Chapter 1
1. Introduction

1.1 Rice

Rice (*Oryza sativa*) is the most important food crop for human nutrition and is the primary source of food for over half of the world's population. It is the model cereal crop belongs to the family Poaceae. There are about 120,000 varieties of rice are known to exist. Two of the types sequenced are Indica and Japonica, the japonica varieties have narrow dark green leaves, medium-height tillers, and short to intermediate plant height. It is usually grown in cooler subtropics and temperate climates, such as Japan, Portugal, Spain, USSR, Italy, and France. The traditional indica rice varieties, widely grown throughout the tropics and subtropics, are tall and heavy tillering with long, narrow, light green leaves (Source: www.moffindia.org/Downloadble/POP/PADDY.doc).

*Oryza sativa* is the cereal selected to be sequenced as a priority and has gained the status "model organism". It has the smallest genome of all the cereals: 430 million nucleotides and it can serve as a model genome for one of the two main groups of flowering plants, the monocotyledons. Because it has been the subject of studies on yield, hybrid vigor, genetic resistance to disease and adaptive responses, scientists have taken advantage of the existence of a multitude of varieties that have adapted to a very wide range of environmental conditions, from dry soil in temperate regions to flooded cultures in tropical regions.

*Oryza sativa* (rice) is one of the most important food crops in the world which is grown in almost all the countries. It is the staple food for over 2.7 billion people and also provides the main resource of energy for more than half of the world population. Rice is exclusive in that it is the only cereal crop consumed directly by humans. Total global area covered under rice is 150.8 million ha with total output of 573 million tones of paddy at an average yield of 3.83 tons per ha. More than 90 percent of global rice area is in Asia. Out of 2.7 billion rice consuming people, more than 95 percent live in Asia. China and India are two major rice producers which account for 49.1 percent of the total rice area (74.1 m ha) and 55.2 percent of world's production (310.1 m tons). India is the largest rice growing country, while China is the largest rice producer. In India, area under rice is 44.6 m ha with
total output of 80 m tones (paddy) with an average productivity of 1855 kg/ha. It is grown in almost all the states. West Bengal, Uttar Pradesh, Madhya Pradesh, Bihar, Orissa, Andhra Pradesh, Assam, Tamilnadu, Punjab, Maharashtra and Karnataka are major rice growing states and contribute to total 92 percent of area and production. (Source: www.moffindia.org/Downloadable/POP/PADDY.doc). According to the Food and Agriculture Organization (FAO) of the U.N., 80 percent of the world rice production comes from 7 countries. FAO data shows that in 2009-2010, China has produced 166,417,000 Mt (32.7 percent) of total world’s rice production, ranked first and India has produced 132,013,000 (26.0 percent) and ranked second (Source: http://www.rice-trade.com/articles/rice-production.html).

1.2 Micronutrients and Plants

Plants require a range of heavy metals as essential micronutrient for normal growth and development. These heavy metals like iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), cobalt (Co), nickle (Ni), boron (B), molybdenum (Mo) and chlorine (Cl) are essential for plants, acting especially as structural or functional cofactors in proteins, although these essential metals can also cause toxic effects similar to those of toxic metals, if present in excess. Some non-essential heavy metals like Cd, Pb or Hg are toxic to plants and disturb the plant metabolism already in very low concentrations, leading to reduced plant growth, lower yield and toxicity symptoms.

1.3 The Physiological Role of Essential Heavy Metals/Micronutrients in Plants

Heavy metals serve for a range of essential functions in plants. These metals have important roles in various pathways in plants. Zn, for example is an essential element, plays a special role in plants, as Zn$^{2+}$ has pronounced Lewis acid characteristics because of its small radius-to-charge ratio, and thus forms strong covalent bonds with Sulphur (S), Nitrogen (N) and Oxygen (O) donors, making it an essential component in thousands of plant proteins (Frausto da Silva and Williams, 2001). As Zn only appears as a divalent cation (Zn$^{2+}$) in biological systems, it does not participate in redox reactions and does not induce the formation of radicals. Therefore Zn ions are key structural or catalytic components in hydrolytic enzymes (e.g. Cu-Zn superoxide dismutase, alcohol dehydrogenase) and DNA-binding proteins like RNA polymerase (Marschner 1995;
Fe is required for several life-sustaining processes in plants. Similar to Cu, Fe is highly redox active, making it important for different electron transfer processes through its cycling between Fe$^{2+}$ and Fe$^{3+}$. Fe is a component of haem proteins (e.g. cytochromes, catalase, and Fe-S proteins such as ferredoxin) and a range of other enzymes, and is essential for chlorophyll production (reviewed in Kim and Guerinot 2007). Pb is potentially toxic to plants, due to their similarity (especially in ionic radii) to some essential heavy metals, such as, Zn and Fe. Cu belongs, in contrast to Zn, to the redox active transition metals. Due to its two oxidation states Cu(I) and Cu(II) it is often involved in electron transfer reactions, making it essential for photosynthesis and respiration e.g. in enzymes like plastocyanin and cytochrome c oxidase (Frausto da Silva and Williams 2001). Cu is an integral component of certain electron transfer proteins in photosynthesis (e.g. plastocyanin) and respiration (e.g. cytochrome c oxidase) and is also involved in lignification (laccase).

