# Interaction of root endophytic fungus *Piriformospora indica* with Chickpea and its role in plant defence response against pathogen *Botrytis cinerea*

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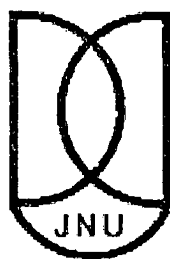

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## CERTIFICATE

The research work embodied in this thesis entitled "**Interaction of root endophytic fungus *Piriformospora indica* with Chickpea and its role in plant defence response against pathogen *Botrytis cinerea*"** has been carried out by **Om Prakash Narayan** for the degree of Master of Philosophy (M.Phil.) in the School of Life Sciences. Jawaharlal Nehru University, New Delhi. It is certified that the work presented is original and has not been submitted in part or fulfilment of any degree or diploma to 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 |
| Km | :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 |
| PDA | :Potato dextrose Agar |
| 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 |
| Vmax | :Maximum velocity |
| U | :Unit |
| UV | :Ultraviolet |
| v/v | :Volume/Volume |
| w/v | :Weight/Volume |
| w/w | :Weight/Weight |

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# 1. Introduction

There are several types of plant microbe interaction, among them some symbiotic and some are non symbiotic. The plant fungus beneficial interaction, referred as mycorrhizal symbiosis is a symbiotic relationship between plant and fungus. Arbuscular Mycorrhiza (AM) is a widely founding mycorrhizal symbiosis with wide range of plant species. It also takes part role in reducing root disease cause by number of soil borne bacteria. *Piriformospora indica* has been discovered from Indian Thar desert. The name *Piriformospora indica* based on its characteristics pear shaped chlamydospore and they mimic the capabilities of typical AM fungus (Verma *et al.*, 1998). This fungus provides a model organism for studying the plant microbe interactions. *P.indica* was found to be involved in strong growth-promoting activities, high salt tolerance, bioregulator, biofertilizer, bio-protectant, disease resistance and also enhances nutritional requirement (like phosphate) of the plants (Waller *et al.*, 2005, Aschheim *et al.*, 2005, Kumar *et al.*, 2009, Yadav *et al.*, 2010).

*P. indica* has potency to grow on number of complex and synthetic media (Hill and Kaefer., 2001). Its mycelium is mostly flat and submerged in to the substratum. Hyphae are thin walled and of different diameters ranging from 0.7-3.5μm. The hyphae are highly interwoven often showed anastomoses and are irregularly septate. In order cultures hyphae are irregularly inflated, showing a nodose to coralloid shape. Hypha is mostly coenocytic and septa are laid infrequently and contain more than one nucleus. Chlamydospores are formed from thin walled vesicle at the tips of the hyphae. Inter and intracellular hyphae in the root cortex often differentiating into dense hyphal coils and chlamydospore. The chlamydospores appeared singly or in cluster and were distinctive due to their pear width. 8-25 nuclei present in cytoplasm of chlamydospores. Like AMF, hyphae multiply inside the host cortical tissues and never traverse through the endodermis. Similarly they also do not invade the aerial portion of the plant i.e. stem and leaves. The host spectrum of *P. indica* is very similar to AMF. *P. indica* vastly improves the growth and overall biomass of various host including legumes (Varma *et al.*, 1999; Varma *et al.*, 2001), medicinal and other plants of economic importance (Peskan-Berghofer *et al.*, 2004; Das *et al.*, 2012).

*Botrytis cinerea* is a parasitic fungus and causative agent of Botrytis Grey Mould (BGM) disease in variety of plants including economically important chickpea plant. The anamorphic stage of *Botrytis cinerea* (Persson ex., Fries) causes serious pre

and post harvest disease in more than 235 different plant species including a range of agronomically important crops such as grapevine, tomato, chickpea, strawberry, cucumber, bulb flowers and ornamental plants and yield losses reported up to 100% under conducive conditions (Jarvis, 1977; De Kock & Holz, 1992; Berrie, 1994; Elad, 1998; Droby & Lichter, 2004). This fungus is widely distributed in the temperate areas of the world where it can infect an extremely wide range of host plants (Elad *et al*., 2004). As mentioned *B. cinerea* causes damage of several highly economically important crop plants while on the other hand *P. indica* has been termed as plant probiotic therefore in the present study we have studied the role of *P. indica* in improvement of plant biomass and protection against *B. cinerea* and antioxidant enzymes (e.g. GR, CAT, SOD and GST) activity during the interaction. Therefore for the present study following objectives were undertaken.

1. **Interaction of *P. indica with* chickpea plant to check growth promoting activity.**
2. **Role of *P. indica* in enhancement of resistance to plant towards fungal pathogen (*B. cinerea*).**
3. **Role of *P. indica* in regulation of plant defence response (antioxidant enzymes) against pathogen during the interaction.**

# 2. Review of Literature

Soil inhabiting microorganisms takes part an active role in regulation of terrestrial ecosystem. Terrestrial ecosystem contain heavy amount of microbial biomass. It has world largest microbial diversity temporarily and spatially soil provides heterogeneous environment to soil microbes. Growth of microbial population in soil influenced by so many factors including presence of solid surface, moisture condition and variable level of organic and inorganic substrates. It also influenced by interaction of other soil born organism.

Rhizosphere is an area around the plant root which differ the environment of root free soil in so many aspects like the physical and chemical condition of these two types of soils. So the soil zone is influenced by plant roots. Microbes have a selective force due to this plant roots. These plant roots are helping microbes in providing substrate for biomass and energy to them in soil. So plants have effect on soil born microbial community and there is also a counter significance of microorganism to plants. These microorganisms also show beneficial relation with plants. Microbial community of rhizosphere helps plants in establishing growth and succession. Microorganisms help plants and plat community in various ways by provision of nutrients and direct hormonal stimulation of growth. They also affect negatively to the organism of rhizosphere as a causative agent of so many disease. So plant microbe interaction in rhizosphere is important to functioning of terrestrial ecosystem.

## PLANT MICROBE INTERACTION

The environmental condition for microorganism interaction with plants considerably differs from highly variable aerial plant part than more stable root system of plants. Plants represent a good ecosystem for microorganism. Microbes interact with plant tissues and cells with different degrees of dependence (Montesinos *et al.*, 2002). Since humans began to rely extensively on cultivated crops for food. The plant microorganism interaction have major role in development of civilization. There are several strategies have been developed by micro organism them self to adapt in to the plant environment. They are showing either beneficial or harm full 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 mycorrhiza. Harmful or pathogenic interactions with plant of microorganism involve viroids, viruses, bacteria and fungi, and lead to infectious diseases affecting the host plant (Montesinos

*et al.*, 2002). In beneficial interaction like symbiosis, the microorganism assists plant with nutrient absorption or contributes biochemical activities that the plant lacks. In turn a plant contributes photosynthate to the competitive advantage of the related microbial symbiont in the rhizosphere. Symbiotic association between plant and microorganism also help plants from disease causing microorganism by altering the balance of microflora in rhizosphere. For a microorganism successfully colonization in host plant they 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. If plant evolving a novel resistance to pathogen, the pathogen must 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 type of relationship, the emergence of disease symptoms, *i.e.* compatibility is the result in most cases and results from the ability of the pathogens to defeat 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

Non symbiotic interaction refers to harmful and pathogenic interaction of host and pathogen. There are the three major categories of disease-causing organisms, viruses and viroids, bacteria, mycoplasma-like organisms, and fungi (Jones *et al.*, 1991).

### Viruses

Plant viruses are excellent entities for the explanation of host– microbe interactions. Viruses mainly depend on the host cell protein and cellular metabolism for their multiplication and movement. Viruses are obligate intracellular pathogenic particle. They require living host for their replication and multiplication. Plant viruses face mainly two types of fundamental challenges during the establishment of systemic infection in their plant host. First invading the host and using their machinery for

development for new protein related to formation of new viral particle. Second is plant virus requiring cell to cell movement through plasmodesmata and through phloem in systemic zone. Plant viruses cause disease in part by causing a reallocation of photosynthates and a disruption of normal cellular processes as they replicate. Since beginning virologist has been studied plant immune response, including hypersensitive (HR), systemic required resistance (SAR), and gene for gene resistance response. Existing immune response paradigms were discovered more than 50 years ago studying plant-infecting viruses (Samuel, 1934; Holmes, 1937, 1938, 1954; Ross, 1961a, 1961b).

**Prokaryotes**

Bacteria and mycoplasma –like organism cause many important disease in the plant host. They show a wide range of symptoms phenotype of disease in plant by the prokaryotic organism. Prokaryotes exhibits diverse life style that ranges from obligate pathogen to facultative pathogen (Agrios, 1988). Group of this organism have different type of mode of pathogenesis. They are either biotrophic or necrotrophic pathogen. Biotrophic pathogens kill their host slowly and during this they have more opportunity to replicate them self where as necrotrophic pathogens shows 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,* and *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 mass attack response is proposed to be mediated by quorum sensing, a 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.

**Fungi**

Almost all fungal group including lower fungi, such as Plasmodiophoromycetes, Chitridomycetes, and Oomycetes to higher fungi, such as Ascomycetes, Basidiomycetes and Deuteromycetes causing major and serious plant diseases (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. Fungal pathogen altered the growth and morphology of the plant in a very sophisticated manner. The penetration processes through which fungi get entry into plant tissue are complex events. 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). However in case of mechanically penetrating fungi, penetration is characterized by 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 (Mendgen and Deising, 1993). During penetration, the hydrolytic enzymes and plant defense responses generate fragments of fungal and plant cell walls. Often compound like 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 them 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, the effectiveness of the corresponding *R* gene 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.

## SYMBIOTIC INTERACTIONS

This type of interaction helps in development of sustainable agro ecosystem. Direct and mutual interaction among member of different microbial type often benefiting plant growth and health. All these interaction taking place in rhizosphere and mediated by plants. Bacteria and fungi have beneficial symbiotic interaction with higher plants with their complexity and by their specificity. They are also of enormous importance for global agricultural productivity (Stacey *et al.*, 1992) like in most nitrogen- fixing symbiosis, soil bacteria or unrelated genera *Rhizobium* and *Frankia* induce cell divisions in fully differentiated (and quiescent) cells in the root cortex or pericycle of plants. 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. Mycorrhiza is an example of beneficial association between root of higher plant and soil fungal hyphae. These hyphae serve to increase the absorptive surface area of the root, thus facilitating the uptake of nutrients and minerals, particularly phosphorus. This association also protect plant roots from infection by pathogenic organisms in the soil.

**Mycorrhizal symbiosis**

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 endomycorrhizal and ectomycorrhiza (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). There is no gross change in the morphology but sub cellular modifications are extensive while 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 symbiont 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 for example members of Brassicaceae, caryophyllaceae, Chenopodiaceae, do not engage in AM interactions (Vierheilig *et al.*, 1996). In AM symbiosis the fungi involved are obligate biotrophs. They reproduce asexually and having multinucleate spores (Trouvelot *et al.*, 1999). During this symbiotic association fungal hypha contacts the root of the host plant where it differentiates to form an appressorium because of stimulation of components of root exudates. They stimulate 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 leads to 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). 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). 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. 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 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.*, 1989). AM fungi cant cultured axenically consequently we are unable to use it for sustainable agriculture because it is unable to make symbiosis with several group of plants.

**O2, ROS, AND OXYGEN TOXICITY**

In plants Reactive Oxygen Molecule (ROS) serve as signalling molecule for normal biological metabolic processes to control various processes including pathogen defense, programmed cell death (Foyer *et al.*, 1994). ROS generates in different cellular compartment like mitochondria, chloroplast and peroxisomes. Very commonly different types of ROS cause the damage of proteins, DNA, and lipids. In response to balance the cellular damage by ROS, cells also produces different types of antioxidative scavengers under physiological steady state conditions are often confined to particular compartments (Alscher *et al.*, 1997). In response to certain environmental changes (biotic or abiotic stresses) plants produces ROS by activating various oxidases and peroxidises (Bolwell *et al.*, 2002, Bolwell *et al.*, 1998, Doke

1985, Schopfer *et al.*, 2001). A rapid increase in ROS level referred as "oxidative burst" and it is a rapid defence response by plant to pathogen attack and so called oxidative burst. Reactive oxygen species are mainly in three forms, superoxide ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), and the oxidant $H_2O_2$. NADPH- dependent oxidase is one of the enzymes which are responsible for generation of ROS. These oxidants are used for killing of microorganism by phagocytes cell but at the same time it harms the surrounding cell also. Transition metal ion like Fe and Cu helps in ROS generation by reducing $H_2O_2$ in to hydroxyl radical by superoxide. There is no any scavenger for hydroxyl radical so there is only one way to detoxify these radical by tight control on generation of these radicals.

Non enzymatic ROS scavenging system contain non enzymatic antioxidants which includes cellular redox buffers ascorbate, glutathione (GSH), and alkaloids, flavonoids, carotenoids, and tocopherol (Creissen *et al.*, 1999). Enzymatic ROS scavising system in plants include the various enzymes like superoxide dismutase (SOD), glutathione peroxidise (GPX), CAT and ascorbate peroxidise (APX). The list of some biologically most important ROS is listed in table (**Table 1**). 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**).

