SAITAMA UNIVERSITY

Doctoral Dissertation

ROLE OF ARBUSCULAR MYCORRHIZAL FUNGI ON THE PERFORMANCE OF FLOODPLAIN PLANTS UNDER NUTRIENT-LIMITED AND HEAVY METAL STRESS CONDITIONS (貧栄養や重金属ストレス下での河川氾濫原植生の動態に対するアーバスキュラーマイコライザの役割)

Author:
Animesh SARKAR

Supervisor:
Professor Takashi ASAEDA

&

Assoc. Professor Qingyue WANG

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Animesh Sarkar
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Abstract

Mycorrhizal fungi are species of fungi that intimately associate with plant roots forming a symbiotic relationship, with the plant providing sugars for the fungi and the fungi providing nutrients to the plants. A vesicular arbuscular mycorrhiza (VA mycorrhiza), now known as arbuscular mycorrhiza (AM), plays a very important role in enhancing plant growth and yield due to an increased supply of nutrients to the host plant. Arbuscular mycorrhizal fungi (AMF) are ubiquitous components of terrestrial ecosystems across the world with multiple functions from the level of individual plants to the ecosystem. Mycorrhizal fungi occur in riparian areas but their functions in promoting plant growth are unknown across floodplain chronosequences. Mycorrhizal fungi can absorb, accumulate, and transport large quantities of nutrients within their hyphae which are then release and translocate to the host plant cells in root tissue. Arbuscular mycorrhiza (AM) is the predominant mycorrhizal type found in early stages of primary succession; for example, in sand dunes and river floodplains though distribution and functions of AMF at transitional zones between aquatic and terrestrial ecosystems, such as flood plains, remain less studied. In such areas, however, the level of colonized plant species has a wide range. Some species, such as*Miscanthus sacchariflorus* or *Phragmites japonica*, grow in stony sterile as well as low nutrient conditions, while other species prefer relatively fine sediment with nutrient rich conditions. Response to mycorrhizal inoculation is linked to the level of soil fertility, and it is well documented that P is the most influential element in mycorrhizal development and efficiency. In nutrient-deficient soils, the yields of horticultural and field crops were found to be largely dependent on their mycorrhizal status under field and greenhouse conditions. Since soils in the early stages of primary succession generally have low nutrient contents, it is possible that AM fungi play an important role in the growth and establishment of pioneer plants.

A preliminary survey was conducted on the banks of the Ara River, Saitama, Japan (36°4′58.02″ N and 139°26′28.85″ E) to identify suitable, experimental plant materials for this study. Five meter × four meter transects (henceforth referred as S1∼ S5, respectively) were randomly selected along the river and marked with poles and rods. The distance between these transects ranged between 10 and 30

m. All plant species within a transect were identified, and their percent coverage was calculated. Though the species composition varied between the transects, *M. sacchariflorus* and *P. japonica* appeared to be the most common species and covered a higher percentage of the transects areas whereas *P. cuspidatum* covered a lower percentage. Therefore, based on the survey and previous literature, we chose *M. sacchariflorus*, *P. japonica* and *P. cuspidatum* as our experimental plants.

The potential effects of arbuscular mycorrhizal fungi (AMF) on growth, nutrient uptake, and inoculation effectiveness of AMF on the dominant pioneer plants *Miscanthus sacchariflorus*(C4), *Phragmites japonica* (moderately C4) and *Polygonum cuspidatum* (C3) were evaluated.Spores of AMF strains (*Gigaspora margarita* Becker & Hall) were collected from the commercial product 'Serakinkon' . Four treatments such as natural soil, natural soil inoculated by AM fungi, sterilized soil inoculated by AM fungi, and sterilized soil without AM fungi inoculation were selected to determine the effects of applied and indigenous AMF by performing pot experiments in greenhouse Saitama University, Japan. The average colonization level of *M. sacchariflorus* was 23–28%, *P. japonica* was 24-33% and *P. cuspidatum* was 0.2%-0.5% whereas no colonization was found in sterilized soil. AMF colonization increased the chlorophyll content, plant dry mass, N, P, K, Mg, Fe, Cu, and Zn concentration of the *M. sacchariflorus* plant' s roots, stems, and leaves when AMF was applied with natural and sterilized soil. Mn concentration decreased in *M. sacchariflorus* roots and stems but increased in leaves in natural soil, and AMF with natural soil treatment. AMF colonization also increased the chlorophyll content ($r= 0.84$, $p<0.01$), plant dry mass ($r=0.89$, $p<0.01$), and N, P, K, Mg, and Fe concentration of the *P. japonica* plant' s roots, stems, and leaves with natural and sterilized soil. Mn concentration decreased in the *P. japonica* roots but increased in the leaves. Cu concentration was not significantly affected by treatments. In all cases, maximum values showed when both plants were applied with natural soil in combination with AMF, but Ca concentration decreased as colonization level increased. N loss minimization from the soil was significant when colonization level was high. Therefore, AMF have some potential effects for growth of the *M. sacchariflorus* and *P. japonica*. Nitrogen retention from the soil was also significant when the colonization level was high in aquatic-terrestrial inter-faces (river banks) whereas *P. cuspidatum* showed very less or a negative response to AMF colonization in all cases.

In addition, the unfavorable oxidative effects adversely influence plant growth under heavy metal stress. However, AM are able to enhance production of antioxidant enzymes, which can alleviate the stress of heavy metals like zinc (Zn) or Lead (Pb). Zinc is an essential micronutrient for plant growth. Conversely, Zn is also an important environmental contaminant in some situations, often reaching phytotoxic concentrations. The feasibility of employing AM in soil re-vegetation

and remediation has elicited great interest, and numerous studies have focused on the functions of AM fungi in metal-contaminated soils. Although AM are most often considered important for uptake of immobile nutrients, they also play an important role in reducing uptake of heavy metals, including Zn, where soil concentrations are high. Thus, AMF have various roles in terms of plant-Zn interactions. To understand these roles, there is a need to study responses of AM across a range of soil types, Zn concentrations and plant species. Therefore, we studied the effects of arbuscular mycorrhizal association on growth and survival capabilities of *Miscanthus sacchariflorus* under different Zn concentrations in soil. Considering the type of mycorrhizal inoculation and addition of Zn, six treatments were taken, viz. (1) soil inoculated by AM fungi (2) soil inoculated by AM fungi and addition of zinc (Zn) 100 mg Kg^{-1} (3) soil inoculated by AM fungi and addition of zinc (Zn) 1000 mg Kg^{-1} (4) soil without AM fungi inoculation (5) soil without AM fungi inoculation but addition of zinc (Zn) 100 mg Kg^{-1} and (6) soil without AM fungi inoculation but addition of zinc (Zn) 1000 mg Kg^{-1} . The Zn0 treatment received no additional Zn, while the Zn100 and Zn1000 treatments were established by adding $ZnSO_4.7H_2O$ to give Zn additions of 100 mg Zn Kg^{-1} and 1000 mg Zn Kg^{-1}.The Zn addition treatments were selected on the basis of the reported existence of soil zinc.The experiment was conducted in the form of pot cultures of *M. sacchariflorus* in a greenhouse at Saitama University, Japan. The pots were laid out in complete randomized design (CRD) in the greenhouse, with four replicates per treatment.

In our experiment, addition of Zn did not show significant effect of mycorrhizal colonization. Even, when the Zn addition level was as high as 1000 mg kg^{-1}, the mycorrhizal infection rate slightly decrease compared to the control receiving no Zn but not statistically significant different. This may imply that Zn has no or little effect on spore germination and AM colonization. Inoculation of AMF (*Gigaspora margariata*) with Zn (100mg kg^{-1}) increased chlorophyll content, Fv/Fm, total dry mass, IAA, TN, TP and Zn concentration and H_2O_2 level, IAAO activity, POD activity was low compare to other two treatments whereas with Zn (1000mg kg^{-1}) induces lower concentrations of these metals in the aerial part of the plant and consequently a beneficial effect on plant growth. In addition, AMF can able to accumulate Zn in plant root. When approaching the inner part of the root, heavy metals are located in the parenchyma cells. Accordingly, it can be stated that AM are able to keep heavy metals out of plant or reduce concentration of areal parts of plants especially for Zn.

Lead (Pb) is a heavy metal that is present in the soil in very small amounts, but anthropogenic activities have increased its content in some locations, which can make these areas unproductive or inappropriate for crop production. Under heavy metal stress, the unfavorable oxidative effects adversely influence plant growth.

However, AM are able to enhance production of antioxidant enzymes, which can alleviate the stress of heavy metals and finally plant growth. In the present experiment, we studied the effects of arbuscular mycorrhizal fungal(AMF) association on growth, survival capabilities, nutrients and Pb uptake of *Miscanthus sacchariflorus* under different Pb concentrations in soil.The experiment was conducted in the form of pot cultures of *M. sacchariflorus*. The pots were laid out in complete randomized design (CRD) with three replicates per treatment. The treatments were composed of the inoculation or no inoculation of the AM fungus, *Gigaspora margarita*, and the addition of three Pb concentrations in the soil (0, 100 and 1,000 mg kg^{-1}). Addition of Pb significantly decreased mycorrhizal colonization. Inoculation of AMF with Pb increased chlorophyll content, Fv/Fm, total dry mass, IAA, TN, and TP whereas H2O2 level, IAAO activity, POD activity was low compare to non-inoculated treatments. Moreover, application of AMF with Pb doses induces concentrations of Pb in the plant where at higher dose Pb (1000mg $^{-1}$) induces lower content of Pb in the aerial part of the plant but higher content in root. AMF enhanced the tolerance of *M. sacchariflorus* against Pb toxic condition and accumulate Pb in plant root whereas translocation to the shoots was inhibited in higher dose Pb (1000mg kg^{-1}).

Chapter 1

General Introduction

1.1 Arbuscular mycorrhiza in flood plain

Arbuscular mycorrhiza is the predominant mycorrhizal type found in early stages of primary succession; for example, in sand dunes (Corkidi and Rincon, 1997; Logan et al., 1989) and river floodplains (Miller and Sharitz, 2000; Nakatsubo, 1997). Since soils in the early stages of primary succession generally have low nutrient contents, it is possible that AM fungi play an important role in the growth and establishment of pioneer plants. Mycorrhizal fungi also occur in riparian areas (Helm et al., 1996; Jacobson, 2004; Piotrowski et al., 2008),but their functions in promoting plant growth across floodplain chronosequences are unknown. Concurrent with vegetation development are changes in soil properties, such as increased concentration of silt relative to sand and increased organic matter content, which influence availability of nutrients to support plant growth (Cleve et al., 1993). Plant species colonizing such areas show a wide range of site requirements. Some species, such as *Miscanthus sacchariflorus* or *Phragmites japonica*, grow in stony sterile as well as low nutrient conditions (Asaeda et al., 2011a), while other species prefer relatively fine sediment with nutrient rich conditions.

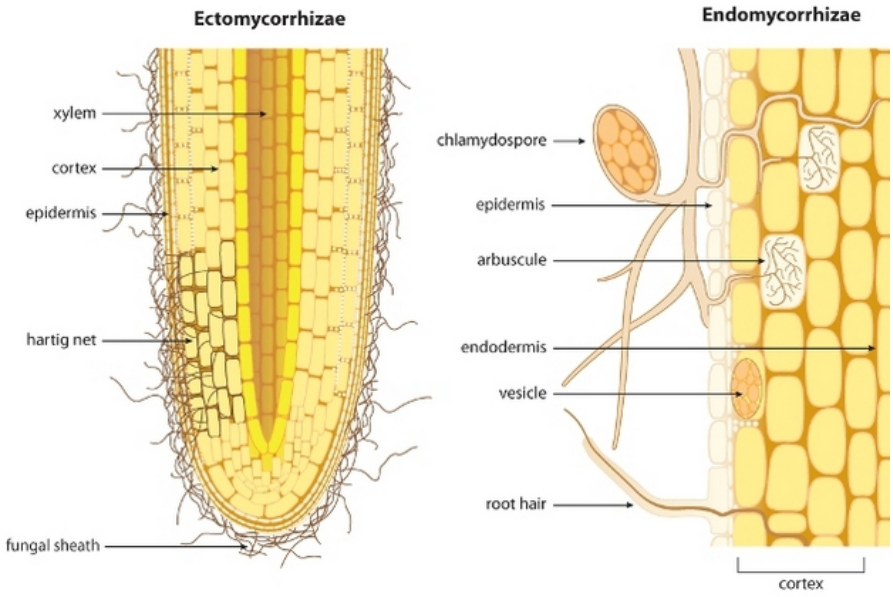

Figure 1.1: Figure showing the absorption mechanism of mycorrhizal fungus

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Arbuscular mycorrhizal fungi (AMF) are ubiquitous components of terrestrial ecosystems across the world with multiple functions from the level of individual plants to the ecosystem (Smith and Read, 2008). In P-deficient soils, the yields of horticultural and field crops were found to be largely dependent on their mycorrhizal status under field (Ortas, 2008) and greenhouse conditions (Ortas, 2003). Soils in floodplains are generally coarse and low in nutrients. These characteristics inhibit the development of dense plant communities (Giller and Malmqvist, 1998; Nilsson, 1987; Pinay et al., 1992). Nutrient enrichment of soil results from plant succession and is essential for the establishment of large herbaceous plant biomass (Carson and Barrett, 1988; Foster and Gross, 1998). Terrestrial systems with infrequent disturbances tend to exhibit a one-way progression of community succession. In contrast, floodplains are subjected to flood disturbances; the nutrient-rich surface sediments are often flushed away, exposing the underlying coarse sediment surfaces, or coarse sediments transported by flood flows accumulate and cover the original surfaces (Asaeda et al., 2011a; Cui and Caldwell, 1997). In floodplain soil, plant growth is often restricted by a shortage of nitrogen and phosphorus (Asaeda and Rashid, 2012; Bedford et al., 1999). Under these conditions, mycorrhiza can increase the capability of plants for assimilating the nutrients from low nutrient soil such as sediments of floodplains (Harner et al., 2011; Miller and Sharitz, 2000; Nakatsubo, 1997). Thus, flood plains along rivers with natural or near natural flow regimes are model systems for testing hypotheses about interactions among

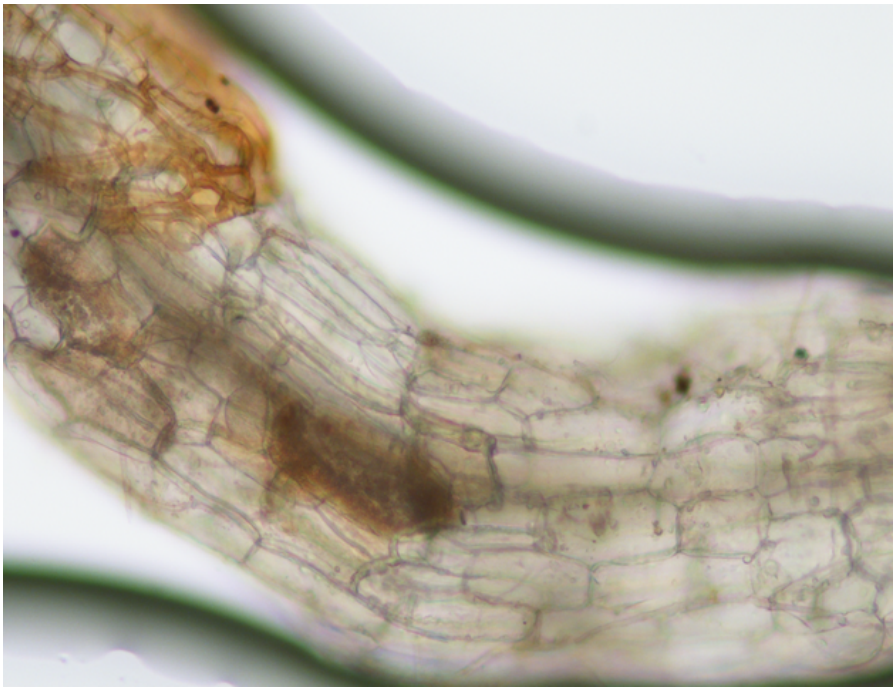

Figure 1.2: Figure represents the absorptive hyphae, arbuscule and vesicule present in our experimental plant

plants, soil, and mycorrhizal fungi because of the diversity of habitats and disturbance regimes in close proximity to one another. Therefore, we examined the effects of AM colonization on the growth and nutrient assimilation of pioneer plants growing in the early stage of primary succession area of river bank in this study.

1.2 Arbuscular mycorrhiza for Zn uptake

Soil stresses such as heavy metals, compaction, salinity and drought can decrease plant growth and hence production. AM can significantly increase plant growth and production under stress due to the formation of extensive hyphal networks. Such abilities can result in enhanced water and nutrient uptake. Under heavy metals stress, the diversity of AM spores decreases compared with stress-free conditions. Hence, a limited number of spores are usually found in the rhizosphere of e.g., Zn-tolerant plant species (Gonzalez-Guerrero et al., 2008; Pawlowska et al., 1997; del Val et al., 1999). Under heavy metal stress, the unfavorable oxidative effects adversely influence plant growth. However, AM are able to enhance production of antioxidant enzymes, which can alleviate the stress of heavy metals (Avery, 2001; Ruiz-Lozano, 2003). It is interesting to examine how some species

develop mycorrhizal symbiosis and how this ability can be improved under conditions including heavy metal stress. AM symbiosis with plants has been observed in soils containing heavy metals (Khan et al., 2000). Most plants that are tolerant to heavy metal stress, intensify their symbiosis (higher root colonisation) with AM at the stage of high-nutrient demand, e.g., at the reproductive stage. The gene products can stabilise and rearrange the structure of proteins that are denatured due to the oxidative stress of heavy metals. The enhanced tolerance of AM plants is related to the simultaneous regulation of AM stress genes and plant tolerance genes (Hildebrandt et al., 2007; Ruiz-Lozano, 2003). It should be mentioned that high levels of stress may turn the symbiosis between the two partners into a parasitic relationship, as unfavourable conditions may adversely influence AM performance (Hildebrandt et al., 2007; Miransari et al., 2008, 2009). The adverse effects of AM on plant growth under stress conditions can be through unfavourable effects of the stress on AM functioning and development. These effects include decreased colonisation rate and spore germination, as well as decreased fungal hyphal growth especially under salt stress condition (Evelin et al., 2009; Jahromi et al., 2008).

Zinc is an essential micronutrient for plant growth. Conversely, Zn is also an important environmental contaminant in some situations, often reaching phytotoxic concentrations (Christie et al., 2004). Common sources of Zn (as a toxicant) in soils include mine spoilings, and runoff from galvanized metal surfaces and roadways. Understanding the mechanisms by which plants maximize acquisition and utilization of Zn under low concentrations, and deal with toxic Zn concentrations in soils, has been studied previously (Cavagnaro et al., 2008; Christie et al., 2004). However, few studies have considered plant responses to low and high soil Zn concentrations in the same study (Chen et al., 2003). Although the functions of AM under conditions of micronutrient deficient conditions have been widely studied and are well understood, much information is required on the contrasting conditions of excessive trace elements (Leyval et al., 1997; Weissenhorn et al., 1995a). The widespread existence of AM fungi in metal contaminated sites has also provided evidence of adaptation and tolerance of microorganisms to toxic metals(Kaldorf et al., 1999; Pawlowska et al., 1997), and metal tolerant fungi have been isolated (Weissenhorn et al., 1993). The feasibility of employing AM in soil revegetation and remediation has elicited great interest, and numerous studies have focused on the functions of AM fungi in metal-contaminated soils (Leyval et al., 1997). Although AM are most often considered important for uptake of immobile nutrients, they also play an important role in reducing uptake of heavy metals, including Zn, where soil concentrations are high (Christie et al., 2004; Hildebrandt et al., 2007). Thus, AMF have various roles in terms of plant-Zn interactions. To understand these roles, there is a need to study responses of AM across a range of soil types, Zn concentrations and plant species(Cavagnaro et al., 2008). Soil

Zn conditions not only influence the functioning of AM, but also their formation. Addition of Zn to soils in amounts ranging from deficient through to toxic, can have positive (Heggo et al., 1990; Hetrick et al., 1994; Lee and George, 2005; Zhu et al., 2001), negative (Bi et al., 2003; Chen et al., 2003) and neutral(Diaz et al., 1996; Ortas et al., 2002) effects on root colonization by arbuscular mycorrhizal fungi (AMF). Given the complex roles of AM in plant-Zn dynamics, there is a need to assess Zn effects on them under both high and low soil Zn conditions in the same study. Here we report results of an experiment in which we assessed the effects of Zn addition on the mycorrhizal colonization, growth, nutrition and related biochemical parameters of the *Miscanthus sacchariflorus* plants responding to these conditions.

1.3 Arbuscular mycorrhiza for Lead (Pb) uptake

1.4 Hypothesis

On the basis of above background, we formulated two hypothesis in relation to the effects of arbuscular mycorrhizal fungi (AMF) on growth, nutrients and heavy metal uptake of pioneering plants under nutrients and heavy metal stress conditions in the floodplain of Ara River, Japan. First, inoculation of AMF enhances plant growth and nutrient uptake of common species growing in nutrient-limited river bank. Second, AMF play an important role for stress minimization and uptake of heavy metals under zinc and lead stressed condition.

1.5 General objectives

Therefore, the general objective of the study is to explore the application of AMF strains *(Gigaspora margarita* Becker & Hall) with *Miscanthus sacchariflorus*, *Phragmites japonica* and *Polygonum cuspidatum* for growth and nutrients assimilation under nutrient-limited condition with special emphasis on Zn and Pb uptake under heavy metal stress condition of *M. sacchariflorus* in the flood plain of Ara River, Japan.

1.6 Thesis outline

The project combined five experimental approaches: i) The potential effects of arbuscular mycorrhizal fungi (AMF) on growth, nutrient assimilation, and inoculation effectiveness of AMF on the pioneer C4 plant *Miscanthus sacchariflorus*

which is dominant in river banks were evaluated; ii) Role of arbuscular mycorrhizal fungi on the performance of floodplain *Phragmites japonica* (moderately C4) under nutrient stress condition were evaluated; iii) Comparative study was done between dominant *Phragmites japonica* (moderately C4) and *Polygonum cuspidatum* (C3); and iv) Contribution of arbuscular mycorrhizal symbiosis on growth and allied biochemical parameters of *Miscanthus sacchariflorus* under zinc (Zn) stress were evaluated; and v)Effects of AMF on growth, growth regulating parameters and lead (Pb) uptake of *Miscanthus sacchariflorus* under lead (Pb) stress were assessed.

Chapter 1: Arbuscular mycorrhizal influences on growth, nutrient uptake, and use efficiency of *Miscanthus sacchariflorus* growing on nutrient-deficient river bank soil

In floodplain soil, plant growth is often restricted by a shortage of nutrients. Under these conditions, mycorrhiza can increase the capability of plants for assimilating the nutrients from low nutrient soil such as sediments of floodplains. Flood plains along rivers with natural or near natural flow regimes are model systems for testing hypotheses about interactions among plants, soil, and mycorrhizal fungi because of the diversity of habitats and disturbance regimes in close proximity to one another. In this study, we examined the effects of AM colonization on the growth and nutrient concentration of a pioneer plant. Therefore, the present study was conducted with the following objectives: i) to determine the effect of AMF on growth and nutrient assimilation of *M. sacchariflorus* and ii) to estimate the role of AMF on nutrient loss minimization.

Chapter 2: Role of arbuscular mycorrhizal fungi on the performance of floodplain *Phragmites japonica* under nutrient stress condition

Soils in floodplains are generally coarse and low in nutrients. These characteristics inhibit the development of dense plant communities. Terrestrial systems with infrequent disturbances tend to exhibit a one-way progression of community succession. Mycorrhiza can increase plants' ability to assimilate the nutrients from low-nutrient soil, such as the sediments of floodplains. Therefore, the present study was conducted to evaluate the effectiveness of AM symbiosis in enhancing *Phragmites japonica* growth and nutrient uptake in nutrient-deficient flood plain soils.

Chapter 3: Arbuscular mycorrhizal association for growth and nutrients assimilation of *Pharagmites japonica* and *Polygonum cuspidatum* plants growing on river bank soil

AM colonization level, growth and nutrient uptake not only depend on the density of AM fungi but also the plant species at the same environment. Considering the above statement, comparison between *Pharagmites japonica* (moderately C4) and *Polygonum cuspidatum* (C3) plants performance under low nutrient condition was evaluated. This study was planned with objectives: i) to determine whether AMF change floodplain river bank vegetation development by increasing growth and nutrient assimilation of *P. japonica* appeared to be the most common species and covered higher percent areas of the transects and *P. cuspidatum* plants, ii) to estimate the role of AMF on nutrient loss minimization for increasing river bank vegetation growth, iii) to find out the effect variation of AMF when applied with *P. japonica* and *P. cuspidatum* species.

Chapter 4: Response of *Miscanthus sacchariflorus* to zinc stress mediated by arbuscular mycorrhizal fungi

Zinc is an essential micronutrient for plant growth. Conversely, Zn is also an important environmental contaminant in some situations, often reaching phytotoxic concentrations. AMF have various roles in terms of plant-Zn interactions. To understand these roles, there is a need to study responses of AM across a range of soil types, Zn concentrations and plant species. Given the complex roles of AM in plant-Zn dynamics, there is a need to assess Zn effects on them under both high and low soil Zn conditions in the same study. Here we report results of an experiment in which we assessed the effects of Zn addition on the mycorrhizal colonization, growth, nutrition and related biochemical parameters of the *Miscanthus sacchariflorus* plants responding to these conditions.

Chapter 4: Arbuscular mycorrhiza confers Pb tolerance and uptake in *Miscanthus sacchariflorus*

we report results of an experiment in which we assessed the effects of Pb addition on the mycorrhizal colonization, growth, nutrition, Pb uptake, and related biochemical parameters of the *M. sacchariflorus* plant responding to these conditions.