Mn, which has less redox potential than Cu has an important role in photosynthesis, where a cluster of Mn atoms forms the catalytic centre for light-induced water oxidation in PS II (photosystem II). Mn is also required as a cofactor in various enzymes, such as the Mn$^{2+}$-dependent superoxide dismutase (MnSOD) (Marschner 1995, for a review see Pittman 2005). Ni is a cofactor of urease and other enzymes such as hydrogenases and Ni-superoxide dismutase (Dalton et al. 1988). Ni is considered as an essential plant nutrient primarily due to its function as an irreplaceable component of the urea hydrolyzing enzyme urease (Gerendas et al. 1999). The involvement in urea breakdown is the only proven nutritional function of Ni in higher plants. Urease is the only Ni- dependent metalloenzyme identified yet in plants, whereas in bacteria there are several reports on additional Ni-dependent enzymes, like different hydrogenases or methyl coenzyme M reductase (Walsh and Orme-Johnson 1987). Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide (Dixon et al. 1975). Thus, the primary role of the plant urease is to allow the use of external or internally generated urea as a nitrogen (N) source (Sirko and Brodzik 2000). Urea N can be assimilated exclusively by urease in higher plants, the released ammonia is
then incorporated into organic compounds mainly by glutamine synthetase. Therefore, the addition of Ni to Murashige and Skoog medium in plant tissue culture was reported to activate the urease activity and to reduce metabolic stress (Witte et al. 2002).

Co is not considered as essential but is beneficial for plants. The term essential mineral element (or mineral nutrient) was proposed by Arnon and Stout (1939). They concluded that certain criteria must be fulfilled for an element to be considered essential, including: 1. A plant must be unable to complete its life cycle in the absence of the mineral element. 2. The function of the element must not be replaceable by another mineral element. Therefore, beneficial elements are those that can compensate for toxic effects of other elements or may replace other mineral nutrients in some cases, for example when the essential nutrient is not available to the plant. Co is considered as being beneficial for plants, because it is required by bacteria for symbiotic nitrogen fixation in legumes (Reisenauer 1960; Gad 2006; Delwiche et al. 1961) and in root nodules of nonlegumes (Hallsworth et al. 1965). For certain nonlegumes like Alnus and Myrica, Co is essential when they are nodulated and if no Co is supplied the plants develop symptoms of nitrogen deficiency, whereas no Co requirement was detected in non-nodulated plants (Hewitt and Bond 1966). Co also has protective effects under conditions of osmotic stress (Li et al. 2005). Bacteria, fungi and algae contain a number of Co-dependent metalloenzymes. For example, methionine aminopeptidase, the enzyme that cleaves the N-terminal methionine from newly translated polypeptides, is Co dependent in animals, yeast and bacteria (Kobayashi and Shimizu 1999), but by now there is no known essential function for Co in plants.

Mo forms together with a pterin compound the Mo cofactor (Moco), which is the active site of eukaryotic Mo enzymes. There are four plant enzymes that depend on Mo: nitrate reductase catalyzes the first step in nitrate assimilation, peroxisomal sulphite oxidase detoxifies sulfite, aldehyde oxidase catalyzes the last step of abscisic acid biosynthesis and xanthine dehydrogenase is essential for purine degradation and stress response (Schwarz and Mendel 2006). Cadmium (Cd), arsenic (As) are potentially toxic to plants, due to their similarity (especially in ionic radii) to some essential heavy metals, such as, Zn and Fe.
1.4 Metal Transport in Plants
Transport of metals and alkali cations across plant plasma and organelar membranes is essential for plant growth, development, signal transduction, nutrition, and also for the use of plants in toxic metal phytoremediation. Plants require a range of transition metals such as Fe, Cu, Mn, and Zn essential micronutrients for normal growth and development. These metals are essential for most redox reactions which, in turn, are fundamental to cellular function. In general, metal deficiencies cause an altered expression and or function of proteins at the metabolic level that leads to different physiological symptoms such as reduced growth, leaf necrosis and chlorosis (Kriedmann et al. 1985; Terry and Abadia 1986). On the other hand, excessive amounts of these metals can cause serious damage to the plant, due to uncontrolled binding of the metals to proteins (Van Assche and Clijsters 1986a, b) or due to their redox activity that can lead to the generation of oxygen radicals (Pahlsson 1989; Price and Hendry 1991). Therefore, plants must carefully regulate both metal acquisition from the soil and cellular partitioning within the plant in order to prevent excess accumulation while obtaining adequate intake.

