C). After 15 days, colonized plant with *P. indica* re-inoculated with *F. moniliforme* (P→F), Fusarium infected plants re-inoculated with *P. indica* (F→P) and both *P. indica* and *F. moniliforme* (P+F-II) inoculated in a set of control plant and the plants were allowed to grow for 35 more days. Shoot and Root morphology of 50 days control (C) and 15+35 days P+F-II (Ia,b), 50 days Pi and 15+35 days P→F (IIa,b), 50 days Fm and 15+35 days F→P (IIIa,b,c,d) and 50 day control (C) and 50 day P+F-I (IVa,b). Black arrows are indicating the remains of root branches and blue are indicating the intact branches.

The effect of *P. indica* on colonization of *F. moniliforme* in the plant root was dynamic and shows an induced variation in roots integrity, "Branching index" (number of root branches/cm.) and biomass yield. We found that the inoculation of *P. indica* in plants pre-colonized with *F. moniliforme* recovers the biomass that is 170% and 187% for fresh and dry weight respectively than *Fusarium* infected control and is comparable to control plant without any fungus (Fig. 6 c), similarly there is improvement in the root system integrity (Fig. 7) and Branching index (number of root branches/cm.). Same effect was also observed in plants simultaneously inoculated with *P. indica* and *F. moniliforme* with 113% and 143% increase in fresh and dry weight respectively (Fig. 6 c), however inoculation of *F. moniliforme* in plants pre-colonized with *P. indica* dose not affect the root integrity and branching index significantly while the biomass yield was affected with 12% decrease in dry weight (Fig. 6 c). *P. indica* show no growth inhibitory effect against *F. moniliforme* and vise versa (Fig. 8).

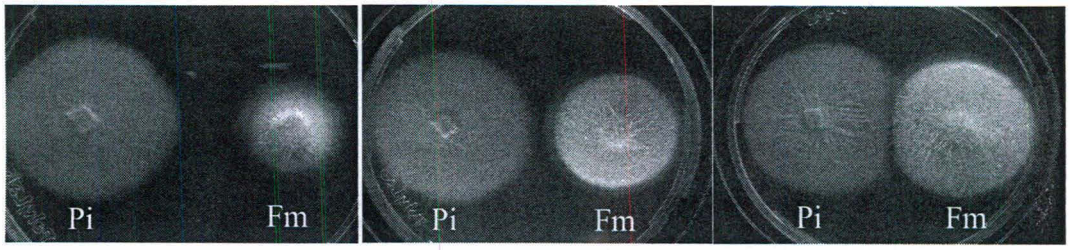

Figure 8. Antibiosis assay for antibiotic secretion and growth inhibition effect of *P. indica* (Pi) and *F. moniliforme* (Fm).

Change in Activity of Antioxidant enzymes in root and shoot of maize plant colonized with *P. indica* and effect inoculation of *F. moniliforme* on enzyme activity

Inoculation of *P. indica* in maize plants significantly alters the level of antioxidative enzymes and is depend on colonization. We have found the activities of CAT (Fig. 9 a), GR (Fig. 9 c), GST (Fig. 10 a) and SOD (Fig. 10 c) were constantly increases during the colonizing process till 25 days in the root and were 26-360, 2-12 , 4-25 and 1.5-10 fold higher than control respectively, interestingly it was found that this unusual increase in enzyme activity did not continued after the 25 days but was significantly higher than control. In case of shoot, the activities were 1-4 fold till the colonizing process for all enzymes while they were observed 20-225, 2-92 and 3-60 fold

higher after 25 days than control for CAT (Fig. 9 b), GST (Fig. 10 b) and SOD (Fig. 10 d) respectively, at the same time GR activity (Fig. 9 d) was not found altered significantly. Inoculation of *F. moniliforme* in 10 days old plant pre-inoculated with *P. indica* show 5-6 and 9-30 fold for CAT, 2-7 and 1.5-9 fold for GR, 2-16 and 3-27 fold for GST and, 1.5-10 and 9-28 fold increase for SOD Activity than control plant without any fungus in root and shoot respectively (Table 8).

Table 8. Activity of antioxidant enzymes in root and shoot of plants alternate (P→F and F→P) and simultaneous (P+F) inoculated with *P. indica* (P) and *F. moniliforme* (F). (Activities are expressed in folds compared to control plant)

State of affairs of inoculation	CAT		GR		GST		SOD	
	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Pi	26-360	20-225	2-12	1-4	4-25	2-92	1.5-10	3-60
Fm	24-60	1.5-27	2-21	1.3- 3.6	3-44	2-15	1.5-5	1.8-8
P→F	5-6	9-30	2-7	1.5-9	2-16	3-27	1.5-10	9-28
F→P	14-41	20-23	2-5	1-10	1-17	3-29	2-16	9-30
P+F	8-80	4-53	0.7-16	1-17	1.8-34	1.6-72	3-16	3-100

Antioxidant enzyme activity in root and shoot of maize plant infected with *F. moniliforme* in response to colonization of *P. indica*

Inoculation of *P. indica* in 10 days old plant pre-inoculated with *F. moniliforme* restore the all enzyme activity non-significantly in root and found increased in shoot than control plant inoculated with *F. moniliforme*, however CAT, GST and SOD activities were follow a continuous increase till 10 days while in case of GR, it was till 15 days in root. The enzyme activities were found increased 14-41 and 20-23 fold for CAT, 2-5 and 1-10 fold for GR, 1-17 and 3-29 fold for GST and, 2-16 and 9-30 fold for SOD Activity, than control plant without any fungus in root and shoot respectively (Table 8). Inoculation of *F. moniliforme* in maize plants significantly alters the activities and were enhanced 24-60 and 1.5-27 fold for CAT (Fig. 9 a and b), 2-21 and 1.3- 3.6 fold for GR (Fig. 9 c and d), 3-44 and 2-15 fold for GST (Fig. 10 a and b), 1.5-5 and 1.8-8 fold for SOD (Fig. 10 c and d) than control respectively in root and shoot.