Chapter 2

Arbuscular mycorrhizal influences on growth, nutrient uptake, and use efficiency of *Miscanthus sacchariflorus* growing on nutrient-deficient river bank soil

Abstract

The potential effects of arbuscular mycorrhizal fungi (AMF) on growth, nutrient assimilation, and inoculation effectiveness of AMF on the pioneer plant *Miscanthus sacchariflorus* which is dominant in river banks were evaluated. A pot experiment was performed in a greenhouse at Saitama University, Japan. Spores of AMF strains were collected from the commercial product 'Serakinkon'. The average colonization level of *M. sacchariflorus* was 23–28%, whereas no colonization was found in sterilized soil. AMF colonization increased the chlorophyll content, plant dry mass, N, P, K, Mg, Fe, Cu, and Zn concentration of the *M. sacchariflorus* plant's roots, stems, and leaves when it was applied with natural and sterilized soil. In all cases, maximum values showed when the *M. sacchariflorus* plant was applied with natural soil in combination with AMF, but Ca concentration decreased as colonization level increased. Mn concentration decreased in roots and stems but increased in leaves in natural soil, and AMF with natural soil treatment. N loss minimization from the soil was significant when colonization level was high. Therefore, AMF have some potential effects for growth of the C4 grass

Miscanthus sacchariflorus and nitrogen retention in aquatic-terrestrial inter-faces (river banks).

2.1 Introduction

Arbuscular mycorrhiza is the predominant mycorrhizal type found in early stages of primary succession; for example, in sand dunes (Corkidi and Rincon, 1997; Logan et al., 1989) and river floodplains (Miller and Sharitz, 2000; Nakatsubo, 1997). Since soils in the early stages of primary succession generally have low nutrient contents, it is possible that AM fungi play an important role in the growth and establishment of pioneer plants. Mycorrhizal fungi also occur in riparian areas (Helm et al., 1996; Jacobson, 2004; Piotrowski et al., 2008), but their functions in promoting plant growth across floodplain chronosequences are unknown. Concurrent with vegetation development are changes in soil properties, such as increased concentration of silt relative to sand and increased organic matter content, which influence availability of nutrients to support plant growth(Cleve et al., 1993). Plant species colonizing such areas show a wide range of site requirements. Some species, such as *Miscanthus sacchariflorus* or *Phragmites japonica*, grow in stony sterile as well as low nutrient conditions (Asaeda et al., 2011a), while other species prefer relatively fine sediment with nutrient rich conditions.

Arbuscular mycorrhizal fungi (AMF) are ubiquitous components of terrestrial ecosystems across the world with multiple functions from the level of individual plants to the ecosystem (Smith and Read, 2008). In P-deficient soils, the yields of horticultural and field crops were found to be largely dependent on their mycorrhizal status under field (Ortas, 2008) and greenhouse conditions(Ortas, 2003). Soils in floodplains are generally coarse and low in nutrients. These characteristics inhibit the development of dense plant communities (Giller and Malmqvist, 1998; Nilsson, 1987; Pinay et al., 1992). Nutrient enrichment of soil results from plant succession and is essential for the establishment of large herbaceous plant biomass (Carson and Barrett, 1988; Foster and Gross, 1998). Terrestrial systems with infrequent disturbances tend to exhibit a one-way progression of community succession. In contrast, floodplains are subjected to flood disturbances; the nutrient-rich surface sediments are often flushed away, exposing the underlying coarse sediment surfaces, or coarse sediments transported by flood flows accumulate and cover the original surfaces (Asaeda et al., 2011b; Cui and Caldwell, 1997). In floodplain soil, plant growth is often restricted by a shortage of nitrogen and phosphorus (Asaeda and Rashid, 2012; Bedford et al., 1999). Under these conditions, mycorrhiza can increase the capability of plants for assimilating the nutrients from low nutrient soil such as sediments of floodplains (Harner et al., 2011; Miller and Sharitz, 2000; Nakatsubo, 1997). Thus, flood plains along rivers with natural or near natural

flow regimes are model systems for testing hypotheses about interactions among plants, soil, and mycorrhizal fungi because of the diversity of habitats and disturbance regimes in close proximity to one another.

In this study, we examined the effects of AM colonization on the growth and nutrient concentration of a pioneer plant. Therefore, the present study was conducted with the following objectives: i) to determine the effect of AMF on growth and nutrient assimilation of *M. sacchariflorus* and ii) to estimate the role of AMF on nutrient loss minimization.

2.2 Materials and Methods

2.2.1 Soil and plant propagate collection from study sites

A preliminary survey was conducted on the banks of the Ara River, Saitama, Japan (36°4′58.02″ N and 139°26′28.85″ E) to identify suitable, experimental plant materials for this study (Figure 6.4). Five meter × four meter transects (henceforth referred as S1∼S5, respectively) were randomly selected along the river and marked with poles and rods. The distance between these transects ranged between 10 and 30 m. All plant species within a transect were identified, and their percent coverage was calculated. Though the species composition varied between the transects, *Miscanthus sacchariflorus* appeared to be the most common species and covered a higher percentage of the transects areas. Azami et al. (2004), Asaeda et al. (2010), and Sekine et al. (2012) also reported that this plant is the most common on the Arakawa River bank. Therefore, based on the survey and previous literature, we chose *M. sacchariflorus* as our experimental plant species. Soil was collected from up to a depth of 20 cm from the surface of the previously selected transects using a shovel. Soils collected from different transects were composited to make a homogenous soil pool. The properties of the soils collected from the transects were homogenous, and there were no significant differences between the individual soil samples from the transects and the composite soil in terms of soil particle size (D25), pH, percentage organic matter content, total carbon (TC), total nitrogen (TN), total phosphorus (TP), and available phosphorus concentrations and water holding capacity (all $p > 0.05$). The soil utilized for the experiment had an average pH of 5.8, with an average of 0.61 mm of D25, organic matter content 0.25%, TP 0.45 mg/g, available phosphorus 0.02 mg/g, TN 0.9 mg/g, water holding capacity 27%, potassium (K) 1.7 mg/g, calcium (Ca) 0.4 mg/g, magnesium (Mg) 1.1 mg/g, iron (Fe) 0.55 mg/kg, copper (Cu) 0.05 mg/kg, manganese (Mn) 0.40 mg/kg, and zinc (Zn) 0.27 mg/kg. Propagates (rhizomes) of *M. sacchariflorus* for planting in pots were collected from the same sites.

2.2.2 Spore isolation from collected soil

Spores were extracted from the same composite soil by wet sieving and centrifugation (Gerdemann and Nicolson, 1963). For each 100 g of collected soil, 3 L of tap water were used to suspend the soil for subsequent passage through a pair of sieves (860 and 36 μm mesh). The material retained in the 36 μm sieve was centrifuged in water (900× g for 4 min), and the resulting pellet was resuspended in 45% sucrose solution and centrifuged again (900 × g for 1 min). The supernatant was washed through a sieve of 36 μm, washed with tap water, and the spores were transferred to Petri dishes. Spores were manipulated under a stereomicroscope and spore morphotypes separated according to spore morphology and color.

2.2.3 Experimental procedure and design

Considering the type of mycorrhizal inoculation, four treatments were taken, viz. (1) natural soil (NS) (2) natural soil inoculated by AM fungi (NS+AM) (3) sterilized soil inoculated by AM fungi (SS+AM) and (4) sterilized soil without AM fungi inoculation (SS). The experiment was conducted in the form of pot cultures of *M. sacchariflorus* in a greenhouse at Saitama University, Japan. The pots were laid out in complete randomized design (CRD) in the greenhouse, with four replicates per treatment.

In each pot (diameter 20.96 cm, vol. 2.7 L), 2 kg (natural or sterilized) of soil was used. Initial moisture content and bulk density of the soil were estimated. For sterilizing, soil was autoclaved twice at 121° C for 1 h to eliminate any indigenous AM fungi. To inoculate soils of respective treatments, AM fungi spores were applied in the form of a commercial inoculant namely 'Serakinkon' powder (The Central Glass Company, Tokyo, Japan). The inoculant was composed of 50 *Gigaspora margarita* Becker & Hall spores per gram powder (Higo et al., 2010; Kaneko and Tanimoto, 2009; Tawaraya et al., 1996) as *Gigaspora* was the most commonly isolated AMF on river bank sand with higher spore densities than those of other species; previous studies revealed the same result (Koske and Gemma, 1997; Kowalchuk et al., 2002; Rose, 1988). 20 g serakinkon powder/kg soil was evenly mixed with soil before being used in a pot. Rhizomes with three nodes (∼2 cm) were employed as propagating materials. A temperature of 25 ± 3° C and 75% relative humidity were maintained in the greenhouse day and night throughout the experimental period. Pots were watered as necessary. The greenhouse culture lasted for 110 days.

2.2.4 Chlorophyll content measurement

At harvest, chlorophyll was extracted from the leaves of *M. sacchariflorus* using N,N dimethylformamide (DMF). The leaves were cut, and fresh weight (∼5 mg) was measured. Pigments were then extracted into 5 ml DMF by incubating leaf cuttings 24 h in a refrigerator at 4° C. The chlorophyll concentration was determined via the method described by Porra et al. (1989). Absorbance at 646.8 nm and 663.8 nm was measured with a UV-mini 1200-spectrophotometer (Shimadzu, Japan). Chlorophyll concentrations were calculated as μg/mg fresh weight (FW) basis.

2.2.5 Harvesting and processing

Plants were harvested at 110 days after transplanting at their senescence stage. After separating from the pot, the shoots were separated from the roots, and fresh weights were taken separately for the roots, stems, and leaves. Soil particles were carefully removed from plant roots using forceps. The whole root system was then divided into two parts: one part for AM colonization observation and the other part for estimating dry mass and analyzing nutrients. Stems, leaves, and roots (fraction) were cut into small pieces and dried to a constant weight at 80° C for 3 days, and final weights (dry mass) were recorded. Since not the whole root was dried, the fraction of oven-dried root was taken into consideration for estimating belowground dry mass. Shoot (including stem and leaf) dry mass was considered as aboveground dry mass. Oven-dried plant tissues (root and shoot) were ground separately, and after removing from the pot and plant roots, the soil was weighed for nutrient budgeting. Moisture content by using the gravimetric method (Black, 1965) and bulk density were also estimated. A fraction of soil was oven-dried at 80° C for 3 days, and the final weight was recorded. Ground plant tissues and soils were kept in air-tight containers for further analysis.

2.2.6 AM colonization determination and nutrient analyses from soil and plant samples

To estimate the extent of AM colonization, roots were cleaned, cut into small segments (2 cm/segment), rinsed and cleared for at least 12 h in 10% KOH solution at room temperature, and then stained with 0.05% trypan blue solution (Koske and Gemma, 1989). Ten randomly selected root fragments from each treatment were mounted on slides, and intersecting vertical gridlines were observed at 100X magnification to determine the presence and absence of AM colonization (internal hyphae, vesicles, or arbuscules) (McGonigle et al., 1990).

Total nitrogen (TN) concentrations of soil and plant tissues were determined with a CHN analyzer (CHN Corder MT-5, Yanoco, and Kyoto, Japan). Powdered samples (2–10 mg) were placed in a crucible and ashed in a muffle furnace for 2 h at 500° C, then dissolved in 1 ml of 5 M HCl, and partially neutralized with 5 M NaOH solution. Orthophosphate was then determined using the phosphomolybdate method according to Murphy and Riley (1962). Total phosphorus in the soil and plant samples was determined by perchloric acid digestion, and available phosphorus was determined after extraction with bray extract solution (Bray and Kurtz, 1945). The concentrations of Ca, Mg, K, Fe, Mn, Cu, and Zn in plants were analyzed after oven drying to a constant weight at 72° C followed by digestion in a perchloric acid mixture, as described by Tandon (1993). Nutrient concentrations in the tissue digest were quantified using an atomic absorption spectrophotometer (AA-6300; Shimadzu, Japan) using the method described in standard methods for the examination of water and wastewater by Gilcreas (1966).

2.2.7 Calculation of inoculation effectiveness

Inoculation effectiveness (IE) was calculated using the following equations based on aboveground dry mass (IEa), belowground dry mass (IEb), and nutrient assimilation (IE_{Nutrient}) as suggested by Conversa et al. (2013),Ortas (2012) and Watts-Williams et al. (2013):

$$IE_a = \left[\frac{(ADM_{+M} - ADM_{-M})}{ADM_{+M}}\right] \times 100 \tag{2.1}$$

$$IE_b = \left[\frac{(BDM_{+M} - BDM_{-M})}{BDM_{+M}}\right] \times 100 \tag{2.2}$$

$$IE_{\text{Nutrient}} = \left[\frac{(\%\text{Nutrient}_{+M} - \%\text{Nutrient}_{-M})}{\%\text{Nutrient}_{+M}}\right] \times 100 \tag{2.3}$$

where, IE_a, IE_b, and IE_{Nutrient} designate inoculation effectiveness based on above ground dry mass (ADM), belowground dry mass (BDM), and nutrient (nitrogen or phosphorus) assimilation (IENutrient), and subscript $+M$ and $-M$ designate inoculated and non-inoculated plants, respectively.

2.2.8 N and P budget calculation

Inputs and outputs of nitrogen (N) and phosphorus (P) in the soil-plant system were estimated for this experiment. Nutrients from the soil before planting

propagules (henceforth 'initial soil') and propagating organs (rhizomes) were considered as input, and output estimates included nutrients removed by crop harvest and nutrient status of the remaining soil after crop harvest (henceforth 'final soil').

2.2.9 Statistical analyses

Prior to commencing statistical analyses, all data were checked for normality, and equal variance was checked using the Levene's test. All data are presented as mean ± SE (n = 4). The effects of mycorrhizal treatment on chlorophyll concentration in the leaves, root colonization, dry weight, nutrient (N, P, K, Ca, Mg, Fe, Mn, Cu, and Zn) concentration of each plant component, and soil were analyzed by two-way ANOVA followed by Tukey's posthoc test at 0.05 significant levels. Correlations between parameters were judged by Pearson's correlation coefficient at the 0.05 significant level. All statistical analyses were performed using SPSS for Windows (Version 13.0, SPSS, Inc., Chicago, IL, USA) and R package (Team, 2010).

2.3 Results

2.3.1 Root colonization

Microscopic observations revealed that almost all root samples of *M. sacchariflorus* in AM treatments were colonized by AM fungi both in collected and sterilized soil. Colonization level of roots in the NS+AM treatment was significantly higher in comparison with the NS treatment. The average colonization level of *M. sacchariflorus* was 23–38% in different treatments (Figure 2.1), whereas in sterilized soil (AM fungi absent), no colonization was found.

2.3.2 Chlorophyll content in leaves

Plants inoculated with *Gigaspora margarita* had significantly higher leaf chlorophyll concentrations than non-inoculated plants. Chlorophyll concentration significantly increased in AM plants both in combination with natural (NS+AM) and sterilized soil (SS+AM), compared to plants in natural soil and sterilized soil without *Gigaspora margarita* (Figure 2.2).

2.3.3 Changes in dry mass production due to AM fungi

AM fungi showed a significant effect on dry mass (DM) production of the *M. sacchariflorus* plants. For roots, maximum dry mass (8.78 g) was produced in

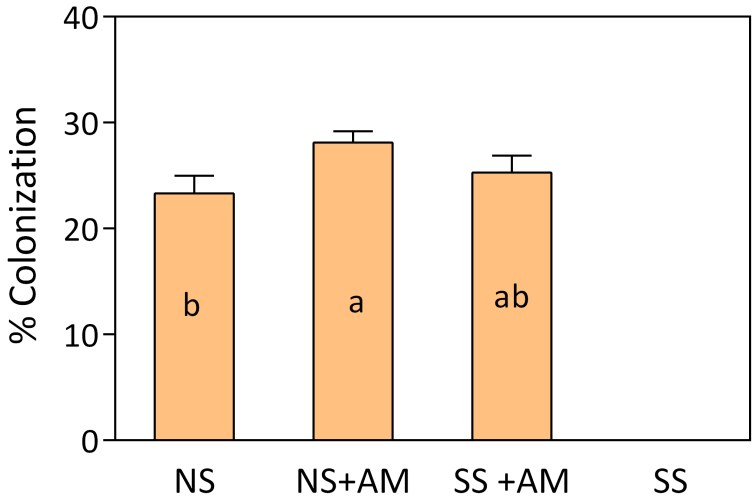

Figure 2.1: Arbuscular mycorrhizal (AM) colonization levels in the herbaceous species (*M. sacchariflorus*) growing on primary succession soils collected from the Arakawa River bank where natural soil (NS), natural soil inoculated by AM fungi (NS+AM), sterilized soil inoculated by AM fungi (SS+AM) and sterilized soil without AM fungi inoculation (SS). Values represent means of four replicates, and 10 root segments per replicate were selected for observation. Error bars indicate ± standard error (SE) of mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

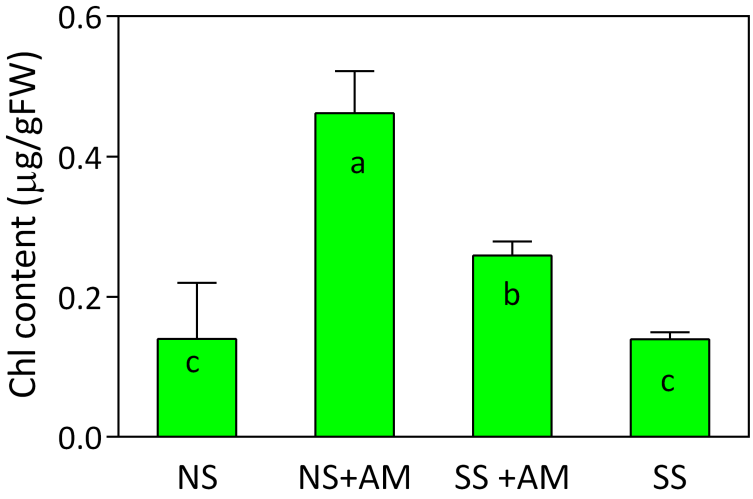

Figure 2.2: Chlorophyll content in leaves from different treatments where natural soil, natural soil inoculated by AM fungi, sterilized soil inoculated by AM fungi and sterilized soil without AM fungi inoculation denoted as NS, NS+AM, SS+AM and SS . Error bars indicate ± SE of mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

the NS+AM treatment, whereas the lowest one was in the SS treatment (3.15 g), and the SS+AM treatment showed better performance than the NS and SS treatment. In the case of stem dry mass production, the NS+AM treatment revealed a significantly higher value in comparison with other treatments, but for leaf dry mass production, all treatments showed statistically similar values except for the SS treatment which produced the lowest value. The trend of total dry mass production was NS+AM > SS+AM ≥ NS ≥ SS (Figure 2.3), and the values in the NS+AM treatment were significantly higher than for all other treatments.

2.3.4 Total phosphorus (TP) and total nitrogen (TN) concentrations in plants

Inoculation by AM fungi showed a positive effect on P concentration of plants and was directly related to the colonization level. Plant P concentration was higher when the colonization level was more pronounced, and this relationship was significant across all trials (Figure 2.4). P concentration in plants (roots, stems, and leaves) was the highest in AM fungi inoculated with natural soil treatment; whereas it was the lowest in the sterilized soil (absence of AM fungi) treatment. When AM fungi were applied with sterilized soil, P concentration in plants was

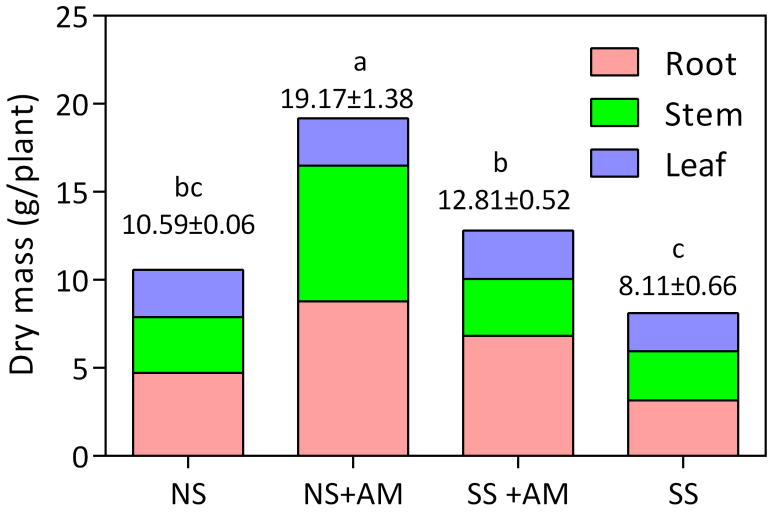

Figure 2.3: Dry mass production of *M. sacchariflorus* where natural soil (NS), natural soil inoculated by AM fungi (NS+AM), sterilized soil inoculated by AM fungi (SS+AM) and sterilized soil without AM fungi inoculation (SS). Values represent the total dry mass ± SE of mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

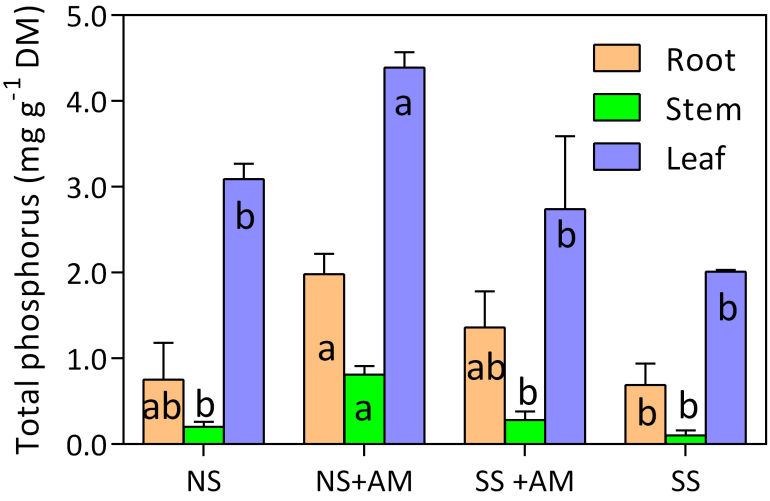

Figure 2.4: TP (mg g^{-1} DM) concentration of plant in different treatments where natural soil (NS), natural soil inoculated by AM fungi (NS+AM), sterilized soil inoculated by AM fungi (SS+AM) and sterilized soil without AM fungi inoculation (SS). Error bars indicate ± SE of mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

statistically similar to natural soil (Figure 2.4). TN concentration in plants varied with the colonization level of AM fungi as well. Nitrogen and P concentrations in the roots, stems, and leaves of the plants were maximum in the NS+AM treatment. Minimum N concentration was shown in sterilized soil. When AM fungi were inoculated with sterilized soil, N concentration was higher in the roots and stems than with natural soil (Figure 2.5).

2.3.5 Total phosphorus, available phosphorus and total nitrogen content in post-harvest soils

Soil TP concentration was the lowest in NS+AM treatments, where mycorrhiza colonization level as well as TP concentrations in the plant were highest, but there was no significant difference among different treatments; the same results were also found for available phosphorus and total nitrogen concentration in the soil after harvesting the plant (data not shown).

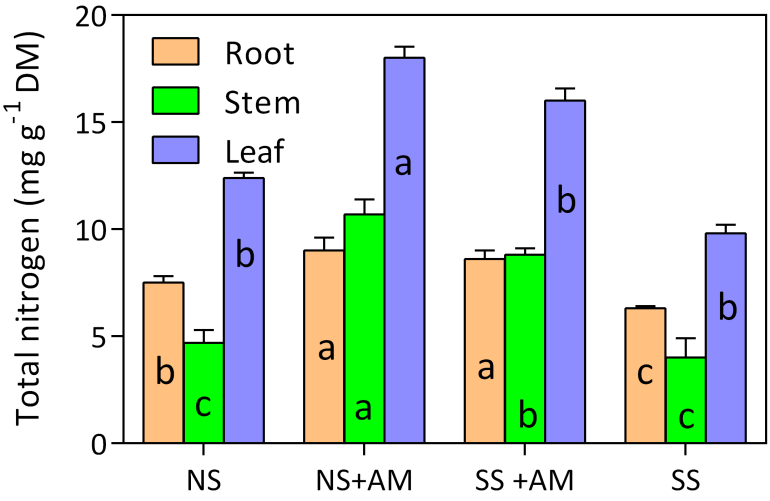

Figure 2.5: TN (mg g^{-1} DM) concentration of plant in different treatments.where natural soil (NS), natural soil inoculated by AM fungi (NS+AM), sterilized soil inoculated by AM fungi (SS+AM) and sterilized soil without AM fungi inoculation (SS). Error bars indicate ± SE of mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

2.3.6 Nitrogen and phosphorus budgeting

Minimum nitrogen loss was found in the NS+AM treatment (19.8%), and maximum nitrogen loss was observed in the SS treatment (35.9%); however, loss in the SS+AM treatment was lower than the NS treatment (Table 2.1). According to these results, there was no significant effect of AM fungi on P loss minimization in different treatments Figure 6.1. For all cases, about a 1% loss was determined.

2.3.7 Major and trace elements content in plants

With the increase of colonization level, major nutrients (K, Mg) and trace elements (Fe, Cu, and Zn) concentration increased in the plant' s roots (Cu), stems (K, Mg, Fe, and Cu), and leaves (K, Mg, Fe, Cu and Zn), and maximum concentrations were seen in the NS+AM treatment followed by the SS+AM and NS treatment. Though the nutrient concentrations showed an increasing trend with colonization level, significant differences were recorded primarily in the leaves and stems with a few exceptions. On the other hand, Ca concentration significantly decreased with increased colonization level in the roots and leaves but not in the stems. However, Mn concentration decreased in the roots and stems but increased in leaves in natural soil and AM fungal inoculation in natural soil treatment compared to sterilized soil treatments; Mn concentration was reduced in root infected with AM fungi in sterilized soil compared to only sterilized soil (Table 2.2).

2.3.8 Inoculation effectiveness

Inoculation of AM fungi remarkably affected dry mass production and nutrients (N, P assimilation) of the plant. Inoculation efficiency on growth in terms of total DM production of aboveground (IEa) and belowground (IEb) were 51.7% and 67.0%, respectively, in AMF inoculated with natural soil (NS+AM treatment), which were the highest among all treatments. When AM fungi were applied with sterilized soil (SS+AM, without indigenous AM), plants showed better performance for above- and belowground dry mass (IEa) production than natural soil (indigenous AM), but the effects were not statistically significant (Figure ??). Assimilation of phosphorus (IE_P) by *M. sacchariflorus* plants was also significantly affected by inoculation of applied mycorrhiza (NS+AM treatment), but in the case of nitrogen (IE_N), there were no significant differences among different treatments though the trend was NS+AM >SS+AM>NS (Figure 2.6).