The regulation of metal uptake across the plasma membrane into the cell appears to be the primary control for point for metal homeostasis (Guerinot 2000). Plants have evolved mechanisms that allow the transport of metal ions, through different categories of metal transporters such as the ZIP (ZRT, IRT-like proteins) family (Guerinot 2000), cation diffusion facilitators (CDFs) (Williams et al. 2000), heavy metal (or CPx-type) ATPases, the natural resistance-associated macrophage proteins (Nramps) and the cation antiporters (Gaxiola et al. 2002), found to be located in different organelles within the cell.

1.5 Heavy Metal Toxicity
Over the past 200 years emissions of toxic heavy metals have risen tremendously and significantly exceed those from natural sources for practically all metals. Heavy metal contamination of soil results from anthropogenic as well as natural activities. Anthropogenic activities such as mining, manufacturing, smelting operation, municipal waste disposal and fertilization have locally increased the levels of heavy metals in soils up to dangerous levels (Sharma and Agrawal 2005).
The properties like redox-activity and Lewis acid strength makes heavy metal ions essential for many biological reactions are also a reason why they can easily be toxic when present in excess. There are three different molecular modes of heavy metal toxicity: (1) Redox-active metal ions in plants can participate in Haber–Weiss and Fenton reactions and thereby trigger the formation of reactive oxygen species (Halliwell and Gutteridge 1986, 1990; reviewed in Clemens 2006), (2) uncontrolled high affinity binding to sulphur-, nitrogen- and oxygen-containing functional groups in biological molecules can cause their inactivation or damage (Chrestensen et al. 2000) and (3) heavy metals can displace essential elements from biomolecules, for example the exchange of essential metal ions from the active centres of enzymes (El-Jaoual and Cox 1998).

The consequences of unbalanced metal concentrations within plant cells can be severe. Antioxidants (both enzymatic and nonenzymatic) provide protection against metal mediated free radical attacks. But mechanisms for cellular tolerance to heavy metals appear to be involved primarily in avoiding the build-up of toxic concentrations at sensitive sites within the cell, rather than in radical scavenging or in the development of less sensitive proteins. To avoid an increased production of heavy metal-mediated ROS, plants have developed a range of mechanisms to avoid the accumulation of toxic metal concentrations in the cytoplasm. All plants including A. thaliana possess a basic metal tolerance, allowing the adjustment of metal homeostasis to fluctuations in soil metal concentrations and cellular metal influx rates within narrow concentration ranges (Clemens 2001; Clemens et al. 2002).

1.6 Heavy Metal Homeostasis and Detoxification
As the physiological range for essential metals, within which plants are unaffected by deficiency or toxicity, is extremely narrow, plants had to develop a tightly controlled metal homeostasis network to adjust to changes in micronutrient concentrations, and also had to develop mechanism for detoxification of heavy metals. These mechanisms include the immobilization of metals in the apoplast, chelation of heavy metals, transport of metals or metal-chelates out of the cell and intracellular sequestration. Compared to other organisms, plants have expanded families of transporters that are involved in the uptake and efflux of metals. The application of genetic and molecular techniques has now identified a range of
families of metal transporters that vary in their substrate specificities, expression patterns, and cellular localization to govern metal translocation throughout the plant. These include the ZIP family proteins, the cation diffusion facilitators (CDFs), heavy metal (or P1B type) ATPases, the natural resistance-associated macrophage proteins (Nramps) and others (Hall and Williams 2003). Some of these transporter gene families are large and comprise several genes. For example, in Arabidopsis there are 15 ZIP family transporters (Maser et al. 2001), eight P1B-ATPases (Mills et al. 2003) and six members of the Nramp family (Williams et al. 2000). Additionally, mycorrhizas that are characteristics of trees and shrubs can be effective in ameliorating the effects of metal toxicity on the host plant (Marschner 1995; reviewed in Hall 2002).

Protection of the plants by Arbuscular Mycorrhizal Fungi (AMF) that colonise plant roots and considerable reduction in the uptake of heavy metals into plant cells may be one of the means that allow metallophytes to thrive on heavy metal-polluted sites (Weissenhorn et al. 1995; Leyval et al. 1997; Kaldorf et al. 1999; Berreck and Haselwandter 2001; Ouziad et al. 2005; Vogel-Mikus et al. 2006). Progress toward this goal has been hindered by a lack of understanding of the basic molecular, biochemical and physiological mechanisms involved in heavy metal hyperaccumulation.