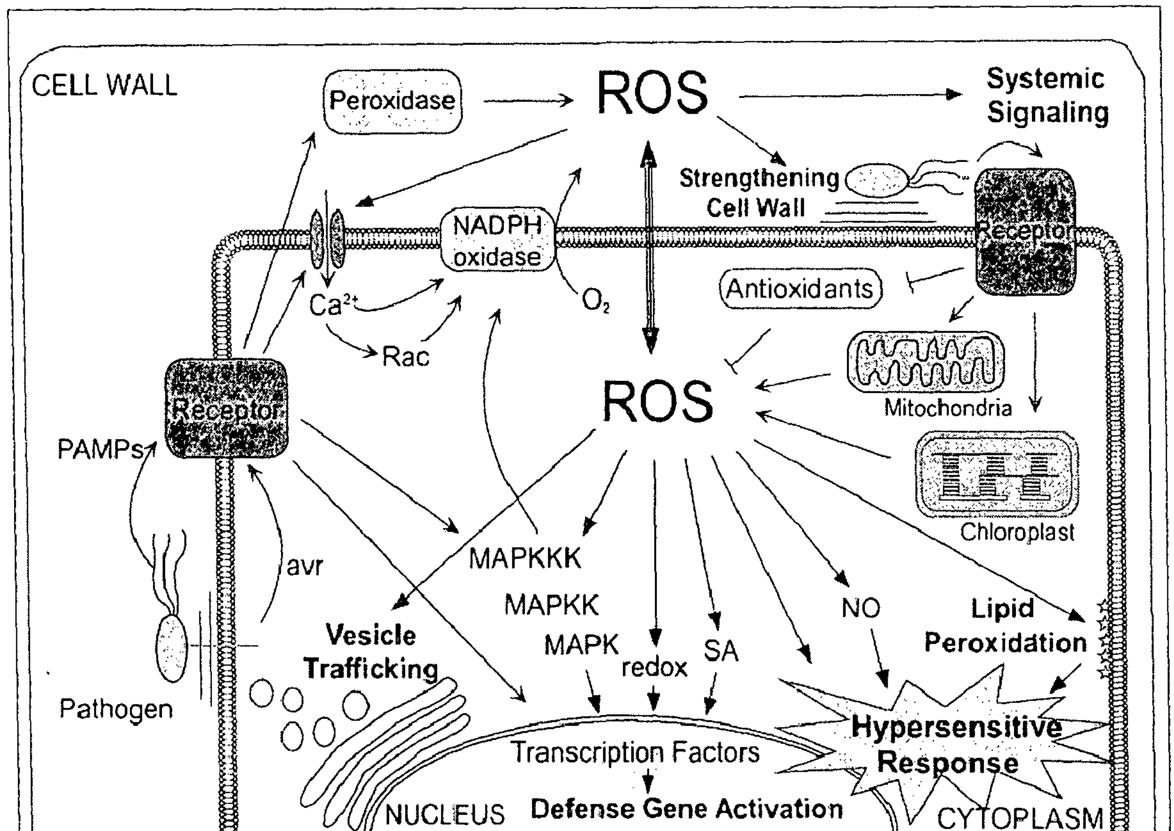



**Figure 1. Function of ROS in plant defense.** (Physiologica Plantarum 138: 414-429.2010).

**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 singletstate)* | ${}^1O_2$: ${}^1\Delta$ | *Photoinhibition; UV irradiation; PS II e- transfer reactions (chloroplasts).* |
| *Superoxide anion* | $O_2^{\circ -}$ | *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. In apoplastic space; defense against pathogens; oxidation of xenobiotics.* |
| *Hydrogen peroxide* | $H_2O_2$ | *Formed from $O_2^{\circ -}$ by dismutation; photorespiration; β-oxidation; proton-induced decomposition of $O_2^{\circ -}$ ; defense* |

| | | |
|---|---|---|
| | | *against pathogens* |
| *Hydroxyl radical* | *OH* | *Decomposition of $O_3$ in apoplastic space; defense against pathogens; reactions of $H_2O_2$ with $O_2^-$ (Haber-Weiss); reactions of $H_2O_2$ with Fe2+ (Fenton); highly reactive with all macromolecules* |
| *Perhydroxyl radical* | *$O_2H$* | *Protonated form of $O_2^-$ ; reactions of O3 and OH. 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* |

**Table2. 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.*<br>• *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)*<br>• *Fragmentation of the peptide chain*<br>• *Aggregation of cross-linked reaction products*<br>• *Altered electrical charge*<br>• *Increased susceptibility to proteolysis*<br>• *Oxidation of Fe-S centers by $O_2^-$ destroys enzymatic function*<br>• *Oxidation of specific amino acids .marks. proteins for degradation by specific proteases*<br>• *Oxidation of specific amino acids (e.g., Try) leads to cross-linking* |
| **Oxidative damage to DNA** |
| • *DNA deletions, mutations, translocations*<br>• *Base degradation, single-strand breakage*<br>• *Cross-linking of DNA to proteins* |

## DEFENSE RESPONSE IN PLANTS

Plant does not have immune system equivalent to animals but they have developed a nice array of structural, chemical and protein based defence mechanism to detect or stop invading organism before any extensive damage. In plants disease can be caused by biotic (living) factor like bacteria, fungi, viruses etc., or by abiotic (non living) factor like pollution, radiation, temperature, draught, salt stresses and nutrients deficiency. Plants have develops a wide verity of **constitutive** and **inducible** defence responses. In constitutive response there is a permanent mechanical barrier like bark, cuticles, waxy epidermis and cell wall. These barriers protect plants from invading pathogens and also provide strength and rigidity to plants. In inducible response plant wait till pathogen produces any toxic harmful chemical and defence related proteins, they produce toxin and pathogen degrading proteins (enzymes) to counter act with bacteria.

Plant pathogen mainly categorize in to three forms **biotrophs, necrotrophs and hemobiotrophs.** Biotrophs keep their host plant alive and feed up on them, for example *Xanthomonas oryzae*, nectrotrophs produces toxin or tissue degrading enzyme and results in cell death of host plant, for example *Botrytis cinerea.* Some pathogens are biotrophic in early stage and later become necrotrophic called as hemibiotrophs for example *Magnaprothe grisea.* Every pathogen have specific **host range** on which a pathogen capable to cause disease. Non host plant species does not cause disease by pathogen by which they are infected. If any pathogen infect any plant host there is two possibilities, in one case interaction result in disease referred as compatible response and in second case interaction result in no disease referred as incompatible response. A particular plant species is susceptible to particular pathogen, among them some plant may have some gene that help in recognising the presence of pathogen and inducing defence against pathogen, some are recognising the pathogen and limit disease via resistance. Disease resistance depend on range of responses ranging from complete lack of any disease symptom (immunity) to some disease symptom (highly resistance) to significant disease symptom (highly susceptible).

There are several layer of defence mechanism in plants to recognise dangerous pathogens. The first line of inducible defence response that protect plant from entire group of organism is called as **Basal resistance** and it trigger when plant recognise the microbe- associated molecular pattern (MAMP$_S$). MAMP$_S$ contain cell wall

content present in microbe like lipopolysaccharides and cell wall specific proteins (Dow *et al.*, 2000). Both type of pathogenic and non-pathogenic microbe are capable of trigger of this basal level of plant resistance. Pathogen also counter act on basal level of defence attack. If pathogen capable to suppress the basal level of plant defence then plant trigger another specific line of defence in which plant cell suicide deliberately at the site of infection to limit nutrients and water to pathogen consequently saving rest of plant cells called as **hypersensitive response (HR)** (Heath *et al.*, 2000. Zhang *et al.*, 2004). HR response trigger in plant when plant specific proteins recognise the presence of pathogen introduced disease causing specific molecule in host plant called as **effector.** 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 appear 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. Host plant become more resistance to a broad range of pathogen if HR once trigger for more time period is called as **systemic acquired resistance (SAR).** Artificial chemical may also trigger SAR called as **activators.** Systemic Acquired resistance 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. Plants also defend themselves from viral pathogen by more sophisticated mechanism called as RNA silencing. Plants release volatile organic compounds ($VOC_S$) (monoterpenoids, sesquiterpenoids and homoterpenoids) in response to insect feeding/damage by sensing elicitors molecule contained in saliva of insect (Pare *et al.*, 1999). These compounds repel insects.

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 hypersensitive reaction and defense (Flor *et al.,* 1955, Baker *et al.,* 1997).

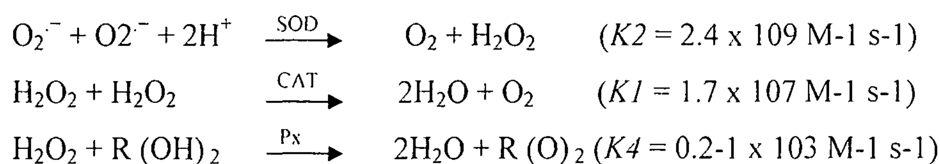
Plant structural defence includes tough bark and cell wall which contain lignin, pectin that provide cell wall rigidity. They have also deposition of cutin, suberin and waxes. Plant epidermal cells also contains throne, spine and prickles that help them protect. Chemical defence include different type of metabolites. Primary metabolites are substances produced by all plant cells that directly involve in plant growth and defence. Secondary metabolites not directly involve in plant growth but they are involved in plant defence for example terpenoids. Phenolics are another large class of secondary metabolites that help plant from pathogen for example flevenoids. Some proteins and enzymes are directly involved in plant defence for example Defencin: a cystein rich protein that have broad antimicrobial activity (Lay *et al.,* 2005). Ricin is a powerful toxin produce in caster seed. Proteses inhibitors, chitinases, glucanases and lysozymes are also directly involved in plant defence against microbial pathogens (Lee *et al.,* 1999).

## ANTIOXIDANTS IN PLANT DEFENSE

Several biotic and abiotic stresses including microbial pathogens are responsible for the production of Reactive Oxygen Intermediate (ROI) like hydrogen peroxide and the superoxide anion radical in plants during certain redox reactions and incomplete reduction of oxygen or oxidation of water by mitochondrial or chloroplast electron transfer chains. ROIs consist of superoxide radical anion $O_2^{\circ}$, the

hydroperoxyl radical $HO_2^{\circ}$, hydrogen peroxide $H_2O_2$, hydroxyl radical $HO^{\circ}$. superoxide, its protonated form $HO_2^{\circ}$ and $HO^{\circ}$ are relatively short-lived whereas $H_2O_2$ is comparatively stable and can cross membranes. $H_2O_2$ formed outside the cell cross plasma membrane and enters the plant cell and eventually removed from cells by conversion to water by certain antioxidant enzymes. Hydroxyl radicals are more toxic among all ROIs because they have ability to react with organic molecules such as phenols, fatty acids, proteins and nucleic acids (Huckelhove 2003). The ascorbate-glutathione cycle is the major antioxidant pathway in plastids, where ROI are generated during normal biochemical processes.

Both enzymic and nonenzymic mechanisms **(Table 3 and 4)** 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). The primary components of this system include carotenoids, ascorbate, glutathione, tocopherols and enzymes such as superoxide dismutase, catalase, glutathione peroxidise and the enzymes involved in the ascorbate glutathione cycle, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase (Noctor and Foyer, 1998). Non-enzymatic defenses include compounds of intrinsic antioxidant properties, such as vitamins C vitamin E, glutathione, and β-carotene. Enzymatic defenses, such as superoxide dismutases (SOD), catalases (CAT) and peroxidases, protects through directly scavenging the superoxide radicals and hydrogen peroxide or through converting them to less reactive species. SODs catalyze the dismutation of $O2^{\circ-}$ 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, $O2^{\circ-}$ 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 defences:



$$O_2^{\cdot -} + O2^{\cdot -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \quad (K2 = 2.4 \times 109\ M\text{-}1\ s\text{-}1)$$

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

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

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

**Glutathione-S-Transferase (GST)**

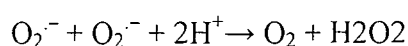
Glutathione-S-transferases plays role in detoxification of xenobiotics compounds and herbicides in plant. Classification of Glutathione S-transferases enzyme is E.C. 2.5.1.18 and it catalyze the conjugation of the tripeptide glutathione (GSH) to a variety of hydrophobic, electrophilic, and cytotoxic substrates (Mannervik *et al.*, 1988). It first discovered in 1960s in animal and 1970 in plant (Wilce and Parker. 1994, Edwards *et al.*, 2000). GST is soluble protein having two polypeptide subunit. molecular mass is around 50kDa but in native form its molecular mass is around 30kDa. GST enzyme has an optimum pH of range 7.5 to 8.0 and its Km value is 4.4 and 1.9 mM for reduced glutathione and p-coumaric acid, respectively (Dean *et al.*, 1995). 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 such as binding of flavonoids natural products in the cytosol prior to their deposition in the vacuole. Recent studies have also implicated GSTs as components of ultraviolet-inducible cell signalling pathways and as potential regulators of apoptosis. 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). Key role of GST is inactivation of toxic compounds plant synthesise some harmful secondary metabolites in response to defense against herbivores and pathogens. Detoxification of such harmful compounds is essential for the survival of cells or organisms and GST helping in inactivation of those compounds. GST also protect tissues from oxidative damages damage initiated by hydroxyl radicals which are highly toxic to cells. For GST assay 1-chloro-2, 4- dinitrobenzene (CDNB) used as substrate for most of the GSTs. CDNB conjugates with GST (by chlorosubstitution) and results in absorbanace change of compound at 340 nm in normal spectrophotometric assay (Mannervik *et al.*, 1988).

**Catalase (CAT)**

Catalse ($H_2O_2$ :$H_2O_2$ oxidoreductase, EC 1.11.1.6;) is a tetrameric enzyme which degrade $H_2O_2$. It found in almost all aerobic bacteria, plant and animals. CAT is most active catalyst, it decompose $H_2O_2$ at most rapid rate.CAT maintain the homeostasis $H_2O_2$ in cells which produces in stress condition (Asada and Takahashi 1987). In plant CAT found in multiple isoforms. 30 years ago CAT multiple isoform was isolated from Maize plant were the products of distinct, unlinked genes and was considered by many to be an isolated case. CAT action in plant and animal tissues was first observed in 1818 by Thenard, who noted that such tissues readily degraded hydrogen peroxide, a substance he had also discovered some years earlier (Aebi and Sutter 1971). During oxidative stress the intermediate reactive oxygen species (ROS) reacts with various biomolecules and altering their biological activity. To minimise this damage plants evolved a variety of enzymatic and nonenzymatic mechanisms that can reduce oxidative stress by detoxifying harmful oxygen species. CAT comes under the enzymatic defence and act as antioxidant enzyme which convert harmful $H_2O_2$ in to $O_2$ and $H_2O$. 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. Plant catalases are generally localized in microbodies (Huang *et al.*, 1983, Scandalios, 1994, Willekens *et al.*, 1995). 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).