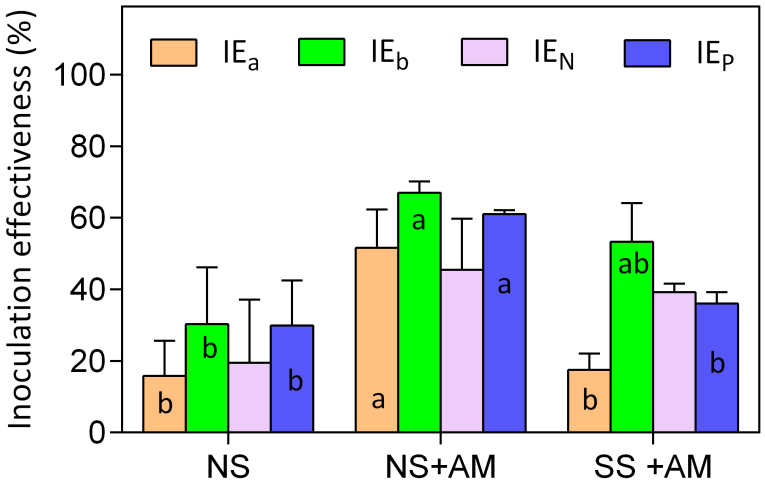

Figure 2.6: Inoculation effectiveness of AM fungi for dry mass production and nutrients (N, P) assimilation of *M. sacchariflorus* where natural soil (NS), natural soil inoculated by AM fungi (NS+AM) and sterilized soil inoculated by AM fungi (SS+AM). Inoculation effectiveness for aboveground dry mass (IEa); belowground dry mass (IEb); total nitrogen assimilation (IE_N) and total phosphorus assimilation (IE_P). Error bars indicate ± SE of mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

2.4 Discussion

2.4.1 Percent colonization of root, chlorophyll content, and dry mass production

Maximum colonization was found in *M. sacchariflorus* plant roots when AM fungi were applied with natural soil because in natural soil, some indigenous AM fungi strains existed, which also facilitated colony development. Moreover, AM fungi with sterilized soil produced a higher colonization level compared with natural soil (without AM fungi) though the value was not significantly different; conversely, no colonization was found in sterilized soil. At the same time, chlorophyll content of the plant leaves increased with the increase in colonization level. According to the results, it can be concluded that colonization level depends on the density of AM fungi, and chlorophyll content of plant leaves is positively related with the colonization level (Figure 2.7). Some previous studies also documented the same results (Feng et al., 2002; Pinior et al., 2005; Tsimilli-Michael et al., 2000; Zuccarini, 2007). Plants inoculated with AM fungi in natural soil showed the best performance for root and stem dry mass as well as total dry mass production of plants, and the lowest one was in sterilized soil. However, in some cases, AM fungi with sterilized soil treatment (SS+AM) outperformed natural soil treatment (NS). Therefore, we can say that AM fungi can increase total dry mass as well as root, stem and leaf dry mass if the colonization level is high, reiterating previous results (Fujiyoshi et al., 2006; Neagoe et al., 2013).

Table 2.1: Nitrogen budget of soil and plant as affected by treatments where natural soil (NS), natural soil inoculated by AM fungi (NS+AM), sterilized soil inoculated by AM fungi (SS+AM) and sterilized soil without AM fungi inoculation (SS).

Treatment	**N in rhizome (g)**	**Initial soil N (g)**	**Distribution of N in plant**			**Leftover N in rhizome (g)**	**N recovery (g)**	**N loss (%)**
			Root/rhizome	**Stem**	**Leaf**			
NS	1.45×10^{-05}	1.86	0.035	0.012	0.030	9.5×10^{-05}	1.24	33.6
NS+AM	1.46×10^{-05}	1.86	0.079	0.083	0.042	9.7×10^{-05}	1.49	19.8
SS+AM	1.46×10^{-05}	1.86	0.059	0.011	0.035	9.5×10^{-05}	1.31	29.5
SS	1.45×10^{-05}	1.86	0.020	0.011	0.021	9.1×10^{-05}	1.19	35.9

2.4.2 Nutrient assimilation and budgeting

After harvesting the plants, concentration of total phosphorus (TP) in both the plants and soil was determined to calculate the P budget to know whether AM fungi have an effect on P loss minimization. Total P concentration of leaves, stems and roots of *M. sacchariflorus* plants was positively related to AM colonization (Figure 2.7), and this result is in agreement with other works (Fujiyoshi et al., 2006; Govindarajulu et al., 2005; Johansen et al., 1994; Tobar et al., 1994; Treseder, 2013). On the contrary, TP in remaining soil (after harvesting the plants) was the lowest in the NS+AM treatment, where both the colonization and TP levels in the plants were maximum. From these results, it can be concluded that AM fungi have a significant effect on P absorption of the C4 plant *M. sacchariflorus*. These results also coincide with previous findings that C4 grasses and non-N-fixing plants tend to be more responsive to AM fungi colonization than C3 grasses and N-fixing plants (Hoeksema et al., 2010).

However, the accumulation rate of TP by *M. sacchariflorus* plants inoculated with AM fungi in natural soil treatment (NS+AM) was much higher than for plants inoculated with AM fungi in sterilized soil (SS+AM) because some phosphate solubilizing microorganisms (bacteria, fungi) were also present in natural soil. These microorganisms played a vital role in making the phosphorus available from an unavailable form (Sarkar et al., 2012), and finally the plants assimilated the available phosphorus with the help of AM fungi. In order to test the AM fungal effect on phosphorus availability, concentration of available phosphorus of soils after harvesting the plants was determined (data not shown); however, AM fungi had no significant positive effect on phosphorus availability in the remaining soil. These results may indicate that AM fungi can play a role in assimilation of P by plants when available phosphorus is high but not on P availability when it is in an unavailable form despite having high root colonization; conversely, the efficient uptake of available P by plants in the presence of AMF might be the possible reason for low level of available phosphorus. Generally, this potential mechanism is consistent with the characterization of the mutualistic relationship between plants and AM fungi (Brundrett, 2004; Mosse, 1957, 1973; Smith and Smith, 2011a,b, 2012). However, a fair amount of evidence has accumulated indicating that AM fungi are in a balanced mutualism with their host plants through a form of regulated exchange: plants should support high levels of root colonization only if the AM fungi provide benefits (Brundrett, 2004). Conversely, there is physiological evidence for large differences in P transfer by different AM fungi, which influence the size (and possible direction) of AM-mediated growth responses and total P uptake (Smith and Smith, 2012). Total nitrogen concentrations of the soil and plants were determined to quantify the N assimilation rate of the plants from the soil. The results of plant and soil TN concentrations indicate that AM fungi have a

Table 2.2: Major and trace elements content in different treatments where natural soil (NS), natural soil inoculated by AM fungi (NS+AM), sterilized soil inoculated by AM fungi (SS+AM) and sterilized soil without AM fungi inoculation (SS). Error bars indicate ± standard error (SE) of mean. Different letters indicate significant differences at P=0.05 according to the LSD test

		NS	NS+AM	SS+AM	SS	p-value
K	Root	5.31±0.05a	17.8±0.82 a	5.40±0.19 a	5.01±0.00 a	0.17
	Stem	1.34±0.02b	2.90±0.06 a	1.60±0.01 ab	1.12±0.02 b	0.02
	Leaf	13.2±0.02 b	24.4±0.01 a	13.4±0.11 b	8.72±0.03 c	<0.001
Mg	Root	3.63±0.06 a	10.6±0.44a	4.10±0.06 a	3.34±0.00 a	0.14
	Stem	0.94±0.00 b	1.30±0.01 a	1.10±0.01 ab	0.63±0.01 b	<0.001
	Leaf	12.8±0.02 c	17.4±0.03 a	14.40±0.06 b	11.21±0.01 d	<0.001
Ca	Root	2.14±0.03 a	1.20±0.01 b	2.20±0.01 a	2.62±0.00 a	<0.001
	Stem	0.81±0.01 a	0.40±0.00 a	1.01±0.03 a	0.54±0.01 a	0.14
	Leaf	7.71±0.01 b	5.30±0.02 d	6.30±0.03 c	8.83±0.01 a	<0.001
Fe	Root	0.85±0.03 a	0.98±0.37 a	0.61±0.14 a	0.46±0.00 a	0.33
	Stem	0.11±0.00 b	0.13±0.00 a	0.12±0.00 ab	0.08±0.00 c	<0.001
	Leaf	0.78±0.01 c	1.07±0.01 a	0.83±0.00 b	0.57±0.02 d	<0.001
Cu	Root	0.05±0.00 b	0.10±0.00 a	0.09±0.01 a	0.03±0.00 b	<0.001
	Stem	0.06±0.00 b	0.08±0.01 a	0.07±0.00 ab	0.04±0.00 c	<0.001
	Leaf	0.08±0.00 a	0.26±0.11 a	0.08±0.01 a	0.06±0.01 a	0.09
Zn	Root	0.07±0.01 a	0.12±0.05 a	0.07±0.01 a	0.06±0.00 a	0.39
	Stem	0.04±0.01 a	0.05±0.01 a	0.05±0.00 a	0.03±0.00 a	0.27
	Leaf	0.06±0.01bc	0.12±0.01 a	0.10±0.01 ab	0.04±0.01 c	<0.001
Mn	Root	0.13±0.01 b	0.30±0.06 ab	0.19±0.01b	0.46±0.08 a	0.01
	Stem	0.04±0.00 b	0.08±0.01 ab	0.10±0.02 ab	0.13±0.02 a	0.02
	Leaf	0.96±0.01 a	0.97±0.05 a	0.54±0.02 b	0.58±0.02 b	<0.001

The unit of K, Mg and Ca concentration is per cent (mg/g) and that of Fe, Cu and Mn is mg/kg dry mass.

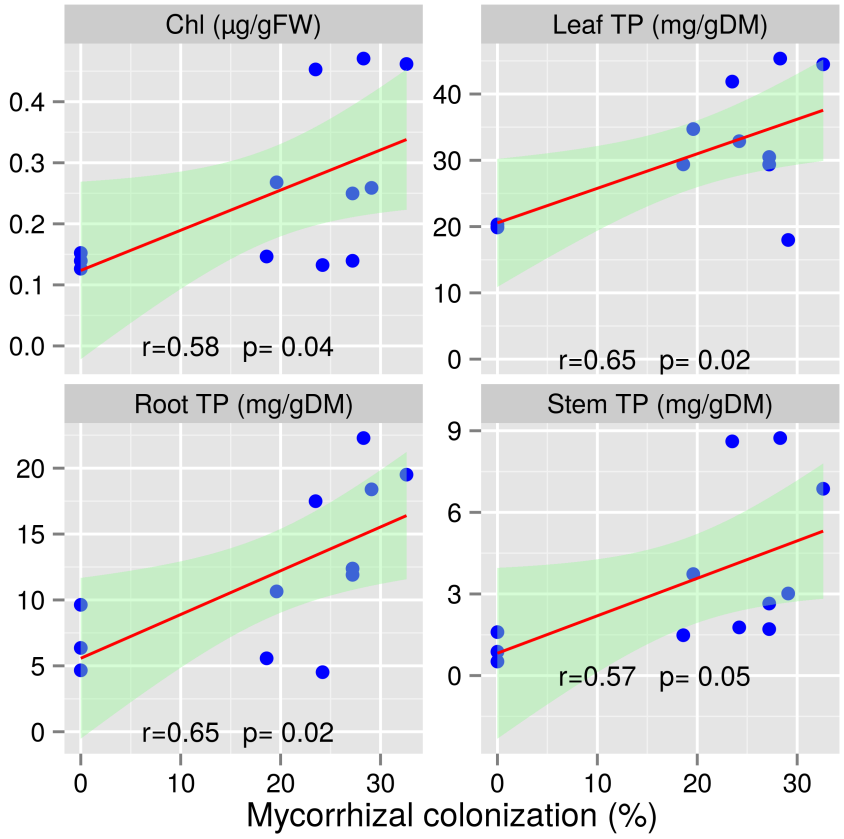

Figure 2.7: Correlation between percent colonization, and chlorophyll and phosphorus concentrations in plants. The shaded ribbons in the plots indicate confidence region of the regression lines (P=0.05).

significant effect on nitrogen assimilation in the C4 plant *M. sacchariflorus*. Arbuscular mycorrhizal fungi assimilate N either exclusively (Tanaka and Yano, 2005) or predominantly (Govindarajulu et al., 2005) in the form of $NH4^+$. Moreover, AM fungi have the ability to mobilize N from organic sources (Atul-Nayyar et al., 2009; Barrett et al., 2011; Hodge et al., 2001, 2010; Leigh et al., 2009). Several studies have revealed that the N mobilized from patches can account for up to 32% of the total N present in the patch (Leigh et al., 2009). Moreover, N mobilization may also take place even when an AM fungus fails to stimulate plant growth(Hodge et al., 2010). On the contrary, some studies indicated that isolates from *Gigasporaceae* had an inferior ability to contribute to plant N nutrition (Reynolds et al., 2005; Veresoglou et al., 2011).

Nitrogen budget was measured to determine the amount of nitrogen loss before and after the AM fungi application. Minimum nitrogen loss (19.8%) occurred in plants inoculated with AM fungi with natural soil treatment, whereas maximum loss was found in only sterilized soil (35.9%) treatment, which was the most prominent finding of this research. These results indicate that AM fungi (*Gigaspora margarita*) can play a direct role not only in nitrogen assimilation, but also may represent an effective way to limit N losses from an ecosystem if the colonization level is high. Amora-Lazcano et al. (1998) studied the interactive effect of AM fungi on the denitrifying community and reported that AM fungi were able to demonstrate a decrease in counts of denitrifying bacteria. Some additional studies are also available on the interactive effect of AM fungi and some verified denitrifying strains of *Pseudomonas*, such as *Pseudomonas putida* (Kim et al., 2008) and *Pseudomonas fluorescens* (Samuelsson et al., 1988), even though it is unclear to what degree these results can be generalized to denitrifying microbes at large (Veresoglou et al., 2012).

Arbuscular mycorrhizal fungi may effectively facilitate leaching, but the role of AM fungi in leaching has not been extensively studied. Asghari and Cavagnaro (2011) were able to detect a decline in NO_3^-, NH_4^+ and phosphate concentrations in the leachate, which could partly be attributed to the bigger size of the mycorrhizal plants. Van der Heijden (2010) demonstrated significant differences in the composition of the leachate with respect to phosphate concentration for three plants under low nutrient supply, with respect to NH_4^+ for *Festuca ovina* under low nutrient supply, and for *Poa pratensis* under high nutrient supply. By contrast, Rillig et al. (2006) studied leaching of DOC/DON components that may be related to AMF but detected no measurable amounts of a putatively AMF derived protein; this study only targeted glomalin-related soil protein, and thus it cannot be used as exhaustive evidence against the occurrence of AM fungal derived N-compounds in leachate. These results signify that AM fungi may represent an effective measure to limit N losses from an ecosystem, which again were the

most important findings of this experiment. The P budget was calculated to test whether AM fungi have an effect on P loss recovery by comparing results between the presence and absence of AM fungi, but there was no significant difference between the various treatments (Figure 6.1). The efficient uptake of mobilized P by *M. sacchariflorus* from the nutrient-deficient soil might be the reason for this result.

Little research has yet been completed on nutrient requirements of *Miscanthus sacchariflorus*. Though it has been reported that this species can grow in a wide range of soils in term of nutrient contents (Roncucci et al., 2014; Dufosse et al., 2014), we did not find any report mentioning the critical values of nutrients in soil for this plant. However, (Rutherford and Heath, 1992) reported that for proper growth of *Miscanthus* spp., 50, 9, 38 kg/ha of N, P and K, respectively are to be added to the soil.

Among the other major nutrients, K and Mg concentration in the plants increased significantly in the stems and leaves of plants inoculated with AM *Gigaspora margarita* both in natural and sterilized soil, but Ca concentration decreased. The increased concentrations of chlorophyll in the leaves were strongly correlated with Mg concentration of the plants ($r = 0.96$, $p = 0.03$), and this trend was in agreement with other works (Feng et al., 2002; Pinior et al., 2005; Tsimilli-Michael et al., 2000; Zuccarini, 2007). However, the reasons for the decreases of Ca in the roots and leaves are not clear. It may be either the effect of AM fungi or the antagonistic effect of other nutrients (Adriano et al., 1971; Asaeda et al., 2014; Malvi, 2011). Trace elements like Cu, Fe, and Zn concentration in the plants increased in the presence of AM (*Gigaspora margarita*) in all cases, whereas Mn concentration showed various trends in different treatments. Some reports indicate higher concentration of trace elements in plants owing to AMF and under deficiency conditions, and the ability of AMF to enhance plant uptake of nutrient elements through extraradical hyphal transport has been demonstrated for Cu, Zn and Fe (Diaz et al., 1996; Faber et al., 1990; Kothari et al., 1990; Li et al., 1991; Liao et al., 2003; Neagoe et al., 2013; Weissenhorn et al., 1995a). In contrast, the uptake of other elements, such as Fe, Mn, and Zn is sometimes reduced when plants are colonized by AMF (El-Kherbawy et al., 1989; George et al., 1994).

2.4.3 Efficiency of AMF after inoculation

Soil inoculation with AM fungi positively affected plant growth in terms of dry mass (DM) production in all treatments, and previous literature also tabulated the same results (Conversa et al., 2013). Due to application of AM fungi, aboveground (IEa) and belowground (IEb) DM were significantly increased in plants compared with non-inoculated plants (both in combination with natural and sterilized soil) and plants in natural soil treatment (NS). Furthermore, remaining indigenous

mycorrhiza also showed remarkable performance for aboveground (IEa) DM and belowground (IEb) DM production in comparison with non-inoculated plants (Fujiyoshi et al., 2006; Neagoe et al., 2013). Mycorrhizal inoculation significantly enhanced P and N assimilation in *M. sacchariflorus* in combination with natural and sterilized soil conditions (Ortas, 2012) in comparison with non-mycorrhizal plants, with maximum assimilation being found in the NS+AM treatment due to their combined effect. Previously, Sainz and Arines (1988) showed that indigenous fungi were the most effective in enhancing plant growth and P uptake, which was correlated with a higher root colonization. This result is quite similar with this experiment, but their experimental results also revealed that inoculation with selected AMF species did not improve plant growth. On the contrary, in our experiment, selected AMF species also improved plant growth and nutrient (N, P) acquisition as in some previous reports (Fujiyoshi et al., 2006; Ortas, 2010, 2012; Treseder, 2013). This result may indicate that AMF can increase plant growth and nutrient assimilation, but it varies with AMF species.

2.5 Conclusions

Mycorrhizal plants were more effective than non-mycorrhizal plants at increasing growth (both for above ground and below ground dry mass) and chlorophyll content of leaves. Mycorrhizal plants (root, stem, and leaf) also had higher concentration of N, P, K, Mg, Fe, Cu, and Zn but Ca concentration decreased with increasing colonization level; Mn concentrations decreased in the root, but were unaffected in the leaf and stem. Moreover, AM fungi had some potential effect on N loss minimization. Thus, it can be concluded that AMF (*Gigaspora margarita*) had a significant effect on nutrient assimilation and vegetation growth of the *M. sacchariflorus* plant in nutrient-deficient sandbar soil. *Miscanthus* spp. has drawn much attention for being used as biofuel and its commercial production has been initiated in many parts of the world. Mycorrhizal association and behavior with this species in cultivated soils, where nutrients are usually high, should be thoroughly studied.

Chapter 3

Role of arbuscular mycorrhizal fungi on the performance of floodplain *Phragmites japonica* under nutrient stress condition

Abstract

A pot experiment was conducted to evaluate the potential effects of arbuscular mycorrhizal fungi (AMF) on growth, nutrient uptake, and inoculation effectiveness on *Phragmites japonica*. Spores of AMF strains (*Gigaspora margarita* Becker & Hall) were collected from the commercial product 'Serakinkon'. Four treatments viz. natural soil, natural soil inoculated by AM fungi, sterilized soil inoculated by AM fungi, and sterilized soil without AM fungi inoculation were selected to determine the effects of applied and indigenous AMF on *P. japonica*. The average colonization level of *P. japonica* was 24-33%, whereas no colonization was found in sterilized soil. AMF colonization increased the chlorophyll content ($r = 0.84$, $p<0.01$), plant dry mass ($r=0.89$, $p<0.01$), and N, P, K, Mg, and Fe concentration of the plant's roots, stems, and leaves when AMF was applied with natural and sterilized soil. In all cases, maximum values were found when the plants were applied with natural soil in combination with AMF, but Ca concentration decreased as the colonization level increased. Mn concentration decreased in the roots but increased in the leaves. Cu concentration was not significantly affected by

treatments. N-loss minimization from the soil was significant when the colonization level was high.

3.1 Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous components of terrestrial ecosystems across the world with multiple functions from the level of individual plants to the ecosystem (Smith and Read, 2008). The distribution and functions of AMF at transitional zones between aquatic and terrestrial ecosystems, such as flood plains, remain less studied. Mycorrhizal fungi occur in riparian areas (Piotrowski et al., 2008), but their functions in promoting plant growth are unknown across floodplain chronosequences. Arbuscular mycorrhizal fungi are the predominant mycorrhizal type found in early stages of primary succession, for example, in sand dunes (Corkidi and Rincon, 1997) and river floodplains (Miller and Sharitz, 2000). Because soils in the early stages of primary succession generally have low nutrient contents, AMF might play an important role in the growth and establishment of pioneer plants. In such areas, however, the levels of colonized plant species have a wide range. Certain species, such as *Phragmites japonica* and *Miscanthus sacchariflorus*, grow in stony sterile as well as low nutrient conditions, (Asaeda et al., 2011a), whereas other species prefer relatively fine sediment with nutrient rich conditions. Response to mycorrhizal inoculation is linked to the level of soil fertility, and P is well documented to be the most influential element in mycorrhizal development and efficiency. In P-deficient soils, the yields of crops were found to be largely dependent on their mycorrhizal status under field (Ortas, 2008) and greenhouse conditions (Ortas, 2003). Soils in floodplains are generally coarse and low in nutrients. These characteristics inhibit the development of dense plant communities (Giller and Malmqvist, 1998; Pinay et al., 1992) and plant growth is more often restricted by a shortage of nitrogen and phosphorus (Asaeda et al., 2009; Asaeda and Rashid, 2012; Bedford et al., 1999). Terrestrial systems with infrequent disturbances tend to exhibit a one-way progression of community succession. Mycorrhiza can increase plants' ability to assimilate the nutrients from low-nutrient soil, such as the sediments of floodplains (Harner et al., 2011; Miller and Sharitz, 2000). Flood plains along rivers with natural or near-natural flow regimes are model systems for testing hypotheses about interactions among plants, soil, and mycorrhizal fungi due to the diversity of habitats and disturbance regimes in close proximity to one another. Therefore, the present study was conducted to evaluate the effectiveness of AM symbiosis in enhancing *Phragmites japonica* growth and nutrient uptake in nutrient-deficient flood plain soils.

3.2 Materials and Methods

3.2.1 Plant propagule collection from study sites

Azami et al. (2004),Asaeda et al. (2010), and Sekine et al. (2012) reported that *P. japonica* is the most common plant on the Arakawa River bank, and Kang et al. (2002) showed that *P. japonica* is also the most common plant in riverine wetlands in South Korea which is perennial grass and it can breed in water side of a mountain valley, river bank etc. Width of leaf is 1.5−2 cm, and flower blossoms between September and October. According to previous literature, we also conducted a preliminary survey on the banks of the Ara River, Saitama, Japan (36°4′58.02″N and 139°26′28.85″E) to identify a suitable, experimental plant for this study (Figure 6.4). Five meter × four meter transects were randomly selected along the river and marked with poles and rods. The distance between these transects ranged between 10 and 30 m. All plant species within a transect were identified, and their percent coverage was calculated. Though the species composition varied between the transects, *P. japonica* appeared to be the most common species and covered a higher percentage of the transects areas. Therefore, based on the survey and previous literature, we chose *P. japonica* as our experimental plant, and rhizomes of *P. japonica* were collected for planting in pots.

3.2.2 Soil analysis and spore isolation from collected soil

Soil was collected to a depth of 20 cm from the surface of previously selected transects using a shovel. Soils collected from different transects were composited to create a homogenous soil pool, and the required amount of soil from the composite bulk was used for the experiments. The properties of the soils collected from the transects were homogenous, and there were no significant differences between the individual soils from the transects and the composite soil in terms of soil particle size (D25), pH, % organic matter content, total carbon (TC), total nitrogen (TN), total phosphors (TP), available phosphorus concentrations or water holding capacity (all $p \geq 0.05$). The soil utilized for the experiment had an average pH of 5.8, with an average of 0.61 mm of D25, 0.25% organic matter content, 0.045% TP, 0.002% available phosphorus, 0.09% TN, 27% water holding capacity, 0.17% potassium (K), 0.04% calcium (Ca), 0.11% magnesium (Mg), 0.55 mg/kg iron (Fe), 0.05 mg/kg copper (Cu), and 0.40 mg/kg manganese (Mn). The initial moisture content and bulk density of the soil were estimated using the gravimetric method (Black, 1965). The total nitrogen (TN) concentrations of the soil were determined with a CHN analyser (CHN Corder MT-5, Yanoco, and Kyoto, Japan). Soil samples were placed in a crucible and ashed in a muffle furnace for 2 h at 500° C, dissolved in 1 ml of 5 M HCl, and partially neutralized with 5 M NaOH solu-

tion. Orthophosphate was then determined using the phosphomolybdate method according to Murphy and Riley (1962). The total phosphorus in the soil was determined by perchloric acid digestion, and the available phosphorus was determined after extraction with bray extract solution (Bray and Kurtz, 1945). After removing plant roots, the soil was dried and weighed for nutrient budgeting. A fraction of soil was oven-dried at 80° C for 3 days, and the final weight was recorded. The soil was kept in air-tight containers for further analysis.

Spores were extracted from the same composite soil by wet sieving and centrifugation (Gerdemann and Nicolson, 1963). For each 100 g of collected soil, 3 L of tap water was used to suspend the soil for subsequent passage through a pair of sieves (860 and 36 μm mesh). The material retained in the 36-μm sieve was centrifuged in water (900 g for 4 min), and the resulting pellet was resuspended in 45% sucrose solution and centrifuged again (900 g for 1 min). The supernatant was washed through a 36-μm sieve and washed with tap water, and the spores were transferred to Petri dishes. The spores were manipulated under a stereomicroscope and spore morphotypes were separated according to spore morphology and colour.