1.7 Role of Mycorrhizae in Heavy Metal Detoxification

1.7.1 Arbuscular Mycorrhizal Fungi

Soil microorganisms like mycorrhiza fungi are known to play a key role in the mobilization and immobilization of metal cations, thereby changing their availability to plants. Mycorrhizal fungi establish mutual symbioses with the majority of higher plants, providing a direct physical link between soil and plant roots. They colonize the cortical tissue of roots during periods of active plant growth. Generally, these symbioses are often characterized by bi-directional exchange of plant-produced carbon to the fungus and fungal-acquired nutrients to the plant thereby providing a critical linkage between the plant root and soil. AMF occur in almost all habitats and climates, including in disturbed soils such as those derived from mine activities but soil degradation usually produces changes in the diversity and abundance of AMF populations. AMF can help to alleviate metal toxicity to plants by reducing metal translocation from root to shoot (Leyval et al. 1997).
Mycorrhizal colonization of roots results in an increase in root surface area for nutrient acquisition. The extramatrical fungal hyphae can extend several cm into the soil and uptake large amounts of nutrients, including heavy metals, to the host root. The effectiveness of AM root colonization in terms of nutrient acquisition differs markedly between AM fungi and host plant genotype. The AMF belongs to the group of Mycorrhizal fungi. Mycorrhizae have also been reported in plants growing on heavy metal contaminated sites indicating that these fungi have evolved a heavy metal tolerance and that they may play a role in the phytoremediation of the site. Mycorrhiza has a significant impact on the expression of several plant genes coding for proteins presumably involved in heavy metal tolerance/detoxification (Repetto et al. 2003; Rivera-Becerril et al. 2005).

### 1.7.2 Functions of AM Fungi

AMF, the root-interacting predominant micro-biota play an indispensable role in upgrading plant growth vigour and survival (Singh et al. 2000). Studies have shown that associations with mycorrhizal fungi influence plant fitness in complex ways, which are not directly related to the improved nutritional status of mycorrhized plants. They were found to increase plant fitness by increasing tolerance of extreme drought conditions (Ruiz-Lozano and Azcon 1995; Ruiz-Lozano et al. 1996; Ruiz-Lozano et al. 2001) and heavy metals (Kaldorf et al. 2005). AM fungi are found under all climates and in all ecosystems, regardless of the type of soil, vegetation or growing conditions. AM fungi, which are microscopic soil fungi, simultaneously colonize the roots and their rhizosphere and spread out over several centimeters in the form of ramified filaments. This filamentous network dispersed inside as well as outside the roots allows the plant to have access to a greater quantity of water and soil minerals required for its nutrition. In return, the plant provides the fungus with sugars, amino acids and vitamins essential to its growth (Harley and Smith 1983). As a result of its improved nourishment, a mycorrhiza-colonized plant has better growth. It fructifies abundantly and, above all, acquires increased resistance to environmental stresses such as drought, cold and root pathogens (Elsen et al. 2001; Sylvia and Williams 1992).
1.8 *Piriformospora indica*: Applications and Diverse Functions