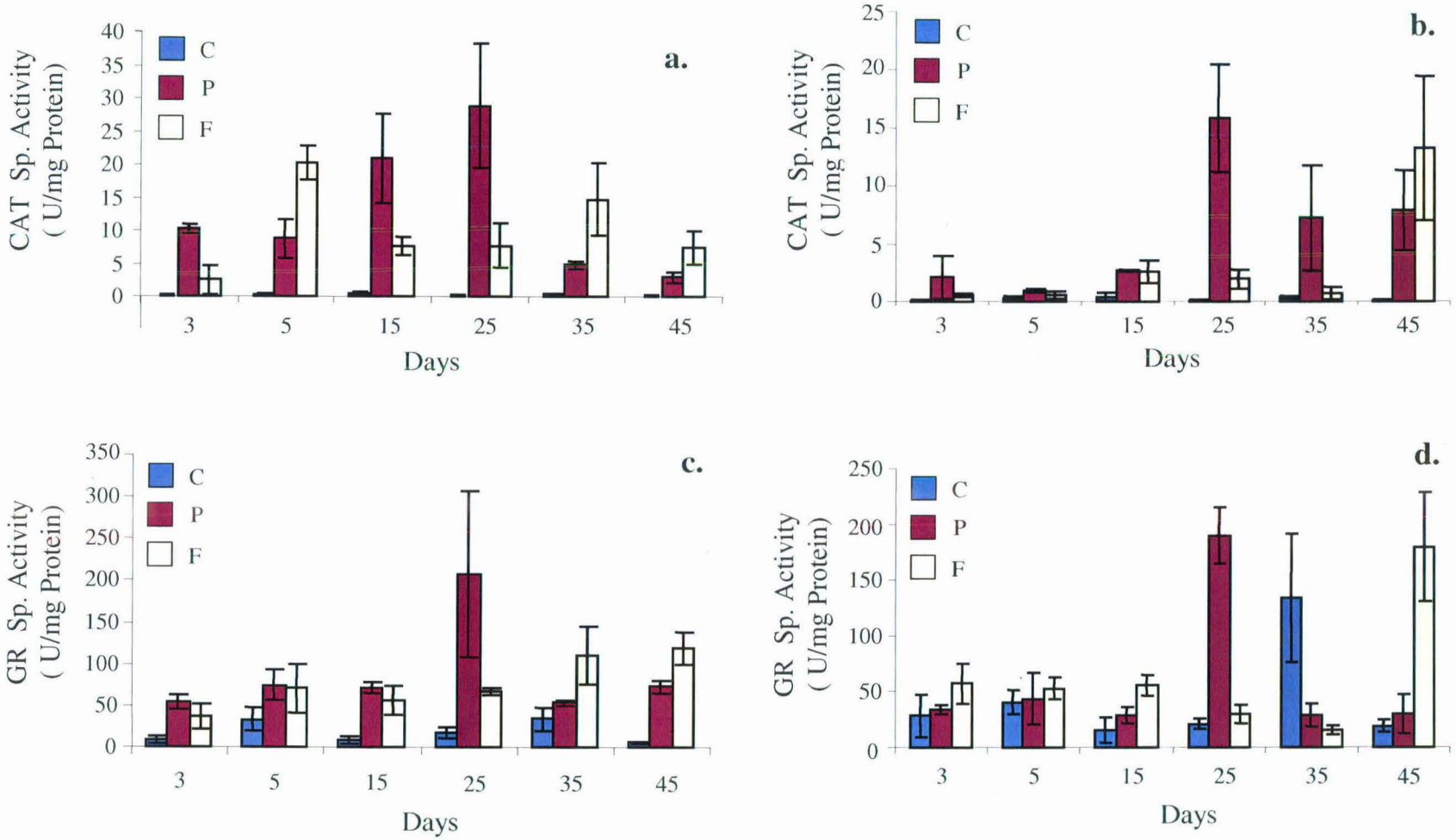

Figure 9. Effect of inoculation of *P. indica* and *F. moniliforme* on Catalase and Glutathione Reductase specific activity. Catalase Activity in Root (a) and shoot (b) and GR activity in root (c) and shoot (d) of plants inoculated with *P. indica* (P) and *F. moniliforme* (F) and in control (C) without any fungus.

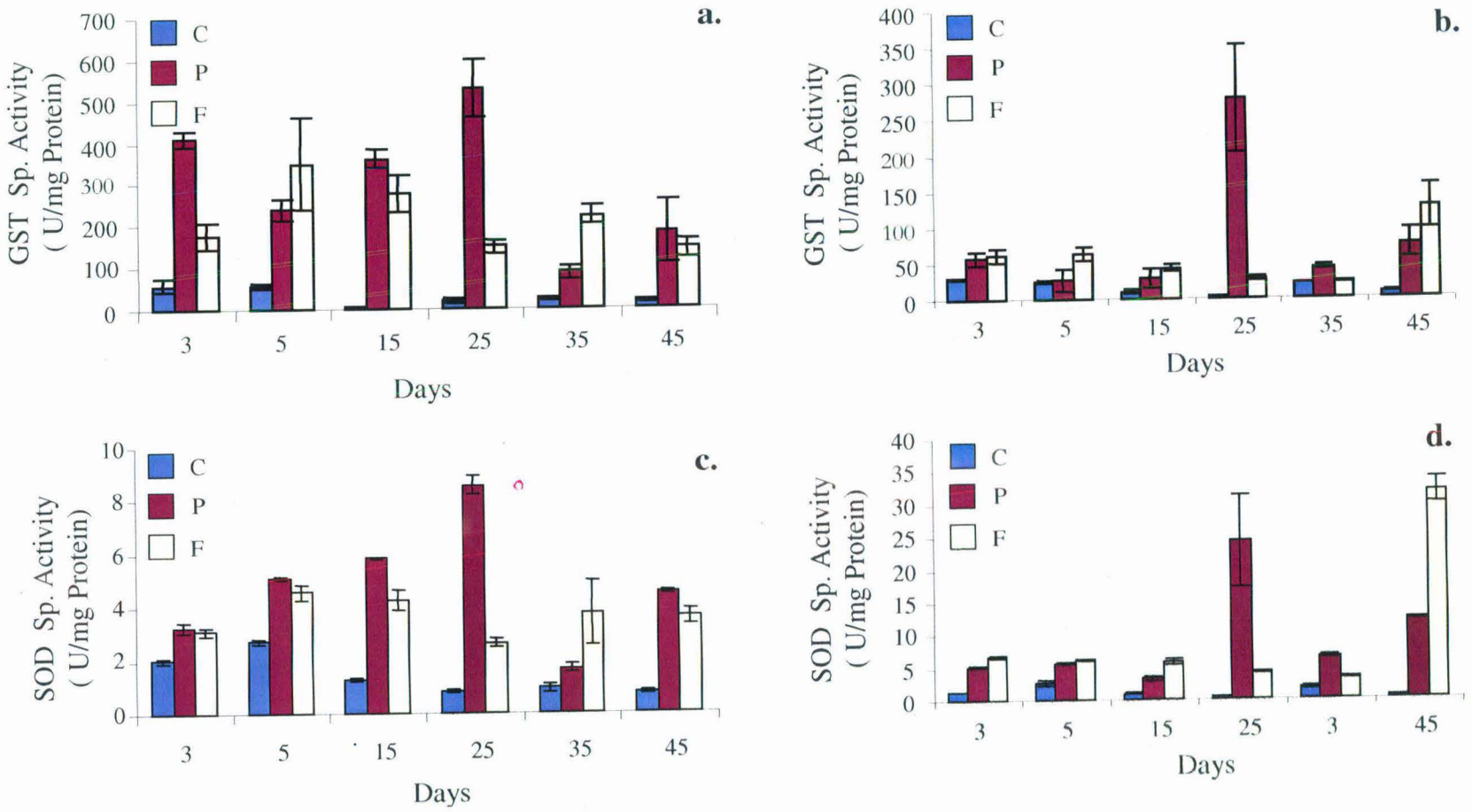

Figure 10. Effect of inoculation of *P. indica* and *F. moniliforme* on GST and SOD specific activity. GST Activity in Root (a) and shoot (b) and SOD activity in root (c) and shoot (d) of plants inoculated with *P. indica* (P) and *F. moniliforme* (F) and in control (C) without any fungus.

Effect of simultaneous inoculation of *P. indica* and *F. moniliforme* on the activity of antioxidant enzyme in root and shoot

Inoculation of both *P. indica* and *F. moniliforme* simultaneously in 10 day old plant continuously increases the activities of all four enzymes till 5 days in root and this was comparable to enzyme activities in plants colonized with *P. indica*. Activities were found 8-80 and 4-53 folds for CAT, 0.7-16 and 1-17 folds for GR, 1.8-34 and 1.6-72 fold for GST and 3-16 and 3-100 folds for SOD than control respectively in root and shoot (Table 8). GR activity in plants inoculated with both *P. indica* and *F. moniliforme* simultaneously did not show significant increase in root and shoot.

Change in Proline Content

Plants inoculated with *P. indica*, interestingly, having high concentration of proline accumulation than non-colonized plants. The change in proline content was seems dependent on colonization. Initially (1 and 2 week) the proline content was increases continuously and become static later (3rd week). *P. indica* stimulated proline accumulation and it was found 1.93, 1.55 and 1.3 times the content of non-colonized plants, 1, 2 and 3 week respectively (Fig. 11).

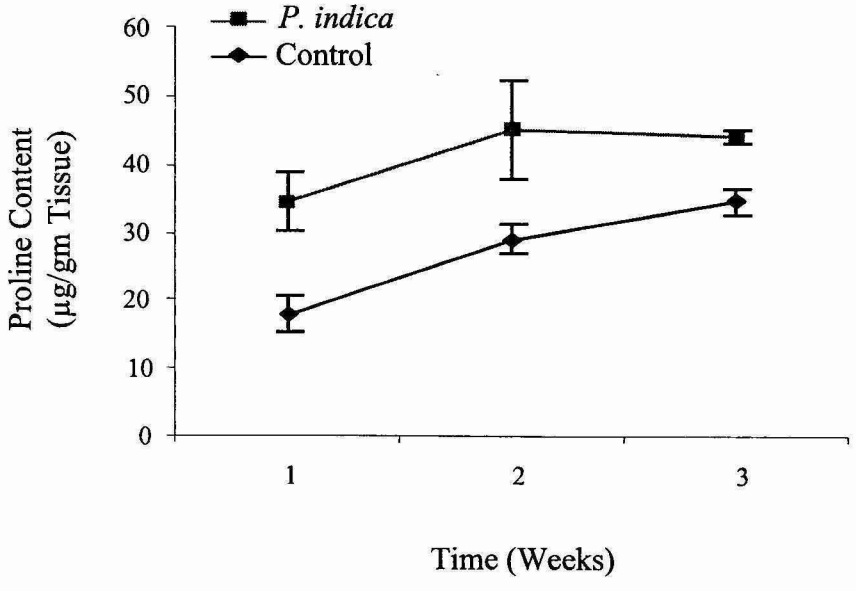

Figure 11. Change in Proline content in plants colonized with *P. indica*. Proline content of *P. indica* colonized and control plants were determined for three weeks. All experiment done in triplicate. Error bars, SE.