3.2.3 Experimental procedure and design

Considering the type of mycorrhizal inoculation, four treatments were used, viz. (1) natural soil (NS), (2) natural soil inoculated by AM fungi (NS+AM), (3) sterilized soil inoculated by AM fungi (SS+AM), and (4) sterilized soil without AM fungi inoculation (SS). The experiment was conducted in the form of pot cultures of *P. japonica* in a greenhouse at Saitama University, Japan. The pots were laid out using a complete randomized design (CRD) in the greenhouse.

In each pot, 2 kg (natural or sterilized) of soil was used. For sterilizing, the soil was autoclaved twice at 121° C for 1 h to eliminate any indigenous AM fungi (Vogel-Mikus et al., 2006). To inoculate the soil of the respective treatments, AM fungi spores were applied in the form of a commercial inoculant namely 'Serakinkon' powder (The Central Glass Company, Tokyo, Japan). The inoculant was composed of 50 *Gigaspora margarita* Becker & Hall spores per gram of powder (Higo et al., 2010; Tawaraya et al., 1996; Kaneko and Tanimoto, 2009) as *Gigaspora* was the most commonly isolated AMF in river bank sand with a higher spore density than those of other species; previous studies revealed the same result (Koske and Gemma, 1997; Kowalchuk et al., 2002). Twenty gram Serakinkon powder/kg soil was evenly mixed with soil before being used in a pot. Rhizomes with three nodes (∼2 cm) were employed as propagating materials. From this rhizome, plants were grown and considered as one-treatment plants and every treatment had four replications. A temperature of 25±3° C and 75% relative humidity were maintained in the greenhouse throughout the experimental period. Pots were wa-

tered as necessary. The greenhouse trial lasted 110 days.

3.2.4 Harvesting and processing

Plants were harvested 110 days after transplanting at their senescence stage. After separation from the pot, the shoots were separated from the roots and fresh weights were taken separately for the root, stem, and leaf. Soil particles were carefully removed from plant roots using forceps. The whole root system was then divided into two parts: one part for AM colonization observation and the other part for estimating dry mass and analysing nutrients. The stems, leaves, and roots (fraction) were cut into small pieces and dried to a constant weight at 72° C for 3 days, and the final weights (dry mass) were recorded. The dry mass of the shoot (including stem and leaf) was considered to be the aboveground dry mass, and oven-dried root was used to estimate the belowground dry mass. The oven-dried plant tissues (roots, stems and leaves) were ground separately and kept in air-tight containers for further analysis.

3.2.5 Chlorophyll content measurement

At harvest, chlorophyll was extracted from the leaves of *P. japonica* using N,N dimethylformamide (DMF). The leaves were cut, and the fresh weight (∼5 mg) was measured. Pigments were then extracted into 5 ml DMF by incubation for 24 h in a refrigerator at 4° C. The chlorophyll concentration was determined via the method described by Porra et al. (1989). Absorbance at 646.8 nm and 663.8 nm was measured with a UV-mini 1200-spectrophotometer (Shimadzu, Japan). Chlorophyll concentrations were calculated as μg/mg fresh weight (FW).

3.2.6 AM colonization determination and nutrient analyses from plant samples

The extent of AM colonization was estimated by the magnified intersections method proposed by McGonigle et al. (1990). For this estimation, the roots were cleaned, cut into small segments (2 cm/segment), rinsed and cleared for at least 12 h in 10% KOH solution at room temperature, and then stained with 0.05% trypan blue solution(Koske and Gemma, 1989). Ten randomly selected root fragments from each treatment were mounted on slides, and intersecting vertical gridlines were observed at 100× magnification to determine the presence or absence of AM colonization. The field of view of the microscope was moved using the stage graticule to make four, six or eight complete passes across each slide perpendicular to its long axis. Intersections were counted in the following categories; 'negative' (no

fungal material in root), 'arbuscules', 'vesicles', and ' hyphae only'. The arbuscular colonization (As) and vesicular colonization (Vs) were calculated by dividing the count for the 'arbuscules' and 'vesicles' categories, respectively, by the total number of intersections examined. Hyphal colonization (Hs) was calculated as the proportion of non-negative intersections.

Total nitrogen (TN) concentrations of plant tissues were determined with a CHN analyser (CHN Corder MT-5, Yanoco, and Kyoto, Japan). Powdered samples (2-10 mg) were placed in a crucible and ashed in a muffle furnace for 2 h at 500° C and then dissolved in 1 ml of 5 M HCl and partially neutralized with 5 M NaOH solution. The total phosphorus of the plant samples was determined by perchloric acid digestion after extraction with bray extract solution (Bray and Kurtz, 1945). The concentrations of Ca, Mg, K, Fe, Mn, and Cu in the plants were analysed after oven drying to a constant weight at 72° C followed by digestion in a perchloric acid mixture as described by Tandon (1993). Nutrient contents in the tissue extracts were quantified using an atomic absorption spectrophotometer (AA-6300; Shimadzu, Japan) using the method described in the standard methods for the examination of water and wastewater by Gilcreas (1966).

3.2.7 Calculation of inoculation effectiveness

Inoculation effectiveness (IE) was calculated using the following equations based on aboveground dry mass (IEa), belowground dry mass (IEb), and nutrient assimilation (IE_{Nutrient}) as suggested by Conversa et al. (2013); Ortas (2012) and Sarkar et al. (2015b):

$$IE_a = \left[\frac{(ADM_{+M} - ADM_{-M})}{ADM_{+M}}\right] \times 100 \tag{3.1}$$

$$IE_b = \left[\frac{(BDM_{+M} - BDM_{-M})}{BDM_{+M}}\right] \times 100 \tag{3.2}$$

$$IE_{\text{Nutrient}} = \left[\frac{(\%\text{Nutrient}_{+M} - \%\text{Nutrient}_{-M})}{\%\text{Nutrient}_{+M}}\right] \times 100 \tag{3.3}$$

where, IE_a, IE_b, and IE_{Nutrient} designate inoculation effectiveness based on above ground dry mass (ADM), belowground dry mass (BDM), and nutrient (nitrogen or phosphorus) assimilation (IE_{Nutrient}), and subscript $+M$ and $-M$ designate inoculated and non-inoculated plants, respectively.

3.2.8 N and P budget calculation

Nutrients from the soil before planting propagules (henceforth 'initial soil') and propagating organs (rhizomes) were considered as input, and output estimates included nutrients removed by crop harvest and the nutrient status of the remaining soil after crop harvest (henceforth 'final soil'). The concentrations of total phosphorus (TP) and total nitrogen (TN) in both the plants and soil were determined, and, finally, the inputs and outputs of nitrogen (N) and phosphorus (P) in the soil-plant system were estimated for calculating the budget.

3.2.9 Statistical analyses

Prior to commencing the statistical analyses, all data were checked for normalcy, and equal variance was measured using Levene's test. All data are presented as the mean ± SD (n=4). The effects of mycorrhizal treatment on the chlorophyll content in the leaf, root colonization, dry weight, nutrient (N, P, K, Ca, Mg, Fe, Mn, and Cu) content of plants, and soil were analysed by two-way ANOVA followed by Tukey's post hoc test at 0.05 significance levels. The correlations between parameters were judged by Pearson's correlation co-efficient at the 0.05 significance level. All statistical analyses were performed using SPSS for Windows (Version 13.0, SPSS, Inc., Chicago, IL, USA) and R package (Team, 2010).

3.3 Results

3.3.1 Root colonization

Microscopic observations revealed that the *P. japonica* in AM treatments were colonized by AM fungi both in the collected and sterilized soil. The colonization level of roots was significantly higher in the NS+AM treatment (33%) than in the NS treatment (24 %), whereas in the sterilized soil (AM fungi absent), no colonization was found (Figure 3.1).

3.3.2 Leaf chlorophyll concentration

The chlorophyll concentration significantly increased in AM plants in combination with both the natural (NS+AM) and sterilized soil (SS+AM), but the plants in the natural soil and those in the sterilized soil without *Gigaspora margarita* did not have a remarkable effect on chlorophyll content (Figure 3.2).

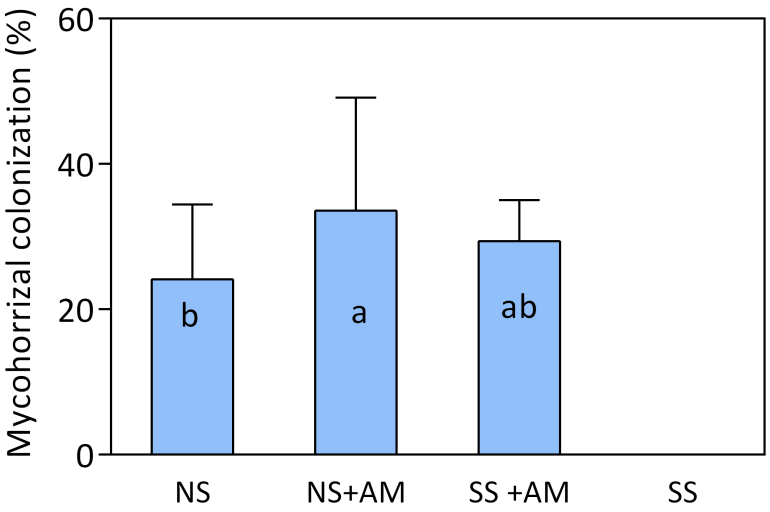

Figure 3.1: Arbuscular mycorrhizal (AM) colonization levels in the herbaceous species *P. japonica*. Values represent the means of four replicates, and 10 root segments per replicate were selected for observation. Error bars indicate the ± standard deviation (SD) of the mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

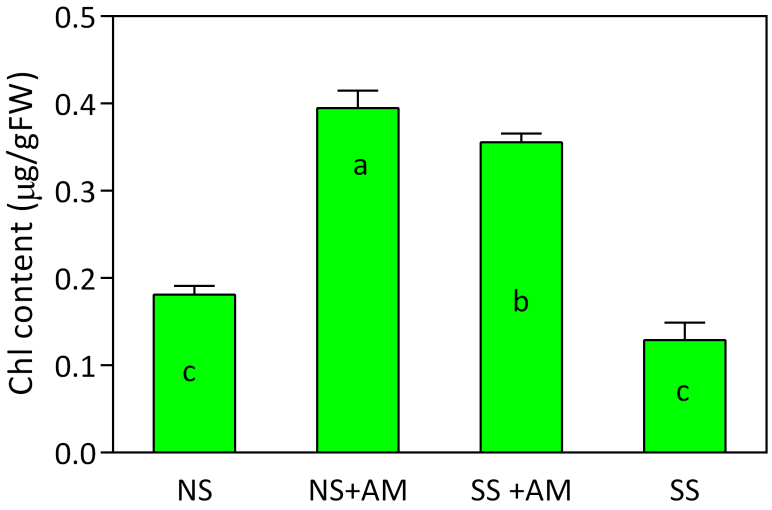

Figure 3.2: Chlorophyll content in leaves from different treatments as affected by native and inoculated AM fungi in sterilized and unsterilized soil. Error bars indicate ± SD of the mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

3.3.3 Plant dry weight

AM fungi showed a significant effect on the dry mass production of the *P. japonica* plant. For root dry mass production, the maximum dry weight (11.06 g) was produced in the NS+AM treatment, whereas the lowest one was produced in the SS treatment (0.67 g). The SS+AM treatment produced more dry weight than the SS treatment but statistically similar to NS. In the case of leaf dry mass production, the NS+AM treatment revealed a significantly different result than the other treatments, and stem dry mass was statistically similar for the SS+AM treatment but significantly different for the other two treatments (Figure 3.3).

3.3.4 Nutrient concentration

The nitrogen and phosphorus concentration in the plants (roots, stems, and leaves) was the highest in the AM fungi inoculated with natural soil treatment but was the lowest in the sterilized soil (absent of AM fungi) treatment. The P concentration of the sterilized soil with AM fungi was statistically similar to that of the natural soil (Figure 3.4). The minimum N concentration was found in the sterilized soil. When AM fungi were inoculated with sterilized soil, the N content was higher in the root and stem than with AM fungi inoculated with natural soil (Figure 3.5).

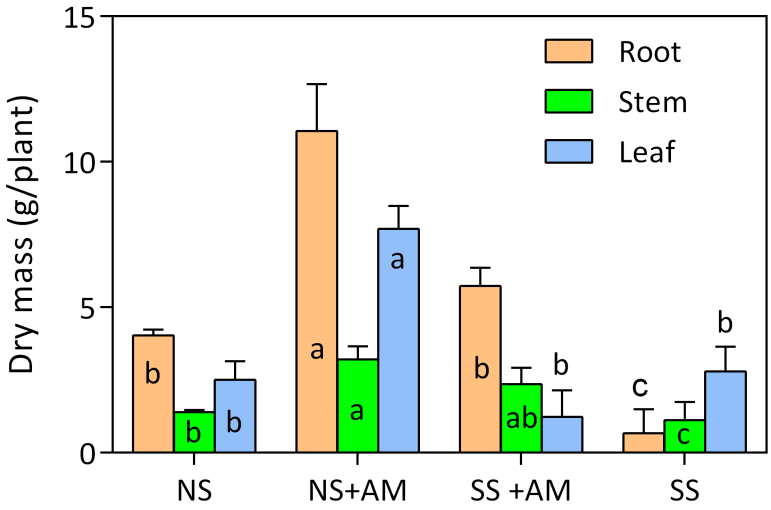

Figure 3.3: Dry mass production of *P. japonica* as affected by native and inoculated AM fungi in sterilized and unsterilized soil. Values represent the total dry mass ± SD of the mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

Among the major nutrients (K, Mg) and trace elements (Fe, Cu), the concentrations in the plant' s roots, stems, and leaves were the highest in the NS+AM treatment followed by the SS+AM and NS treatments. In contrast, the Ca concentration significantly decreased in the leaves of the NS+AM treatment but not in the root and stem. The Mn concentration significantly decreased in the root but increased in the leaf, though there were no significant differences in the cases (Table 3.1).

3.3.5 Nitrogen and phosphorus budgeting

Minimum nitrogen loss was found in the NS+AM treatment (12.4%), and maximum nitrogen loss was observed in the SS treatment (34.9%); however, the loss in the SS+AM treatment was lower than the loss in the NS treatment (Figure 3.6). According to these experimental results, there was no significant effect of AM fungi on P loss minimization in different treatments Figure 6.2. For all cases, an approximate 1% loss was determined.

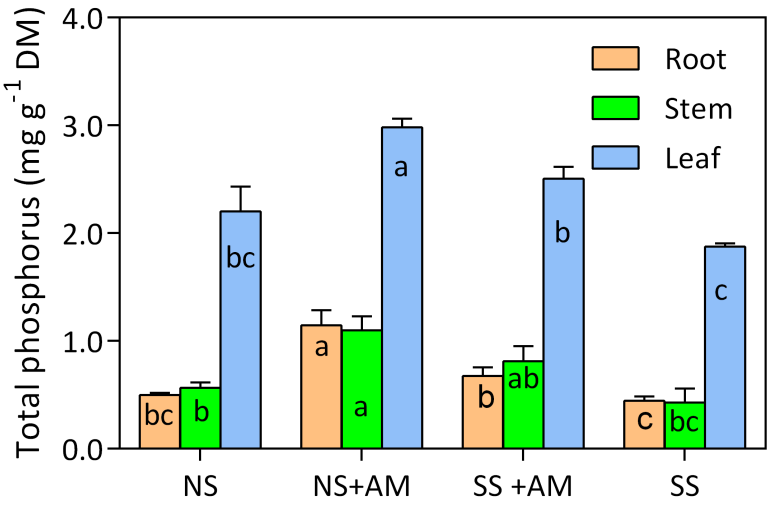

Figure 3.4: TP (mg g^{-1} DM) concentration of plants in different treatments as affected by native and inoculated AM fungi in sterilized and unsterilized soil. Error bars indicate ± SD of the mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

3.3.6 Inoculation effectiveness

The inoculation effectiveness on growth in terms of the total DM production of aboveground (IEa) and belowground (IEb) were 67.8% and 65.0%, respectively, in the AMF inoculated with natural soil (NS+AM treatment), which were the highest among all treatments, though (IEa) was not significantly different than in other treatments. When the AM fungi were applied with sterilized soil (SS+AM, without indigenous AM), the plants showed better performance than in the natural soil (indigenous AM) for aboveground and belowground dry mass production, but the values were not statistically significant (Table 2). The uptake of phosphorus (IE_P) and nitrogen (IE_N) by the *P. japonica* plants was also significantly affected by the inoculation of the applied mycorrhiza (NS+AM treatment), whereas the trend was NS+AM> SS+AM>NS (Table 3.2).

3.4 Discussion

Per cent colonization of root, chlorophyll content, and dry mass production Maximum colonization was found in the *P. japonica* plant roots when AM fungi were applied with natural soil because several indigenous AM fungi strains existed in

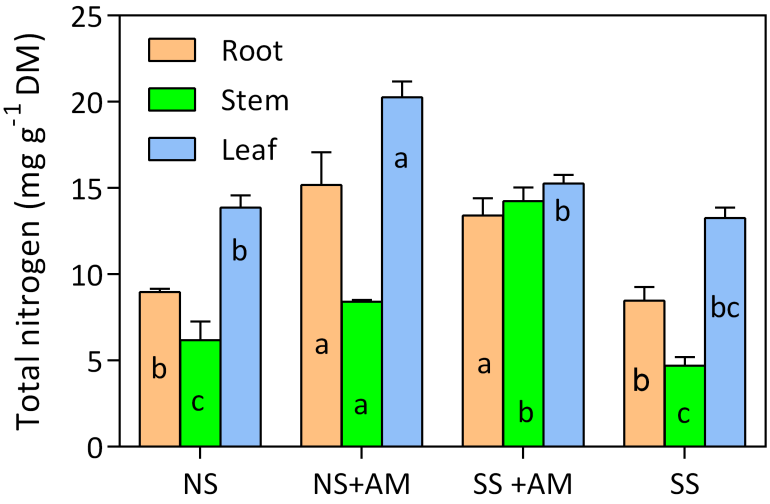

Figure 3.5: TN (mg g^{-1} DM) concentration of plants in different treatments as affected by native and inoculated AM fungi in sterilized and unsterilized soil. Error bars indicate ± SD of the mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

the natural soil, which also facilitated colony development. Moreover, the AM fungi with sterilized soil produced a higher colonization level than the natural soil, though the value was not significantly different; conversely, no colonization was found in the sterilized soil. At the same time, the chlorophyll content of the plant leaves increased with the increase in colonization level. Based on the results, it can be concluded that colonization level depends on the density of AM fungi and that the chlorophyll content of plant leaves is positively correlated with the colonization level (Figure 3.7). Previous studies documented the same results (Pinior et al., 2005; Sarkar et al., 2015b; Zuccarini, 2007). The plants inoculated with AM fungi in natural soil showed the best performance for total dry mass production, and the worst performance occurred in the sterilized soil. However, in some cases, the AM fungi with sterilized soil treatment (SS+AM) outperformed natural soil treatment (NS). Therefore, we can say that AM fungi can increase total dry mass as well as root, stem and leaf dry mass if the colonization level is high, reiterating previous results (Fujiyoshi et al., 2006; Neagoe et al., 2013).

3.4.1 Nutrient uptake and budgeting

Inoculation by AM fungi showed a positive effect on P concentration of the plants and was directly related to the colonization level (Figure 3.7). The increases in

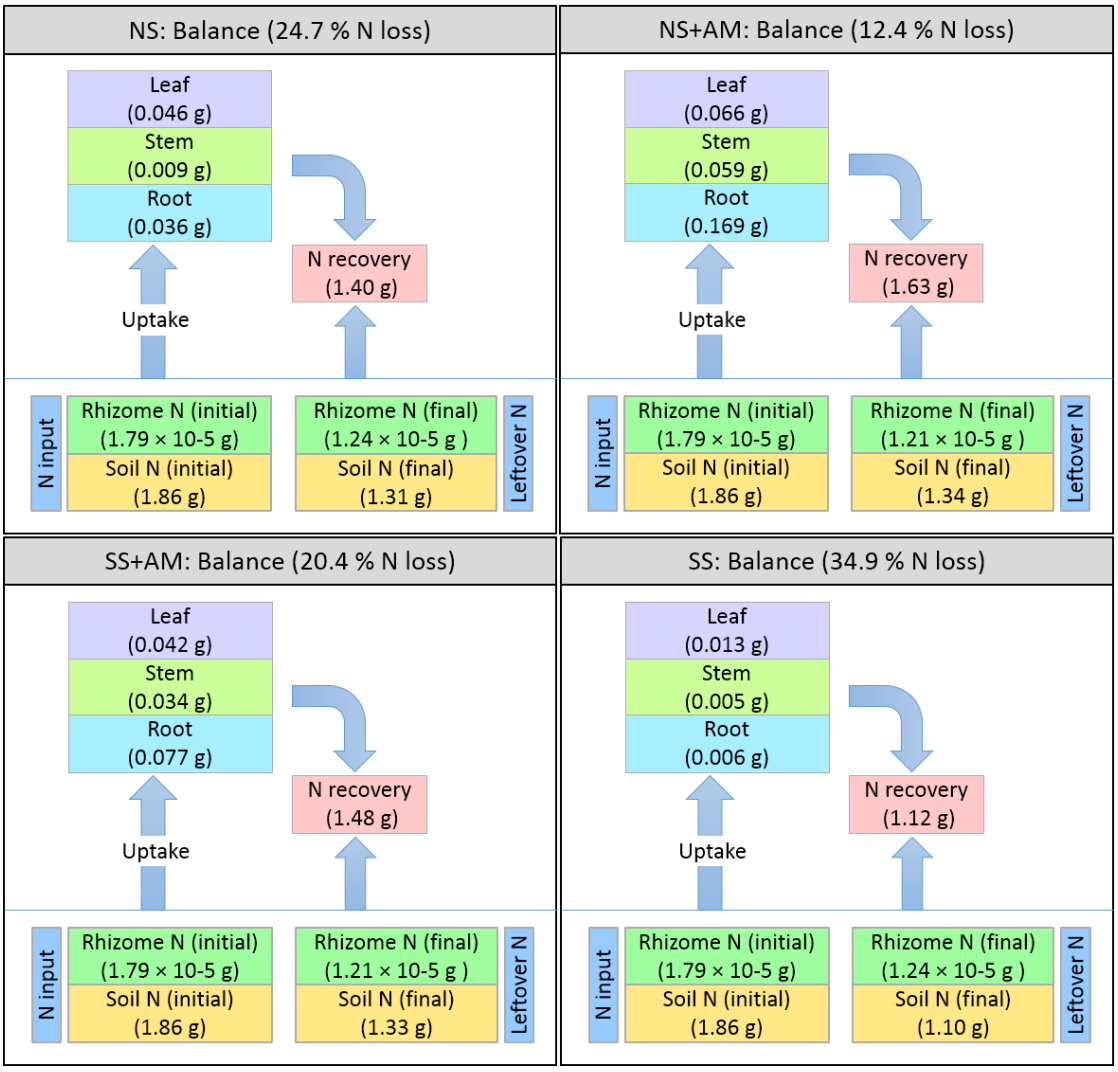

Figure 3.6: Nitrogen budgets of soil and plants as affected by native and inoculated AM fungi in sterilized and unsterilized soil.

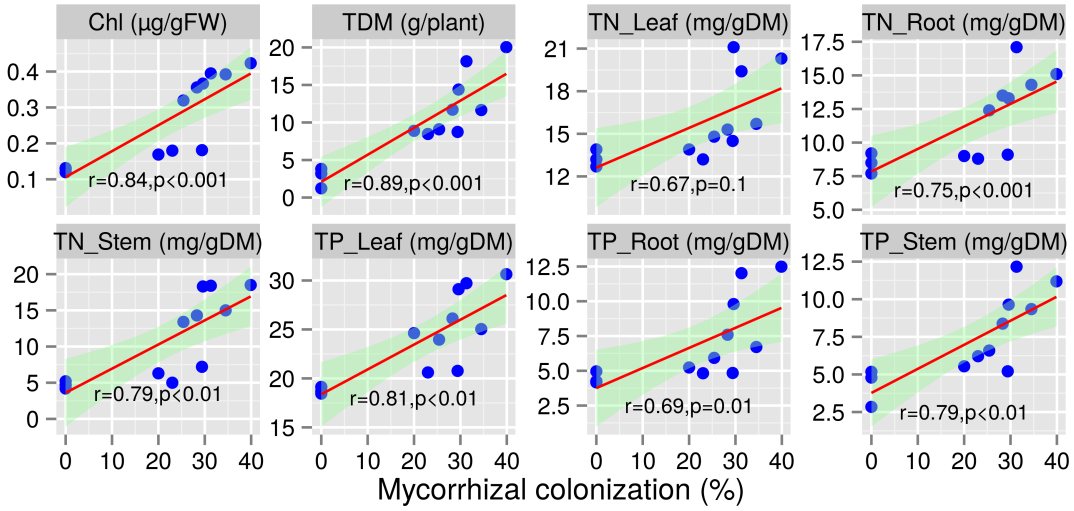

Figure 3.7: Correlation between percent colonization, and chlorophyll content, total dry mass (TDM) and plant nutrients

plant P content were greater when the colonization level was more pronounced, and this relationship was significant across all trials (Figure 3.4). This result is in agreement with those of other works (Fujiyoshi et al., 2006; Govindarajulu et al., 2005; Sarkar et al., 2015b; Treseder, 2013). On the contrary, TP in the remaining soil (after harvesting the plants) was the lowest in the NS+AM treatment, where both the colonization and TP levels in the plants were optimal. These results show that AM fungi have a significant effect on the P absorption of the *P. japonica* (C4) plant. These results also coincide with previous findings that C4 grasses and non-N-fixing plants tend to be more responsive to AM fungi colonization than C3 grasses and N-fixing plants (Hoeksema et al., 2010; Sarkar et al., 2015a).

However, the accumulation rate of TP by *P. japonica* plants inoculated with AM fungi in natural soil treatment (NS+AM) was much higher than for plants inoculated with AM fungi in sterilized soil (SS+AM) because some phosphate solubilizing microorganisms (bacteria, fungi) were also present in natural soil. These microorganisms played a vital role in making the phosphorus available from an unavailable form (Sarkar et al., 2012), and finally, the plants assimilated the available phosphorus with the help of AM fungi. These results may indicate that AMF can play a role in the assimilation of P by plants when available phosphorus is high but do not play a role in P availability when the P is in an unavailable form despite having high root colonization. Generally, this potential mechanism is consistent with the characterization of the mutualistic relationship between plants and AM fungi (Brundrett, 2004; Smith and Smith, 2011a,b, 2012). However, there is a fair

amount of evidence indicating that AM fungi are in a balanced mutualism with their host plants through a form of regulated exchange: plants should support high levels of root colonization only if the AM fungi provide benefits (Brundrett, 2004). The P budget was calculated to test whether AM fungi have an effect on P loss recovery by comparing results between the presence and absence of AM fungi, but there was no significant difference between the various treatments (Figure 6.2).