Prof. Varma and his Collaborators, from the School of Life Sciences, Jawaharlal Nehru University, New Delhi, have screened a novel endophytic root colonizing fungus which mimics the capabilities of a typical AM fungus. The exceptional feature is that this fungus is axenically culturable and this is a milestone achieved for the scientist dealing with the mycorrhizal research. The fungus has been termed as *Piriformospora indica* (*P. indica*) because of its characteristic pear shaped chlamydospores, related to the Hymenomycetes of the Basidiomycota (Verma *et al.* 1998). The properties of *P. indica* have been patented (Varma and Franken, 1997, European Patent Office, Muenchen, Germany, Patent number 97121440.8-2104, Nov. 1998). The culture has been deposited at Braunsweich, Germany (DMS number 11827) and 18S rDNA fragment deposited with GenBank, Bethesda, USA (AF 014929). *P. indica* can grow axenically on a number of complex and semi-synthetic media (Hill and Käfer 2001; Pham *et al.* 2004b). The mycelium is mostly flat and submerged into the substratum. The hyphae are highly interwoven often showed anastomosis and are irregularly septated. It is phylogenetically close to mycorrhizal endosymbionts of orchids and ericoid root (Verma *et al.* 1998; Varma 1999; Weiss *et al.* 2004). The fungus is able to associate with the roots of various plant species in a manner similar to arbuscular mycorrhizal fungi and promotes plant growth (Varma *et al.* 1999, 2001; Singh *et al.* 2003; Shahollari *et al.* 2004; Pham *et al.* 2004a). The fungus *P. indica* has been observed to have exceptional properties as a biofertilizer, bioprotector and immunoregulator. This fungus plays a crucial role in promoting plant growth, vigour and survival by producing a positive impact on the nutritional and hydraulic status of the plant (Davies *et al.* 1992). The root-colonizing fungal symbiotic, *P. indica* was discovered in association with woody shrubs in the Indian Thar desert in 1997. Since then, the fungus has been shown to confer growth promotion to a broad spectrum of host plants (Schafer *et al.* 2007). *P. indica* is a newly described axenically cultivable phyto-promotional endosymbiont, which mimics the capabilities of AMF. The fungus has a broad host spectrum showing pronounced growth-promotional effects (Bothe *et al.* 2010). It mobilizes the insoluble phosphates and helps in translocation of phosphorus to the host, in an energy-dependent process. As a biological hardening agent of micropropagated plants, it renders more than 90 per cent survival rate for laboratory to field transferred plantlets (Singh *et al.* 2000). Depending on the ultra structure of hyphae (presence of dolipore septa) and 18s-
rRNA gene sequence, *P. indica* was grouped in the class Hymenomycetes (Basidiomycota) (Verma et al. 1998). Weiss et al. (2004) classified the fungus as a member of the order *Sebacinales* depending on nuclear rDNA sequence for the 5’ terminal domain of the ribosomal large subunit (nucLSU). *P. indica* infests roots of a broad range of mono- and dicotyledonous plants (Verma et al. 1998; Pham et al. 2004a). Endophytic root colonization by this fungus confers enhanced growth to the host plant (Varma et al. 1999; Peskan-Berghoefer et al. 2004) and provides protection against biotic and abiotic stresses. *P. indica* enhances salt stress tolerance, confers disease resistance and increases grain yield in barley (Waller et al. 2005). Disease resistance is provided not only to the roots but also to the shoot. As endophytic growth of *P. indica* is restricted to the root, the fungus is able to provide systemic protection due to a yet unknown mechanism of induced resistance. As *P. indica* can easily be cultured without a host plant (Varma et al. 1999), it is suitable as a model system to study compatible plant–microbe interactions. Like other mutualistic endophytes, *P. indica* colonizes roots in an asymptomatic manner. PCR-based quantification of *P. indica* revealed that root colonization gradually increases with tissue maturation. The endophyte *P. indica* has an encouraging influence on growth and development on host plants. It also promotes nutrient uptake, allows plants to survive under water, temperature and salt stress, confers (systemic) resistance to toxins, heavy metal ions and pathogenic organisms and stimulates growth and seed production (Verma et al. 1998; Varma et al. 1999, 2001; Sahay and Varma 1999; Oelmüller et al. 2004, 2005; Pham et al. 2004a, b; Peskan-Berghoefer et al. 2004; Kaldorf et al. 2005; Shahollari et al. 2005, 2007, Sherameti et al. 2005, 2008a, b; Vadassery et al. 2008, 2009a, b; Waller et al. 2005, 2008). Like AM fungi, *P. indica* functions as bioregulator, biofertilizer and bioprotector as well as delays wilting and withering of the leaves. In addition, it also prolongs life-span of callus tissues. Several studies have demonstrated that *P. indica* may be used for phyto-remediation, because it accumulates heavy metals and prevents their uptake into the plants (Oelmüller et al. 2009). Kaldorf et al. (2005) demonstrated that when the plantlets of *Populus* Esch5 explants with roots were inoculated with *P. indica*, the root biomass and the number of second order roots increased. However, when the plantlets were exposed to a medium with pre-grown fungus, plant and root growth was completely blocked. Prolonged incubation of the plantlets with the fungus caused even colonization of the aerial parts of poplar. Application of ammonium to the medium leads to
bleaching and withering of the plantlets in the presence of the fungus. Fungal toxin formation or the extension of the colonization to the shoots may be responsible for the antagonistic interaction. Deshmukh et al. (2006) and Schafer et al. (2007) reported that *P. indica* requires cell death for the proliferation during mutualistic interaction with barley. They found that the majority of the hyphae were present in dead rhizodermal and cortical cells. This suggested that *P. indica* either actively kills cells or senses cells that undergo endogenously programmed cell death. Thus, the endophyte interferes with the host cell death program to form a mutualistic interaction with the plants. More detailed analysis with other plant species are required to find out whether this is a general phenomenon or specific for barley, a host that strongly interacts with *P. indica* (Oelmüller et al. 2009).