Change in expression profile of β- Glucanase and detection of isoforms

Separation of acidic and neutral proteins from maize root and shoot extracts using the Davis system enabled detection of one main bands with β-1,3-glucanase activity in maize roots colonized with *P. indica* or *F. moniliforme*, corresponding to constitutively expressed isoforms. The activity of constitutive isoforms appears to increase with *P. indica* (Fig. 12 a, lanes PR and PS). Additionally, two new isoforms were induced in *P. indica*-colonized roots and shoot respectively (Fig. 12). No β-1,3-glucanase activity was detected in protein extracts from plant shoot infected with *F. moniliforme*. The isoform which has higher electrophoretic mobility than constitutive isoforms, induced highly only in root and shoot colonized with *P. indica* and the activity of this isoform dose not observed in case of *F. moniliforme* or control plant (fig. 12 a). In figure 12 b it is very clear that one isoform with high mobility expressed only in plants colonized with *P. indica*. The observation indicates the relationship of this isoform with *P. indica* colonization.

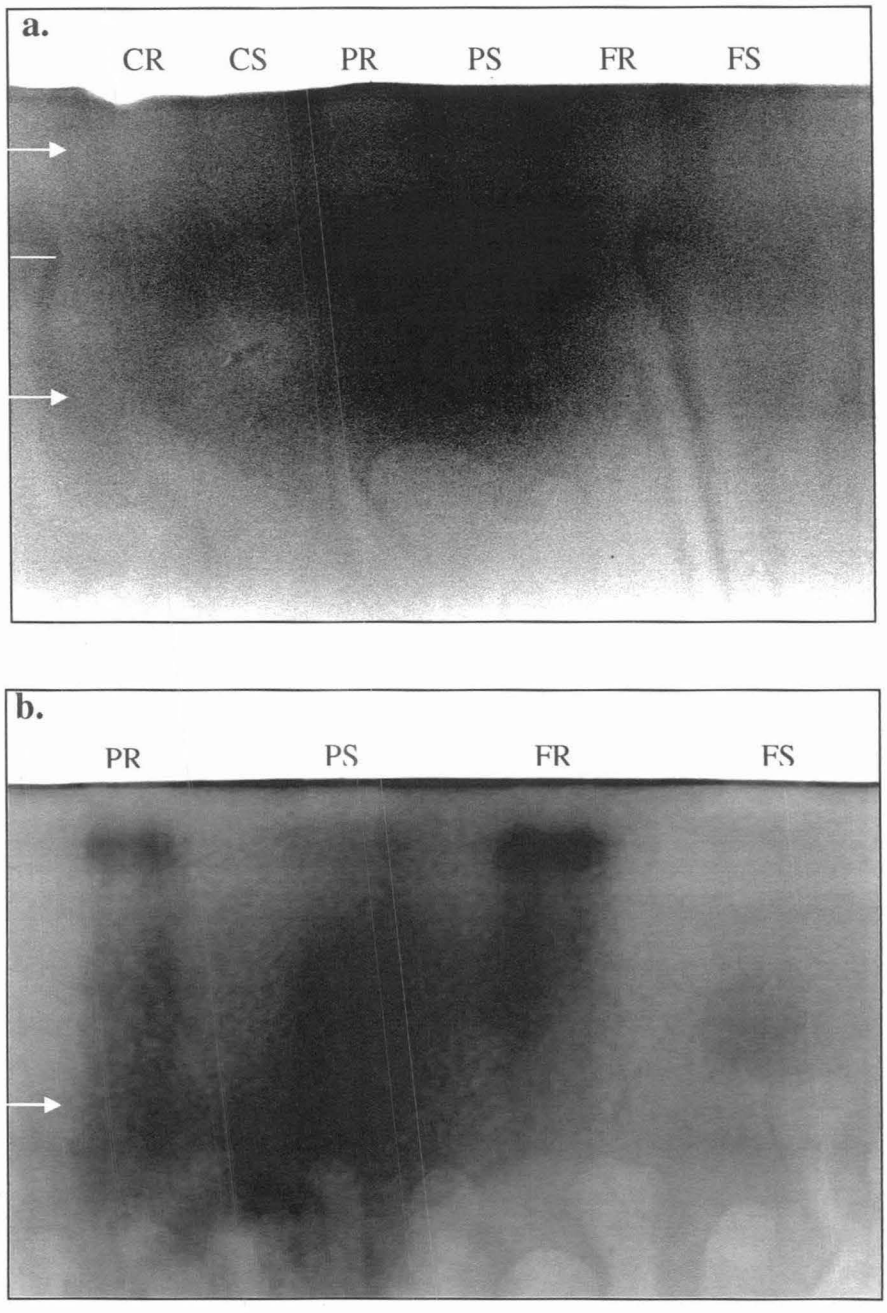

Figure 12. β-Glucanase activity in root and shoot of plant colonized with *P. indica* and *F. moniliforme*. Acidic β-1,3-glucanase activities after separation of proteins by the Davis system in 10% (w/v) with 20µg protein (a) and 15 % (w/v) with 15µg protein (b) polyacrylamide gels containing soluble β-glucans (Laminarin from *Laminaria digitata*, SIGMA) as substrate. Extracts from *P. indica* colonized root (PR), shoot (PS), *F. moniliforme* infected root (FR) and Shoot (FS), control root (CR) and shoot (CS) of maize plants after 7 weeks of growth, either inoculated with *P. indica* or *F. moniliforme*. Constitutive isoforms are indicated by bars and additional isoforms by arrows.

5. Discussion

The inoculation of maize plants with *P. indica* stimulated biomass production of the plant. The biomass is of importance when studying the degree of *P. indica* effectiveness on the host plant. Increased biomass of the inoculated maize plants, compared with non-inoculated plants, indicates mycorrhiza-like growth promotion activity in relation to plant biomass production. Plant fungus symbiosis, like mycorrhizal association causes imperative changes in plant metabolism and growth (Arines et al., 1994), although very little is known of the biochemical mechanisms involved in this symbiotic association. Increased growth associated with *P. indica* and AM infection has been attributed to enhanced nutrient uptake, especially of Phosphorus and Nitrogen (Yadav et al., 2007 unpublished data, Sherameti et al., 2005, Toro et al., 1998, Requena et al., 2001). Altered root morphology or contributions by soil hyphae of *P. indica* increases the total absorption area and that leads to enhanced nutrients and water absorption as in case of mycorrhiza (Auge, 2001). Thus, the increased biomass of *P. indica* inoculated plants could be partly related to the increase in water and nutrient uptake.

The colonization of *P. indica* in maize roots lead to significantly lower the susceptibility to *F. moniliforme* infections than observed in non-inoculated plants. The effect of alternate and simultaneous inoculation of *P. indica* and *F. moniliforme* in maize plants on biomass and susceptibility varied with state of affairs of inoculation of two fungi and level of colonization of *P. indica*. *F. moniliforme* is a major parasite of members of Gramineae. It causes stalk rot in maize (Ayers and Nelson, 1970; Christensen and Wilcoxson, 1966; Hooker, 1972) and poor crop stand, reduced root and shoot weight and reduced plant growth and emergence in maize seedlings (Futrell and Kilgore, 1969; Van wyk et al., 1988; Palmer and MacDonald, 1974; Scott and Futrell, 1970). Improvement in stand, plant growth, biomass and root integrity in response to *P. indica* force to make conclusion about its biotic-tolerance and disease resistance. Besides its tolerance to root parasite, *P. indica* is resistance for leaf parasite (Waller et al., 2005) suggesting the induction of systemic resistance towards plant fungal pathogen and/or parasite. We performed antibiosis assay for fungus which revels no secretion of effective antibiotics by *P. indica* that can inhibit the growth of *F. moniliforme*, suggesting the biotic-tolerance is due to reprogrammed and induced resistance of plant by *P. indica* and not due to antibiotic secretion. The increase in proline content in plants colonized with *P.*

indica might provide the salt and drought tolerance to the plant as describes by Waller et al. (2005).