The total nitrogen concentration in plants varied with the colonization level of AM fungi. The total nitrogen content of the soil and plants was determined to quantify the N assimilation rate of the plants from the soil. The results of plant and soil TN concentration indicate that AMF have a significant effect on nitrogen assimilation in the C4 plant *P. japonica*. AMF assimilate N either exclusively (Tanaka and Yano, 2005) or predominantly (Govindarajulu et al., 2005) in the form of NH_4^+. Moreover, AMF have the ability to mobilize N from organic sources (Atul-Nayyar et al., 2009; Barrett et al., 2011; Hodge et al., 2001; Leigh et al., 2009). Several studies have revealed that the N mobilized from patches can account for up to 32% of the total N present in the patch (Leigh et al., 2009). Moreover, N mobilization may also take place even when an AM fungus fails to stimulate plant growth (Hodge et al., 2010). The nitrogen budget was measured to determine the amount of nitrogen loss before and after the AM fungi application. Minimum nitrogen loss (13.4%) occurred in plants inoculated with AM fungi with natural soil treatment, whereas maximum loss was found in only the sterilized soil (34.9%) treatment, which was the most prominent finding of this research. These results indicate that AM fungi (*Gigaspora margarita*) not only can play a direct role in nitrogen assimilation but also may represent an effective way to limit N-losses from an ecosystem.

AMF may effectively facilitate leaching, but the role of AMF in leaching has not been extensively studied. Asghari and Cavagnaro (2011) were able to detect a decline in NO_3^-, NH_4^+, and phosphate concentrations in the leachate, which could partly be attributed to the bigger size of the mycorrhizal plants. Van der Heijden (2010) was able to demonstrate significant differences in the composition of the leachate with respect to phosphate concentration for three plants under low nutrient supply, with respect to NH_4^+ for *Festuca ovina* under low nutrient supply, and for *Poa pratensis* under high nutrient supply. By contrast, Rillig et al. (2006) studied the leaching of DOC/DON components that may be related to AMF but detected no measurable amounts of a putatively AMF derived protein; this study targeted only glomalin-related soil protein and thus cannot be used as exhaustive evidence against the occurrence of AM-fungal-derived N-compounds in leachate. These results indicate that AM fungi may represent an effective measurement to limit N-losses from an ecosystem, which again, was the most important finding of this experiment.

Table 3.1: Nutrients concentration in *P. japonica* roots, stems and leaves as affected by native and inoculated AM fungi in sterilized and not-sterilized soil. Different letters in the row indicate significant difference at P=0.05 according to the LSD test.

		NS	NS+AM	SS+AM	SS	p-value
K	Root	0.53±0.04bc	0.73±0.10 a	0.56±0.09 ab	0.40±0.03 c	<0.001
	Stem	0.32±0.01b	0.36±0.01 a	0.33±0.01b	0.21±0.00 c	<0.001
	Leaf	2.24±0.04 b	4.12±0.96 a	2.55±0.40b	1.84±0.38 b	<0.001
Mg	Root	0.28±0.01 c	0.41±0.02 a	0.32±0.01b	0.22±0.00 d	<0.001
	Stem	0.16±0.02ab	0.18±0.02 a	0.13±0.02bc	0.12±0.00 c	<0.001
	Leaf	1.28±0.04 c	1.74±0.05 a	1.44±0.10 b	1.12±0.02 d	<0.001
Ca	Root	0.22±0.07 a	0.18±0.06 a	0.17±0.07 a	0.33±0.18 a	0.33
	Stem	0.07±0.00 b	0.11±0.01 ab	0.11±0.03 ab	0.20±0.06 a	0.01
	Leaf	0.77±0.05 b	0.59±0.03 c	0.90±0.04b	1.26±0.09 a	<0.001
Fe	Root	0.69±0.05 b	0.90±0.04 a	0.70±0.08 b	0.36±0.01 c	<0.001
	Stem	0.05±0.01 b	0.07±0.00 a	0.05±0.00 b	0.03±0.01b	<0.001
	Leaf	0.70±0.04 c	3.37±0.09 a	1.68±0.09 b	0.43±0.02 d	<0.001
Cu	Root	0.07±0.00 a	0.08±0.01 a	0.08±0.00 a	0.08±0.00 a	0.16
	Stem	0.05±0.02 a	0.08±0.01 a	0.05±0.02 a	0.05±0.00 a	0.13
	Leaf	0.30±0.04 a	0.34±0.02 a	0.33±0.01 a	0.28±0.07 a	0.44
Mn	Root	0.13±0.03 b	0.29±0.06 b	0.26±0.02 b	0.77±0.08 a	<0.001
	Stem	0.07±0.02a	0.08±0.01a	0.22±0.13 a	0.07±0.00 a	0.06
	Leaf	2.20±0.11 b	2.98±0.09 a	2.48±0.20ab	2.69±0.38ab	0.02

The unit of K, Mg and Ca concentration is per cent (%) and that of Fe, Cu and Mn is mg/kg dry mass.

Table 3.2: Inoculation effectiveness of AM fungi for dry mass production and nutrients (N, P) assimilation of *P. japonica* as affected by native and inoculated AM fungi in sterilized and not-sterilized soil where inoculation effectiveness for above ground (IEa) and below ground dry mass production (IEb); total nitrogen assimilation (IE_N); total phosphorus assimilation (IE_P). Different letters indicate significant differences at P=0.05 according to the LSD test.

Treatments	IEa (%)	IEb (%)	IE_N (%)	IE_P (%)
NS	55.5±1.34a	20.0±0.92b	73.7±0.11c	74.5±0.39b
NS+AM	67.8±0.98a	65.0±1.23a	91.3±0.20a	90.2±0.43a
SS+AM	59.1±1.84a	52.5±0.60b	84.5±0.14b	79.9±0.50b

Among the other major nutrients, K and Mg concentration increased significantly in the plants inoculated with AM *Gigaspora margarita* both in natural and sterilized soil, but Ca concentration decreased. Though the nutrient concentration showed an increasing trend with colonization level, significant differences were recorded primarily in the leaf and stem with a few exceptions. The increased concentrations of chlorophyll in the leaves were strongly correlated with the Mg content of the plants ($r=0.79$, $p<0.01$), and this trend was in agreement with the findings of other works (Pinior et al., 2005; Zuccarini, 2007). However, the reasons for the decreases of Ca in the leaves are not clear. Either the effect of AMF or the antagonistic effect of other nutrients may be a cause(Asaeda et al., 2014; Malvi, 2011). Trace elements, like Fe concentration, in the plants increased in the presence of AM (*Gigaspora margarita*), whereas Mn concentration showed various trends in different treatments. Several reports indicate higher concentration of trace elements in plants owing to AMF and deficiency conditions, and the ability of AMF to enhance the plant uptake of nutrient elements through extraradical hypal transport has been demonstrated for Cu, Zn and Fe (Liao et al., 2003; Neagoe et al., 2013). In contrast, the uptake of other elements, such as Fe, Mn, and Zn is sometimes reduced when plants are colonized by AMF (El-Kherbawy et al., 1989; George et al., 1994).

3.4.2 Effectiveness of AMF after inoculation

Soil inoculation with AMF positively affected plant growth in terms of dry mass (DM) production in all treatments, and previous literature tabulated the same results (Conversa et al., 2013). In the plants with AMF, belowground (IEb) DM were significantly increased compared with those of the non-inoculated plants (both in combination with natural and sterilized soil) and the plants in natural soil treatment (NS). Furthermore, remaining indigenous mycorrhiza also showed remarkable

performance for aboveground (IEa) DM and belowground (IEb) DM production in comparison with those of non-inoculated plants (Fujiyoshi et al., 2006; Neagoe et al., 2013).

Mycorrhizal inoculation significantly enhanced P and N assimilation in *P. japonica* in combination with natural and sterilized soil conditions (Ortas, 2012) in comparison with non-mycorrhizal plants, where maximum assimilation was found in NS+AM treatment due to their combined effect. Previously,Sainz and Arines (1988) showed that indigenous fungi were the most effective in enhancing plant growth and P uptake, which was correlated with a higher root colonization. This result is quite similar to those of this experiment, but their experimental results also revealed that inoculation with selected AMF species did not improve plant growth. On the contrary, in our experiment, selected AMF species also improved the plant growth and nutrient (N, P) acquisition, as in some previous reports (Fujiyoshi et al., 2006; Ortas, 2012; Treseder, 2013). This result may indicate that AMF can increase plant growth and nutrient assimilation but that it varies with AMF species.

3.5 Conclusions

This study was carried out to evaluate the effect of AMF on vegetation growth and nutrient assimilation of a pioneering plant (*P. japonica*). If the AM colonization was high, then the growth and nutrient absorption capacity was also high in all cases. All root samples of *P. japonica* in AM treatments (applied and indigenous) were colonized by AM fungi both in collected soil and sterilized soil. The average colonization level of *P. japonica* was 24-33%, whereas no colonization was found in the SS treatment. The DM production and chlorophyll content of leaves were the highest in plants inoculated with AM fungi in NS, where the AM colonization level was also high; the lowest AM colonization level occurred in the SS (where AM fungi was absent). N, P, K, Mg, Fe, and Cu concentration in the plants (root, stem, and leaf) showed a similar trend to that of dry mass production, but Ca concentration decreased with increasing colonization level; Mn concentration decreased in the root but increased in the leaf. Moreover, AM fungi had a potential effect on N-loss minimization. Thus, it can be concluded that AMF had a significant effect on nutrient assimilation and vegetative growth of the *P. japonica* plant in nutrient-limited riverbank soil. In the current study we could not analyse the community structure of AM fungi in the experimental soil. Therefore, the results reflect the case of a small subset of AMF interacting with the target plant species. Further study is needed to understand the behaviour of AMF community on the same.Pinpoint of the internal mechanism of AM fungi for nutrient uptake and their role in limiting nutrient loss also demand detailed investigation.

Chapter 4

Arbuscular mycorrhizal association for growth and nutrients assimilation of *Pharagmites japonica* and *Polygonum cuspidatum* plants growing on river bank soil

Abstract

Effects of arbuscular mycorrhizal fungi (AMF) on the growth, nutrient absorption and inoculation effectiveness of AMF on pioneer plants, *Pharagmites japonica* (C4) and *Polygonum cuspidatum* (C3) were evaluated by performing pot experiments in a greenhouse Saitama University, Japan. AMF spores were collected from the commercial product namely 'Serakinkon' . The average colonization level of *P. japonica* and *P. cuspidatum* was 24-33% and 0.2%-0.5% respectively and no colonization found in sterilized soil treatment. AMF colonization increased the plant dry mass, P and N concentration of *P. japonica* plant' s roots stems and leaves when AMF applied with natural and sterilized soil compared with only sterilized and natural soil and also represented the significant effect for N-loss minimization from soil. Maximum value showed when *P. japonica* applied with natural soil in combination with AMF whereas *P. cuspidatum* showed very less or a negative response to AMF colonization in all cases.

4.1 Introduction

Soils in floodplains are generally coarse and low in nutrients. These characteristics inhibit the development of dense plant communities (Giller and Malmqvist, 1998; Nilsson, 1987; Pinay et al., 1992). Nutrient enrichment of soil result from plant succession are essential for the colonization of thick herbaceous plant biomass (Carson and Barrett, 1988; Foster and Gross, 1998). In floodplain soil, plant growth is more often restricted by a shortage of nitrogen and phosphorus (Asaeda and Rashid, 2012) where mycorrhiza can increase the capability of plants for assimilating the nutrients from low nutrient soil like sediments of floodplains (Nakatsubo, 1997; Miller and Sharitz, 2000).

Arbuscular mycorrhiza (AM) plays a very important role on enhancing the plant growth and yield due to an increase supply of phosphorus to the host plant. Mycorrhizal fungi occur in riparian areas (Jacobson, 2004; Piotrowski et al., 2008) as for example, in sand dunes (Logan et al., 1989; Corkidi and Rincon, 1997), river floodplains (Nakatsubo, 1997; Harner et al., 2011) but their functions in promoting plant growth are unknown across floodplain chronosequences and remain studied are scant. Since soils in the early stages of primary succession generally have low nutrient, it is possible that AM fungi play an important role in the growth and establishment of pioneer plants. In such areas, however, the level of colonized plant species has a wide range. Some species, such as *Phragmites japonica*, grow at stony sterile as well as low nutrient condition, (Asaeda et al., 2011a) while *Polygonum cuspidatum* prefer relatively fine sediment with nutrient rich condition. Response to mycorrhizal inoculation is linked with the level of soil fertility and it is well know that P is the most influential element in mycorrhizal development and efficiency. In P-deficient soils, the yields of horticultural and field crops were found to be largely dependent on their mycorrhizal status under field (Ortas, 2008) and greenhouse conditions (Ortas, 2003). Flood plains along rivers with natural or near natural flow regimes are model systems for testing hypotheses about interactions among plants, soil, and mycorrhizal fungi because of the diversity of habitats and disturbance regimes in close proximity to one another.

We examined the effects of AM colonization on the growth and nutrient assimilation of pioneer plants growing in the early stage of primary succession area of river bank in this study. Therefore, the present study was planned with objectives: i) to determine whether AMF change floodplain river bank vegetation development by increasing growth and nutrient assimilation of *P. japonica* and *P. cuspidatum* plants appeared to be the most common species and covered higher percent areas of the transects, ii) to estimate the role of AMF on nutrient loss minimization for increasing river bank vegetation growth, iii) to find out the effect variation of AMF when applied with *P. japonica* and *P. cuspidatum* species.

4.2 Materials and Methods

4.2.1 Soil and plant propagate collection from study sites

A preliminary survey was conducted on banks of Ara River, Saitama, Japan (36°4′58.02″ N and 139°26′28.85″ E) to decide the experimental plant materials for this study (Figure 6.4). Five transects (henceforth referred as S1∼S5, respectively), each of five meter× four meter, were randomly selected along the River and marked with poles and rods. The distance between these transects ranged between 10 and 30 m. All plant species within a transect were identified and their percent coverage were calculated. Though the species composition varied between the transects, *Phragmites japonica* and *Polygonum cuspidatum* appeared to be the most common species and covered higher percent areas of the transects. Azami et al. (2004), Asaeda et al. (2010), Sarkar et al. (2015c) and Sekine et al. (2012) also reported that these plants are the most common in the Arakawa River and Kang et al. (2002) showed that *Phragmites japonica* is the most common species in riverine wetlands in South Korea . Therefore, based on the survey and previous literatures, we decided to choose *P. japonica* and *P. cuspidatum* as our experimental plants. Soil was collected from up to 20 cm depth from the surface of previously selected transects using a shovel. The soils had no profile development known as entisol according to USDA soil taxonomy classification. Soils collected from different transects were composited to make a homogenous soil pool and required amount of soil form the composite bulk was used for the experiments. The properties of the soils collected from the transects were homogenous and there was no significant differences between the individual soils from the transects and the composite soil in terms of soil particle size (D25), pH, % organic matter content, total carbon (TC), total nitrogen (TN), total phosphors (TP), available phosphorus concentrations and water holding capacity (all $p>0.05$). The soil used for experiment had an average pH of 5.85, with an average of 0.61 mm of D25, organic matter content 0.25%, TP 0.045%, available phosphorus 0.002%, TN 0.09% and water holding capacity 27%. Propagules (stem and rhizome) of *P. japonica* and *P. cuspidatum* for planting in pot were collected from the same sites.

4.2.2 Spore isolation from collected soil

Spores were extracted from the same composite soil by wet sieving and centrifugation (Gerdemann and Nicolson, 1963). For each 100 g of river bank soil, 3 L of tap water was used to suspend the soil for subsequent passage through a pair of sieves (860 and 36 μm mesh). The material retained in the 36 μm sieve was centrifuged in water (900 g for 4 min), and the resulting pellet was resuspended in 45% sucrose solution and centrifuged again (900 g for 1 min). The supernatant

was washed through a sieve of 36 μm, washed with tap water and the spores were transferred to Petri dishes. Spores were observed under a stereomicroscope and spore morphotypes separated according to spore morphology and color.

4.2.3 Experimental procedure and design

Considering the type of mycorrhizal inoculation, four treatments were taken, viz. (1) natural soil (NS), (2) natural soil inoculated by AM fungi (NS+AM), (3) sterilized soil inoculated by AM fungi (AM), and (4) sterilized soil without AM fungi inoculation (SS). The experiment was conducted in the form of pot cultures of *Phragmites japonica* and *Polygonum cuspidatum* in a greenhouse at Saitama University, Japan. The pots were laid out in complete randomized design (CRD) in a greenhouse and each treatment had four replications (repeated in space).

In each pot (20.96 cm, vol. 2.7 L), 2 kg (natural or sterilized) soil was used. Initial moisture content by using the gravimetric method (Black, 1965) and bulk density of soil were estimated. For sterilizing, soil was autoclaved twice at 121° C for 1 h to eliminate any indigenous AMF. The composition of spores isolated from the filed soils showed that *Gigaspora* was the most abundant AMF. Previous studies (Koske and Gemma, 1997; Kowalchuk et al., 2002; Rose, 1988) also support that *Gigaspora* is the most common AMF in floodplain soil. Therefore, to inoculate soils of respective treatments, we used a commercial inoculant namely 'Serakinkon' powder (The Central Glass Company, Tokyo, Japan) which is composed of 50 *Gigaspora margarita* Becker & Hall spores per gram powder (Higo et al., 2010; Kaneko and Tanimoto, 2009; Tawaraya et al., 1996). 20 g serakinkon powder/kg soil was evenly mixed with soil before using in a pot. Rhizomes with three nodes (∼2 cm) and stem cuttings with three nodes (∼3 cm) were used as propagating materials for *P. japonica* and *P. cuspidatum*, respectively. A temperature of 25±3° C and 75% relative humidity was maintained in the greenhouse throughout the experimental period. Pots were watered as necessary. The greenhouse culture lasted for 110 days.

4.2.4 Harvesting and processing

Plants (both species) were harvested at 110 days after transplanting at their senescence stage. After separating from the pot, the shoots were separated from the roots and fresh weights were taken separately for roots, stems and leaves. Soil particles were carefully removed from plant roots using forceps. The whole root system was then divided into two parts; one part for AM colonization observation and the other part for estimating dry mass and analyzing nutrients. Stems, leaves and roots (fraction) were cut into small pieces and dried to a constant weight at 80° C for 3 days and final weights (dry mass) were recorded. Since whole root

was not dried, the fraction of root oven-dried was taken into consideration for estimating belowground dry mass. Shoot (including stems and leaves) dry mass will be considered as aboveground dry mass. Oven-dried plant tissues (root and shoot) were ground separately. After removing from pot and plant roots, soil was weighed for nutrient budgeting. Moisture content and bulk density were also estimated. A fraction of soil was oven dried at 80° C for 3 days and final weight was recorded. Grinded plant tissues and soils were kept in air-tight containers for further analysis.

4.2.5 AM colonization determination and nutrient analyses from soil and plant samples

The extent of AM colonization were estimated by magnified intersections method as stipulated by (McGonigle et al., 1990). (1990). For this estimation, roots were cleaned, cut into small segments (2 cm/segment), rinsed and cleared for at least 12 h in 10% KOH solution at room temperature, and then stained with 0.05% trypan blue solution(Koske and Gemma, 1989). Ten randomly selected root fragments from each treatment were mounted on slides, and intersecting vertical gridlines were observed at 100× magnification to determine the presence and absence of AM colonization. The field of view of the microscope was moved using the stage graticule to make four, six or eight complete passes across each slide perpendicular to its long axis. Intersections were counted in the following categories; ' negative' (no fungal material in root), 'arbuscules', 'vesicles', and ' hyphae only'. The arbuscular colonization (As) and vesicular colonization (Vs) were calculated by dividing the count for the 'arbuscules' and 'vesicles' categories respectively by the total number of intersections examined. Hyphal colonization (Hs) was calculated as the proportion of non-negative intersections.

Total nitrogen (TN) concentrations of soil and plant tissues were determined with a CHN analyzer (CHN Corder MT-5, Yanoco, Kyoto, Japan). Powdered samples (2-10 mg) were placed in a crucible and ashed in a muffle furnace for 2 h at 500° C then dissolved in 1 ml of 5 M HCl and partially neutralized with 5 M NaOH solution. Orthophosphate was then determined using the phosphomolybdate method of (Murphy and Riley, 1962). Total phosphorus in soils sample was determined by perchloric acid digestion and available phosphorus was determined after extraction with bray extract solution (Bray and Kurtz, 1945).

4.2.6 Calculation of inoculation effectiveness

Inoculation effectiveness (IE) was calculated using the following equations based on aboveground dry mass (IEa), belowground dry mass (IEb), and nutrient as-

similation (IE_{Nutrient}) as suggested by Conversa et al. (2013), Ortas (2012) and Watts-Williams et al. (2013):

$$IE_a = \left[\frac{(ADM_{+M} - ADM_{-M})}{ADM_{+M}}\right] \times 100 \tag{4.1}$$

$$IE_b = \left[\frac{(BDM_{+M} - BDM_{-M})}{BDM_{+M}}\right] \times 100 \tag{4.2}$$

$$IE_{\text{Nutrient}} = \left[\frac{(\%\text{Nutrient}_{+M} - \%\text{Nutrient}_{-M})}{\%\text{Nutrient}_{+M}}\right] \times 100 \tag{4.3}$$

where, IE_a, IE_b, and IE_{Nutrient} designate inoculation effectiveness based on above ground dry mass (ADM), belowground dry mass (BDM), and nutrient (nitrogen or phosphorus) assimilation (IENutrient), and subscript $+M$ and $-M$ designate inoculated and non-inoculated plants, respectively.

4.2.7 N and P budgeting

Inputs and outputs of nitrogen (N) and phosphorus (P) in the soil-plant system were estimated for this experiment. Nutrients from the soil before planting propagules (henceforth 'initial soil') and propagating organs (rhizomes) were considered as input, and output estimates included nutrients removed by crop harvest and nutrient status of the remaining soil after crop harvest (henceforth 'final soil').

4.2.8 Statistical analyses

Prior to commencing statistical analyses, all data were checked for normalcy, and equal variance was measured using the Levene test. All data are presented as mean ± SD (n=4). The effects of mycorrhizal treatment on root colonization, dry mass, nutrient (N, P) content of each plant species, and soil were analyzed by two-way ANOVA followed by Tukey's posthoc test at 0.05 significant levels. Correlations between parameters were judged by Pearson's correlation co-efficient at the 0.05 significant level. All statistical analyses were performed using SPSS for windows (Version 13.0, SPSS, Inc., Chicago, IL, USA).

4.3 Results

4.3.1 Root Colonization

Microscopic observations represented that *P. japonica* treated with AM were colonized by AM fungi both in collected soil and sterilized soil. Colonization level of roots in NS+AM treatment was significantly different in comparison with NS treatment in *P. japonica* plant. the average colonization level of *P. japonica* was 24-33 % in different treatments whereas *P. cuspidatum* showed very low level of AM colonization (0.2-0.5%). In sterilized soil (AM fungi absent), no colonization was found for both plant species (Figure 4.1).

4.3.2 Changes of dry mass production due to AM fungi

AMF colonization showed the significant effect on dry mass production of *P. japonica* plant. For roots, stems and leaves dry mass production, maximum dry mass were produced in NS+AM treatment whereas the lowest one was SS. SS+AM and NS treatment showed statistically similar performance in all cases. However, the trend of total dry mass production was NS+AM>SS+AM>NS>SS (Figure 4.2). There was no significant positive response was found for dry mass production in case of *P. cuspidatum* whereas the trend of total dry mass production was SS>SS+AM>NS>NS+AM (Figure 4.2).

4.3.3 Total phosphorus content in plants

AM fungi had shown positive effect on P concentration of *P. japonica* and it is directly related with the colonization level. Increases in plant P concentration were greater when the colonization level was more pronounced, and this relationship was significant across all trials (Figure 4.3). P contents in plants (roots, stems and leaves) was the highest in AM fungi with natural soil treatment whereas lowest in sterilized soil (absent of AM fungi). When AM fungi applied with sterilized soil, P concentration was also higher than normal soil (Figure 4.3). *P. cuspidatum* did not show any specific trend but maximum P concentration was found in NS+AM treatment for roots, and in SS+AM treatment for stems and leaves (Figure 4.3).

4.3.4 Total nitrogen content in plants

TN content in different plants varied with the colonization level of AM fungi. Highly colonized plant such as *P. japonica* absorbed remarkable nitrogen (N). N absorption in *P. japonica* (root, stem and leaf) was maximum in NS+AM treatment where P absorption was also maximum. The minimum N absorption was

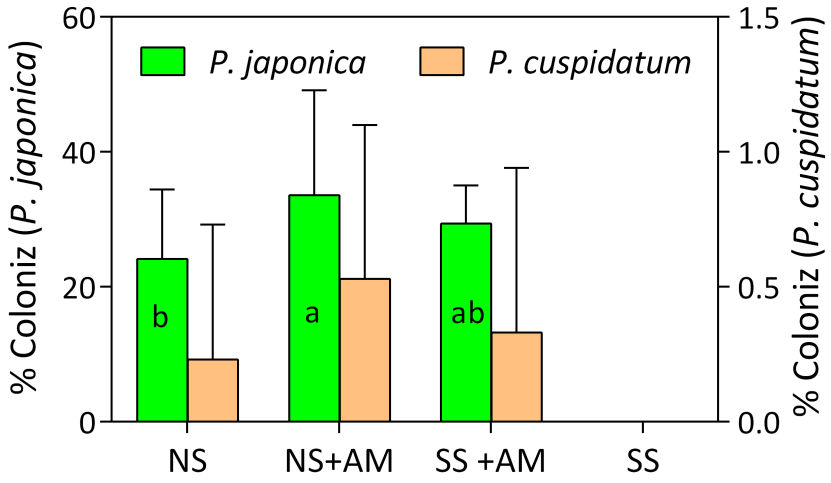

Figure 4.1: Arbuscular mycorrhizal (AM) colonization levels in the two herbaceous species growing on primary succession soils collected from Arakawa river bank. Values represent means of three replications and 10 root segments per replication were selected for observation. Error bars indicate ± standard deviation (SD) of mean. Different letters indicate significant differences at P=0.05 according to the LSD test.