**Super-Oxide Dismutase (SOD)**

Super-Oxide Dismutase (SOD, EC 1·5·1·1) is main antioxidant metalloenzyme enzyme. It found in two form, one complexed with Cu and another with Zn and it is localizes in cytosol are essential for biological defence against the superoxide anion (Fridovich, 1983). SOD catalyses reactive oxygen species, like hydrogen peroxide by converting them to hydrogen peroxide which is then transformed to water 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) which catalyzes the dismutation or disproportionation of superoxide free radical anions.

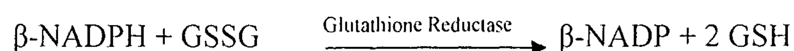


$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H2O2$$

These hydroxyl radicals are thought to be largely responsible for mediating oxygen toxicity *in-ivo*, they can react indiscriminately with DNA, proteins, lipids, and almost any constituent of cells (Halliwell, 1987; Cadenas, 1989; Fridovich, 1989).

**Glutathione reductase (GR)**

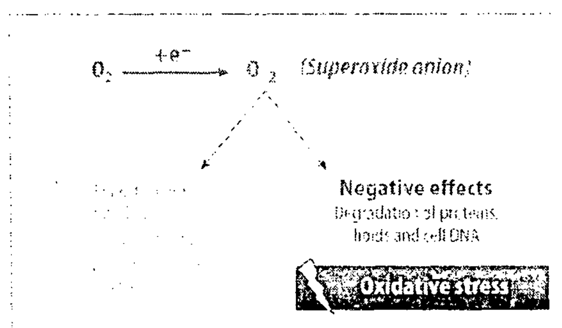
Glutathione reductase (GR; EC 1.6.4.2) catalyzing the reduction of oxidized glutathione disulfide (GSSG) to the reduced glutathione (GSH) by the help of NADPH as an electron donor (Meister and Anderson, 1983). It found both in eukaryotes and prokaryotes and it up regulate in presence various stresses like salinity, drought, high light intensity, mechanical wounding, chilling, exposure to heavy metals, and herbicides (RomeroPuertas *et al.*, 2006). Isoforms of glutathione reductase is also reported some species of plants such as mustard ((Drumm-Herrel *et al.*, 1989), pea (Creissen *et al.*, 1992). The tripeptide glutathione (γ-Lglutamyl- L-cysteinyl-glycine) is the major free 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 (Foyer and Noctor, 2001). Reduced glutathione participates in signalling processes by itself, and also as nitrosoglutathione (GSNO) after its reaction with nitric oxide (del Rio *et al.*, 2003; Barroso *et al.*, 2006). This has been also shown that GR was found upregulated and is 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*. The principle of enzymatic assay of glutathione reductase assay is summarizing as given below in form of equation.



$$\beta\text{-NADPH} + \text{GSSG} \xrightarrow{\text{Glutathione Reductase}} \beta\text{-NADP} + 2\ \text{GSH}$$

When the antioxidant systems of defense are excess with free radicals of oxidative stress may finally add to the development of inflammatory or degenerative diseases

(**Figure 2**). Role of antioxidant enzymes in the inactivation process of superoxide ion is indicated in given figure (**Figure 3**).



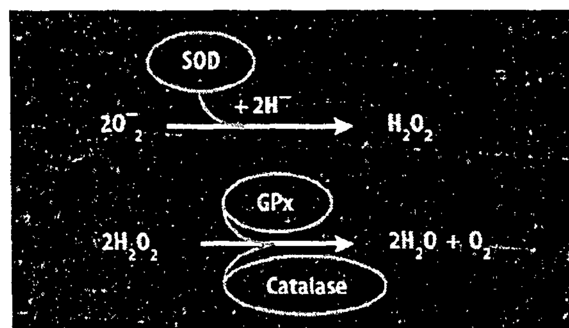




**Figure 2. Effects of oxidative stress**

**Figure 3. Role of antioxidant enzymes in the inactivation process of superoxide ion**

(Adopted from Phytothérapie (2005) Numéro 3: 1-4.)

## PATHOGENESIS RELATED PROTEINS (PR PROTEINS)

Plant does not have immune system as like animals, they don't have any phagocytic cell or circulatory system instead they have tough bark and cell wall. When pathogen attack the plants they shows innate immune response specific to pathogens such as oxidative burst, synthesis of compounds like phytoalexin and pathogenesis-related proteins (PR proteins). All these responses trigger by various bacterial, fungal, viral and non-pathogen attack. It also trigger artificially by using following substances such as salicylic acid, jasmonate (Delaney *et al.*, 1994 ;) and 2, 6-dichloro-isonicotinic acid under Systemic Acquired Resistance (SAR). In these induced responses PR proteins have crucial role in boosting the resistance power of plant against pathogenic attack (Adrienne and Barbara, 2006).

Various antimicrobial proteins are produces by organism to kill the pathogens. There are more than 13 proteins related to pathogenesis are known. Among them antifungal PR proteins of plants are also present which are more important for agro economic point of view (Dempsey *et al.*, 1998). There are 17 families of PR protein currently (Christensen *et al.*, 2002).The PR-1 family consists of proteins with small size (usually 14-17 kD) and antifungal activity. The PR-2 family consists of β-1, 3-glucanases, which are able to hydrolyze β-1, 3-glucans, a biopolymer found in fungal cell walls. The PR-3, -4, -8 and -11 families consist of chitinases belonging to various

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chitinase classes (I–VII) and its substrate. The substrate of chitinases, chitin, is also a major structural component of fungal cell walls. PR-5 family consists of thaumatin-like proteins and osmotin-like proteins. Other PR families include proteinase inhibitors, endoproteinases, peroxidases, 1ribonuclease-like proteins, defensins, thionins, lipid transfer proteins, oxalate oxidases, and oxalate oxidase-like proteins. It have been studied that by over expressing genes of the PR-1, PR-2, PR-3, and PR-5 families mediate host plant resistance to phytopathogenic fungi.

**PR-2 proteins (β-glucanses)**

Plant β-1, 3-glucanases is involved in developmental processes along with pathogen defence. Its molecular mass ranges from 33 to 44 kDa (Hong and Meng, 2004; Saikia *et al.*, 2005). On the basis of amino acid sequence analysis there is three classes of PR-2 protein (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 is processed prior to being enzymatically active. Wound, hormonal signals such as methyl jasmonate and ethylene, pathogen attack such as some fungal elicitors releases from pathogen cell wall (Boller, 1995) can induced β-1,3-glucanases in the various parts of plant (Wu and Bradford, 2003; Saikia *et al.*, 2005). 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 micro molar levels against a wide number of fungi, including human and plant pathogens (e.g., *Rhizoctonia solani, C.albicans*, and *Aspergillus fumigatus*). α-1, 3- glucanases are involves in hydrolytic cleavage of the 1,3-α-D-glucosidic linkages in α-1,3- glucans, a major component of fungi cell wall (Simmons, 1994). So that cell lysis and cell death occur as a result of hydrolysis of glucans present in the cell wall of fungi.

**PR-3 proteins (chitinases)**

PR-3 proteins (chitinases) have a molecular mass range of 26 and 43 kDa (Nielsen *et al.*, 1997; Watanabe *et al.*, 1999). Chitinases can be divided into two categories and five groups. Categories are exochitinases and endochitinases and they

are demonstrating activity only for the non-reducing end of the chitin chain and hydrolyse internal α-1, 4- glycoside bonds respectively. 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. Chitin is the substrate of Chitinases - a natural homopolymer of α-1, 4- inked N-acetylglucosamine residues (Kasprzewska, 2003). PR-3 proteins cleaves the cell wall chitin polymers and resulting in a damaged cell wall (Jach *et al.*, 1995).

**PR-5 (TL) proteins**

PR-5 proteins are also known as Thaumatin-like proteins: a polypeptides class that share homology with thaumatin, sweet protein from Thaumatococcus danielli (Cornelissen *et al.,* 1986). Majority of PR-5 proteins have molecular masses in range of 18- 22 kDa and are stabilized by eight disulfide bonds are highly stabilized structure allows PR-5 proteins to be very resistant to protease degradation (Roberts and Selitrennikoff, 1990). 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. In one study it has been found that thaumatin-like proteins were isolated from cherry, apple and banana shows antifungal activity against Verticillium alboatrum and having endo- α1,3-glucanase activity (Menu-Bouaouiche etal., 2003). The specific function of many thaumatin-like proteins are unkown but they are involve in Acquired Systemic Resistance (ASR) in plants in response to fungal infection causing the inhibition of hyphal growth and reduction of spore germination by a membrane permeabilization mechanism and/or by interaction with pathogen receptors most probably (Thompson *et al.*, 2007).

## ANTIOXIDANTS AND ARBUSCULAR MYCORRHIZAL SYMBIOSIS

Arbuscular Mycorrhizal (AM) symbiosis with plant alters the antioxidant enzymes level during the colonization to build a better plant fungi association. There are several signalling mechanisms are found, which helps plant to recognise pathogen infection and for a proper response against them. Initially 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 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, activation of plasma membrane-bound enzymes, activation of kinases, phosphatases, phospholipases, and production of signal molecules, including active oxygen species and its result in the transcriptional activation of defence-related genes.

During the early stages of AM formation a hypersensitive response and some elements of signal transduction pathways are activated but the plant defense response is weak in early stage of root colonization by AMF and 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). At sites of hyphal tips of AM fungi association in plant where fungi attempted to penetrate a cortical root cell of the plant, an oxidative burst occurs. Accumulation of SA during the early stages of infection also has been observed in the interaction. It is found that $H_2O_2$ level 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 crosslinking 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 peroxidise 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 (Garcia-Garrido and Ocampo. 2002), accordingly 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.

***BOTRYTIS CINEREA: A Cruel fungus***

*Botrytis cinerea* is a very well known pathogenic fungus which is causative agent of Botrytis Grey Mould (**BGM**) disease in several species of plant kingdom. It also causing BGM disease in chickpea (*Cicer arietinum* L.), a very economically important plant especially in areas where cool, cloudy, and humid weather persists. It may leads to complete crop loss in the case of several epidemic conditions (Reddy *et al.* 1988; Pande *et al.* 2002). First case of occurrence of BGM on chickpea was reported in India by Shaw and Ajrekar, 1915. The first epidemic case of BGM was reported by Carranza 1965, in Argentina which resulted in a crop loss up to 95%. Several cases of complete loss of yield of chickpea have been reported in chickpea growing countries. Among various countries this disease is a serious concern in India, Bangladesh. Nepal. Pakistan, Australia. and Argentina (Haware and McDonald 1992, 1993; Bakr *et al.* 1993; Dhar *et al.* 1993; Karki *et al.* 1993; Malik *et al.* 1993; Haware 1998; Pande *et al.* 2002; Davidson *et al.* 2004).

*B. cinerea* is a globally distribute non-specialised pathogen having an extensive host range. More than 235 species of plant from different genera including ornamental plants. vegetables, fruit. field and glasshouse crops, several weeds, and post-harvest produce comes under their host range. It includes species such as black gram, strawberry, grapevine. apple, cabbage, carrot, cucumber, eggplant. lettuce, lentil, mungbean, mustard, paddy, pea. pepper. pigeon pea. squash. tomato, chrysanthemum, dahlia, lily, rose, gladiolus, and tulip (Chand 1997). *B. cinerea* grown on potato dextrose agar media (PDA) having white. cottony appearance, which turns light grey with time. The young hyphae are thin, hyaline, and 8–16 μm wide, and they become brown and septate with age. The conidiophores are light brown, septate, and erect, ramified pseudodichotomically with slightly enlarged tips bearing small pointed sterigmata bearing 1–2-celled, hyaline, oval, or globose conidia forming clusters. Conidia from infected chickpea plants and on PDA measure 4–25×4–18 μm

and 4–16×4–10 μm, respectively (Jarvis 1980; Nene and Reddy 1987; Pande *et al.* 2002).

Asexual stage of necrotrophic fungus *B. Cinerea* is dominant in chickpea plant. They infect mainly aerial part of chickpea and this part is more susceptible with growing tips and flowers being the most vulnerable (Bakr and Ahmed 1992; Grewal *et al.* 1992; Haware and McDonald 1992; Haware 1998; Bakr *et al.* 2002). Although pathological, physiological, and molecular characteristics of *B. cinerea* causing grey mould type diseases on chickpea and several other hosts has been investigated very extensively but the process of infection processes and genetic basis of pathogen variability have not been clearly established.

## *PIRIFORMOSPORA INDICA* - A plant probiotic

*Piriformospora indica* recently discovered from rhizosphere of Thar Desert soil of Rajasthan, India (Verma *et al.*, 1999, Verma *et al.*, 1998). This fungus is axenically cultivable, it colonise with wide range of host plant root without any host specify (Verma *et al.*, 2001). They colonise with all phylum like bryophytes, pteridophytes, gymnosperms and a large number of mono- and dicots plants and the allow them to grow in high physical and nutrient stress (Fakhro *et al.*, 2009, Oelmuller *et al.*, 2009, Prasad *et al.*, 2008, Shahollari *et al.*, 2005, Verma *et al.*, 2001). This fungus invade cortex region of root and colonised there in the rhizosphere zone. They form pear shaped chlamydospores having auto fluorescent in the cortex region during colonization. They grows inter- and intracellular and do not invade endodermis and areal part of plant. *P. indica* after successfully colonization, promote plant to uptake nutrient from soil and allow them to survive in different types of biotic and abiotic stresses, pathogenic attack (Das *et al.*, 2012). This plant fungus association also helps in plant growth like production of biomass, early flowering, seed production (Das *et al.*, 2012, Verma *et al.*, 1998, Yadav *et al.*, 2010).

On the basis of 18S rRNA sequences analysis and electron microscopy it is found that this fungus is related to the Hymenomycetes of the Basidiomycota. It is found that they are showing presence of typical dolipores with continuous non-perforated parenthesomes, which also show that they are belonging to Hymenomycetes of the Basidiomycota. By molecular phylogenetic analysis it is found that *P. indica* is a member of the basidiomycetous order Sebacinales

(Basidiomycota: Agaricomycetes) (Qiang *et al.*, 2012, Weiss *et al.*, 2004). *P. indica* genome has been sequenced and confirmed by blast with eukaryotic conserved genes, they have a complete 24.97 Mb size of genome (Zuccaro *et al.*, 2009, Zuccaro *et al.*, 2011).