Systemically induced antioxidative enzymes in root and shoot of plants infested with *P. indica* suggesting an enhanced antioxidative capacity in plant. Unusual strong induction of antioxidative enzymes during colonization period could be for detoxification of ROS, generated during the colonization and play a protective role (Alguacil et al., 2003) and for the compatibility of symbiotic interaction between plant and fungus. No reinforcement of cell wall and hypersensitive reaction was observed in root colonized by *P. indica*, again the evidence for its recognition as a non-pathogen/parasite by the plant. This compatibility may involve some specific signaling cascade that has to be discovered. Tanaka et al. (2006) suggest a symbiosis mechanism in which ROS production by the *E. festucae* NoxA *in planta* negatively regulates fungal development and hyphal tip growth, thereby preventing excessive colonization of the plant tissue. Increase in the level of antioxidative enzymes in response of alternate and simultaneous inoculation of *P. indica* and *F. moniliforme*, were observed insignificant compared to control in root while significant in case of shoot. The observation suggesting a high antioxidative capacity in shoot than root and this slightly increased level of antioxidant enzymes probably favors the symbiosis and results in improvement in stand, plant growth, biomass and root integrity. The outcome of such an interaction can vary in a seamless manner from mutualism to parasitism. In most cases, the host plant does not suffer; in fact it often gains an advantage from colonization by a fungus. This benefit is based on a fine-tuned balance between the demands of the invader and the plant response. If the interaction becomes unbalanced, disease symptoms appear or the fungus is excluded by induced host defense reactions (Kogel et al., 2006). *P. indica* dose not induces the expression of pathogenesis related or jasmonic-acid induced gene in early phase of symbiosis which generally induces in systemic disease resistance (Waller et al., 2005). Now we have found, in late phase of this symbiosis, there is induced expression of PR-2 protein has been detected and two new isoforms have been detected in root and shoot, associated with *P. indica* colonization. High level of expression of β-glucanase activity is associated with strong defense response in

plant root against *F. moniliforme.* Fungus contain β-1,3-glucans, chitin and/or chitosan and glycoproteins. All these molecules are known to be potent elicitors of plant defence reactions. When colonization by AM fungi is successful, some fungal strategies of self camouflage may occur such as important wall modifications during the colonization process or repression of the induced plant defense mechanisms (Gollote et al., 1997). In fact, the host plant influences wall morphology and composition of AM fungi as they develop within the root tissues. Lemoine et al. (1995) described a progressive disappearance in wall β-1,3-glucans during root colonization. Extraradical hyphal walls contained a large quantity of β-1,3-glucans, but they were less abundant in intercellular hyphae and became undetectable in arbuscules (Gollote et al., 1997). In this context, it can be hypothesized that the new systemic induced acidic glucanase isoforms could help the plant to control the *P. indica* development and defense response against pathogen. Though, we did not analyze the cell wall composition of *P. indica* during colonization process, as repression of oxidative defense mechanism in early phase and not at late phase of colonization of *P. indica* but it can be concluded that some how *P. indica* get protected from this hydrolytic enzyme in late phase and survive inside the root as endophyte.

We have shown for the present work that colonization of *P. indica*, have strong growth promoting effects, systemic resistance to biotic stress, biochemical changes that can provides resistance to salt and drought stress and enhanced antioxidant capacity. Because *P. indica*, unlike arbuscular mycorrhiza fungi, axenically cultivable in absence of host plant (Varma et al., 1999), can be used as a tool in sustainable agriculture, new biocontrol method to control soil borne diseases in eco-agriculture and its use in Agri-management and Agribusiness, without using a transgenic approach, in the near future. It can also serve as a model organism for study the plant symbiose interaction, molecular mechanism of disease resistance and much important for environmental studies.

6. References

Agrios, G. N., 1988. Plant Pathology: 3rd ed. Academic Press, San Diego. pp. 803.

Alexander, T., Toth, R., Meier, R. and Weber, H.C. 1989. Dynamics of arbuscule development and degeneration in onion, bean and tomato with reference to vesicular arbuscular mycorrhizae in grasses. Can. J. Bot. 67: 2505-2513.

Alguacil, M.M., Hernandez, J.A., Caravaca, F., Portillo, B. and Roldan, A. 2003. Antioxidant enzyme activities in shoot from three mycorrhizal shrub species afforested in a degraded semi-arid soil. Physiol. Plant. 118: 562-570.

Ane, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E., Ayax, C., Levy, J., Debelle, F., Baek, J.M. and Kalo, P. 2004. *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. Science. 303: 1364-1367.

Arines, J., Quintela, M., Vilarino, A. and Palma, J.M. 1994. Protein pattern and superoxide dismutase activity in non-mycorrhizal and arbuscular mycorrhizal *Pisum sativum* (L.) Plants. Plant Soil. 166: 37-45.

Armstrong, R.N. 1993. Glutathione S-transferases: structure and mechanism of an archetypical detoxification enzyme. Adv. Enzymol. 69: 1-44.

Arnon, D.I. and Hoagland, D.R. 1940. Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. Soil Sci. 50: 463-483.

Asada, K. 2000. The water-water cycle as alternative photon and electron sinks. Philosophical Transactions of the Royal Society of London. Series B, Biology Sciences. 355: 1419-1431.

Asada, K. and Takahashi, M. 1987. Production and scavenging of active oxygen in photosynthesis. In: Photoinhibition (Kyle, D.J., Osmond, C.B. and Arntzen, C.J. eds). Elsevier Science Publishers B.V. Amsterdam. pp. 227-287.

Auge, R.M. 2001. Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza. 11: 3-42.

Ayers, J.E. and Nelson, P.E. 1970. *Fusarium* species associated with corn stalk rot in Pennsylvania. Phytopathology. 62: 493 (Abstract).

Bacon, C.W., Yates, I.E., Hinton, D.M. and Meredith, F. 2001. Biological Control of *Fusarium moniliforme* in maize. Envt. Healh. Perspet. 109: 325-332.

Baker, B., Zambryski, P., Staskawicz, B. and Dinesh-Kumar, S. P. 1997. Signalling in plant-microbe interactions. Science. 276: 726-733.

Ballantyne, J.S. and Chamberlin, M.E. 1994. Regulation of cellular amino acid levels. In: Cellular and Molecular Physiology of Cell Volume Regulation (Strange, K. ed.), CRC Press, Boca Raton. pp. 111-122.

Barroso, J.B., Corpas, F.J., Carreras, A., Rodriguez-Serrano, M., Esteban, F.J., Fernandez-Ocana, A., Chaki, M., Romero-Puertas, M.C., Valderrama, R., Sandalio, L.M. and del Rio, L.A. 2006. Localization of S-nitrosoglutathione and expression of S-nitrosoglutathione reductase in pea plants under cadmium stress. J. Exp. Botany. 57(8): 1785-1793.

Beers, R.F. Jr. and Sizer, I.W. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195(1): 133-140.

Beffa, R. and Meins, F. Jr. 1996. Pathogenesis-related function of plant β-1, 3-glucanases investigated by antisense transformation-a review. Gene. 179: 97-103.

Blee, K.A. and Anderson, A.J. 2000. Defense responses in plants to arbuscular mycorrhizal fungi. In: Current advances in mycorrhizae research (Podila G.K. and Douds D.D. eds.). Minnesota, USA: The American Phytopathological Society. Pp. 27-44.

Bloch, C., Patel, Jr. S.U., Baud, F., Zvelebil, M.J., Carr, M.D., Sadler, P.J. and Thornton, J.M. 1998. $_1$H NMR structure of an antifungal g-thionin protein SIa1: similarity to scorpion toxins. Proteins. 32: 334-349.

Bodker, L., Kjoller, R., Kristensen, K. and Rosendahl, S. 2002. Interactions between indigenous arbuscular mycorrhizal fungi and *Aphanomyces euteiches* in field-grown peas. Mycorrhiza. 12: 7-12.

Bonfante, P. and Perotto, S. 1995. Strategies of arbuscular mycorrhizal fungi when infecting host plants. New Phytol. 130: 3-21.

Bonfante-Fasolo, P. 1984. Anatomy and morphology of VA mycorrhizae. In: VA Mycorrhizae (Powell, C.L., Bagyaraj, D.J. and Boca Raton, F.L., eds). CRC Press. pp. 5-33.

Bowler, C., VanMontagu, M. and Inze, D. 1992. Superoxide dismutase and stress tolerance. Annu. Rev. Plant. Physiol. Plant Mol. Biol. 43: 83-116.

Boyland, E. and Chasseaud, L.F. 1969. The role of glutathione and glutathione s-transferases in mercaptuic acid biosynthesis. Adv. Enzymol. 32: 173-219.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Bradley, D.J., Kjellbom, P. and Lamb, C.J. 1992. Elicitor-and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein-a novel rapid defense response. Cell. 70: 21-30.

Brady, C.J., Gibson, T.S., Barlow, E.W.R., Spiers, J., Wyn Jones, R.G. 1984. Salt tolerance in plants. I. Ions, compatible solutes and the stability of plant ribosomes. Plant, Cell and Environ. 7: 571-578.

Broekaert, W.F., Terras, F.R., Cammue, B.P. and Osborn, R.W. 1995. Plant defensins: Novel antimicrobial peptides as components of host defense system. Plant Physiol. 108: 1353-1358.