1.8.1 Effect of *P. indica* on Phosphorus (P), Nitrogen (N), Sulphur (S) and Sugar Metabolism

*P. indica* mediate P and N uptake from the soil (Sherameti et al. 2005; Varma et al. 2001). Recent work suggested that *P. indica* stimulates NADH dependent nitrate reductase activity in the roots of *Arabidopsis* and tobacco (Sherameti et al. 2005). *P. indica* mediates nitrate uptake from the soil, which is in contrast to AMF, where nitrogen is preferentially absorbed as ammonium. Malla et al. (2004) have shown that *P. indica* contains substantial amounts of an acid phosphatase which has the potential to solublize phosphate in the soil and delivers it to the host plant. The application of different techniques for characterization of ACPase (Acid phosphatase) in *P. indica* and *Sebacina vermifera* senu which belong to same taxonomic group show similar morphology, functions, protein profiles and isozyme characterization along with close acid phosphatise relationship (Malla et al. 2010).

1.8.2 Active Component of Cell Wall Extract (CWE) of *P. indica*

The endophyte *Piriformospora indica* colonises the roots of many plant species including rice and *Arabidopsis thaliana* (*A. thaliana*) and promotes their growth, development and seed production. The fungus stimulates nutrient uptake and confers resistance to various biotic and abiotic stresses (cf. Verma et al. 1998; Sahay and Varma, 1999; Varma et al. 1999, 2001; Peskan-Berghoefer et al. 2004; Shahollari et al. 2005, 2007; Sherameti et al. 2005, 2008; Waller et al. 2005). *P. indica* functions as bioregulator, biofertilizer and bioprotector against root pathogens; overcome the water stress (dehydration), acidity,
desiccation and heavy metal toxicity; protection from pests delay the wilting of the leaves, prolong aging of callus tissues. Apart from these effects on vegetative and generative plant development, *P. indica* mediates stress tolerance to infested plants (Pham et al. 2004b). It has been reported by Vadassery et al. (2009), that the active fraction of cell wall (CW) of *P. indica* can mimic the presence of the fungus in the initial stages in plants and induce growth promotion. This fraction is also able to induce an elevation of [Ca$^{2+}$]cyt in plant roots stably expressing the Ca$^{2+}$ bioluminescent indicator aequorin. This may point to a role for Ca$^{2+}$ in the early signalling events between *P. indica* and *A. thaliana*, similar to the well-characterised legume–rhizobia and arbuscular mycorrhizal symbiosis. This active fraction has been used in this study to resist the plants against heavy metal stress.

1.9 Heavy Metal Detoxification Using Carbon Nano Tubes (CNTs)

Among the several materials used for the removal of heavy metals, CNT can play a strategic role due to their ability of adsorbing the element Zn, Pb, Ni, Cd, Cr and Hg (Li et al. 2002, 2003; Lia et al. 2003; Lu and Chiu 2006; Lu et al. 2008; Rao et al. 2007; Yang et al. 2009). CNTs have remained in the forefront of intense research for more than a decade, due to their exceptional physical, chemical and electronic properties that have been inherited from the parent in-plane graphite (Dresselhaus et al. 2001). Their small dimensions, closed topology and lattice helicity have enabled nanotubes to influence broad areas of science and technology, ranging from super strong nanocomposites to nanoelectronics (Endo et al. 2008; Balasubramanian and Burghard 2008; Sharma and Ahuja 2008). Two major categories of CNTs are single walled nanotubes (SWNTs) and multi walled nanotubes (MWNTs). Potential practical applications of CNTs such as chemical sensors, field emission (Buldum and Lu 2003), electronic devices (Sharma and Ahuja 2008), high sensitivity nanobalance for nanoscopic particles (Saito 1997), nanotweezers (Kim and Lieber 1999), reinforcements in high performance composites, biomedical and chemical investigations (Lim et al. 2005), anode for lithium ion batteries (Simon and Gogotsi 2008), supercapacitors (Simon and Gogotsi 2008) and hydrogen storage (Dresselhaus et al. 1999; Dillon et al. 1997) have been reported. Even though the challenges in fabrication may prohibit realization of many of these practical device applications, the fact that the properties of CNTs can be altered by suitable surface modifications can be exploited for more imminent realization of practical devices. In this
respect, a combination of CNTs and other nanomaterials, such as nanocrystalline metal/CNTs, nanocrystalline metal oxide/CNTs, polymer/CNTs and metal filled CNTs may have unique properties and research have therefore been focused on the processing of these CNT based nanocomposites and their different applications. Carbon materials are a class of significant and widely used engineering adsorbent. As a new member of the carbon family, CNTs have exhibited great potentials as an attractive adsorbent in waste water treatment [Li et al. 2003]. According to Tofighy (2011), the CNT sheets can be used to remove some divalent heavy metal ions (Cu$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Cd$^{2+}$, Co$^{2+}$) from aqueous solutions.