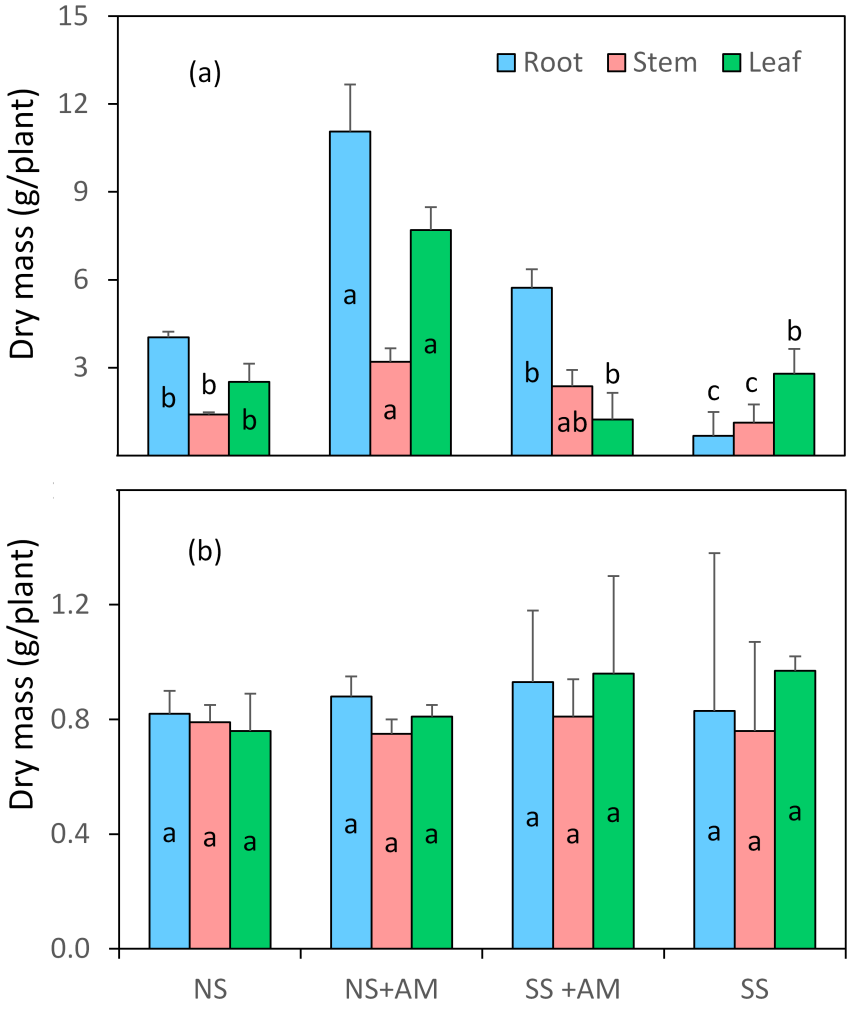

Figure 4.2: Dry mass production of (a) *P. japonica* and (b) *P. cuspidatum*. Error bars indicate ± SD of mean. Different letters indicate significant differences at P=0.05 according to the LSD test

showed in sterilized soil. When AM fungi applied with sterilized soil, N content was higher than natural soil (Figure 4.4). *P. cuspidatum* showed very less effect on N absorption whereas in NS+AM treatment leaves and SS+AM treatment stems assimilated the highest amount (Figure 4.4).

4.3.5 Total phosphorus and available phosphorus content in post-harvest soils

Soil TP contents after harvesting of both plants was statistically similar (Figure 4.5) but available phosphorus concentration in soil after harvesting of *P. japonica* was the highest in SS treatment showed significant difference with NS and NS+AM treatments (Figure 4.5). But there was no significant difference among different treatments for available phosphorus concentration in soil after harvesting of *P. cuspidatum* (Figure 4.5).

4.3.6 Total nitrogen content in soils after harvesting of plants

In case of *P. japonica* plant, soil in NS+AM treatment showed minimum TN content where root colonization level as well as plant TN accumulation rate was also high. Among different treatments, statistically significant differences were found which were negatively co-related with colonization level. But, TN contents in soils after harvesting of *P. cuspidatum* did not have any significant different among different treatments (Figure 4.6).

4.3.7 Nitrogen and phosphorus budgeting

Minimum nitrogen loss was found in NS+AM treatment (13.4%) and maximum nitrogen loss was found in SS (34.9%) for *P. japonica* plant where loss in SS+AM treatment was lower than NS treatment (Table 4.1). But for *P. cuspidatum* plant, there was no significant difference for nitrogen loss percentage among different treatments and % loss was quite similar with SS treatment of *P. japonica* plant. According to these experimental results, there was no significant effect of AM fungi for P loss minimization in different treatments (Figure 6.2 & 6.3).

4.3.8 Inoculation effectiveness

Inoculation of AM fungi remarkably affected on dry mass production and nutrients (N, P assimilation) of *P. japonica* plant. Inoculation efficiency on growth in terms of total DM production of above ground (IEa) and below ground (IEb) were 67.8%

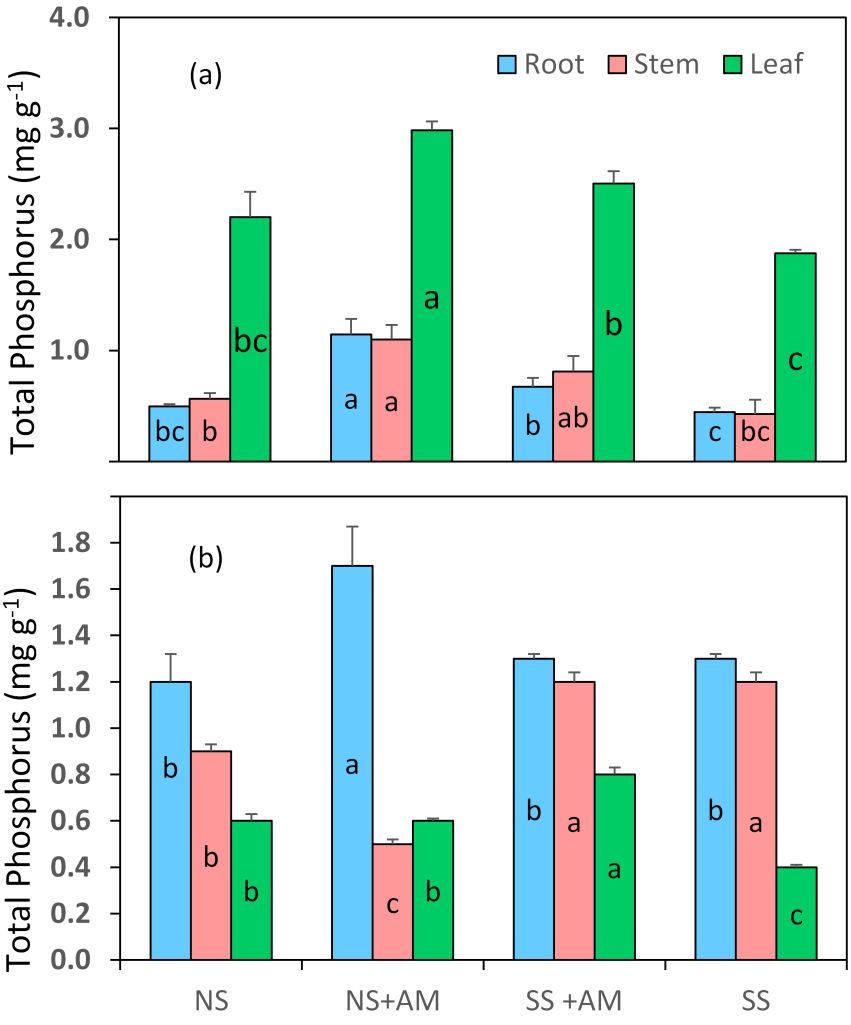

Figure 4.3: TP contents of plants in different treatments (a) *P. japonica* and (b) *P. cuspidatum*. Error bars indicate ± SD of mean. Different letters indicate significant differences at P=0.05 according to the LSD test

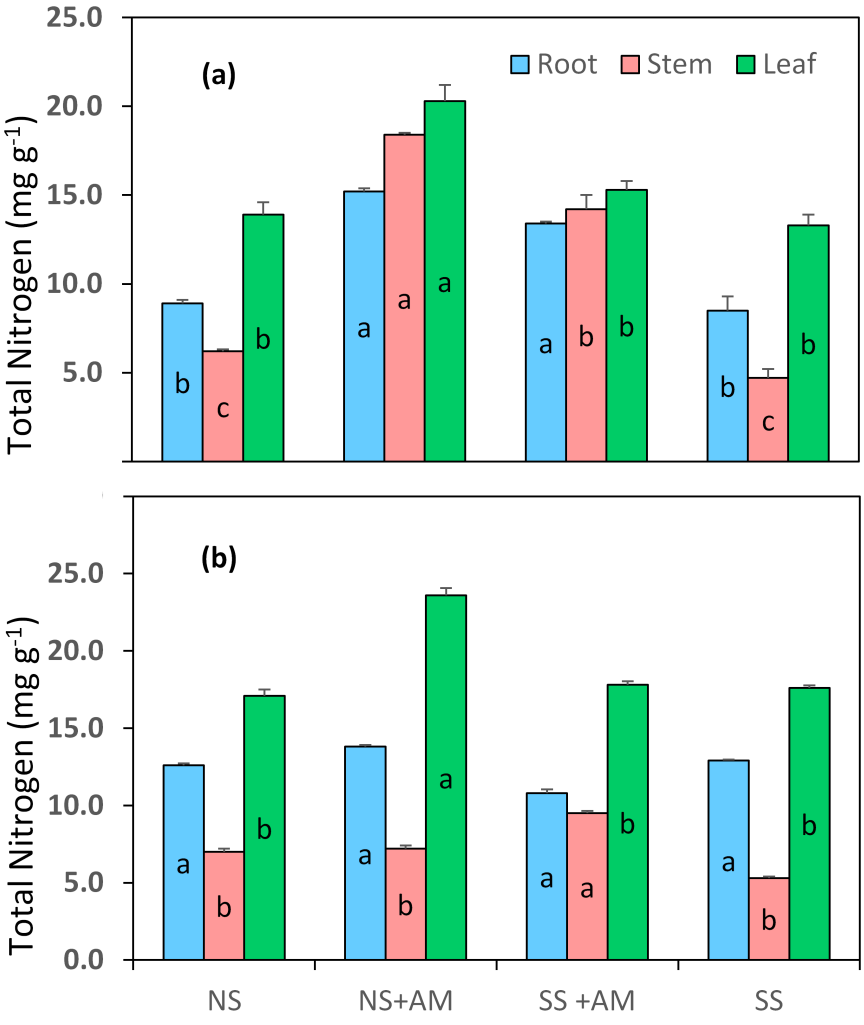

Figure 4.4: TN contents of plants in different treatments (a) *P. japonica* and (b) *P. cuspidatum*. Error bars indicate ± SD of mean. Different letters indicate significant differences at P=0.05 according to the LSD test

and 65% respectively in applied AMF with natural soil (NS+AM treatment) which were the highest among all treatments. When AM fungi applied with sterilized soil (SS+AM, without indigenous AM), showed better performance for above ground dry mass (IEa) and below ground dry mass (IEb) production than natural soil but the value different was not statistically significant (Figure 4.7).

Assimilation of nitrogen IE_{N} and phosphorus IE_{P} by *P. japonica* plant was also significantly affected by inoculation of applied mycorrhiza. Similar trend were found in both cases and the trend was NS+AM>SS+AM>NS (Figure 4.7). But *P. cuspidatum* plant showed no or negative response (data not shown) in all cases.

4.4 Discussion

Percent colonization of root and dry mass production Maximum colonization was found in *P. japonica* plant root when AM fungi were applied with natural soil as because in natural soil some indigenous AM fungi strains were existed which also performed for colony development. Moreover, AM fungi with sterilized soil showed higher colonization level compared with natural soil only. But, initial colonizer *P. cuspidatum* roots did not show remarkable colonization in all treatments and in sterilized soil, no colonization was found for both plant species. According to the results it can be concluded that colonization level depends not only the density of AM fungi but also the plant species. These results agree with previous studies in which many *Polygonum* species were shown to be non-mycotrophic (Fujiyoshi et al., 2006; Harley and Harley, 1987; Neagoe et al., 2013).

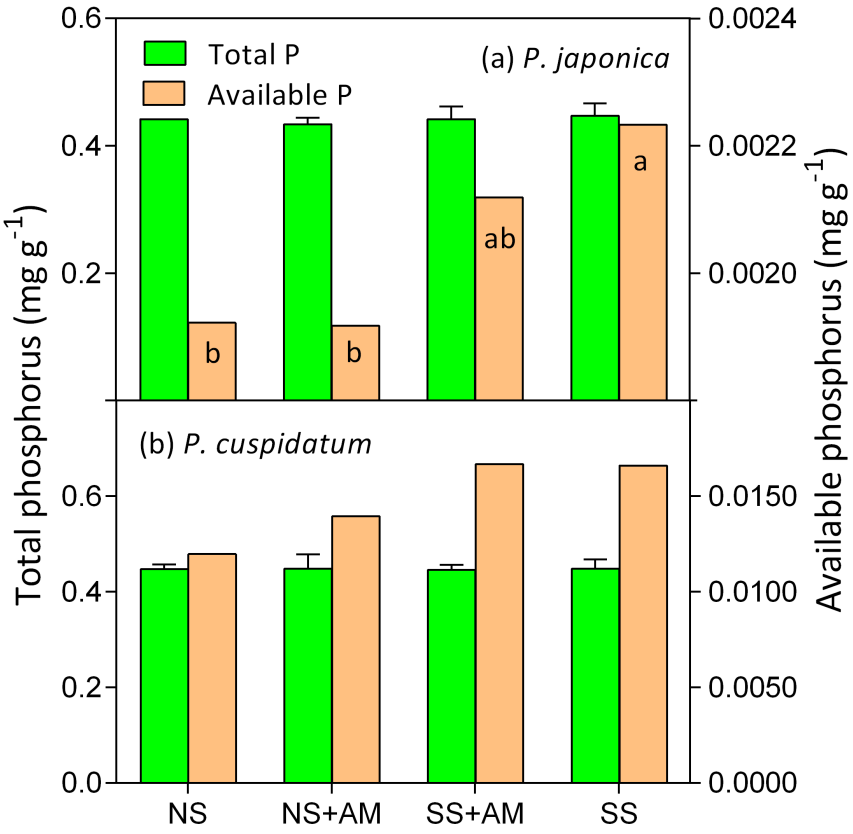

Figure 4.5: TP and available P content in soils after harvesting of plants (a) *P. japonica* and (b) *P. cuspidatum*. Error bars indicate ± SD of mean. A column having no error bar indicates that the SD value of the respective mean was too small or zero. Different letters indicate significant differences at P=0.05 according to the LSD test

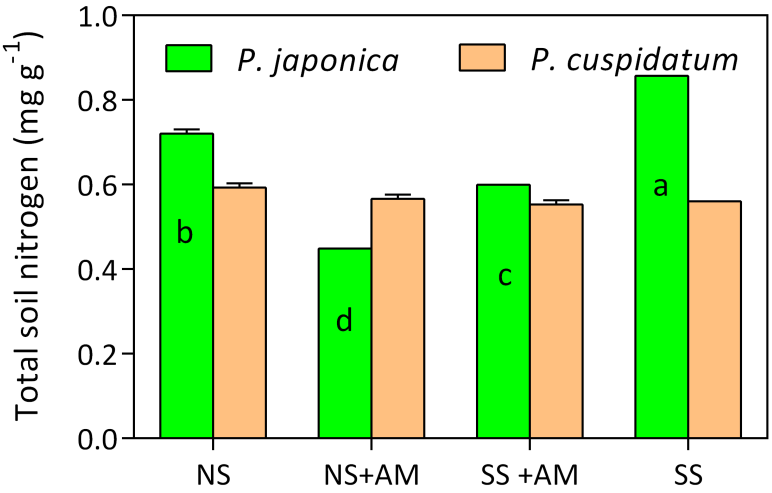

Figure 4.6: TN content in soils after harvesting of plants (a) *P. japonica* and (b) *P. cuspidatum*. Error bars indicate ± SD of mean. A column having no error bar indicates that the SD value of the respective mean was too small or zero. Different letters indicate significant differences at P=0.05 according to the LSD test

Table 4.1: Nitrogen budget of soil and plant as affected by treatments

Treatment	N in rhizome (g)	Initial soil N (g)	Distribution of N in plant			Leftover N in rhizome (g)	N recovery (g)	N loss (%)
			Root/rhizome	Stem	Leaf			
P. japonica								
NS	1.79×10^{-05}	1.86	0.036	0.009	0.046	1.24×10^{-05}	1.40	24.7
NS+AM	1.79×10^{-05}	1.86	0.169	0.059	0.066	1.21×10^{-05}	1.61	13.4
SS+AM	1.79×10^{-05}	1.86	0.077	0.034	0.042	1.21×10^{-05}	1.48	20.1
SS	1.79×10^{-05}	1.86	0.006	0.005	0.013	1.22×10^{-05}	1.21	34.9
P. cuspidatum								
NS	1.64×10^{-05}	1.86	0.004	0.003	0.009	1.19×10^{-05}	1.20	35.4
NS+AM	1.64×10^{-05}	1.86	0.004	0.001	0.009	1.17×10^{-05}	1.14	38.7
SS+AM	1.64×10^{-05}	1.86	0.008	0.005	0.017	1.19×10^{-05}	1.14	38.7
SS	1.64×10^{-05}	1.86	0.011	0.003	0.02	1.21×10^{-05}	1.15	38.1

AM fungi with natural soil showed the best performance for roots, stems and leaves dry mass production of *P. japonica* plant and the lowest one was sterilize soil. However, SS+AM and AM treatment showed statistically similar values in all cases. Finally, total dry mass was the highest in AM fungi with natural soil whereas AM fungi with sterilized soil also higher than other treatments. But there was no significant response for total dry mass production in *P. cuspidatum* plant when AMF was applied. So, we can say that AM fungi can increase total dry mass as well as roots, stems and leaves dry mass if colonization level is high but it varies with plant species (Fujiyoshi et al., 2006; Neagoe et al., 2013).

4.4.1 Nutrients (N, P) assimilation and budgeting

After harvesting of plants, total phosphorus (TP) in plant and soil, was determined to get more idea about P accumulation rate of plants and finally P budget was done to know either AM fungi have some effect on P loss minimization. Effects of P concentration in *P. japonica* plant were positively related to AM colonization (Figure 4.3) (Tanaka and Yano, 2005; Fujiyoshi et al., 2006; Johansen et al., 1994; Tobar et al., 1994; Govindarajulu et al., 2005).On the contrary, TP in remaining soil (after harvesting of plant) was the lowest in NS+AM treatment where colonization level was maximum as well as TP in plant was also maximum. But there was no significant effect on *P. cuspidatum* plant. From these results, it can be mentioned that AM fungi had some significant effect on P absorption of *P. japonica* (C4) plant than *P. cuspidatum* (C3) plant. This results also coincide with previous researchers where they reported that C4 grasses and non-N-fixing plants tended to be more responsive to AM fungi colonization than were C3 grasses and N-fixing plants (Hoeksema et al., 2010). But the accumulation rate of TP by *P. japonica* (C4) plant in AM fungi with natural soil treatment (NS+AM) was much more higher than AM fungi with sterilized soil (SS+AM) as because some phosphate solubilizing microorganisms (bacteria, fungi) were present in natural soil which played a vital role for making the phosphorus available from unavailable form (Sarkar et al., 2012) and finally plant assimilated the available phosphorus by the help of AM fungi. Also to know the AM fungal effect on phosphorus availability, available phosphorus content of soils after harvesting of plant were determined but AM fungi had no significant positive effect on availability of phosphorus in remaining soil. Moreover, phosphorus availability was maximum in sterilized soil treatment (SS) where colonization level of AM fungi was zero. This result may indicate that AM fungi can play role for assimilation of P by plant where available phosphorus is high and finally make it lower in compare to other treatments but not on P availability from unavailable form though the root colonization level is high. Generally this potential mechanism is consistent with the characterization of the mutualistic relationship between plants and AM fungi (Brundrett, 2004;

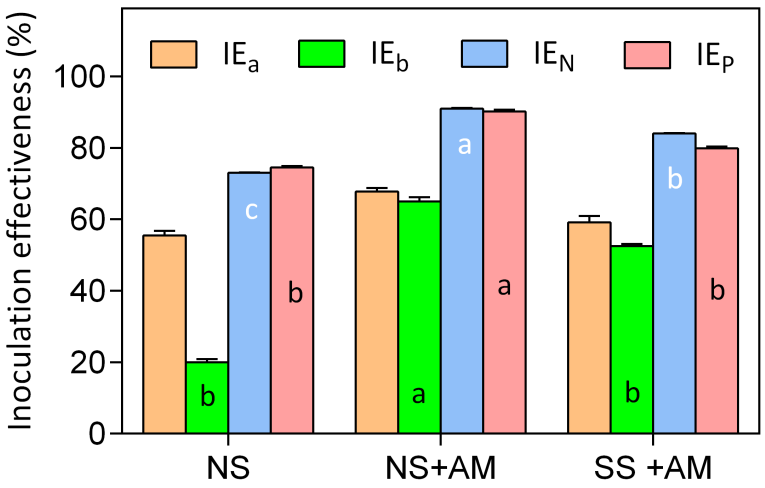

Figure 4.7: Inoculation effectiveness of AM fungi for dry mass production and nutrients (N, P) assimilation of *P. japonica*. Inoculation effectiveness for above ground (IEa) and below ground dry mass production (IEb); for total nitrogen assimilation (IEN); total phosphorus assimilation (IEP). Error bars indicate ± SD of mean. A column having no error bar indicates that the ± SD value of the respective mean was too small or zero. Different letters indicate significant differences at P=0.05 according to the LSD test

Mosse, 1957, 1973; Smith and Smith, 2011a,b, 2012). However, a fair amount of evidence has accumulated indicating that AM fungi are balanced mutualisms that function by a form of regulated exchange, so that plants should support high levels of root colonization only if the AM fungi provide benefits(Brundrett, 2004). But, physiological evidence for large differences in P transfer by different AM fungi that would be expected to influence the size (and possible direction) of AM-mediated growth responses and total P uptake (Smith and Smith, 2012).

Total nitrogen content of soil and plant was determined to know the TN assimilation rate of plants from the soil. From the results of TN content in plant and soil indicate that AM fungi have significant effect on nitrogen assimilation in C4 plant (*P. japonica*) but very less or poor effect on C3 plant (*P. cuspidatum*). As because, AM fungi assimilate N either exclusively (Tanaka and Yano, 2005) or predominantly (Govindarajulu et al., 2005) in the form of NH_4^+ . Moreover, AM fungi have the ability to mineralize N from organic sources (Hodge et al., 2001, 2010; Atul-Nayyar et al., 2009; Leigh et al., 2009; Barrett et al., 2011). Several studies have revealed that the N mobilized from patches can account for up to 32% of the total N present in the patch (Leigh et al., 2009). Moreover, N mo-

bilization may also take place even when an AM fungus fails to stimulate plant growth (Hodge et al., 2010). On the contrary, some studies included that isolates from the *Gigasporaceae* detected an inferior ability of these isolates to contribute to plant N nutrition (Veresoglou et al., 2011; Reynolds et al., 2005).

Nitrogen budget was done to determine the amount of nitrogen loss before and after the AM fungi application. From this experiment, we got some important findings in case of *P. japonica* plant. Minimum nitrogen loss (13.4%) was occurred in AM fungi with natural soil treatment where maximum loss was found in sterilized soil (34.9%) treatment. These results indicate that AM fungi can play direct role not only for assimilation of nitrogen but also may represent an effective way to limit N-losses from an ecosystem if the colonization level is high. Amora-Lazcano et al. (1998) studied on interactive effect of AM fungi on the denitrifying community and reported that AM fungi were able to demonstrate a decrease in counts of denitrifying bacteria. Some additional studies are also available on the interactive effect of AM fungi and some verified denitrifying strains of *Pseudomonas* such as *Pseudomonas putida* (Kim et al., 2008) and *Pseudomonas fluorescens* (Samuelsson et al., 1988), even though it is unclear to what degree these results can be generalized to denitrifying microbes at large (Veresoglou et al., 2012).

AM fungi may have some effective role in leaching but the role of AM fungi in leaching has not been extensively studied yet. Asghari and Cavagnaro (2011) were able to detect a decline in NO_3^- , NH_4^+, and phosphate concentrations in the leachate, which could partly be attributed to the bigger size of the mycorrhizal plants. Van der Heijden (2010) was able to demonstrate significant differences in the composition of the leachate with respect to phosphate concentration for three plants at the low nutrient supply; also with respect to NH_4^+ for *Festuca ovina* at the low nutrient supply and for *Poa pratensis* at the high nutrient supply. By contrast, Rillig et al. (2006) studied leaching of DOC/DON components that may be related to AMF, but detected no measurable amounts of a putatively AMF derived protein; this study only targeted glomalin-related soil protein, and thus it cannot be used as exhaustive evidence against the occurrence of AM fungal derived N-compounds in leachate. These results signify that AM fungi may represent an effective measurement to limit N-losses from an ecosystem and that was the most important findings of this experiment. P budget was done to know either AM fungi have some effect on P loss recovery by comparing results between the presence and absence of AM fungi but there was no significant difference in different treatments considering both of the plants (Figure 6.2 & 6.3).