Cadenas, E. 1989. Biochemistry of oxygen toxicity. Annu. Rev. Biochem. 58: 79-110.

Chen, C. and Dickman, M.B. 2005. Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii.* Proc. Natl. Acad. Sci. USA. 102 (9): 3459-3464.

Chen, C.N. and Pan, S.M. 1996. Assay of superoxide dismutase activity by combining electrophoresis and densitometry. Bot. Bull. Acad. Sin. 37: 107-111.

Christensen, J.J. and Wilcoxson, R.D. 1966. Stalk rot in corn. The American Phytopathological Society, Minnesota, USA. pp. 50.

Cobbett, C. and Goldsbrough, P. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu. Rev. Plant Biol. 53: 159–182.

Cole, R.C., Kirksey, J.W., Cutler, H.G., Doupnik, B.L. and Peckham, J.C. 1973. Toxin from *Fusarium moniliforme*: Effect on plants and animals. Science. 179: 1324-1326.

Cote, F., Cutt, J.R., Asselin, A. and Klessig, D.F. 1991. Pathogenesis related acidic β-1,3-glucanase genes of tobacco are regulated by both stress and developmental signals. Mol. Plant Microbe Interact. 4:173-181.

Davis, B.J. 1964. Disc electrophoresis. II. Method and application to human serum proteins, Ann. New York Acad. Sci. 121: 404-427.

Dean, J.V., Devarenne, T.P., Lee, I. and Orlofsky, L.E. 1995. Properties of a maize glutathione S-transferase that conjugates coumaric acid and other phenyl propanoids. Plant Physiol. 108: 985-994.

del Rio, L.A., Corpas, F.J., Sandalio, L.M., Palma, J.M. and Barroso, J.B. 2003. Plant peroxisomes, reactive oxygen metabolism and nitric oxide. IUBMB Life. 55: 71-81.

Delauney, A.J. and Verma, D.P.S. 1993. Proline biosynthesis and osmoregulation in plants. Plant J. 4: 215-223.

Deshmukh, S., Huckelhoven, R., Schafer, P., Imani, J., Sharma, M., Weiss, M., Waller, F. and Kogel, K.H. 2006. The root endophytic fungus Piriformospora indica requires host cell death for proliferation during mutualistic symbiosis with barley. Proc. Natl. Acad. Sci. U S A. 103 (49): 18450-18457.

Dickman, M.B., Podila, G.K., and Kolattukudy, P.E. 1989. Insertion of cutinase gene into a wound pathogen enables it to infect intact host. Nature. 342: 446-448.

Dickson, S., Mandeep and Smith, S. M. 1998. Evaluation of vesicular arbuscular mycorrhizal colonization by staining. In: Mycorrhiza Manual (Varma, A. ed). Springer-Verlag, Germany. pp. 77-84.

Duncan, D.R. and Widholm, J.M. 1987. Proline accumulation and its implication in cold tolerance of regenerable maize callus. Plant Physiol. 83: 703-708.

Edwards, R., Dixon, D. P. and Walbot, V. 2000. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci. 5: 193-198.

Flor, H.H. 1955. Host-parasite interaction in flax rust - its genetic and other implications. Phytopathology. 45: 680-685.

Foyer, C.H. and Noctor, G. 2001. The molecular biology and metabolism of glutathione. In: Significance of glutathione in plant adaptation to the environment (Grill, D., Tausz, M. and De Kok, L. eds). Dordrecht, Kluwer Academic Publishers Netherlands. pp. 27-57.

Foyer, C.H., Lelandais, M., Galap, C. and Kunert, K.J. 1991. Effects of elevated cystosolic glutathione reductase activity on the cellular glutathione pool and photosynthesis in leaves under normal and stress conditions. Plant Physiol. 97: 863-872.

Foyer, C.H., Lescure, J., Lefebvre, C., Morot-Gaudry, J.F., Vincentz, M. and Vaucheret, H. 1994. Adaptations of photosynthetic electron transport, carbon assimilation and carbon partitioning in transgenic *Nicotiana plumbaginifolia* plants to changes in nitrate reductase activity. Plant Physiol. 104: 171–178.

Fridovich, I. 1995. Superoxide radical and superoxide dismutases. Annual Review of Biochem. 64: 87-112.

Fridovich, I. 1989. Superoxide dismutases. An adaptation to a paramagnetic gas. J. Biol. Chem. 264: 7761-7764.

Futrell, M.C. and Kilgore, M. 1969. Poor stands of corn and reduction of root growth caused by *Fusarium moniliforme*. Plant Dis. Rep. 53: 213-215.

Garcia-Garrido, J.M. and Ocampo, J.A. 2002. Regulation of the plant defense response in arbascular mycorrhizal symbiosis. J. Exp. Botany. 53 (373): 1377-1386.

Gianinazzi, S., Dexhejiner, J., Gianinazzi-Pearson, V. and Marx, C. 1983. Role of host-arbuscular interface in the VA mycorrhizal symbiosis, ultrastructural studies of processes involved in Phosphate and carbohydrate exchange. Plant Soil. 71: 211-215.

Gibson, T.S., Spiers, J., Brady, C.J. 1984. Salt tolerance in plants. II. *In vitro* translation of m-RNAs from salt-tolerant and salt-sensitive plants on wheat germ ribosomes: responses to ions and compatible solutes. Plant Cell Environ. 7: 579-587.

Giovannetti, M., Sbrana, C., Avio, L., Citernesi, A.S. and Logi, C. 1993. Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during preinfection stages. New Phytol. 125: 587–593.

Gollote, A., Cordier, C., Lemoine, M.C. and Gianinazzi-Pearson, V. 1997. Role of fungal wall components in interactions between endomycorrhizal symbionts. In: Eukaryotism and Symbiosis (Schenck, H.E.A., Herrmann, R., Jeon, K.W., Muller, N.E. and Schwemmler. Eds), Springer, Berlin. pp. 412-428.

Grant, J.J. and Loake, G.J. 2000. Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol. 124: 21-30.

Grenier, J. and Asselin, A. 1993. Detection of β-1, 3- Glucanase activity in gels containing alkali-soluble Yeast Glucan. Anal. Biochem. 212: 301-302.

Habig, W.H., Pabst, M.J. and Jacoby, W.B. 1974. Glutathione-S-Transferases. J. Biol. Chem. 249: 7130-7139.

Halliwell, B. 1996. Free radicals in biochemistry and medicine. In: Encyclopedia of Molecular Biology and Molecular Medicine (Meyers, R.A. ed). Vol. 2. Wiley-VCH, Weinheim, Germany. pp.330-337.

Halliwell, B. and Gutteridge, J.M.C. 2000. Free radicals in biology and medicine. Clarendon Press, Oxford, UK.

Halliwell, B. 1987. Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. Chem. Phys. Lipids. 44: 327-340.

Handa, S., Handa, A.K., Hasegawa, P.M. and Bressan, R.A. 1986. Proline accumulation and the adaptation of cultured plant cells to water stress. Plant Physiol. 80: 938-945.

Hanson, A.D. and Hitz, W.D. 1982. Metabolic responses of mesophytes to plant water deficits. Annu. Rev. Plant Physiol. 33: 163-203.

Hare, P.D. and Cress, W.A. 1997. Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regulation. 21: 79-102.

Hare, P.D., Cress, W.A. and Van Staden, J. 1998. Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ. 21: 535-553.

Harrison, M.J. 1996. A sugar transporter from *Medicago truncatula*: Altered expression patterns in roots during vesicular-arbuscular (VA) mycorrhizal associations. Plant J. 9: 491-503.

Hasan, H.M. and Fridovich, I. 1981. Chemistry and biochemistry of superoxide dismutases. Eur. J. Rheumatol. Inflamm. 4: 160-172.

Hausladen, A. and Alscher, R.C. 1994. Cold-Hardiness-Specific Glutathione Reductase isozymes in Red Spruce. Plant Physiol. 105: 215-223.

Hill, T.W. and Kaefer, E. 2001. Improved protocols for aspergillus medium: trace elements and minimum medium salt stock solutions. Fungal Genet. News Lett. 48: 20-21.

Hooker, A.L. 1972. The worst diseases of crops-corn. Crops & Soils 24: 15-18.

Howard, R.J., Ferrari, M.A., Roach, D.H. and Money, D.H. 1991. Penetration of hard substances by a fungus employing enormous turgor pressures. Proc. Natl. Acad. Sci. USA. 88:11281-11284.

Huang, A.H.C., Trelease, R.N. and Moore, T.S. 1983. Plant Peroxisomes. Academic Press, New York.