*P. indica* appears whitish with inconspicuous zonations, hyphae are thin walled having diameters ranging from 0.7 to 3.5 μm and mycilia are often intertwined and overlap each other. Sometimes hyphae show anastomosis and are irregularly septated that's why many cells contain more than one nucleus. Chlamydospores are formed from thin-walled vesicles at the tips of the hyphae. Chlamydospores are pear-shaped structure and they appear like typically single or in clusters. Spore walls are of 1.5 μm thick, spores appear as two layered: smooth and pale yellow. Chlamydospore cytoplasm appears densely packed with granular materials and usually contains 8–25 nuclei (**Figure 4**). Neither any sexual structure nor any clamp connections are found.

*P. indica* grows very nicely in synthetic Hill- Kafer media (Hill *et al.*, 2001, Pham *et al.*, 2004). Optimum cultural condition for its growth is: temperature 30°C, pH 6.5, inoculum size 5 %, agitation speed 200 rpm, and working volume 30 %. Spore started sporulation after 48 h and spore yield is around of $9.25\times10^7$ spores/ml appears after 60 hrs of growth. Maximum spore yield obtained after 8 days of inoculation. As mentioned above that P.indica help plants in various biotic and abiotic stresses, for this there are several genes involved in resistance to biotic, abiotic stress and nutrients transport. From this root endophyte there is a gene encoding a phosphate transporter (PiPT) belongs to high affinity phosphate transporter family (Pht1) is reported (Yadav *et al.*, 2010) which help plant in absorption of P from soil.

**Table 5. Taxonomic position of *P. indica*.**

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

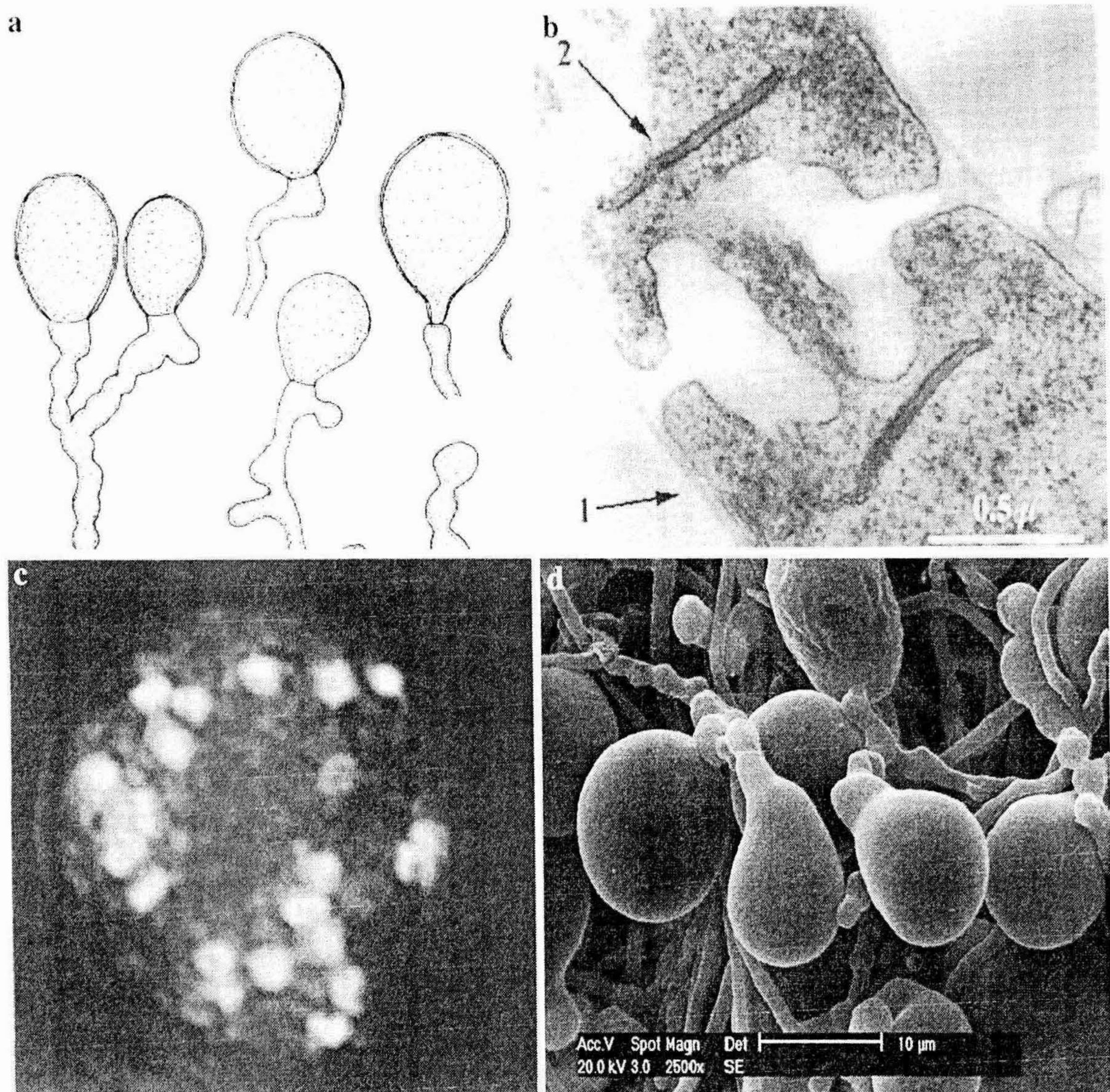



**Figure 4. (a). Chlamydospores of *P. indica* (b). Ultrathin sections of *P. indica* mycelium showing dolipore, parenthesomes (arrow 2) and cell wall (arrow 1) (c). Nuclei in a chlamydospore. Chlamydospores were stained with DAPI and observed by epifluorescense microscopy. Different optical plains were assembled in one picture using the Improvision software package (d). Scanning electron micrograph of chlamydospores of *P. indica*. (Adopted from Agric Res (April–June 2012) 1(2):117–131).**

*P. indica* helps so many plant to overcome abiotic stress like drought in Arabidopsis thaliana and Chinese cabbage (Brassica campestris L. ssp. Chinensis) [Sherameti *et al.*, 2008, Sun *et al.*, 2010] for this following genes are involved in drought and other stresses such as early response to dehydration (ERD1), phospholipase Dd (PLD), salt- and drought-induced ring finger (SDIR1) etc.

*P. indica* helps plants in increased nutrient uptake, confers systemic resistance to toxins, pathogenic organisms, heavy metal ions survival under drought, high- and low-temperature and salt stresses. It also increases the growth and overall biomass production of diverse hosts. In recent study the interaction of this fungus with the plants (Maize plant) in the presence of a pathogen (*Fusarium moniliforme)* is helpful in defense mechanism.

In several studies it has been found that *P.indica* helps plant to cope with biotic and abiotic stresses. *P.indica* associated plants showed a high level of antioxidant enzymes generation in response to increased level of ROS in infected or stressed plants. These antioxidant enzymes save plants from oxidative damages due to generated ROS. *P.indica* inoculated plants showing a strong increase in antioxidative enzymes such as SOD, GR, CAT and GST (Kumar *et al.*, 2009, Harrach *et al.*, 2013). They also reported that per inoculated plant with *P.indica* showed a enhance level of antioxidant enzymes in case of plant post inoculated with Fusarium species as compare to control plant only inoculated with Fusarium. They also proposed that the increased level of antioxidant enzymes helps plants to overcome the infection and stresses and enhance the plant growth by checking the ROS generation in plants.

From the literature cited here in it is clear that role of *P. indica* in protection against *B. cinerea* is not reported, therefore, present study is undertaken to study the mechanism of defense response mediated by *P. indica* in the presence of a plant parasite like *B. cinerea.*

# 3. Work plan

# WORK PLAN

Colonization study of *P.indica* with chickpea plant.

Study of biomass yield of plant in presence of symbiotic fungus *P. indica* and parasitic fungus.

Study of antioxidant enzyme in plant colonized with *P. indica* and effect of parasitic fungus on enzyme activity.

Study of plant associated *P. indica* in induction of strong plant defence response.

# 4. Materials and Methods

**Plant Material**

Chick pea (*Cicer arietinum*) : PUSA 1105 variety (Obtained from IARI, New Delhi).

**Fungal Material**

*Piriformospora indica* : A newly discovered root symbiotic fungus (Obtained from Prof. Ajit Varma).

*Botrytis cinerea* : A parasitic fungus (Obtained from Dr. Rupam Kapoor. Botany Department. University of Delhi, North Campus, Delhi).

**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.

| Material | Source |
|---|---|
| *Coomassie Brilliant Blue, Coomassie blue G 250* | *Sigma* |
| *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* |
| *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 (MillexTM, Millipore, USA).

**Maintenance of Fungal Culture**

*P. indica* and *B. cinerea* were maintained routinely on solidified Aspergillus medium (Hill and Kaefer. 2001) and Potato dextrose agar (PDA) solid media or Potato dextrose broth (1.5%) liquid media respectively. The inoculated Petri plates were incubated for 7-10 days at 30±2 °C for *P. indica* and 15-20 days at 20-22 °C for *B. cinerea* in both solid and liquid media.

**Seed Surface Sterilization and Germination**

Chickpea seeds (*Cicer arietinum*) surface effectively sterilized in 2.5% sodium hypochlorite solution containing 5 - 6 drops of Tween-20 for 30 min. The seeds were then washed in sterile distilled water for 3 - 4 times. Surface sterilized seeds then imbibed in sterile distilled water overnight. The next day, the remaining water is discarded and the seeds were blot dried on a sterile filter paper. Seeds germinated by placing 10 seeds on each Petri plate containing wet germination paper and incubated at 23±2°C (Aktubul *et al.*, 2008).

**Plant Fungus Interaction**

Surface sterilized pre-germinated chickpea 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 Kumar *et al.*, 2009 whereas in control plants, autoclaved $dH_2O$ was used. Initially the plants were inoculated with *P. indica* by direct mixing of culture in sterile soil and by spraying *Botrytis* grown culture suspension on aril part of plant and allow one set of plants to grow without any fungus. In order to study the bioprotection and protective potential, all plants were initially grown for 10 days, and subsequently the following sets of plants were used: (1) chickpea plants grown for 30 days without any fungus (control); (2) chickpea plants inoculated with *P. indica* alone at day 0 and grown for 30 days; (3) chickpea plants infected with *B. cinerea* alone at day 0 and grown for 30 days; (4) chickpea plants first infected with *B. cinerea* at day 0 and at day 10 inoculated with *P. indica* and grown for a total of 30 days; (5) chickpea plants first inoculated with *P. indica* at day 0 and at day 10 infected with *B. cinerea* and grown for a total of 30 days; (6) chickpea plants grown simultaneously with both fungi inoculated/infected at day 0 and grown for 30 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 *B. cinerea* to infected plants with *B. cinerea* . 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**

Chickpea plants were grown in a controlled environment in green house at 23±2°C temperature with 12 hours photoperiod and relative humidity 60-70%, with light intensity of 1000 Lux. Plants were watered twice and fertilized with Hoagland solution (Arnon and Hoagland, 1940) once in week. Plant samples 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 destined in lactophenol (Dickson *et al.*, 1998; Phillips and Hayman, 1970). Observation was done under light microscope (Leica Microscope, Type 020-518.500, Germany). Percent colonization was calculated for the inoculated plants using the following formula (McGonigle *et al.*, 1990, Kumar *et al.*, 2009).

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

**Antibiosis assay of *P. indica* and *B. cinerea***

To demonstrate the antibiotic activity *P. indica* and *B. cinerea* 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.

**Determination of plant biomass yield (Fresh and Dry weight)**

**Fresh weight**: Plant removed from soil was washed properly by water and plants surface moisture were removed by soft tissue paper and weighted on weighing machine immediately.

**Dry weight**: Plants removed from soil were washed properly by water and plant surface moisture was dried by soft tissue paper. Plants were dried in oven at $80^0$C for 72 h and then weighted on weighing machine immediately. For each analysis 6 samples were taken.

**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-Stransferase (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 formula bellow-

**Specific Activity = V. ΔOD420. Dilution / (0.5ΔODblank) .v. protein Concentration**

Whereas v = Sample volume V = Reaction Mixture Volume

**NOTE:** Due to auto-oxidation of Pyrogallol **ΔOD420** is represented as

**ΔOD420 = ΔOD420 sample- ΔOD420 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 formula bellow-

**Specific Activity = V. ΔOD240. / E. v. Protein concentration**

Whereas E= 40 mM-1cm-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 millilitre 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 formula bellow-

**Specific Activity = V. ΔOD340. / E. v. protein concentration**

Whereas E= 9.6mM-1cm-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 formula bellow-

**Specific Activity = V. ΔOD340. / E. v. protein concentration**

Whereas E= 6.22 mM-1cm-1 v = Sample volume V = Reaction Mixture Volume

**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). Laminarins were incorporated at a final concentration of 0.6 mg/ml directly in the separation gel for the Davis system. After electrophoresis gel was incubated in 50 mM sodium acetate buffer, pH 5.0, for 3 h at

37°C. β- 1, 3-Glucanase activities was obtained by gels were revealed by staining the gel 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 Statistical Analysis

### Prediction of theoretical isoelectric point (*pI*) and molecular weight (*Mw*) of β-1,3-Glucanase of Chickpea plant.

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). All graphs were created and statistical calculations were performed using Microsoft office excel 2007. Significance of the data obtained was checked with student *t*- test and ANOVA by using program Sigma plot v11.0

# 5. Results

## Colonization study of *P. indica* with chickpea plant and plant Growth

Surface sterilized chickpea seeds, *P. indica* culture grown in KF media and *B. cinerea* culture grown in PDA media are shown in **Fig. 5 a, b and c.** In case of *B. cinerea* we observed cottony grayish appearance and in case of *P.indica* it's appears ball shaped structure.