4.4.2 Efficiency of AMF after inoculation

Soil inoculation with AM fungi (*Gigaspora margarita*) positively affected plant growth (Conversa et al., 2013) in terms of dry mass (DM) production in all

treatments. Due to application of AM fungi, above ground (IEa) and below ground (IEb) DM were significantly increased in *P. japonica* plant compare to non-inoculated plant. Indigenous mycorrhiza remain in natural soil (NS treatment) and applied mycorrhizal treatment (both in combination with indigenous mycorrhiza and sterilized soil) also showed remarkable performance for above ground (IEa) DM and below ground (IEb) DM as well as total DM production (Fujiyoshi et al., 2006; Neagoe et al., 2013). Mycorrhizal inoculation had enhanced significantly P and N assimilation in *p. japonic*a with combination of natural and sterilized soil condition (Ortas, 2012) in compare to non-mycorrhizal plant where maximum assimilation was found in applied with indigenous mycorrhizal treatment due to their combined effect. Previously, Sainz and Arines (1988) showed that indigenous fungi were the most effective in enhancing plant growth and P uptake, which were correlated with a higher root colonization and that result is quite similar with this experiment results but that experimental results also represented that inoculation with selected AMF species did not improve plant growth but in our experiment, selected AM species also improved the plant growth and nutrients (N, P) acquisition Ortas (2012); Fujiyoshi et al. (2006); Treseder (2013). This result may indicate that AMF can increase plant growth and nutrient assimilation but it varies with AMF species and host plant.

4.5 Conclusion

This study was carried out to evaluate the effect of AMF for the vegetation growth and nutrient assimilation of pioneering plants. If the AM colonization was high then the growth and nutrient absorption capacity was also high for *P. japonica*. Roots of *P. japonica* in AM treatments were colonized by AM fungi both in collected soil and sterilized soil. The average colonization level of *P. japonica* was 24-33% whereas *P. cuspidatum* showed very low level of AM colonization (0.2%-0.5%). Dry mass production was the highest in AM fungi with natural soil treatment where AM colonization level was also high and the lowest DM production was showed in sterilized soil (where AM fungi was absent) in *P. japonica* plant. P and N concentration in plants were showed similar trend like DM production. Moreover, AM fungi have some potential effect to limit N-loss minimization. But for *P. cuspidatum* did not show the significant differences among different treatments due to low level of AM colonization. So, it can be concluded that AMF have much effect on natural vegetation growth and nutrient assimilation but it differs from species to species.

Chapter 5

Response of *Miscanthus sacchariflorus* to zinc stress mediated by arbuscular mycorrhizal fungi

Abstract

Zinc is an essential micronutrient for plant growth and zinc deficiency is one of the most commonly reported plant deficiencies worldwide. Conversely, Zn is also a common environmental contaminant, significantly reducing plant growth. In the present experiment, we studied the effects of arbuscular mycorrhizal association on growth and survival capabilities of *Miscanthus sacchariflorus* under different Zn concentrations in soil. According to this experimental result, Zn did not show significant effect of mycorrhizal colonization. Even, when the Zn addition level was as high as 1000 mgkg^{-1}, the mycorrhizal infection rate slightly decrease compared to the control receiving no Zn but not statistically significant different. This may imply that Zn has no or little effect on spore germination and AM colonization. Inoculation of AMF (*Gigaspora margariata*) with Zn (100 mg kg^{-1}) increased chlorophyll content, Fv/Fm, total dry mass, IAA, TN, TP and Zn concentration and H_2O_2 level, IAAO activity, POD activity was low compare to other treatments whereas with Zn (1000mg kg^{-1}) induces lower concentrations of these metals in the aerial part of the plant and consequently a beneficial effect on plant growth. In addition, AMF can able to accumulate Zn in plant root.When approaching the

inner part of the root, Zn is located in the parenchyma cells. Accordingly, it can be stated that AM are able to keep Zn out of plant or reduce concentration of aerial parts of plant.

5.1 Introduction

Arbuscular mycorrhiza (AM) can significantly increase plant growth and production under stress due to the formation of extensive hyphal networks. Such abilities can result in enhanced water and nutrient uptake. Under heavy metals stress, the diversity of AM spores decreases compared with stress-free conditions. Hence, a limited number of spores are usually found in the rhizosphere of e.g., Zn-tolerant plant species (del Val et al., 1999; Gonzalez-Guerrero et al., 2008; Pawlowska et al., 1997). Under heavy metal stress, the unfavorable oxidative effects adversely influence plant growth. However, AM are able to enhance production of antioxidant enzymes, which can alleviate the stress of heavy metals (Avery, 2001; Ruiz-Lozano, 2003). It should be mentioned that high levels of stress may turn the symbiosis between the two partners into a parasitic relationship, as unfavourable conditions may adversely influence AM performance (Hildebrandt et al., 2007; Miransari et al., 2008, 2009). The adverse effects of AM on plant growth under stress conditions can be through unfavourable effects of the stress on AM functioning and development. These effects include decreased colonisation rate and spore germination, as well as decreased fungal hyphal growth especially under salt stress condition (Evelin et al., 2009; Jahromi et al., 2008).

Zinc is an essential micronutrient for plant growth. Conversely, Zn is also an important environmental contaminant in some situations, often reaching phytotoxic concentrations (Christie et al., 2004). Common sources of Zn (as a toxicant) in soils include mine spoilings, and runoff from galvanized metal surfaces and roadways. Understanding the mechanisms by which plants maximize acquisition and utilization of Zn under low concentrations, and deal with toxic Zn concentrations in soils, has been studied previously (Cavagnaro et al., 2008; Christie et al., 2004). However, few studies have considered plant responses to low and high soil Zn concentrations in the same study (Chen et al., 2003).Although the functions of AM under micronutrient deficient conditions have been widely studied and are well understood, much information is required on the contrasting conditions of excessive trace elements (Leyval et al., 1997; Weissenhorn et al., 1995a).The widespread existence of AM fungi in metal contaminated sites has also provided evidence of adaptation and tolerance of microorganisms to toxic metals (Piotrowski et al., 2008; Kaldorf et al., 1999), and metal tolerant fungi have been isolated (Weissenhorn et al., 1993). The feasibility of employing AM in soil re-vegetation and remediation has elicited great interest, and numerous studies have focused on the

functions of AM fungi in metal-contaminated soils (Leyval et al., 1997). Even though AM are most often considered important for uptake of immobile nutrients, they also play an important role in reducing uptake of heavy metals, including Zn, where soil concentrations are high (Christie et al., 2004; Hildebrandt et al., 2007). Thus, AMF have various roles in terms of plant-Zn interactions. To understand these roles, there is a need to study responses of AM across a range of soil types, Zn concentrations and plant species (Cavagnaro et al., 2008). Soil Zn conditions not only influence the functioning of AM, but also their formation. Addition of Zn to soils in amounts ranging from deficient through to toxic, can have positive (Heggo et al., 1990; Hetrick et al., 1994; Lee and George, 2005; Zhu et al., 2001), negative (Bi et al., 2003; Chen et al., 2003) and neutral (Chen et al., 2003; Diaz et al., 1996; Ortas et al., 2002) effects on root colonization by arbuscular mycorrhizal fungi (AMF). Given the complex roles of AM in plant-Zn dynamics, there is a need to assess Zn effects on them under both high and low soil Zn conditions in the same study. Here we report results of an experiment in which we assessed the effects of Zn addition on the mycorrhizal colonization, growth, nutrition and related biochemical parameters of the *Miscanthus sacchariflorus* plants responding to these conditions.

5.2 Materials and methods

5.2.1 Collection of soil and plant propagate from study sites

Soils collected from different transects (five meter×four meter) on the banks of the Ara River, Saitama, Japan (36°4′58.02″ N and 139°26′28.85″ E) were composited to make a homogenous soil pool (Figure 6.4). The properties of the soils collected from the transects were homogenous, and there were no significant differences between the individual soil samples from the transects (P>0.5) and the composite soil in terms of soil particle size (D25), pH, percentage organic matter content, total carbon (TC), total nitrogen (TN), total phosphorus (TP), and available phosphorus concentrations and water holding capacity (all $p > 0.05$). The soil utilized for the experiment had an average pH of 5.8, with an average of 0.61 mm of D25, organic matter content 0.25%, TP 0.45 mg/g, available phosphorus 0.02 mg/g, TN 0.9 mg/g, water holding capacity 27%, potassium (K) 1.7 mg/g, calcium (Ca) 0.4 mg/g, magnesium (Mg) 1.1 mg/g, iron (Fe) 0.55 mg/kg, copper (Cu) 0.05 mg/kg, manganese (Mn) 0.40 mg/kg, and zinc (Zn) 0.27 mg/kg. Propagates (rhizomes) of *Miscanthus sacchariflorus* for planting in pots were collected from the same sites.

Table 5.1: Summary of AM fungi-Zn interaction and application for growth response

Soil type	**Host plant**	**Zn Conc.**[1]	**M. Fungi**[2]	**Reference**
Contaminated soil	Colver, sunflower, mustard and phacelia	1096	*Glomus intraradices*	Neagoe et al. (2013)
Sewage sludge	*Allium porrum*, *Sorghum bicolor*	294.6	*Glomus claroideum*, *G. mosseae*, *Glomus* sp.	del Val et al. (1999)
Non-contaminated soil	*L. spartum*, *A. cytisoides*	0∼1000	*Glomus mosseae, G. marocarpum*	Diaz et al. (1996)
Heavy-metal polluted soil	leek, maize	1220	*Glomus mosseae*	Weissenhorn et al. (1993, 1995b)
Sewage-sludge amended soil	Maize	199∼1074	NA	Weissenhorn et al. (1995a)
Heavy metal polluted soil	*Thlaspi praecox*	280	NA	Vogel-Mikus et al. (2006)
Calcareous soil	Red clover	0∼1200	*Glomus mosseae*	Chen et al. (2003)
Uncontaminated & contaminated soil	*Cynodon dactylon*	0.08, 438	*Acaulospora* spp., *Glomus* spp.	Wu et al. (2010)
Field soil	Tomato	0.65∼75	NA	Cavagnaro et al. (2010)
Sandy soil	Maize	0∼900	*Glomus mosseae* BEG167	Shen et al. (2006)
Loamy soil	White clover	30∼270	*Glomus* spp.	Vivas et al. (2006)
Sandy clay loam	Red clover	0∼1000	*Glomus mosseae*	Li and Christie (2001)
Sandy clay loam	White clover	0∼400	NA	Zhu et al. (2001)
Farm soil	Rice	5	*Glomus etunicatum*	Purakayastha and Chhonkar (2001)
Raman type brown forest soil	Maize	250	*Glomus intraradices*	Seres et al. (2006)
Soil from industrial site	Sorghum	20μ Zn	*Glomus intraradices*, *G. spurcum*	Toler et al. (2005)
Contaminated agricultural soil	Barrel medics	627	*Glomus intraradices*, *G. intraradices* BEG 141, *G. mosseae* BEG 69, *G. mosseae* BEG 12, *Glomus* spp.	Redon et al. (2009)
Contaminated silt loam soil	*Andropogon gerardii*	0∼1000	*Glomus constrictum*, *Scutellospora pellucida*, *G. ambisporum*, *G. ambisporum*, *S. pellucida*, *G. constriticum*, *G. mosseae*, *G. constrictum*, *S. calospora*	Shetty et al. (1995)

[1] The unit of Zn concentration is mg Kg^{-1} soil, if not specified in the column. The soil is either contaminated or Zn is supplied as experimental treatment.

[2] The mycorrhizal fungi species are either naturally found or inoculated.

[NA] Data not available

5.2.2 Experimental set-up

The experiment was conducted in the form of pot cultures of *M. sacchariflorus* in a greenhouse at Saitama University, Japan. The pots were laid out in complete randomized design (CRD) with four replicates per treatment. The treatments were composed of the inoculation or no inoculation of the AM fungus, *Gigaspora margarita*,and the addition of three Zn concentrations in the soil (0, 100 and 1,000 mg kg-1). Zn was added as an aqueous solution of $ZnSO_4.7H_2O$. The Zn addition treatments were selected on the basis of the reported existence of soil zinc (Table 6.1).

In each pot (diameter 20.96 cm, vol. 2.7 L), 2 kg (natural) of soil was used. Initial moisture content and bulk density of the soil were estimated. To inoculate soils of respective treatments, AM fungi spores were applied in the form of a commercial inoculant namely 'Serakinkon' powder (The Central Glass Company, Tokyo, Japan). The inoculant was composed of 50 *Gigaspora margarita* Becker & Hall spores per gram powder (Higo et al., 2010; Kaneko and Tanimoto, 2009; Tawaraya et al., 1996) as *Gigaspora* was the most commonly isolated AMF on river bank sand with higher spore densities than those of other species; previous studies revealed the same result (Koske and Gemma, 1997; Kowalchuk et al., 2002; Rose, 1988; Sarkar et al., 2015a,b,c). 20 g serakinkon powder/kg soil was evenly mixed with soil before being used in a pot. Rhizomes with three nodes (∼2 cm) were employed as propagating materials. A temperature of 25 ± 3° C and 75% relative humidity were maintained (day and night) in the greenhouse throughout the experimental period. Pots were watered as necessary. The greenhouse culture lasted for 110 days.

5.2.3 Chlorophyll content and chlorophyll fluorescence

At harvest, chlorophyll was extracted from the leaves of *M. sacchariflorus* using N,N dimethylformamide (DMF). The leaves were cut, and fresh weight (∼5 mg) was measured. Pigments were then extracted into 5 ml DMF by incubating leaf cuttings 24 h in a refrigerator at 4° C. The chlorophyll and carotenoid concentration was determined via the method described by (Porra et al., 1989). Absorbance at 646.8 nm and 663.8 nm was measured with a UV-mini 1200-spectrophotometer (Shimadzu, Japan).Chlorophyll concentrations were calculated as μg/mg fresh weight (FW) basis. The plants were incubated in dark for 15 min before the measurements were taken in order to allow complete oxidation of the photosystem II (PSII) reaction centers. Maximum photochemical efficiency of PSII (Fv/Fm) was determined.

5.2.4 Hormone and enzyme analysis

IAA concentration in the tissues was measured using Salowski reagent (Gordon and Weber, 1951). For the analysis of endogenous H_2O_2 concentration, samples were extracted with cold acetone; and the method was followed as described by Cervilla et al. (2007) and Zaman and Asaeda (2013). Phosphate buffer (0.1 mol/L) at pH 6 was used to make extracts suitable for the measurements of POD and IAAO activities. IAA destruction was measured to determine IAAO activity (Zhang et al., 2009). POD was determined according to Goel et al. (2003) and the absorbance differences at 420 nm were plotted in every 30 s for 3 min (Chanjirakul et al., 2006).

5.2.5 Harvesting and processing

Plants were harvested at 110 days after transplanting at their senescence stage. Soil particles were carefully removed from plant roots using forceps. The whole stems, leaves, and roots were then divided into two parts: one part for AM colonization (roots) observation, hormone and enzyme analysis whereas the other part for estimating dry mass and analyzing nutrients. Stems, leaves, and roots (fraction) were cut into small pieces and dried to a constant weight at 80° C for 3 days, and final weights (dry mass) were recorded. Since not the whole roots, stems and leaves were dried, the fraction of oven-dried roots, stems and leaves were taken into consideration for estimating belowground (roots) and aboveground (including stem and leaf) dry mass. Ground plant tissues were kept in air-tight containers for further analysis.

5.2.6 AM colonization determination and nutrient analyses from soil and plant samples

To estimate the extent of AM colonization, roots were cleaned, cut into small segments (2 cm/segment), rinsed and cleared for at least 12 h in 10% KOH solution at room temperature, and then stained with 0.05% trypan blue solution (Koske and Gemma, 1989). Ten randomly selected root fragments from each treatment were mounted on slides, and intersecting vertical gridlines were observed at 100× magnification to determine the presence and absence of AM colonization (internal hyphae, vesicles, or arbuscules) (McGonigle et al., 1990). Total nitrogen (TN) concentrations plant tissues was determined with a CHN analyzer (CHN Corder MT-5, Yanoco, and Kyoto, Japan). Total phosphorus in plant samples was determined by perchloric acid digestion. The concentrations of Zn in plants were analyzed after oven drying to a constant weight at 72° C followed by digestion in a perchloric acid mixture, as described by (Tandon, 1993). Nutrient concentrations

in the tissue digest were quantified using an atomic absorption spectrophotometer (AA-6300; Shimadzu, Japan) using the method described in standard methods for the examination of water and wastewater by Gilcreas (1966).

5.2.7 Scanning electron micrograph (SEM) analyses

Plant segments were excised with a razor blade and rapidly frozen in liquid nitrogen with slush. The frozen sample was set on an Alto 1000 cryo-transfer system, fractured with a cold razor blade, and mounted on the cold stage. After excess water was removed by sublimation, back scarred electron images were obtained with a Hitachi S-3400 SEM in low vacuum mode (30 Pa) at -120° C without any coating. Energy dispersive X-ray (EDX) analysis was performed by a Bruker Quantax attached to the SEM.

5.2.8 Statistical analyses

Prior to commencing statistical analyses, all data were checked for normality, and equal variance was checked using the Levene' s test. All data are presented as mean ± SE (n = 4). The effects of mycorrhizal treatment on chlorophyll concentration in the leaves, fluorescence, root colonization, dry weight, nutrients (N, P) and Zn concentration of each plant component, were analyzed by two-way ANOVA followed by Tukey' s posthoc test at 0.05 significant levels. Correlations between parameters were judged by Pearson' s correlation coefficient . All statistical analyses were performed using SPSS for Windows (Version 13.0, SPSS, Inc., Chicago, IL, USA) and R package (Team, 2010).

5.3 Results

5.3.1 Root colonization

Microscopic observations revealed that almost all root samples of *M. sacchariflorus* in AM treatments were colonized by AM fungi both in treated and non-treated soil with Zn. Colonization level of roots in the control and Zn100 treatment was little higher in comparison with the Zn1000 treatment though it was statistically similar (P>0.05)(Table 7.1). The average colonization level of *M. sacchariflorus* was 22–29% in different treatments (Figure 5.1).

5.3.2 Chlorophyll content and fluorescence in leaves

Plants inoculated with *Gigaspora margarita* had significantly higher leaf chlorophyll concentrations than non-inoculated plants with control (Zn0 treatment) and

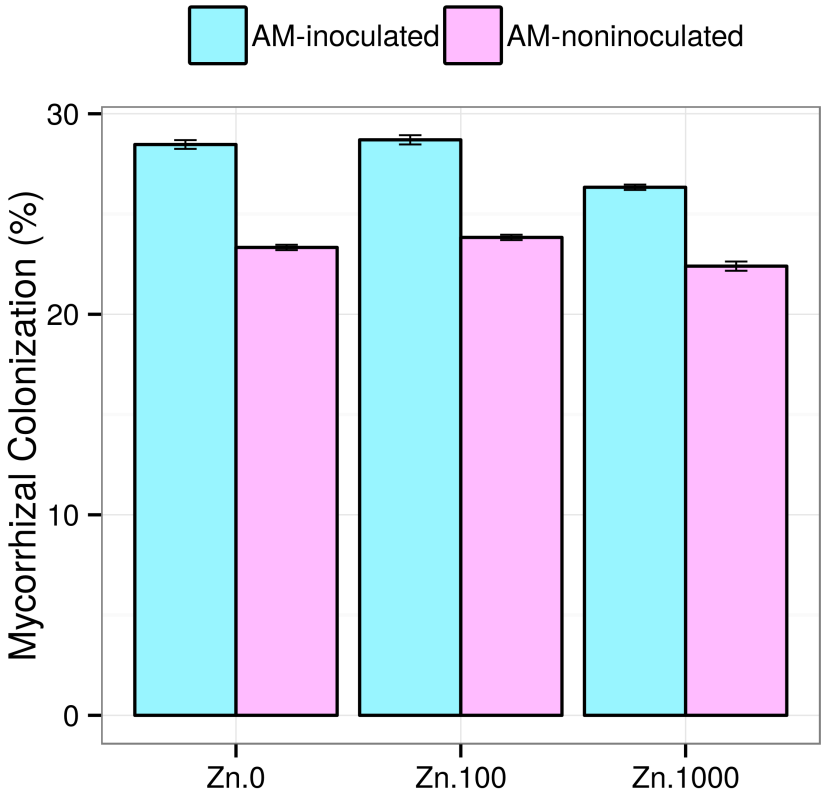

Figure 5.1: Arbuscular mycorrhizal (AM) colonization at harvest of herbaceous specie (*M. sacchariflorus*) plants grown in various doses of Zn with inoculation of AMF (*Gigaspora margarita*) and natural soil condition. Values represent means of four replicates, and 10 root segments per replicate were selected for observation. Error bars indicate ± standard error (SE) of mean.

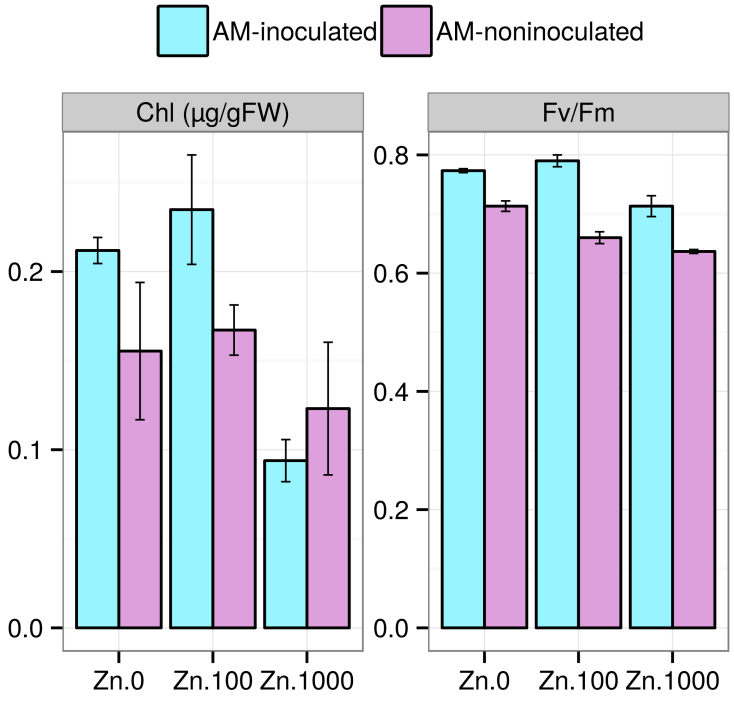

Figure 5.2: Chlorophyll content and chlorophyll fluorescence in leaves from different treatments where various doses of Zn was added with AMF (*Gigaspora margarita*) and natural soil. Error bars indicate ± SE of mean.

medium dose of zinc (Zn100 treatment) whereas high Zn treatment (Zn1000) reduced chlorophyll concentration of leaves compare to non-inoculated plant. Among different levels of Zn application, the Fv/Fm ratio decreased significantly in non-inoculated plants whereas inoculation of AMF increased Fv/Fm ratio (Figure 5.2 & Table 7.1).

5.3.3 IAA concentration and IAA catabolism

The major growth hormone IAA concentration was significantly decreased in plant's roots, and leaves exposed to high zinc application. Inoculation of AMF increased the IAA concentration in plants leaves and roots with medium dose of zinc which is statistically similar to control (Figure 5.3 & Table 7.1). On the other hand, IAAO activity was significantly ($P<0.05$) elevated in plants roots under AMF inoculated conditions (Figure 5.3 & Table 7.1).

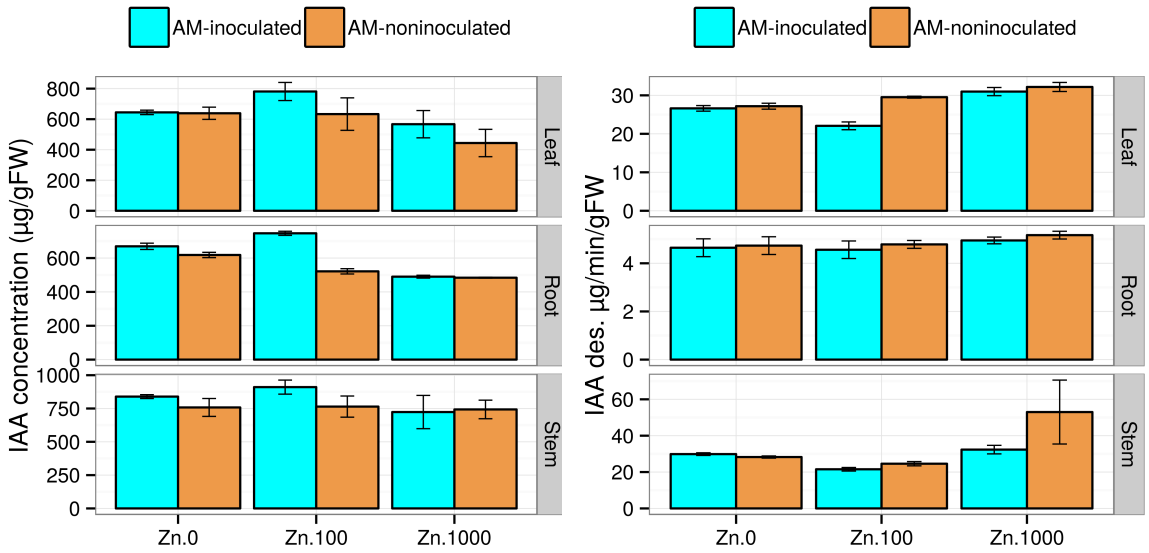

Figure 5.3: IAA concentration (left) and IAAO activity (right) of *M. sacchariflorus* grown under different doses of Zn with AMF (*Gigaspora margarita*) and natural soil. Error bars indicate ± SE of mean.

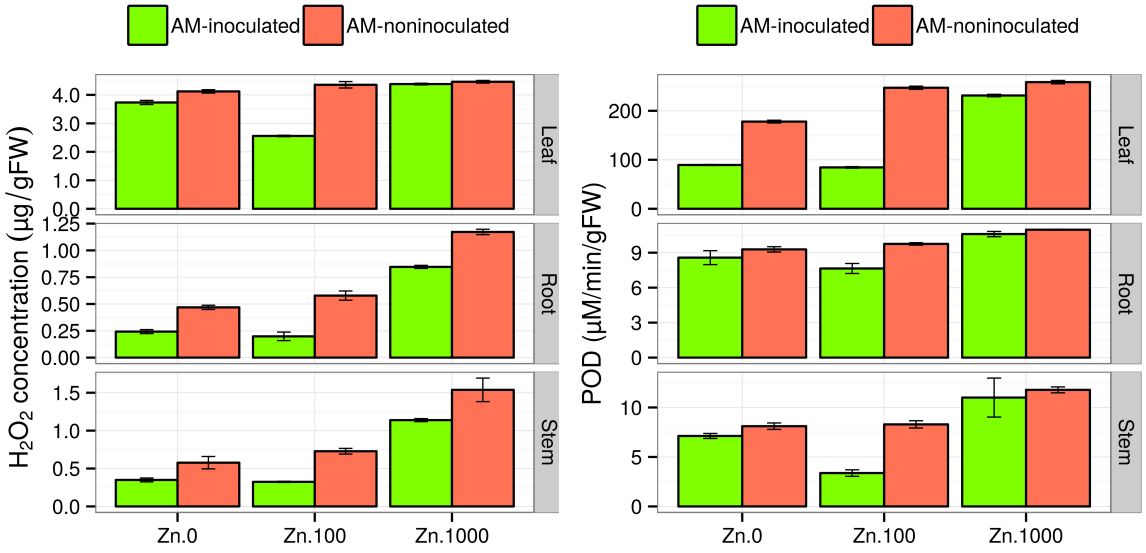

Figure 5.4: H_2O_2 concentration (left) and POD activity (right) of *M. sacchariflorus* grown under different doses of Zn with AMF (*Gigaspora margarita*) and natural soil. Error bars indicate ± SE of mean.