Huckelhoven, R. and Kogel, K.H. 2003. Reactive oxygen intermediates in plant microbe interactions: Who is who in powdery mildew resistance? Planta. 216: 891-902.

Huynh, Q.K., Borgmeyer, J.R. and Zobel, J.F. 1992. Isolation and characterization of a 22 kDa protein with antifungal properties from maize seeds. Biochem. Biophys. Res. Commun. 182:1-5.

Garcia-Romera, I., Garcia-Garrido, J.M., Martinez-Molina, E. and Ocampo, J.A. 1991. Production of pectolytic enzymes in lettuce root colonized by *Glomus mosseae*. Soil Biol. Biochem. 23: 597-601.

Ibeas, J.I., Lee, H., Damsz, B., Prasad, D.T., Pardo, J.M. Hasegawa, P.M. Bressan, R.A. and Narasimhan, M.L. 2000. Fungal cell wall phosphomannans facilitate the toxic activity of a plant PR-5 protein. Plant J. 23:375–383.

Garcia-Garrido, J.M., Garcia-Romera, I. and Ocampo, J.A. 1992. Cellulase production by the vesicular arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol and Gerd) Gerd. and Trappe. New Phytol. 121: 221-226.

Garcia-Garrido, J.M., Garcia-Romera, I., Parra-Garcia, M.D. and Ocampo, J.A. 1996. Purification of an arbuscular mycorrhizal endoglucanase from onion roots colonized by *Glomus mosseae*. Soil Biol. Biochem. 25: 1443-1449.

Jabs, T., Tschope, M., Colling, C., Hahlbrock, K. and Scheel, D. 1997. Elicitor-stimulated ion-fluxes and O_2 from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. Proc. Natl. Acad. Sci. USA. 94: 4800-4805.

Jach, G., Gornhardt, B., Mundy, J., Logemann, J., Pinsdorf, E., Leah, R., Schell, J. and Maas, C. 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J. 8: 97-109.

Jackson, A.O. and Taylorbi, C. 1996. Plant-Microbe Interactions: Life and Death at the Interface. Plant Cell. 8: 1651-1668.

Jackson, C., Dench, J., Moore, A., Halliwell, B., Foyer, C. and Hall, D. 1978. Subcellular localization and identification of superoxide dismutase in the leaves of higher plants. Eur. J. Biochem. 91: 339-344.

Jacquelinet-Jeanmougin, J., Gianinazzi-Pearson, V. and Gianinazzi, S. 1987. Endomycorrhizas in the Gentianaceae. II. Ultrastructural aspects of symbiont relationships in *Gentiana lutea* L. Symbiosis. 3: 269-286.

Jones, J.B., Jones, J.P., Stall, R.E. and Zitter, T.A. 1991. Compendium of tomato diseases. St. Paul, APS Press, MN. pp. 73.

Ketchum, R.E.B., Warren, R.C., Klima, L.J., Lopez-Gutierrez, F. and Nabors, M.W. 1991. The mechanism and regulation of proline accumulation in suspension cultures of the halophytic grass *Distichlis spicata* L. J. Plant Physiol. 137: 368-374.

Ketterer, B., Taylor, J., Meyer, D., Pemble, P., Coles, B., ChuLin, X. and Spencer, S. 1993. Some functions of glutathione transferases. In: Structure and function of glutathione transferases. (Tew, K.D., Pickett, C.B., Mantle, T.J., Mannervik, B. and Hayes, J.D. eds.) CRC Press, Boca Raton, Florida.

Kim, Y.J. and Hwang, B.K. 1997. Isolation of a basic 34 kD β-1, 3-glucanase with inhibitory activity against *Phytophthora capsici* from pepper stems. Physiol. Mol. Plant Pathol. 50: 103-115.

Koehler, B. 1942. Natural mode of entrance of fungi into corn ears and some symptoms that indicate infection. J. Agric. Res. 64: 421-442.

Kogell, K.H., Franken, P. and Huckelhovenl, R. 2006. Endophyte or parasite – what decides? Curr. Opin. Plant Biol. 9(4): 358-363.

Kolattukudy, P.E. 1985. Enzymatic penetration of the plant cuticle by fungal pathogens. Annu. Rev. Phytopathol. 23: 223-250.

Lamb, C. and Dixon, R.A. 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plan. Mol. Biol. 48: 251-275.

Leigh, R.A., Ahmad, N. and Wyn-Jones, R.G. 1981. Assessment of glycinebetaine and proline compartmentation by analysis of isolated beet vacuoles. Planta 153: 34-41.

Lemoine, M.C., Gollotte, A., Gianinazzi-Pearson, V. and Gianinazzi. S. 1995. Localization of β-1, 3 glucan in walls of the endomycorrhizal fungi *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *Acaulospora laevis* (Gerd. and Trappe) during colonization of host roots. New Phytol. 129: 97-105.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C.J. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell. 79: 583-593.

Li, H.Y., Yang, G.D., Shu, H.R., Yang, Y.T., Ye, B.X., Nishida, I. and Zheng, C.C. 2006. Colonization by the Arbuscular Mycorrhizal Fungus *Glomus versiforme* induces a Defense Response against the Root-knot Nematode *Meloidogyne incognita* in the Grapevine (*Vitis amurensis* Rupr.), Which Includes Transcriptional Activation of the Class III Chitinase Gene VCH3. Plant Cell Physiol. 47(1): 154.

Linderman, R.G. 1994. Role of VAM fungi in biocontrol. In Mycorrhiza and Plant Health. (Pfleger, F.L. and Linderman, R.G. eds) The American Phytopathological Society Press, St Paul, MN, USA. pp. 1-27.

Lone, M.I., Kueh, J.S.H., Wyn-Jones, R.G., and Bright, S.W.J. 1987. Influence of proline and glycinebetaine on salt tolerance of cultured barley embryos. J. Exp. Bot. 38: 479-490.

Low, P.S. 1985. Molecular basis of the biological compatibility of nature's osmolytes. In "Transport Processes, Iono- and Osmoregulation" (Gilles, R. and Gilles-Baillien, M. eds), Springer-Verlag, Berlin. pp. 469-477.

Marrs, K.A. 1996. The functions and regulation of glutathione S-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 127-158.

McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L. and Swan, J.A. 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. 115: 495-501.

Meister, A. 1989. On the biochemistry of glutathione. In: Glutathione centennial: molecular perspectives and clinical implications. (Taniguchi, N., Higashi, T., Sakamoto, Y. and Meister, A. eds). Academic Press, San Diego, Ca.

Mendgen, K., and Deising, H. 1993. Infection structures of fungal plant pathogens-A cytological and physiological evaluation. New Phytol. 124, 192-213.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7:405-410.

Mok, M.I., Finger, L.D., Stryker, J.L., Fuqua, C., Eberhard, A. and Winans, S.C. 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science 272: 1655-1658

Money, N. and Harold, F. 1992. Extension growth of the water mold *Achyla*: interplay of turgor and wall strength. Proc. Natl. Acad. Sci. USA. 89:4245-4249.

Montesinos, E., Bonaterra, A., Badosa, E., Frances, J., Alemany, J., Llorente, I. and Moragrega, C. 2002. Plant-microbe interactions and the new biotechnological methods of plant disease control. Int. Microbiol. 5: 169-175.

Mullineaux, P.M. and Creissen, G.P. 1997. Glutathione Reductase: Regulation and role in oxidative stress. In: Oxidative stress and the molecular biology of antioxidants (Scandalios, J.G. ed.). Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press. pp. 667-713.

Nash, D., Paleg, L.G. and Wiskich, J.T. 1982. Effect of proline, betaine and some other solutes on the heat stability of mitochondrial enzymes. Aust. J. Plant Physiol. 9: 47-57.

Nicholson, R.L., and Epsteln, L. 1991. Adhesion of fungi to the plant surface. In: The Fungal Spore and Disease initiation in Plants and Animals (Cole, G.T. and Hoch, H.C. eds). NY: Plenum Press. pp. 3-23.

Nielsen, K.K., Nielsen, J.E., Madrid, S.M. and Mikkelsen, J.D. 1997. Characterization of a new antifungal chitin-binding peptide from sugar beet leaves. Plant Physiol. 113:83–91.

Noctor, G., Gomez, L., Vanacker, H. and Foyer, C.H. 2002. Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. J. Exp. Botany. 53: 1283-1304.

Noctor, G. and Foyer, C. 1998. Ascorbate and GSH: Keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 249-279.

Nordhoff, A., Bucheler, U.S., Werner, D. and Schirmer, R.H. 1993. Folding of the four domains and dimerization are impaired by the Gly446 → Glu exchange in human glutathione reductase. Implications for the design of antiparasitic drugs. Biochemistry. 32(15): 4060-4066.