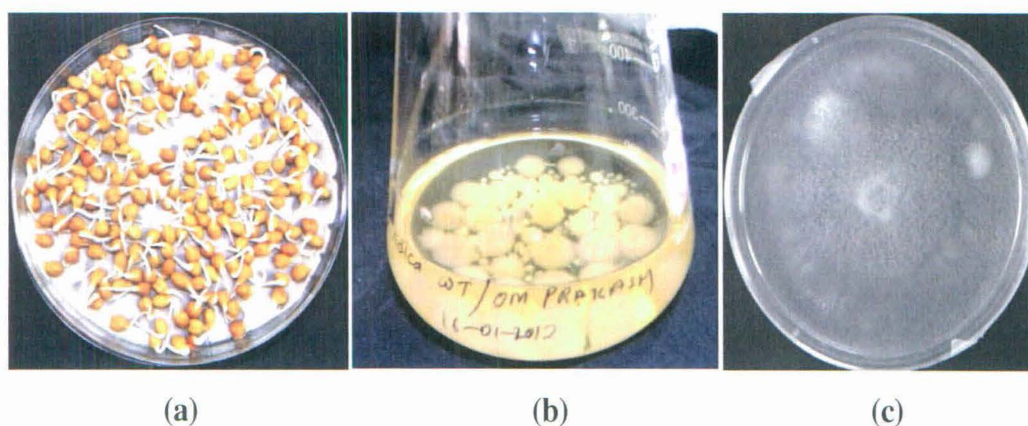
The fungal inoculation treatments showed different levels of effectiveness in improving the growth of the chickpea plant. Initially it was observed that the fungal spore germinates and enter root through root hair or other root surface within 5 to 7 days. After 5 days period it was observed that fungal mycelium grow intercellular in the root cortex and forms a network of hyphae. Colonization of fungus depends on the size of inoculum and time. Using one gram of *P. indica* fresh culture per pot, we have found 7-9 % colonization for 5-7 days, and at the end of 30 days approx 65% colonization was observed **(Table 7)**. Colonization of fungus characterized with intracellular densely packed pear shaped chlamydospores (**Fig. 6**).

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

We have observed dramatic morphological alteration in root of plant initially and later in shoot also colonized *P.indica*. Colonization results in an increase in the length of primary, seminal, crown and lateral roots of chickpea 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. 11a. and 7b.)**. The shoot length and leaves broadness were significantly increases in response to *P. indica* colonization **(Fig. 7a.)**. Effect of colonization *P. indica* stimulate the total fresh and dry weight of plant, which shows 135 % and 110 % increase respectively as compared to control plant (non-colonized) **(Fig. 8a. and 8b)**. Phenotypic growth pattern of plant shoots and roots biomass of chickpea inoculated with *P. indica* also visible at different growth stages of plant **(Fig. 9a-c)**.

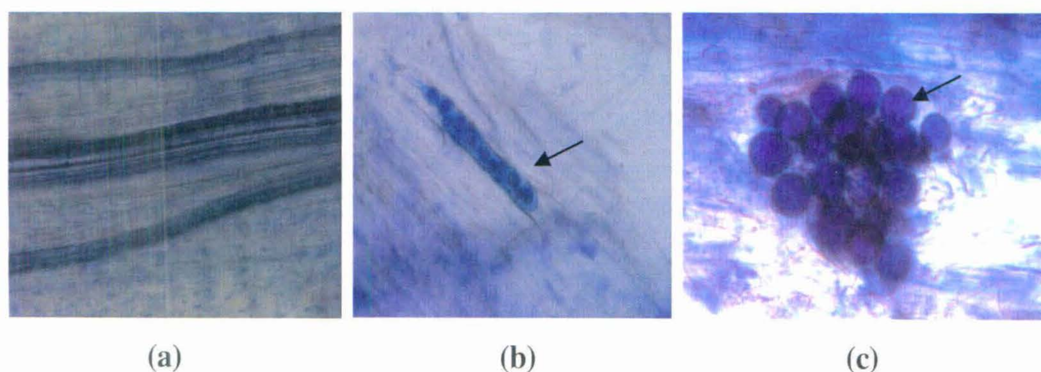
**Table 7. Percent Colonization of *P. indica* in chickpea root.** Plants were inoculated with *P. indica* culture (1 gm/pot) and roots were harvested at 5, 15, 30 days after inoculation. Roots were chopped into ~1 cm pieces and observed for fungal structure (chlamydospores) under light microscope.

| Time After Inoculation (Days) | % Colonization |
|---|---|
| 5 | 8 |
| 15 | 28 |
| 30 | 65 |




(a) (b) (c)

**Figure 5.** (**a**) Surface sterilized & germinated chickpea seeds (**b**) *P. indica* culture grown in KF media (**c**) *B. cinerea* grown in PDA media.



(a) (b) (c)

**Figure 6. Colonization of *P. indica* in chickpea root (Histochemical analysis):(a)** Fungus first establishes itself in root tissue, control (**b**) Trypan blue staining of chickpea plant roots showing intracellular *P. indica* chlamydospores observed at day 5, arrow indicating a few chlamydospore of *P.indica* in plant root after 5 days of inoculation. (**c**) Trypan blue staining of chickpea plant roots showing intracellular *P. indica* chlamydospores observed at day 30, arrow indicating the densely packed chlamydospore of *P. indica* in cortical cells of root tissue after 30 days of inoculation.

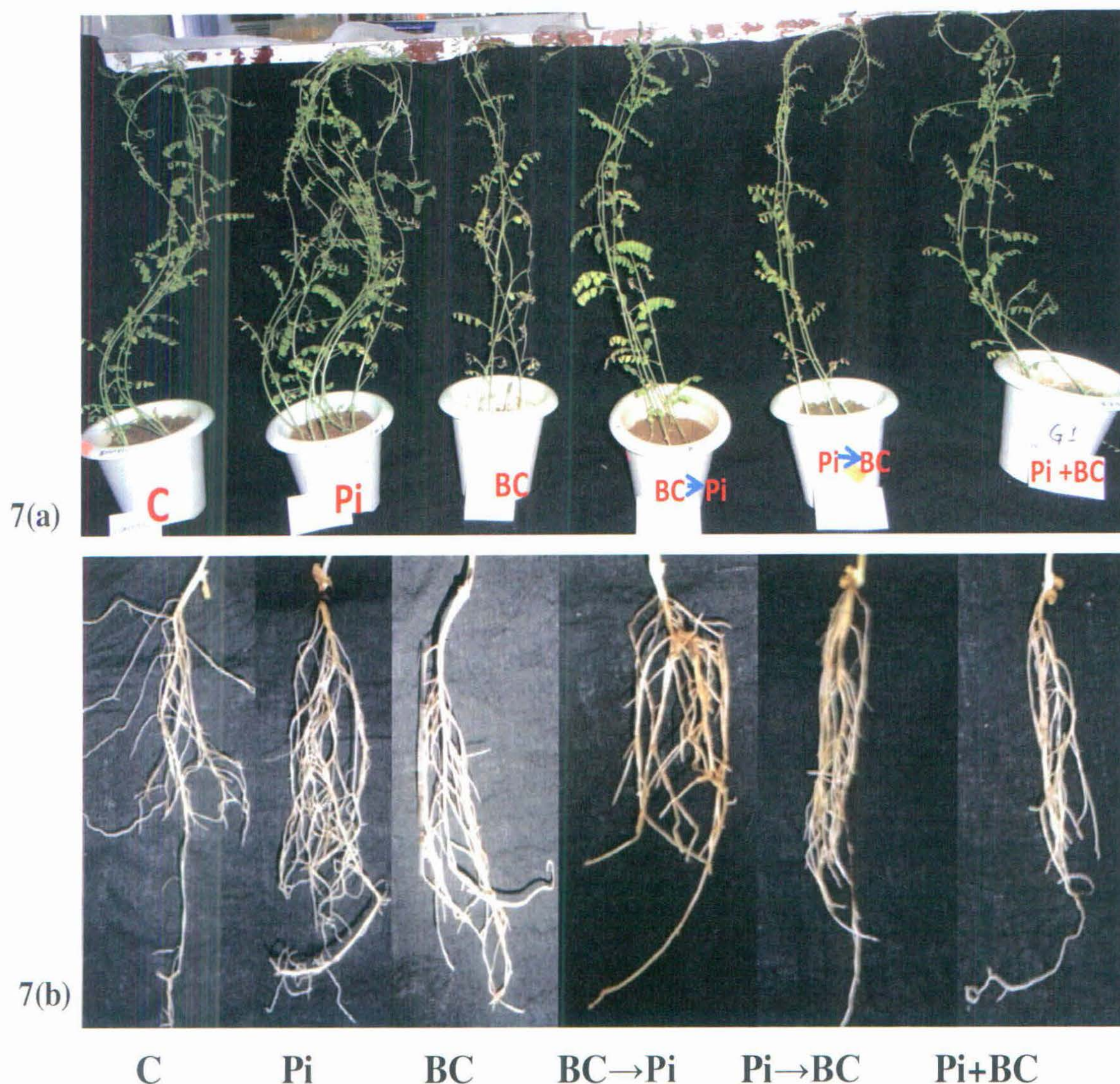
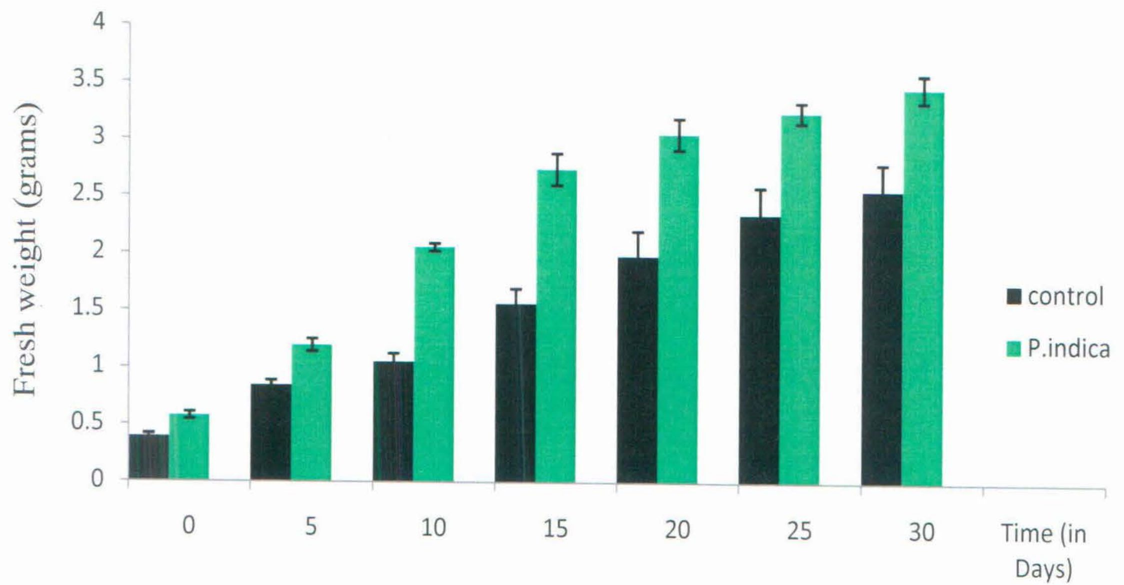
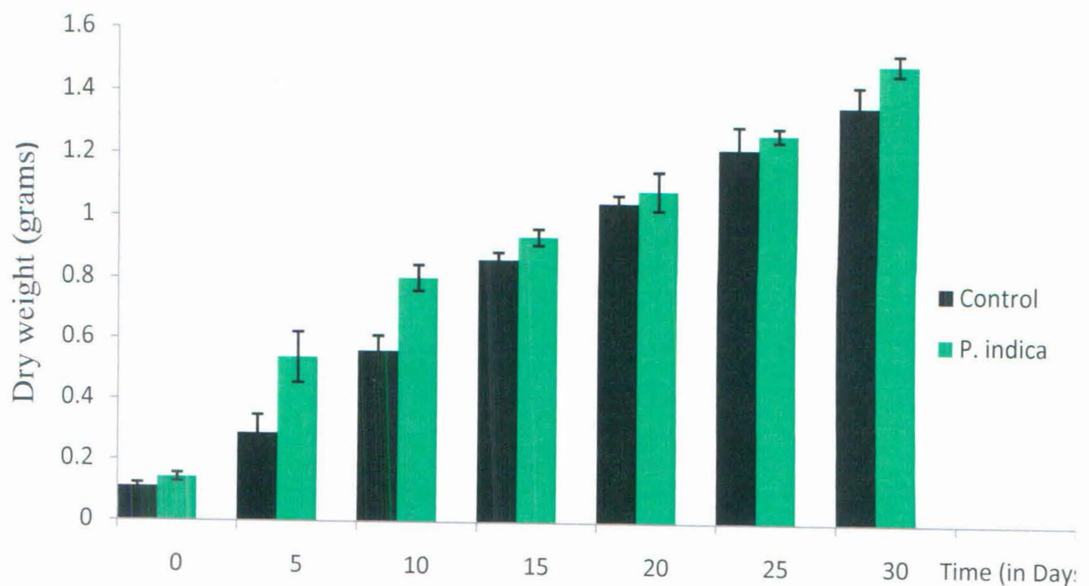



**Figure 7(a). Plant shoot phenotype demonstrating the bioprotection role of *P. indica* against *B. cinerea*** ; **(C)** Chickpea plants grown for 30 days without any fungus were used as control; **(Pi)** Chickpea plants inoculated with *P.indica* alone at day 0 and grown for 30 days. **(BC)** Chickpea plants infected with *B. cinerea* alone at day 0 and grown for 30 days; **(BC→Pi)** Chickpea plants first infected with *B. cinerea* at day 0 and at day 10 inoculated with *P.indica* and grown for a total of 30 days; **(Pi→BC)** Chickpea plants first inoculated with *P.indica* at day 0 and at day 10 infected with *B. cinerea* and grown for a total of 30; **(Pi+BC)** Chickpea plants inoculated/infected simultaneously with fungi at day 0 and grown for 30 days.