1.10 Heavy Metal Transporetrrs

1.10.1 The ZIP (ZRT, IRT-like protein) Transporters

Higher plants take up Zn from the rhizosphere via transporters. In the A. thaliana genome, a large number of cation transporters potentially involved in metal ion homeostasis have been identified (Maser et al. 2001). Several members of the Zn-regulated transporters in the Fe-regulated transporter-like protein (ZIP) gene family (Guerinot 2000) have been characterized and shown to be involved in metal uptake and transport in plants (Eide et al. 1996; Korshunova et al. 1999; Vert et al. 2001, 2002; Connolly et al. 2002). The ZIPs are involved in the transport of Fe, Zn, Mn, and Cd with family members differing in their substrate range and specificity (Guerinot 2000; Maser et al. 2001). About 85 ZIP family members have now been identified from bacteria, archaea and all types of eukaryotes, including 15 genes in Arabidopsis (Maser et al. 2001). Most of the ZIP proteins are predicted to have eight transmembrane domains with the amino- and carboxyl- terminal ends situated on the outer surface of the plasma membrane (Guerinot, 2000). They range quite widely in overall length, this being due to a variable region between TM-3 and TM-4. This region is predicted to be on the cytoplasmic side and is a potential metal-binding domain rich in histidine residues. The most conserved region of these proteins lies in TM-4 and is predicted to form an amphipathic helix containing a fully conserved histidine that may form part of an intramembranous metal binding site involved in transport (Guerinot 2000; Maser et al. 2001); the transport function after heterologous expression in yeast is eliminated when conserved histidines or certain adjacent residues are replaced by mutation.
in different amino acids (Rogers et al. 2000). Some ZIPs can transport several metals, although with differential selectivity for each metal (Korshunova et al. 1999).

AtIRT1 (Fe-regulated transporter 1), the first member of the ZIP family to be identified (Eide et al. 1996), was cloned from Arabidopsis by functional complementation of an Fe-uptake-deficient yeast mutant (fet3 fet4). IRT1 is now thought to be the major transporter for high affinity Fe uptake by roots (Connolly et al. 2002; Vert et al. 2002). IRT1 mRNA is detectable by 24 hour on transfer to Fe-deficient conditions and both mRNA and protein levels peak after 72 hour; IRT1 mRNA and protein are undetectable 12 hour after transfer back to Fe-sufficient conditions (Connolly et al. 2002). Plants overexpressing AtIRT1 also accumulate higher concentrations of Cd and Zn than wild types under Fe deficient conditions, indicating an additional role in the transport of these metals (Connolly et al. 2002) which is also supported by transport studies in yeast (Eide et al. 1996; Korshunova et al. 1999).

OsIRT1 from rice, which has high homology to the Arabidopsis AtIRT1 gene, is also predominantly expressed in roots and is induced by Fe- and Cu-deficiency (Bughio et al. 2002). irt1-1, an Arabidopsis knockout mutant, was chlorotic and showed severe growth defects, although this condition could be rescued by the exogenous application of Fe (Vert et al. 2002); the protein localized to the plasma membrane and, under Fe-deficient conditions, IRT1 was expressed predominantly in the external layers of the root. Mutants of IRT1 also showed significant changes in photosynthetic efficiency and developmental defects that were consistent with a deficiency in Fe transport and homeostasis (Henriques et al. 2002; Varotto et al. 2002). Interestingly, AtIRT2 another member of ZIP family is also expressed in root epidermal cells under Fe deficiency. However, it cannot substitute for loss of IRT1 (Grotz and Guerinot 2002) and it appears to have a greater specificity as regards substrates; although it can complement Fe and Zn uptake mutants it does not seem to transport Cd or Mn in yeast (Vert et al. 2001). This suggests that these transporters have different functions in Arabidopsis. LeIRT1 and LeIRT2 have now been reported in tomato and are both predominantly expressed in roots (Eckhardt et al. 2001). LeIRT1, but not LeIRT2, is strongly enhanced by Fe limitation and, together with particular P and K transporter genes, it is also up-regulated by P and K deficiencies in the root medium,
suggesting a possible coregulation of the transporter genes for certain essential minerals (Wang et al. 2002). Studies in yeast suggest that LeIRT1 and 2 also have a broad range (Eckhardt et al. 2001).