Pahlich, E., Kerres, R. and Jager, H.J. 1983. Influence of water stress on the vacuole/extravacuole distribution of proline in protoplasts of *Nicotiana rustica*. Plant Physiol. 72: 590-591.

Paleg, L.G., Douglas, T.J., van Daal, A. and Keech, D.B. 1981. Proline, betaine and other organic solutes protect enzymes against heat inactivation. Aust. J. Plant Physiol. 8: 107-114.

Paleg, L.G., Stewart, G.R., Bradbeer, J.W. 1984. Proline and glycine betaine influence protein solvation. Plant Physiol. 75: 974-978.

Palmer, L.T. and MacDonald, D.H. 1974. Interaction of *Fusarium spp.* and certain plant parasitic nematods in maize. Phytopathology. 64: 14-17.

Phillips, J.M. and Hayman, D.S. 1970. Improved procedures for clearing roots and staining parasitic and VAM fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55: 158-161.

Pollard, A., Wyn Jones, R.G. 1979. Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta. 144: 291-298.

Pozo, M.J., Cordier, C., Dumas-Gaudot, E. Gianinazzi, S., Barea, J.M. and Azcon-Aguilar, C. 2002. Localised versus systemic effect of arbascular mycorrhizal fungi on defense responses to *Phytopthora* infection in tomato plant. J. Exp. Bot. 53(368): 525.

Pozo, M.J., Azcon-Aguilar, C., Dumas-Gaudot, E. and Barea, J.M. 1998. Chitosanase and chitinase activities in tomato roots during interactions with arbuscular mycorrhizal fungi or *Phytophthora parasitica*. J. Exp. Bot. 49(327): 1729-1739.

Pozo, M.J., Azcon-Aguilar, C., Dumas-Gaudot, E. and Barea, J.M. 1999. β-1,3-Glucanase activities in tomato roots inoculated with arbuscular mycorrhizal fungi and/or *Phytophthora parasitica* and their possible involvement in bioprotection. Plant Science 141: 149-157.

Rai, M.K., Varma, A. and Pandey, A.K. 2004. Antifungal potential of Spilanthes calva after inoculation of *Piriformospora indica*. Mycoses. 47(11-12): 479-481.

Rejon-Palomares, A., Garcia-Garrido, J.M., Ocampo, J.A. and Garcia-Romera, I. 1996. Presence of xyloglucan-hydrolyzing glucanases (xyloglucanases) in arbuscular mycorrhizal symbiosis. Symbiosis. 21: 249-261.

Requena, N., Perez-Solis, E., Azcon-Aguilar, C., Jeffries, P. and Barea, J.M. 2001. Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Appl. Environ. Microbiol. 67: 495-498.

Rhodes, D. 1987. Metabolic responses to stress. In: The Biochemistry of Plants (Davies, D.D. ed). Vol. 12, Academic Press, NY. pp. 201-241.

Rhodes, D., Verslues, P.E. and Sharp, R.E. 1999. Role of amino acids in abiotic stress resistance. In: Plant Amino Acids: Biochemistry and Biotechnology. Marcel Dekker, NY, pp. 319-356.

Roberts, W. and Selitrennikoff, C.P. 1990. Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. J. Gen. Microbiol. 136: 1771-1778.

Rodriguez, R. and Redman, R. 2005. Balencing the generation and elimination of reactive oxygen species. Proc. Natl. Acad. Sci. USA. 102(9): 3175-3176.

Roth, Jr E.F. and Gilbert, H.S. 1984. Pyrogallol assay for SOD: Absence of a glutathione artifact. Anal. Biochem. 137: 50-53.

Rudolph, A.S., Crowe, J.H. and Crowe, L.M. 1986. Effects of three stabilizing agents - proline, betaine and trehalose on membrane phospholipids. Arch. Biochem. Biophys. 245: 134-143.

Sahay, N.S. and Varma, A. 1999. *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol. Let. 181: 297-302.

Salzer, P., Corbiere, H. and Boller, T. 1999. Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by the arbuscular mycorrhizal *Glomus mosseae*. Planta. 208: 319-325.

Samaras, Y., Bressan, R.A., Csonka, L.N., Garcia-Rios, M.G., Paino-D'Urzo, M. and Rhodes, D. 1995. Proline accumulation during drought and salinity. In: Environment and Plant Metabolism: Flexibility and Acclimation (Smirnoff, N. ed). Bios Scientific Publishers, Oxford. pp 161-187.

Sanders, F.E., Tinker, B.P., Black, R.L.B. and Palmerly, S.M. 1977. The development of endomycorrhizal root systems. Speed of infection and growth-promoting effects with four species of vesicular–arbuscular endophyte. New Phytol. 78: 257-268.

Santarius, K.A. 1992. Freezing of isolated thylakoid membranes in complex media. VIII. Differential cryoprotection by sucrose, proline and glycerol. Physiol. Plant. 84: 87-93.

Santoro, M.M., Liu, Y., Khan, S.M.A., Hou, L.X. and Bolen, D.W. 1992. Increased thermal stability of proteins in the presence of naturally occurring osmolytes. Biochemistry. 31: 5278-5283.

Scandalios, J.G. 1997. Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press, Plainview, NY, USA.

Scandalios, J.G. 1994. Regulation and properties of plant catalases. In: Causes of photooxidative stress and amelioration of defense systems in plants (Foye,r C.H. and Mullineaux, P.M. eds). CRC Press, Boca Raton. 275-315.

Scandalios, J.G. 2005. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Brazilian J. Med. Biol. Res. 38: 995-1014.

Scandalios, J.G., Guan, L.M. and Polidoros, A. 1997. Oxidative Stress and the Molecular Biology of Antioxidant Defenses, (Scandalios, J.G. ed). Cold Spring Harbor Lab. Press, Plainview, NY. pp. 343–406.

Scott, G.S. and Futrell, M.C. 1970. Response of maize seedlings to Fusarium moniliforme and a toxic material extracted from this fungus. Plant Dis. Reporter. 54: 483-486.

Segura, A., Moreno, M. and Garcia-Olmedo, F. 1993. Purification and antipathogenic activity of lipid transfer proteins (LTPS) from the leaves of *Arabidopsis* and spinach. FEBS Lett. 332: 243-246.

Shahollari, B., Vadassery, J., Varma, A., Oelmuller, R. 2007. A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J. 50(1): 1-13.

Shaw, J.G. 1996. Plant viruses. In: Fundamentals of Virology (Fields, B.N., Knipe, O.M. and Howley, R.M. eds) Philadelphia: Lippincott-Raven. pp. 367-400.

Sherameti, I., Shahollari, B., Venus, Y., Altschmied, L., Varma, A. and Oelmuller, R. 2005. The Endophytic fungus *Piriformospora indica* stimulates the expression of Nitrate Reductase and the starch–degrading enzyme Glucan-water Dikinase in Tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. J. Biol. Chem. 280 (28): 26241-26247.

Smirnoff, N. and Cumbes, Q.J. 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry. 28: 1057-1060.

Smith, S.E. and Gianinazzi-Pearson, V. 1988. Physiological interactions between symbionts in vesicular-arbuscular mycorrhizal plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39: 221-44.

Smith, S.E. and Read, D.J.1997. Mycorrhizal symbiosis. Academic Press, London.

Smith, S.E. 1993. Transport at the mycorrhizal interface. Mycorrhiza News. 5:1-3

Somero, G.N. 1986. Protons, osmolytes, and fitness of internal milieu for protein function. Am. J. Physiol. 251: R197-R213.

Songstad, D.D., Duncan, D.R. and Widholm, J.M. 1990. Proline and polyamine involvement in chilling tolerance of maize suspension cultures. J. Exp. Botany. 41: 289-294.

Srinivas, V. and Balasubramanian, D. 1995. Proline is a protein-compatible hydrotrope. Langmuir. 11: 2830-2833.

Stacey, G., Burris, R.H. and Evans, H.J. 1992. Biological nitrogen fixation. Chapman and Hall, NY. 763-834.

Stewart, C.R. 1981. Proline accumulation: Biochemical aspects. In: Physiology and Biochemistry of Drought Resistance in Plants (Paleg, L.G. and Aspinall, D. eds) Academic Press, Sydney. pp. 243-259.

Stewart, G.R. and Larher, F. 1980. Accumulation of amino acids and related compounds in relation to environmental stress. In: The Biochemistry of Plants (Miflin, B.J. ed), Vol. 5, Academic Press, NY. pp. 609-635.

Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdioglu, S., Kauffmann, S., Geoffroy, P., Legrand, M. and Fritig, B. 1993. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie. 75: 687-706.

Tanaka, A., Christensen, M.J., Takemoto, D., Park, P. and Scotta, B. 2006. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. Plant Cell. 18: 1052-1066.

Taylor, C.B. 1996. Proline and water deficit: ups and downs. Plant Cell. 8: 1221-1224.

The American Phytopathological Society. 1973. A compendium of corn diseases. pp. 64.

Thompson, J.F. 1980. Arginine synthesis, proline synthesis, and related processes. In: The Biochemistry of Plants (Miflin, B.J. ed). Vol. 5, Academic Press, NY. pp. 375-403.

Timasheff, S.N. 1993. The control of protein stability and association by weak interactions with water: how do solvents affect these processes? Annu. Rev. Biophys. Biomol. Struct. 22: 67-97.

Toro, M., Azcon, R. and Barea, J.M. 1998. The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotype, mycorrhizal fungi, phosphate-solubilizing rhizobacterias and rock phosphate on nitrogen and phosphorus acquisition by *Medicago sativa*. New Phytol. 138: 265-273.

Trouvelot, S., van-Tuinen, D., Hijri, M. and Gianinazzi-Pearson, V. 1999. Visualization of ribosomal DNA loci in spore interphasic nuclei of glomalean fungi by fluuorescence *in situ* hybridization. Mycorrhiza. 8: 203-206.

Trudel, J., Grenier, J., Potvin, C. and Asselin, A. 1998. Several thaumatin-like proteins bind to β-1,3-glucans. Plant Physiol. 118: 1431-1438.

Tsang, E.W.T., Bowler, C., Herouart, D., Van Camp, W., Villarroel, R., Genetello, C., Van Montagu, M. and Inze D. 1991. Differential regulation of superoxide dismutases in plants exposed to environmental stress. The Plant Cell. 3: 783-792.

van Swaaji, A.C., Jacobsen, E. and Feenstra, W.J. 1985. Effect of cold hardening, wilting and exogenously applied proline on leaf proline content and frost tolerance of several genotypes of *Solanum*. Physiol. Plant. 64: 230-236.

Van wyk, P.S., Scholtz, D.J. and Marasas, W.F.O. 1988. Protection of maize seedlings by *Fusarium moniliforme* against infection by *Fusarium raminearum* in the soil. Plant and Soil. 107: 251-257.

Varma, A. and Schuepp, H. 1994. Mycorrhizae, their applications in micropropagated plantlets. Critical Reviews in Biotechnology. CRC Press, Florida, USA. 15: 313-328

Varma, A., Singh, A., Sudha, Sahay N.S., Sharma, J., Roy, A., Kumari, M., Rana, D., Thakran, S., Deka, D., Bharti, K., Hurek, T., Blechert, O., Rexer, K.H., Kost, G., Hahn, A., Maier, W., Walter, M., Strack, D. and Kranner, I. 2001. *Piriformospora indica*: an axenically culturable mycorrhiza-like endosymbiontic fungus. In: The Mycota IX - Fungal Associations (Hock, B., Maier, W., Walter, M., Strack, D., Kranner, I. eds) Springer-Verlag, Berlin Heidelberg. pp. 125-150.

Varma, A., Verma, S., Sudha, Sahay, N., Butehorn, B. and Franken, P. 1999. *Piriformospora indica*, a Cultivable Plant-Growth-Promoting Root Endophyte. Appl. Env. Microbiol. 65 (6): 2741-2744.

Vierheilig, H., Iseli, B., Alt, M., Raikhel, N., Wiemken, A. and Boller, T. 1996. Resistance of *Urtica dioica* to mycorrhizal colonization: a possible involvement of *Urtica dioica* agglutinin. Plant Soil. 183: 131-136.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Huckelhoven, R., Neumann, C., Wettstein, D., Franken, P. and Kogel, K.H. 2005. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc. Natl. Acad. Sci. USA. 102: 13386-13391.

Watanabe, T., Kanai, R., Kawase, T., Tanabe, T., Mitsutomi, M., Sakuda, S. and Miyashita, K. 1999. Family 19 chitinases of *Streptomyces* species: characterization and distribution. Microbiology. 145: 3353-3363.

Webster, J. 1980. Introduction to Fungi. Cambridge University Press, Cambridge.

Wilce, M.C.J. and Parker, M.W. 1994. Structure and function of glutathione Stransferases. Biochim. Biophys. Acta. 1205: 1-18

Willekens, H., Inze, D., Montagu, M.V. and vanCamp, W. 1995. Catalases in plants. Mol. Breeding. 1: 207-228.

Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Van Montagu, M., Inze, D. and Van Camp, W. 1997. Catalase is a sink for H_2O_2 and is indispensable for stress defense in C3 plants. E.M.B.O. 16 (16): 4806-4816.

Withers, L.A. and King, P.J. 1979. Proline: a novel cryoprotectant for the freeze preservation of cultured cells of *Zea mays*. Plant Physiol. 64: 675-678.

Wyn Jones, R.G., Storey, R., Leigh, R.A., Ahmad, N., Pollard, A. 1977. A hypothesis on cytoplasmic osmoregulation. In: Regulation of Cell Membrane Activities in Plants(Marre, E. and Cifferi, O. eds). Elsevier, Amsterdam. pp. 121-136.

Yadav, V. et al. 2007. A Phosphate Transporter from Axenically Cultivable Arbascular Mycorrhiza like Fungus *Piriformospora indica* and its Role in the Phosphate Transfer to the Plants. Unpublished.

Yancey, P.H. 1994. Compatible and counteracting solutes. In: Cellular and Molecular Physiology of Cell Volume Regulation (Strange, K. ed), CRC Press, Boca Raton. pp. 81-109.

Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D. and Somero, G.N. 1982. Living with water stress: evolution of osmolyte systems. Science. 217: 1214-1222.

7. Appendix

Appendices

All the reagents and buffer used and not mentioned in appendix were prepared according to "Molecular cloning: A laboratory manual" 3rd edition (Sambrook and Russel, 2001)

Table 1. Aspergillus Medium (Hill and Kaefer, 2001)

Constituents	**Composition (g/L)**
Glucose	10
Peptone	2
Yeast extract	1
Casamino acid	1
Vitamins stock solution	1ml
Macroelements from stock	50ml
Microelements from stock	1ml
Agar	10
pH	6.5
Macroelements (Major Elements) Stock (g/L)	
$NaNO_3$	120.0
KCl	10.4
$MgSO_4.7H_2O$	10.4
KH_2PO_4	16.3
K_2HPO_4	20.9
Microelements (Trace Elements) Stock (g/L)	
$ZnSO_4.7H_2O$	22
H_3BO_3	11
$MnCl_2.4H_2O$	5
$FeSO_4.7H_2O$	5
$CoCl_2.6H_2O$	1.6
$CuSO_4.5H_2O$	1.6
$(NH_4)_6 Mo_7O_{27}.4H_2O$	1.1
Na_2EDTA	60
Vitamins Stock (%)	

Biotin	0.05
Nicotinamide	0.5
Pyridoxal phosphate	0.1
Amino benzoic acid	0.1
Riboflavin	0.25

The stocks were stored at 4°C and vitamin was stored at -20 °C in aliquots. The stock of $FeSO_4$. $7H_2O$ was prepared separately.

Note: The modified aspergillus media used for enzyme activity contains 10 times reduction of peptone, yeast extract and casamino acid without vitamins

Table 2. Lactophenol (Phillips and Hayman, 1970)

Phenol	150 g
Water	150 ml
Lactic acid	125 ml
Glycerol	125 ml

Table 3. Trypan Blue (Phillips and Hayman, 1970)

Trypan blue	0.1 g
Lactophenol	100 ml

Table 4. Aniline blue (Phillips and Hayman, 1970)

Aniline blue	0.2 g
Na-P buffer (0.07 M, pH 9)	100 ml

Table 5. Bradford Reagent (Bradford, 1976)

Coomassie brilliant blue G 250	100 mg
90 % ethanol	50 ml
85 % o-phosphoric acid	100 ml

Table 6. Sodium Acetate Buffer (50 mM), pH 3-6.5

Sodium acetate	6.8 g

Dissolved in 1 liter deionized water and pH adjusted by acetic acid glacial

Table 7. 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al. 1974)

CDNB	366 mg
Ethanol (90%)	6 ml

Table 8. Glutathione (reduced) (Habig et al. 1974)

Glutathine	45 mg
MQ water	5 ml

Table 9. Pyrogallol (Roth and Gilbert, 1984)

Pyrogallol	10.2 mg
MQ water	10 ml