**Figure 7(b). Plant root phenotype demonstrating the bioprotection role of *P. indica* against *B. cinerea***:-**(C)** Chickpea plants grown for 30 days without any fungus were used as control; **(Pi)** Chickpea plants inoculated with P.indica alone at day 0 and grown for 30 days; **(BC)** Chickpea plants infected with *B. cinerea* alone at day 0 and grown for 30 days; **(BC→Pi)** Chickpea plants first infected with *B. cinerea* at day 0 and at day 10 inoculated with P.indica and grown for a total of 30 days; **(Pi→BC)** Chickpea plants first inoculated with P.indica at day 0 and at day 10 infected with *B. cinerea* and grown for a total of 30; **(Pi+BC)** Chickpea plants inoculated/infected simultaneously with fungi at day 0 and grown for 30 days.

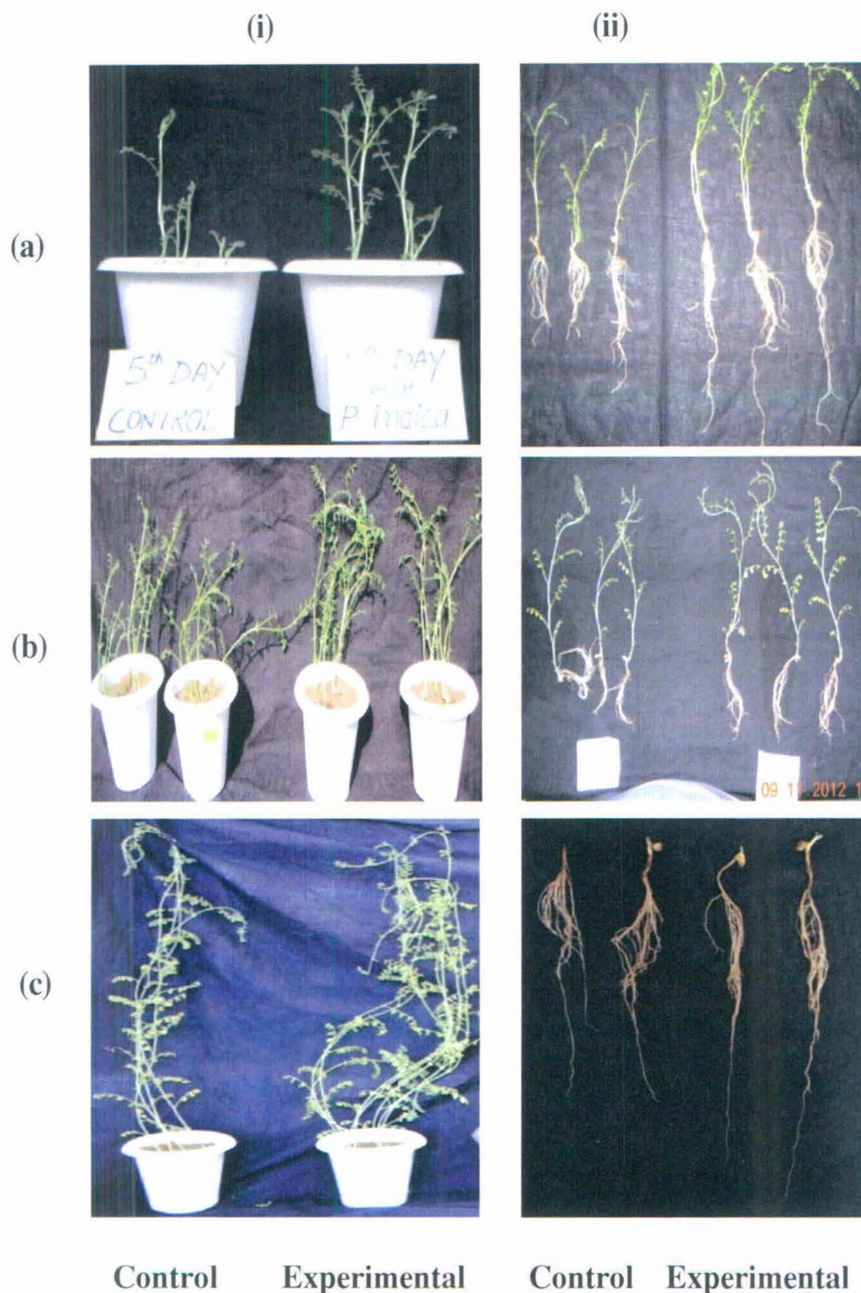



(a)




(b)

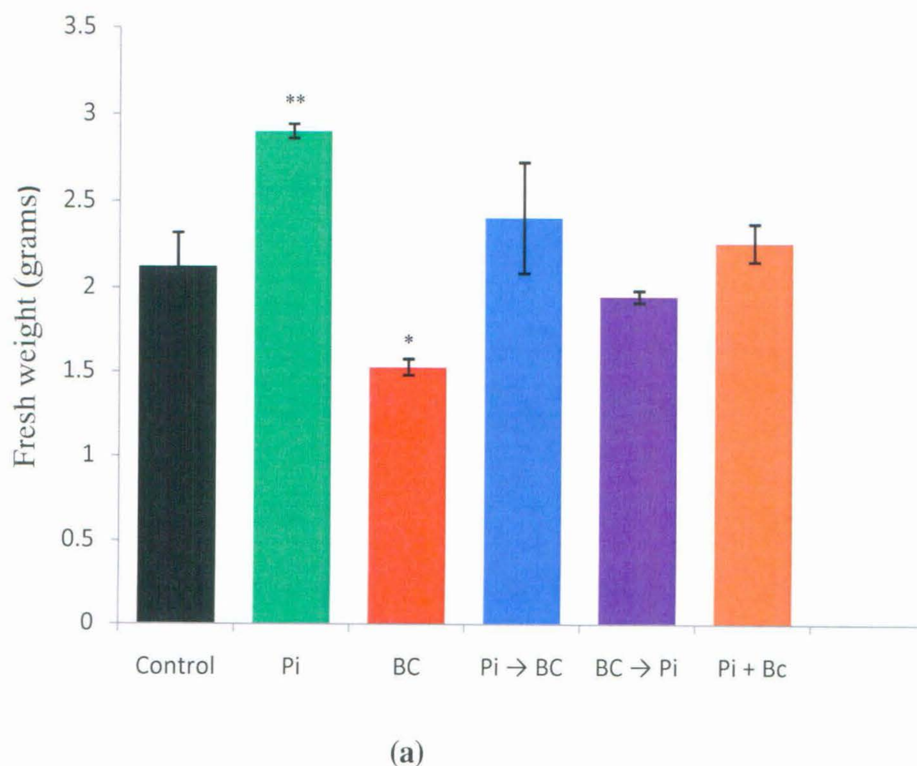
**Figure 8. Impact of inoculation of *P. indica* on biomass yield.** Growth promoting effect of *P. indica* on Fresh weight (**a**) and Dry weight (**b**) of *P. indica* colonized and control plants were determined every 5 days; shows value significantly different from those of the controls ($P$<0.05).




**Control-** Chickpea plant without *P. indica*
**Experimental**- Chickpea plant inoculated with *P. Indica*

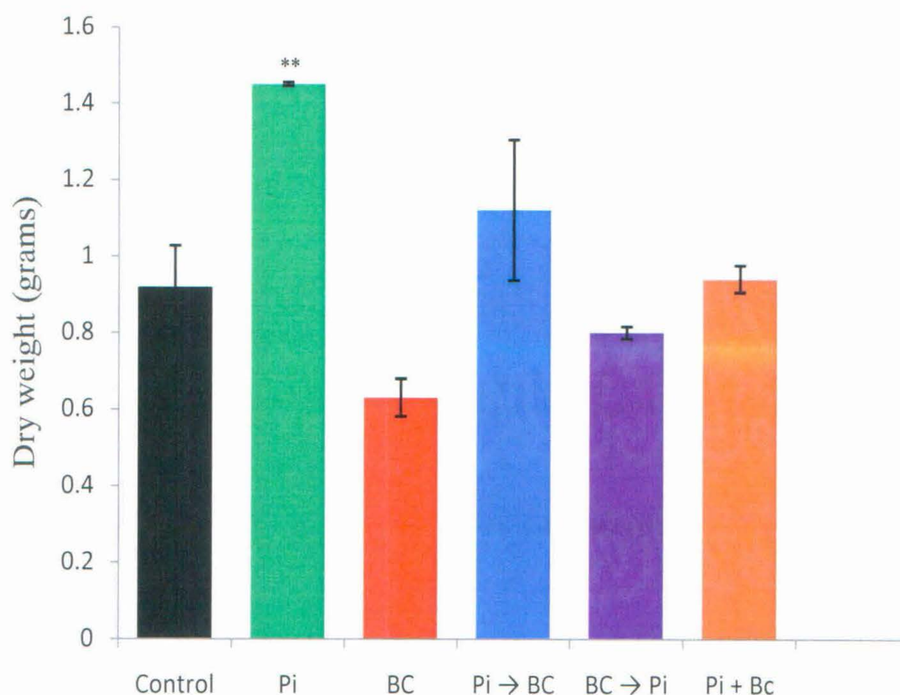
**Figure 9. Chickpea plant interaction with *P. indica*.** **(a)(i) &(a)(ii) ;** Plant shoot and root phenotype demonstrating the biomass growth enhancement of *P. indica* with chickpea. Plants grown for 5 days without any fungus were used as a control and chickpea plants inoculated with *P. indica* alone at day 0 and grown for 30 days). **(b)(i) &(b)(ii);** Plant shoot and root phenotype demonstrating the biomass growth enhancement of *P. indica* with chickpea. Plants grown for 15 days without any fungus were used as a control and chickpea plants inoculated with *P. indica* alone at day 0 and grown for 15 days. **(c)(i) &(c)(ii);** Plant shoot and root phenotype demonstrating the biomass growth enhancement of *P. indica* with chickpea, plants grown for 30 days without any fungus were used as a control and chickpea plants inoculated with *P. indica* alone at day 0 and grown for 30 days).

The effect of *P. indica* on colonization of *B. cinerea* in the plant root was dynamic and shows an induced variation in roots integrity, Branching index. (Number of root ranches/cm.) and biomass yield. We found that the inoculation of *P. indica* in plants pre-colonized with *B. cinerea* recovers the biomass that is 128% and 127% for fresh and dry weight respectively than *Botrytis* infected control with comparable to control plant without any fungus **(Fig.10a and 10b.)**, similarly there is improvement in the root system integrity and Branching index (number of root branches/cm.) **(Fig.7 and 11)**. We have observed 148% and 149% increase in fresh and dry weight respectively of plants simultaneously inoculated with *P. indica* and *B. cinerea* as compared to the plants infected with *B.cinerea* **(Fig.10a and 10b.)**. However inoculation of *B. cinerea* in plants pre-colonized with *P. indica* does not affect the root integrity and branching index significantly while the biomass yield was affected with 23% decrease in dry weight as compared to the plants colonized with *P.indica* only **(Fig.10a and 10b.)**. *P. indica* show no growth inhibitory effect against *B. cinerea* and vise -versa **(Fig 12)**.



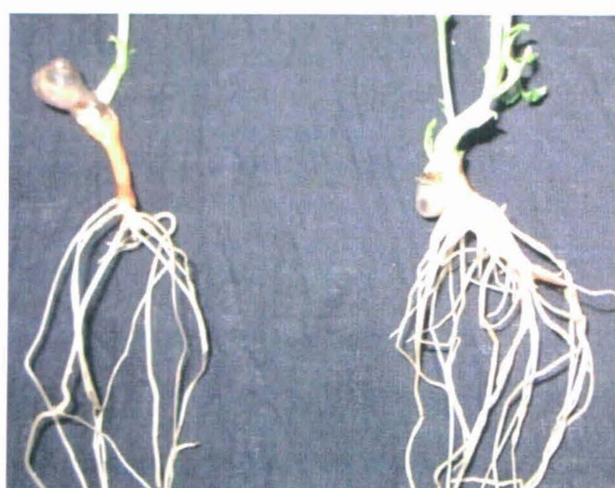

(a)

**Figure 10. (a) Impact of simultaneous and alternate inoculation of *P. indica* and *B. cinerea* on Biomass yield.** Change in biomass yield (Fresh weight) relative to *P. indica* and *B. cinerea* colonized and control plants were determined at 30 days. All experimental conditions were the same as described for fig. **7a** and **7b;** asterisks shows value significantly different from those of the controls (**$P$<0.01, *$P$<0.05).



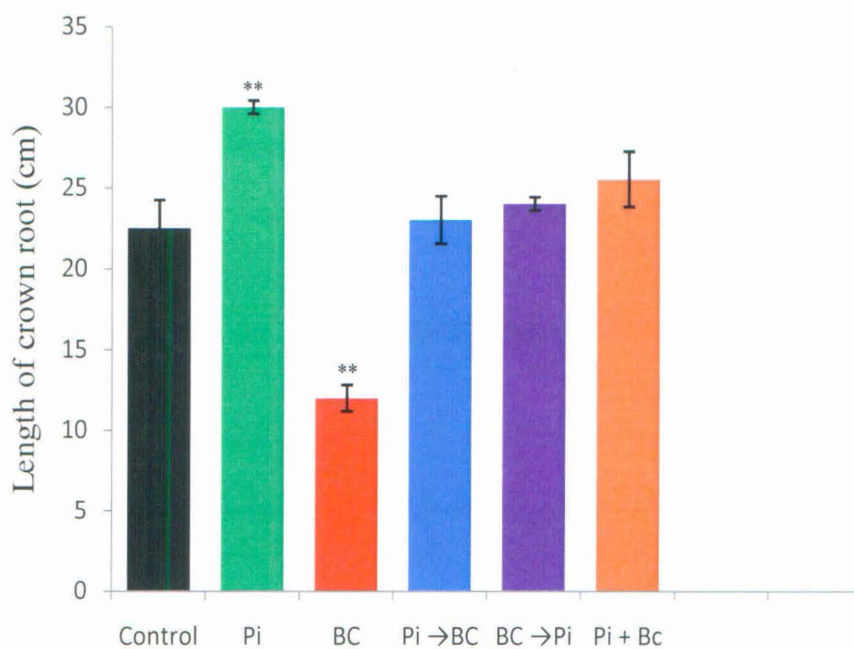

(b)

**Figure 10. (b) Impact of simultaneous and alternate inoculation of *P. indica* and *B. cinerea* on Biomass yield.** Change in biomass yield (Dry weight) relative to *P. indica* and *B. cinerea* colonized and control plants were determined at 30 days. All experimental conditions were the same as described for fig. **7a** and **7b;** asterisks shows value significantly different from those of the controls (**$P<0.01$).