1.10.2 The Cation Diffusion Facilitator Family (CDFs)

Proteins belonging to the cation-efflux family also known as the cation diffusion facilitator family, were first described by Nies and Silver in 1995 (Nies and Silver 1995), and have been implicated in conferring Zn$^{2+}$, Cd$^{2+}$, Co$^{2+}$ or Ni$^{2+}$ tolerance in a range of organisms. Proteins of the CDF family from diverse sources have the following features in common: (1) they share an N-terminal signature sequence that appears to be specific to the family; (2) the proteins possess six transmembrane-spanning regions; (3) they share a cation efflux domain; and (4) most of the eukaryotic members possess an intracellular His-rich domain that is absent from the prokaryotic members (Paulsen and Saier 1997). Members of this family were first identified in bacteria, but can also be found in archaea and eukaryotes, where they encode proton antiporters that efflux heavy metals like Zn, Co or Cd out of the cytoplasm (Paulsen and Saier, 1997; Eide, 1998; Gaither and Eide, 2001). Some of the best characterised members of this family are the ZnT Zn efflux transporters of human and rodents, but in the last few years two Arabidopsis CDFs have been identified and studied. The first member of the CDF family characterized in Arabidopsis was ZAT1 (Zn transporter gene 1), later renamed MTP1 (metal tolerance protein 1) (Delhaize et al. 2003). Overexpression of MTP1 confers Zn tolerance in Arabidopsis (van der Zaal et al. 1999), whereas plants lacking the expression of MTP1 are more sensitive to Zn, due to a function of MTP1 in sequestration of Zn to the vacuole (Kobae et al. 2004; Desbrosses-Fonrouge et al. 2005). Another member of the CDF family in Arabidopsis, AtMTP3, contributes to basic cellular Zn tolerance and controls Zn partitioning, particularly under conditions of high rates of Zn influx into the root symplasm, for example at conditions of Zn oversupply or Fe deficiency (Arrivault et al. 2006). Members of the CDF family are also supposed to be involved in the hyperaccumulation of metals in species like Arabidopsis halleri, a Zn/Cd hyperaccumulator. A. halleri contains three MTP1 genes, two MTP1 loci co-segregate with Zn tolerance (Drager et al. 2004). Cross-species microarray revealed an increased expression of MTP1 in shoots of Arabidopsis halleri. Enhanced expression of CDF transporters also determines the metal tolerance of the Ni/Zn hyperaccumulator.
Thlaspi goesingense (Persans et al. 2001). The T. goesingense TgMTP1 functions as a Zn exporter at the plasma membrane (Kim et al. 2004). The CDF transporter ShMTP1 of the tropical legume Stylosanthes hamata confers Mn2+ tolerance when expressed in yeast and Arabidopsis (Delhaize et al. 2003).

1.11 Objectives of the thesis
This study aims at the following:

1. Promotion of higher uptake of Nutrients into the plant: Effect of Nano-embedded (TNP, AgNP, CNT, Active charcoal) P. indica culture on Rice
2. Effect of Nanoparticles on Rice tissue
3. In silico analysis of ZIP and CDF family genes

Essential transition metals are required in all plant cells for the activities of numerous metal-dependent enzymes and proteins, but can become toxic when present in excess. The maintenance of metal homeostasis in plants depends on membrane transport proteins, which keep whole plant and cellular metal concentrations in the physiological range. Despite a considerable number of metal transporters that have been identified to date, most of their physiological functions remained unclear. In this thesis we have studied the phenotypic effect of the heavy metals on the rice plants and we have used different strategy to cope up with the problem of heavy metals toxicity effect on the plant. As the arbuscular mycorrhizal fungi have an important role in the detoxification of heavy metals from soil by helping the plants at contaminated sites, we have isolated the active fraction of the fungus and used it in minimizing the heavy metal effect on plants. The wsCNT have also been used for this purpose as they can adsorb the heavy metal ions and help plant in detoxification. We have studied the role of metal transporters in metal homeostasis in plants.

Chapter 1 Summarises the current state of knowledge on the heavy metal as plant micronutrients and metal homeostasis in plants
Chapter 2 Summarizes the materials and methods used in the research
Chapter 3 The effect of Cell wall extract isolated from the endophytic fungus Piriformospora indica studied on Oryza sativa under Heavy Metal Stress
Chapter 4 The effect of water soluble Carbon Nanotubes on *Oryza sativa* plants under Heavy metal stress is described

Chapter 5 explores the use of wsCNT in enhancing the growth of endophytic fungus *P. indica*

Chapter 6 In silico analysis of ZIP and CDF family transporters is described

Then summarizes the previously described results and discusses them in the context of previous work on metal transport and metal detoxification strategies in plants.