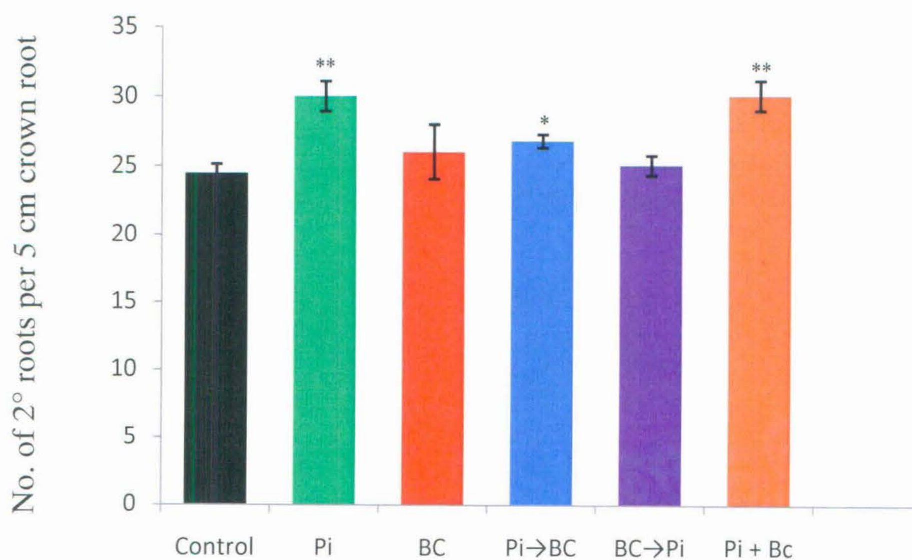


**11(a) Control** **Experimental**

**Figure 11(a). Different morphological root growth pattern in case of Chickpea plant inoculated with *P. indica* and *B. cinerea* in different experimental condition as described for figure 8a and 8b.** Control- chickpea plant without *P. indica*. Experimental – Chickpea plant inoculated with *P. Indica*.

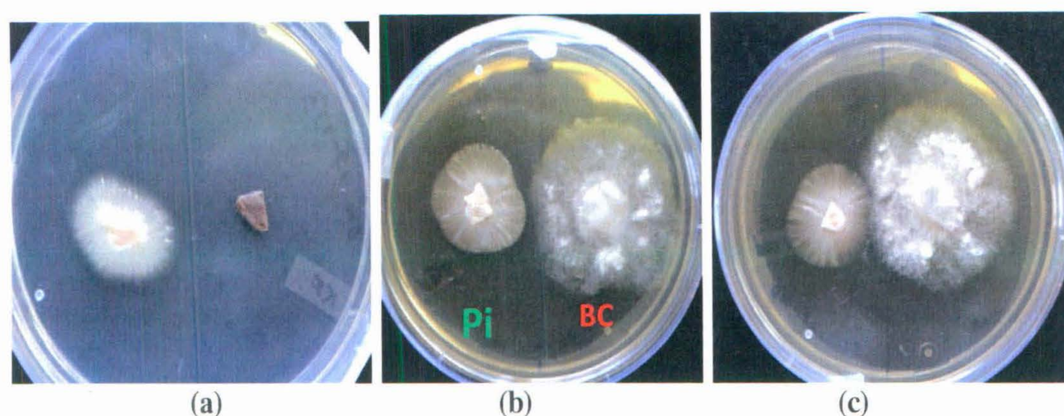



**11(b)**




**11(c)**

**Figure 11(b),(c). Different morphological root growth pattern in case of Chickpea plant inoculated with *P. indica* and *B. cinerea* in different experimental condition as described for figure 8a and 8b. (b)** Change in length of crown roots. **(c)** Number of secondary roots; asterisks shows value significantly different from those of the controls (**$P$<0.01, *$P$ <0.05).




(a) (b) (c)

**Figure 12. Antibiosis assay for antibiotic secretion and growth inhibition effect of *P. indica* (Pi) and *B. cinerea* (BC).** (From left to right, images were taken at 5, 10 and 15 days).

## Change in Activity of Antioxidant enzymes in root and shoot of chickpea plant colonized with *P. indica* and effect inoculation of *B. cinerea* on enzyme activity

Inoculation of *P. indica* in chickpea plants significantly alters the level of antioxidative enzymes and is depend on colonization. We have found the activities of GST **(Fig. 13a),** GR **(Fig. 13b),** CAT **(Fig. 13c)** and SOD **(Fig. 13d)** were constantly increases during the colonizing process till 30 days in the root and were 34, 76, 26 and 19 fold higher than control respectively, interestingly it was found that this unusual increase in enzyme activity remain constant after the 30 days but was significantly higher than control. In case of shoot, the activities were observed are 48, 14, 18 and 18 fold higher after 30 days than control for GST **(Fig. 14a),** GR **(Fig. 14b),** CAT **(Fig. 14c)** and SOD **(Fig. 14d)** respectively. Inoculation of *B. cinerea* in 10 days old plant pre-inoculated with *P. indica* show 18 and 26 fold for GST, 44 and 10 fold for GR, 10 and 15 fold for CAT, 19 and 40 fold increase for SOD Activity than control plant without any fungus in root and shoot respectively **(Table 8).**

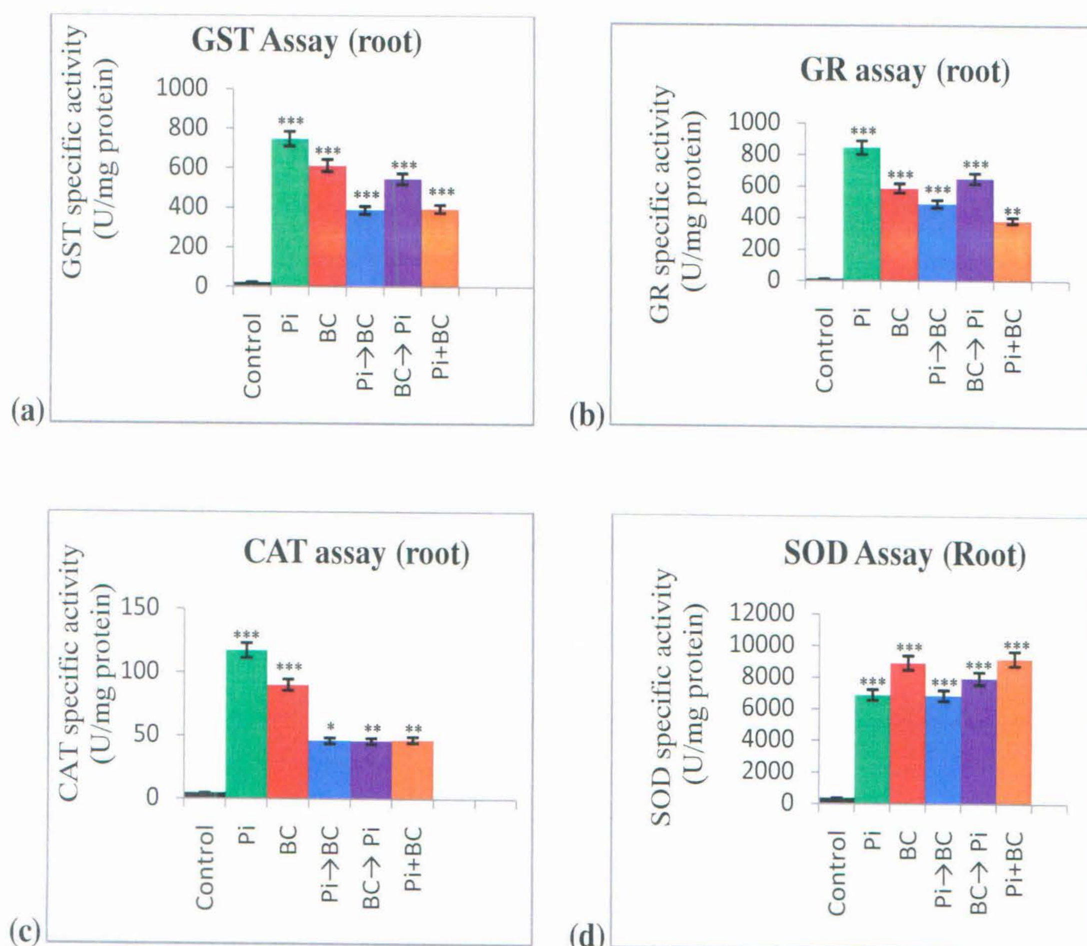



**Figure 13. Effect of inoculation of *P. indica* and *Botrytis cinerea on* antioxidant enzyme activities in chick pea roots.** (**a**) GST, (**b**) GR, (**c**) CAT, and (**d**) SOD specific activities compared with those of control plants; asterisks shows value significantly different from those of the controls(***$P$<0.001,**$P$<0.01, *$P$<0.05).

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

| State of Affairs of inoculation | GST | | GR | | CAT | | SOD | |
|---|---|---|---|---|---|---|---|---|
| | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot |
| Pi | 34 | 48 | 76 | 14 | 26 | 28 | 19 | 18 |
| BC | 28 | 38 | 52 | 15 | 20 | 29 | 24 | 113 |
| Pi → BC | 18 | 26 | 44 | 10 | 10 | 15 | 19 | 40 |
| BC → Pi | 25 | 31 | 59 | 11 | 10 | 14 | 21 | 55 |
| Pi + BC | 18 | 30 | 35 | 7 | 10 | 23 | 25 | 47 |

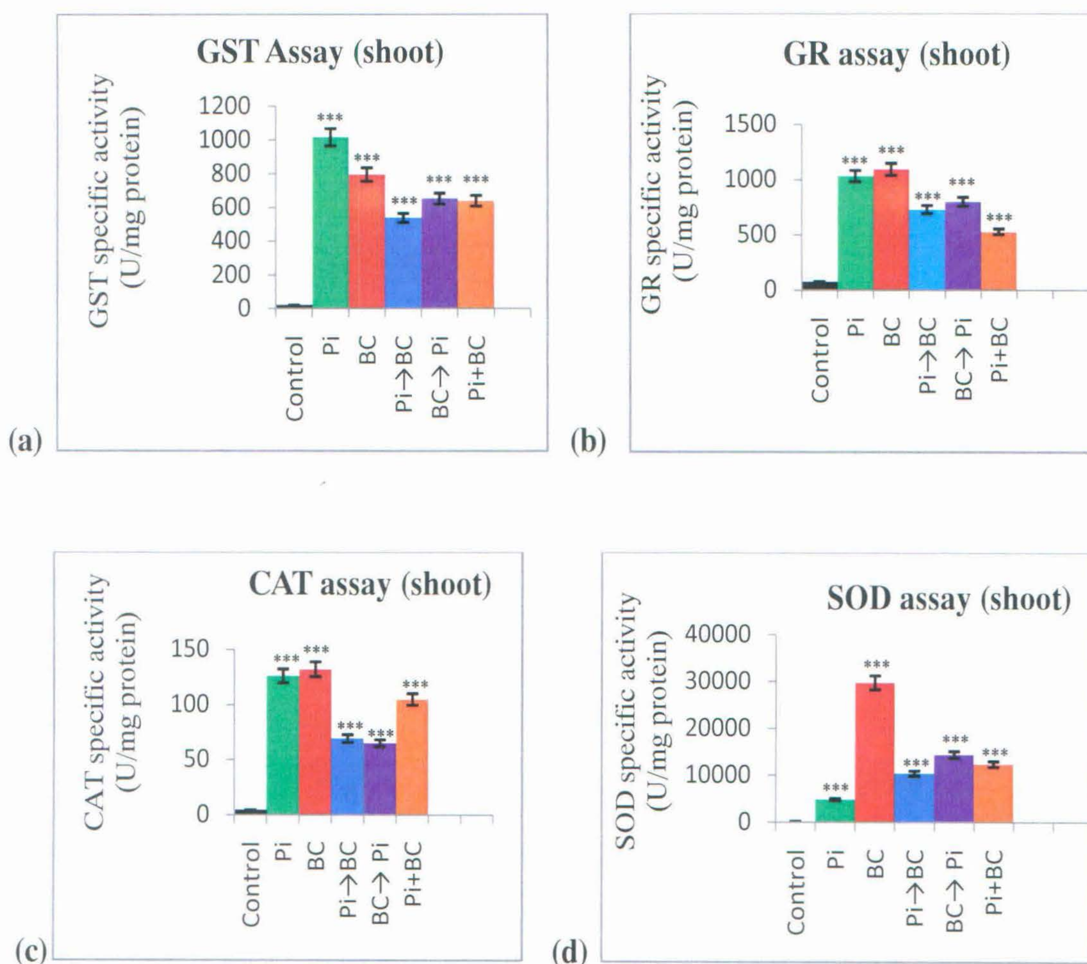



**Figure 14. Effect of inoculation of *P. indica* and *Botrytis cinerea* on antioxidant enzyme activities in chick pea shoots.** (a) GST, (b) GR, (c) CAT, and (d) SOD specific activities compared with those of control plants; asterisks shows value significantly different from those of the controls(***$P$<0.001).

**Antioxidant enzyme activity in root and shoot of chickpea plant infected with *B. cinerea* in response to colonization of *P. Indica*.**

In case of root it was observed that chickpea plant colonised with *B. cinerea* showed a 52 fold increased GR activity as compared with non-colonised plant (**Fig.13**). Under similar condition we found increased activity i.e. 28, 20, and 24 fold for GST, CAT, and SOD respectively (**Fig.13**).Inoculation with *P. indica* of plant previously colonised with *B. cinerea* resulted in a 10 and 3 fold decrease in CAT and SOD activity respectively, which was found significant (**Fig.13**) However, we did not

find a significant difference in SOD activities in plant previously colonised with *P.indica* and later colonised with *B. cinerea* and plants colonised with *P. indica* alone.

In case of shoot chickpea plant colonised with *B. cinerea* showed a 113 fold increased SOD activity as compared with non-colonised plant with significance (**Fig.14**). Under similar condition we found increased activity i.e. 38, 15, and 29 fold for GST, GR, and CAT respectively **(Fig.14)** as compare with non- colonised plants.

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

In case of 10 days old plants inoculated with both *P. indica* and *B. cinerea* simultaneously, continuously increases in the activities of all four enzymes till 30 days in root and this was comparable to enzyme activities in plants colonized with *P. indica*. Activities were found 10 and 23 folds for CAT, 35 and 7 folds for GR, 18 and 30 fold for GST and 25 and 47 folds for SOD as compare to control respectively in root and shoot (**Table 8**).

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

By using the Davis system, separation of acidic and neutral proteins from chickpea root and shoot extracts enables detection of one main band with β -1, 3-glucanase activity in chickpea roots colonized with *P. indica* or *B. Cinerea* corresponding to constitutively expressed isoforms. The activity of constitutive isoforms appears to increase with *P. indica* (**Fig. 15, lanes BS and BR).** New isoforms were induced in *P. indica*-colonized roots and shoot respectively (**Fig. 15)** No β -1, 3-glucanase activity was detected in protein extracts from plant root infected with *B. Cinerea.* The isoforms which has higher electrophoretic mobility than constitutive isoforms, induced highly only in shoot colonized with *B. cinerea.* Isoforms with high mobility expressed in plants colonized with *P. indica* and *B. Cinerea.*

We observed Botrytis Grey Mould (BGM) disease in chickpea plant caused by *B. cinerea* infection **(Fig 16).**

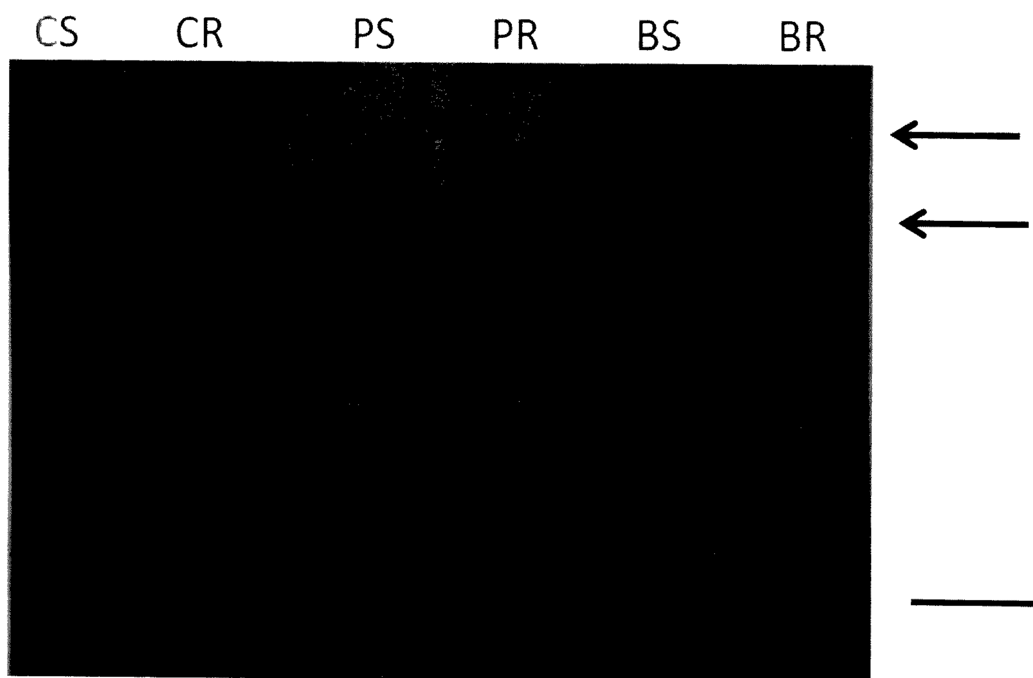



**Figure15. Β-Glucanase activity in root and shoot of plant colonized with *P. indica* and *B. cinerea*.** Acidic β-1, 3-glucanase activities after separation of proteins by the Davis system in 15 % (w/v) with 15μg protein. Polyacrylamide gels containing soluble β-glucans (Laminarin from *Laminaria digitata*, SIGMA) as substrate. Extracts from *P. indica* colonized root (PR), shoot (PS), *B. cinerea* infected root (BR) and Shoot (BS), control root (CR) and shoot (CS) of chickpea plants after 30 days of growth, either inoculated with *P. indica* or *B. cinerea* . Constitutive isoforms are indicated by bars and additional isoforms by arrows.

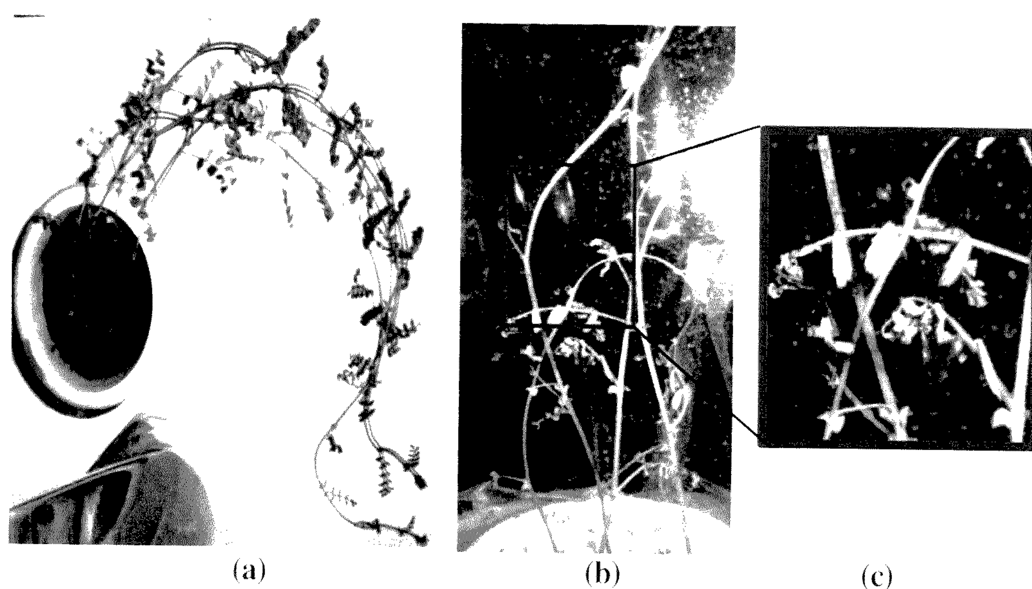



**Figure 16. Chickpea plant infected with *B. cinerea* showing symptom of Botrytis grey mould (BGM) disease (a)** Control plant, **(b)** *Botrytis* infected chickpea plant **(c)** close up of picture b.

# 6. Discussion

The microbial symbiotic relation with plants is very important for both the organism which helps them to cope with the environmental adverse condition. Microbes help host plant from both biotic and abiotic stresses. Particularly Arbuscular Mycorrhizal fungi (AMF) association enhance the host growth and tolerance to various diseases, soil borne pathogens, biotic and abiotic stresses. (Gosling *et al.*, 2006; Harrier & Watson, 2004). They also help in improving the crop yield. Therefore microbial interaction is beneficial for better survival of host plant. Some fungi compensate the use of chemical fertilizers for increasing the crop productivity and hence promote the bio safety. *P.indica* mimics the AMF and has strong association with a large band of plant species. It is newly discovered and it will be powerful innovative symbiotic component for enhancement of plant growth activities. Presently plant nutrition, defence and tolerance with various stresses is our top challenges and it has been studied that *P. indica* is helping effectively. *P indica* has ability to colonize with variety of unrelated host and it will be a putative bio control agent and bio fertilizer (Varma *et al.*, 1999; Waller *et al.*, 2005).

In present study we have studied the role of *P. indica* in growth promoting activities i.e. biomass yield as well as it role in bioprotection against the fungal pathogen *Botrytis cinerea* which causes Botrytis Grey Mould (BGM) disease in very economically important chickpea plant. In this study we found that chickpea plant colonised with *P. indica* showed increased biomass production as compared with non-colonised plants. This indicates the mycorrhiza like growth promoting activity of *P. indica*. Similar results have been also found by Kumar *et al.*, (2009) during the interaction between *P. indica* and maize plant infected with *Fusarium*, which supports our data.

The colonization of *P. indica* with chickpea plant lowers the infection pressure of *B. cinerea* as compare with non colonised plants. We found that simultaneous and alternate inoculation of *P. indica* and *B. cinerea* helped in improvement of plant growth, biomass increments. The role of *P. indica* against the leaf parasite *B. graminis* has been shown by Waller *et al.*, (2005). In present work we observed that chickpea pre inoculated *B. cinerea* and at day 10 inoculated with *P. indica* showed interesting bio protective property of *P. indica*. Our result showed the decreasing disease load of shoot infecting parasite *B. cinerea* over chickpea plant due to

colonization of *P. indica*. This will possible by systemic induction of defence mechanism of chickpea plant by symbiotic fungi *P. indica*.

We also performed antibiosis assay to confirm that there is no any antibiotic secretion by *P. indica* to inhibit the growth of *B. cinerea* suggesting that biotic tolerance is due to induction of resistance of the plant by root colonising fungus *P. indica*. Similar finding have been also observed by Kumar *et al*., (2009) and thus support our data. We also observed increased antioxidant enzyme activity in root and shoot of chickpea plant colonised with *P. indica*. It was found that GR activity was more in chickpea root colonised with *P. indica* (76 fold) and CAT activity was also higher (34 fold) which suggesting that GR and CAT playing important role in regulation of fungal in root. It is suggested that CAT induction attenuate the elicitation of plant defence response by scavenging $H_2O_2$ (Wu *et at*., 1997) and promotes intra radical fungal growth. Enhanced GR activity is associated with maintaining the enhanced level of reduced glutathione which involve in maintaining antioxidant ability (Waller *et al*., 2005). Along with above enzyme, GST and SOD level was also enhanced. It shows all these enzymes helps in colonization of *P. indica* by reducing the plant oxidative defence by detoxification of ROS. GST was found induced during the infection of *Nicotiana benthamiana* with *Colletotrichum orbiculare* in another study by Dean *et al*., (2005).

In chickpea shoot induction of antioxidant enzyme also observed in plant colonised with *P. indica* and inoculate with *B. cinerea* in different combination of inoculation. But interestingly it was found that antioxidant enzyme activity was comparatively more enhanced in plant colonised with *P.indica* than in plant inoculated with *P. indica* and *B. cinerea* in alternate and simultaneous manner. These observations suggest that there may be systemic induction of antioxidant defence in the case of plant colonised by *P. indica*. The plant colonised with *P. indica* showing enhanced SOD level responsible for accumulation of more $H_2O_2$. may be responsible for reduced infection of *B. cinerea* in chickpea so the plant recover biomass yield and normal root proliferation because in a previous study it have been observed that $H_2O_2$ level was increased in infection of *B. graminis* f. sp. *tritici* in wheat plant. (Serfling *et al*., 2007) and it support our results.

We have found induced expression of PR-2 protein in late stage of symbiosis. We have observed two new isoforms of PR-2 protein in root and shoot of chickpea

plant colonised with *P.indica*. High level of expression of β-glucanase activity is associated with strong defense response in plant root against *B. cinerea.* Fungus contains β-1, 3-glucans, chitin and/or chitosan and glycoproteins, all these molecules are known to be potent elicitors of plant defence reactions. 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. Extra-radical 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.

A recent study (Harrach *et al.,* 2013) showed that *P.indica* protects plants from necrotrophic pathogens by activating the plant antioxidant capacity. They have found that in *Fusarium culmorum*-infected barley (*Hordeum vulgare*) roots, there is a significant reduction in the activities of antioxidative enzymes like GR, DHAR, SOD, MDHAR and APX. But pre-inoculation of roots with *P. indica* almost completely vanish the *Fusarium*-induced decrease in antioxidant capacity significantly. They also reported that *P. indica* colonised root of barley plant showed a high antioxidant defence response to *F. Culmorum* infection. *Botrytis cinerea* is a necrotrophic pathogen which causes pathogenicity by utilizing the production of ROS by enhancing cell death of host plant (Govrin and Levine 2000). Recently in *Arabidopsis,* it was found that inhibition of oxidative burst resulted in *Botrytis cinerea* infection (Yang *et al.,* 2011). In contrast to the (Harrach *et al.,* 2013) report we observed increased activity of CAT, GR, SOD in plants infected with the *B.cinerea* as compared to the control plants.

Present work showing colonization of *P. indica* with chickpea plant have strong growth promoting effects, systemic resistance to biotic stress, biochemical changes that can provides enhanced antioxidant capacity. *P. indica*, unlike AMF, axenically cultivable in absence of host plant (Varma *et al.,* 1999), can be used as a tool in sustainable agriculture. So association of *P. indica* is very use full in upcoming agricultural challenges related to crop yield, biomass production, bio control, agro management, and disease resistance to plant by overcoming the use of pesticides and chemical fertilizers.

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# 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)**

| | |
|---|---|
| $ZnSO_4$. 7 $H_2O$ | 22 |
| $H_3BO_3$ | 11 |
| $MnCl_2$. 4 $H_2O$ | 5 |
| $FeSO_4$. 7 $H_2O$ | 5 |
| $CoCl_2$. 6 $H_2O$ | 1.6 |

| | |
|---|---|
| $CuSO_4 . 5 H_2O$ | 1.6 |
| $(NH4)_6 Mo_7O_{27} . 4 H_2O$ | 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 $FeSO_4 . 7 H_2O$ 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 | 0.1g |
| Lactophenol | 100 ml |

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

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

**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 litre deionised 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 |