5.3.4 Reactive oxygen species (ROS) production and peroxidase (POD) activity

A significantly higher H_2O_2 concentration ($P<0.05$)(Table 7.1) was found in roots, stems and leaves of plants under high level Zn application which was reduced with inoculation of AMF.The activity of POD also showed similar trend with exposure of Zn and inoculation of AMF (Figure 5.4).

5.3.5 Above and below ground dry mass (DM) production

AMF showed a significant effect on dry mass production of the *M. sacchariflorus* plants with exposure of Zn doses.Above ground and below ground dry mass significantly increased with the inoculation of AMF in control (Zn0) and medium dose of zinc (Zn100) treatment though there was no statistically significant difference in combined effect (Figure 5.5 & Table 7.1).

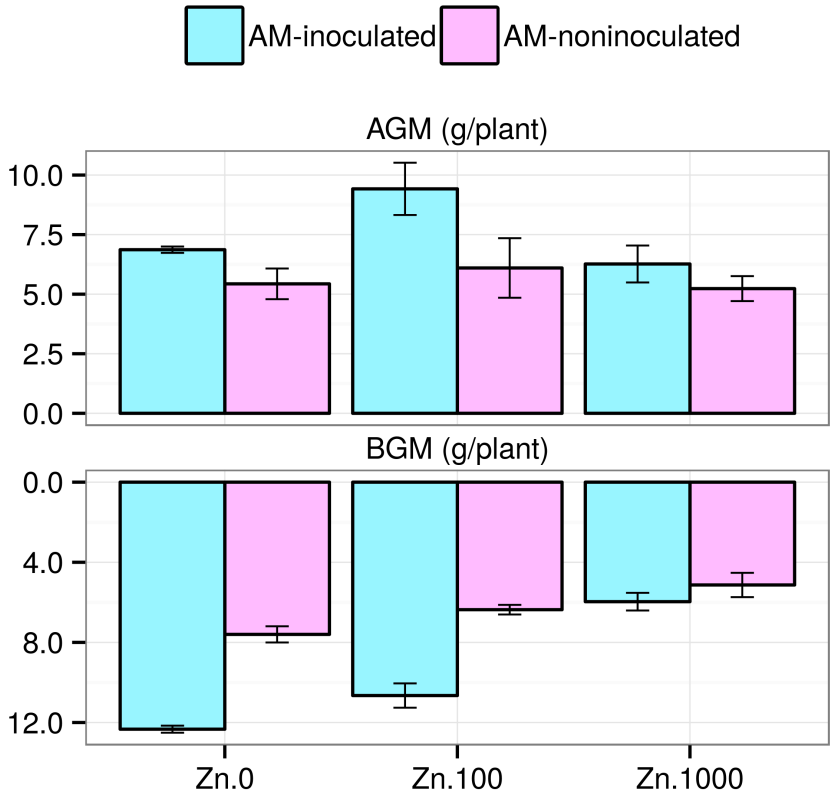

Figure 5.5: Above and below ground dry mass production of *M. sacchariflorus* where various doses of Zn was added with AMF (*Gigaspora margarita*) and natural soil. Values represent the total dry mass ± SE of mean.

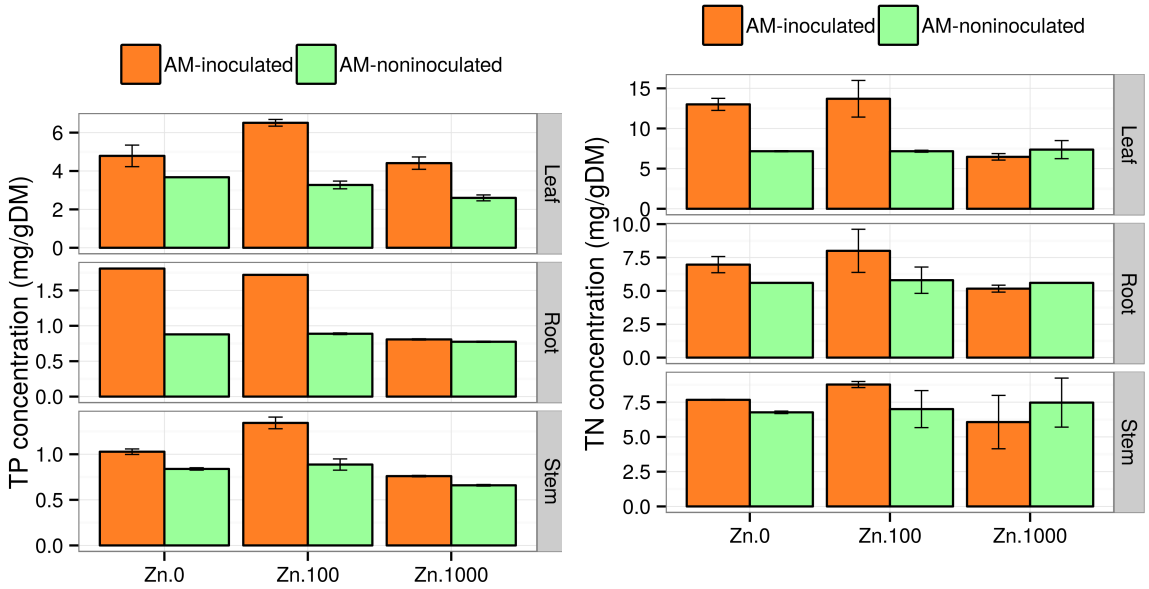

Figure 5.6: TP (mg g^{-1} DM) (left) and TN (mg g^{-1} DM) (right) concentration of*M. saccharifiorus* in different treatments where various doses of Zn was added with AMF (*Gigaspora margarita*) and natural soil. Error bars indicate ± SE of mean.

5.3.6 Total phosphorus (TP) and total nitrogen (TN) concentration in plants

Inoculation by AM fungi showed a positive effect on P concentration of plants whereas exposure of Zn decreased P concentration in plants (Table 7.1) . Plant P concentration was higher when medium dose of zinc (Zn100) was added with AMF though it showed decreasing trend in high dose of zinc (Zn1000) treatment (Figure 5.6). P concentration in plants (roots, stems, and leaves) was the highest in AM fungi inoculated with medium dose of zinc (Zn100) treatment; whereas it was the lowest in the of zinc (Zn1000) treatment of non-inoculated plant (absence of AM fungi) (Figure 5.6). TN concentration in plants varied with the AM fungi application as well. Nitrogen concentrations in the roots, stems, and leaves were maximum in the medium dose of zinc (Zn100) with AMF treatment though it was statistically similar with zinc control (Zn0 treatment)(Table 7.1). Exposure of Zn did not have significant effect on N concentration in plants under non-inoculated treatments (Figure 5.6).

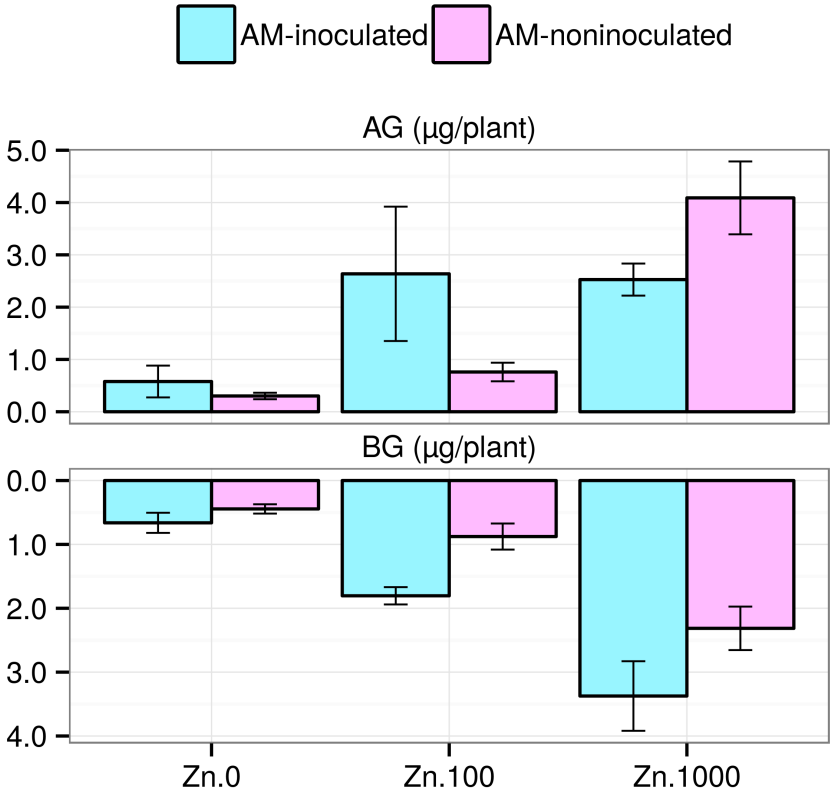

Figure 5.7: Zn content of plants in different treatments where various doses of Zn was added with AMF (*Gigaspora margarita*) and natural soil. Error bars indicate ± SE of mean.

5.3.7 Zinc content in plants

Zn content in plants (above and below ground) was increased with the application of higher doses of zinc (Table 7.1). Inoculation of AMF with different doses of Zn also showed remarkable effects. Zinc content was higher both in above and below ground dry mass than non-inoculated plant under medium dose of zinc (Zn100) treatment whereas below ground zinc content increased but above ground zinc content decreased compared to non-inoculated plant in high dose of zinc (Zn1000) treatment (Figure 5.7).

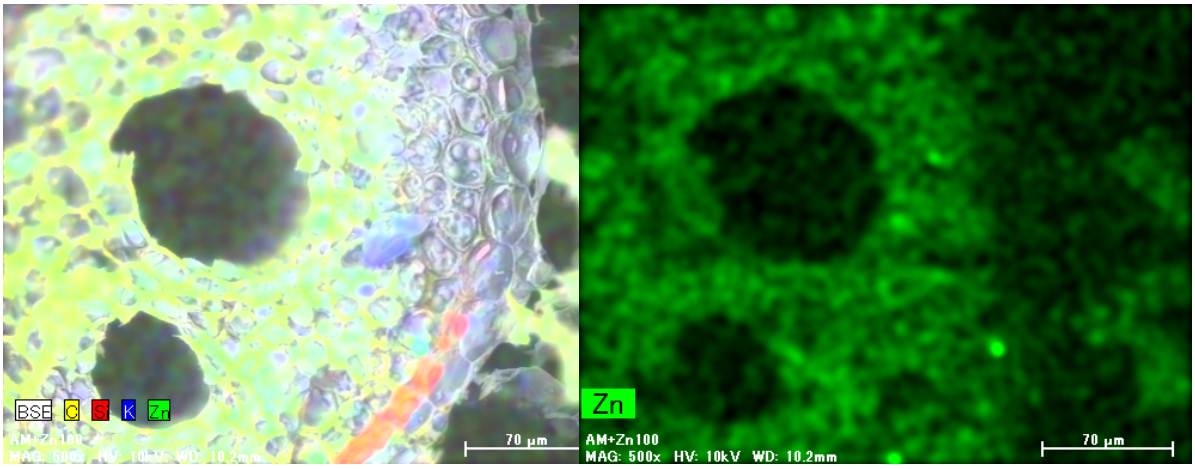

Figure 5.8: Scanning electron microscopic micrograph (back scattered electron image) of cross-sections of *M. sacchariflorus* root cells under exposure of Zn with AMF. Left side figure represent main elements mapping (C, Si, K, Zn) where measurement time was 600sec.) and the right side figure represent the evidence of Zn present in parenchyma cell.

5.3.8 Zinc crystal in mycorrhizal infected root

Scanning electron micrograph (SEM) observations revealed that Zn crystal was present in parenchyma cells of root of AMF plants when grown in higher dose of Zn. However, we did not observe any crystal formation in non-mycorrhizal plant (Figure **??**).

5.4 Discussion

5.4.1 Root colonization, growth, hormone and enzyme activity of plants

Performance of floodplain *M. sacchariflorus* to Zn contamination by the AM fungal strain were evaluated in the present study. Even when the Zn addition level was as high as 1000 $mgkg^{-1}$, the mycorrhizal infection rate slightly decrease compared to the control receiving no Zn though it was not statistically remarkable. This may imply that Zn has no or little effect on spore germination and AM colonization (Chen et al., 2003; Ortas et al., 2002). Under low or moderate Zn application levels, plant growth showed no decrease as Zn addition increased. A slight growth inhibition was observed only at the very high Zn levels above 1000

mg kg^{-1}. Mycorrhizal infection consistently improved plant yield. This can easily be explained by enhanced nutrients uptake via the extrametrical mycelium (Sarkar et al., 2015a,b,c). The mycorrhizal plants showed a more than two fold increase in P uptake compared with corresponding non-mycorrhizal plants(Sarkar et al., 2015b) though AMF did not have any significant effect on phosphate solubility like other bacteria or fungi suggested by Sarkar et al. (2012).The improved P nutrition of host plants might be one of the major mechanisms involved in the alleviation of metal toxicity as a result of mycorrhizal colonization. Inoculation of AMF also increased the IAA concentration in plants under low or moderate level of Zn though it was not statistically significant. The high concentration of cellular H_2O_2 and the elevated POD activity in our experimental plants suggest that the ROS scavenging system was activated there under the Zn stress conditions, and this result is in agreement with other works (Chaoui et al., 1997; Prasad et al., 1999; Rao and Sresty, 2000) which was reduced by AMF application. Previous research results also suggest that AM are able to enhance production of antioxidant enzymes, which can alleviate the stress of heavy metals (Avery, 2001; Ruiz-Lozano, 2003) and our result is the reprint of previous results.

The alleviating potential of AM on heavy metal stress is determined by different factors: type and concentration of heavy metal, plant specification and growth conditions (Hildebrandt et al., 2007). Molecular analyses have indicated mechanisms involved in heavy metal tolerance of AM. Root AM colonization of plants under heavy metal stress results in expression of specific genes responsible for production of proteins (including metallothioneins) that increase the tolerance of plants to stress (Rivera-Becerril et al., 2005). Metallothioneins are metal-binding proteins produced in many different organisms when exposed to high concentrations of heavy metals such as copper (Cu), Zn and Cd. There are many AM and plant genes involved in this tolerance to heavy metal stress, including metal transporter genes, which are expressed at different levels, and AM symbiosis can regulate the transcription of such genes (Hildebrandt et al., 2007; Lanfranco et al., 2002; Gonzalez-Guerrero et al., 2005).

5.4.2 Nutrients and Zinc uptake

Inoculation of AMF increased the TP concentration in plants. Some previous research results prove that plants produce organic root exudates such as malic and citric acids and or /acid phosphatase when P is deficient, resulting in enhancement of nutrient uptake (Khan et al., 2000). In addition, the interactive effects of plant roots and microbial populations in the rhizosphere increase root exudation of organic products and hence activity of soil microorganisms, and eventual plant nutrients uptake including P, NH_4^+, NO_3^-, K, Mg, Fe, Cu and Zn (Khan et al., 2000; Cavagnaro et al., 2006; Sarkar et al., 2015a,b,c). There are many

microorganisms in the soil that can enhance the solubility of different P sources (such as rock phosphate) by producing organic acids, including AM, *Aspergillus* sp., *Bacillus* sp., *Enterobacter* sp., *Pseudomonas* sp.(Sarkar et al., 2012) though *Gigaspora margarita* Becker & Hall spores did not have any role for enhancing the solubility of P sources(Sarkar et al., 2015b). TP and TN concentration in plants (roots, stems, and leaves) was the highest in AM fungi inoculated under moderate dose of zinc and lowest in high dose of zinc. The possible reason for inhabitation of P uptake by the additional Zn is mutually antagonistic relationship(Shetty et al., 1995). The mycorrhizal hyphae in the outer compartments were able to grow well even at the added Zn level of 200 mg kg^{-1}, and furthermore they were active and efficient at absorbing P from the hyphal zone and transferring it to the plant, as evidenced by the comparatively high P acquisition by the mycorrhizal plants compared with non-mycorrhizal controls receiving 300 $mgkg^{-1}$ of additional P (Chen et al., 2003). Similar results were also obtained by Li and Christie (2001), and lend further evidence that uninhibited fungal growth under elevated Zn additions is consistent with a high mycorrhizal infection rate.

Enhanced uptake of Zn at low Zn application levels was found in the present study, and the protective effects of AM fungi against Zn toxicity of *M. sacchariflorus* plants under conditions of Zn contamination were also observed.Zinc mobility is greatly affected by soil pH. It is therefore quite possible that the immobilization of Zn by fungal activity is partly due to changes in soil pH and this could contribute to the inhibition of Zn uptake by mycorrhizal plants under conditions of high Zn contamination(Christie et al., 2004). Although plant Zn concentration increased with increasing Zn levels irrespective of inoculation, much Zn was retained in the mycorrhizal roots (Figure **??**) and translocation to the shoots was inhibited. In contrast, non-mycorrhizal plants experienced higher Zn concentrations in their shoots. In the present study, the absence of an effect on the Zn concentration of non-mycorrhizal plants and gradually increasing Zn concentrations of mycorrhizal plants with increasing Zn application level support the hypothesis that Zn is taken up and transferred to the host plant via the extraradical hyphae. Khan et al. (2000) observed similar results for Zn, and stated that Zn absorbed by AM hyphae is crystallised in these hyphae and cortical cells of mycorrhizal roots. The large specific surface area of AM hyphae allows the fungus to absorb high levels of nutrients, even beyond the growing zone of the plant roots. This process is called phytostabilisation, by which AM increase plant ability to immobilise heavy metals in the soil through absorbing such metals in their hyphae and consequently decreasing translocation from plant roots to shoots (Chen et al., 2003).When approaching the inner part of the root, heavy metals are located in parenchyma cells (Figure **??**), and for most AM structures, in hyphae, arbuscules and vesicles. While the fungal cytoplasm remains free of Zn, Cu or Cd accumulation,

the cell wall and electron-dense granules contain high amounts of these elements (Gonzalez-Guerrero et al., 2008, 2005). Accordingly, it can be stated that AM are able to keep heavy metals out of plants or reduce concentrations in aerial parts of plants (Hildebrandt et al., 2007).Though there are some research related to Zn levels and AMF inoculation, to our knowledge, this is the first report to evaluate the effects of *Gigaspora margarita* on specific host plant (*M. sacchariflorus*) under soil Zn regime and Zn crystal evidence showing in parenchyma cell of mycorrhizal infected plant' s root.

5.5 Conclusion

Exposure of Zn did not have remarkable effect on root colonization of *M. sacchariflorus* plant though Zn uptake of plant increased with increased doses of Zn. AMF (*Gigaspora margarita*) inoculation showed significant effect for stress minimization, growth and growth enhancing parameters and finally Zn uptake in the present study. The rate of uptake is very much dependent on AM species, soil condition and plant genotype. Considering that the AM fungus is a non-host specific symbiont, and that the host plant always plays the dominant role in the symbiosis, much attention also should be paid to host plants. More systematic research on the mechanisms involved in arbuscular mycorrhizal metal absorption and transportation processes in plants is necessary for the application of this symbiosis in future.

Chapter 6

Arbuscular mycorrhiza confers Pb tolerance and uptake in *Miscanthus sacchariflorus*

Abstract

Lead (Pb) is a heavy metal that is present in the soil in very small amounts, but anthropogenic activities have increased its content in some locations, which can make these areas unproductive or inappropriate for crop production. Under heavy metal stress, the unfavorable oxidative effects adversely influence plant growth. However, AM are able to enhance production of antioxidant enzymes, which can alleviate the stress of heavy metals and finally plant growth. In the present experiment, we studied the effects of arbuscular mycorrhizal fungal(AMF) association on growth, survival capabilities, nutrients and Pb uptake of *Miscanthus sacchariflorus* under different Pb concentrations in soil.The experiment was conducted in the form of pot cultures of *M. sacchariflorus*. The pots were laid out in complete randomized design (CRD) with three replicates per treatment. The treatments were composed of the inoculation or no inoculation of the AM fungus, *Gigaspora margarita*, and the addition of three Pb concentrations in the soil (0, 100 and 1,000 mg kg^{-1}). Addition of Pb significantly decreased mycorrhizal colonization. Inoculation of AMF with Pb increased chlorophyll content, Fv/Fm, total dry mass, IAA, TN, and TP whereas H2O2 level, IAAO activity, POD activity was low compare to non-inoculated treatments. Moreover, application of AMF with Pb doses

induces concentrations of Pb in the plant where at higher dose Pb (1000mg $^{-1}$) induces lower content of Pb in the aerial part of the plant but higher content in root. AMF enhanced the tolerance of *M. sacchariflorus* against Pb toxic condition and accumulate Pb in plant root whereas translocation to the shoots was inhibited in higher dose Pb (1000mg kg^{-1}).

6.1 Introduction

Soil contamination by heavy metals (HMs) has increased because of the increased intensity of anthropic activities, mainly due of mining, industrial activities, utilisation of sewage sludge and the application of fertilisers or pesticides in agricultural environments (Nriagu et al., 1988). Lead (Pb) is a toxic HM with low solubility in soils and occurs in the soil solution predominantly as Pb^{2+} and, more rarely, Pb^{4+}. This element is potentially toxic, and it is known that Pb can be taken up from the soil by plants and may be transferred up in the food chain. Plants that are not able to tolerate high concentrations of HMs in the soil can become tolerant or increase their performance when associated with arbuscular mycorrhizal (AM) fungi (de Souza et al., 2012). Under heavy metal stress, the unfavorable oxidative effects adversely influence plant growth. However, AM are able to enhance production of antioxidant enzymes, which can alleviate the stress of heavy metals (Avery, 2001; Ruiz-Lozano, 2003).Lead has not been shown to be essential in plant metabolism and soil contamination with this metal can cause a diversity of damages for the plant, including loss of vegetation cover (Watanabe, 1997). Evidences that several plant species can absorb and accumulate Pb from soil have been found (Kabata-Pendias, 2004). The concentrations of 100–500 mg kg^{-1} of Pb in soils are considered to be toxic for most plants (Kabata-Pendias, 2004; Ross, 1994). However, plant sensitivity to Pb varies according to the different plant species; some of them can accumulate high concentrations of heavy metals and can be used in experimental assays for phytoremediation of contaminated soils (Huang and Cunningham, 1996; McGrath et al., 2002). *Miscanthus sacchariflorus* is a grass species with a wide plasticity to grow in polluted or marginal soils and is able to accumulate much higher contents of the heavy metals (Kalembasa and Malinowska, 2009).

Soil microorganisms play an important role in plant health, nutrient uptake and resistance against heavy metals influence (Andrade et al., 2004; Chen et al., 2005; de Souza et al., 2012). AM fungi not only provide nutrient to the plant but also play an important role in plant tolerance to heavy metals (Khan et al., 2000; Lin et al., 2007). However, AM fungi increase tolerance of plants to Pb but the role of these fungi on Pb resistance and plant Pb uptake is not clear (Gaur and Adholeya, 2004). Most of the previous researches have been carried out related

with trees, ectomycorrhizal fungi and heavy metals but a few with AM fungi and grass like *Miscanthus sacchariflorus*. In the present study, we report results of an experiment in which we assessed the effects of Pb addition on the mycorrhizal colonization, growth, nutrition, Pb uptake, and related biochemical parameters of the *M. sacchariflorus* plant responding to these conditions.

6.2 Materials and methods

6.2.1 Collection of soil and plant propagate from study sites

Soils collected from different transects (five meter×four meter) on the banks of the Ara River, Saitama, Japan (36°4′58.02″ N and 139°26′28.85″ E) were composited to make a homogenous soil pool (Figure 6.4). The properties of the soils collected from the transects were homogenous, and there were no significant differences between the individual soil samples from the transects (P>0.5) and the composite soil in terms of soil particle size (D25), pH, percentage organic matter content, total carbon (TC), total nitrogen (TN), total phosphorus (TP), and available phosphorus concentrations and water holding capacity (all $p > 0.05$). The soil utilized for the experiment had an average pH of 5.8, with an average of 0.61 mm of D25, organic matter content 0.25%, TP 0.45 mg/g, available phosphorus 0.02 mg/g, TN 0.9 mg/g, water holding capacity 27%, potassium (K) 1.7 mg/g, calcium (Ca) 0.4 mg/g, magnesium (Mg) 1.1 mg/g, iron (Fe) 0.55 mg/kg, copper (Cu) 0.05 mg/kg, manganese (Mn) 0.40 mg/kg, zinc (Zn) 0.27 mg/kg and lead (Pb) 0.12 mg/kg. Propagates (rhizomes) of *Miscanthus sacchariflorus* for planting in pots were collected from the same sites.

Table 6.1: Summary of AM fungi-Zn interaction and application for growth response

Soil type	**Host plant**	**Zn Conc.**[1]	**M. Fungi**[2]	**Reference**
Contaminated soil	Colver, sunflower, mustard and phacelia	1096	*Glomus intraradices*	Neagoe et al. (2013)
Sewage sludge	*Allium porrum*, *Sorghum bicolor*	294.6	*Glomus claroideum*, *G. mosseae*, *Glomus* sp.	del Val et al. (1999)
Non-contaminated soil	*L. spartum*, *A. cytisoides*	0∼1000	*Glomus mosseae, G. marocarpum*	Diaz et al. (1996)
Heavy-metal polluted soil	leek, maize	1220	*Glomus mosseae*	Weissenhorn et al. (1993, 1995b)
Sewage-sludge amended soil	Maize	199∼1074	NA	Weissenhorn et al. (1995a)
Heavy metal polluted soil	*Thlaspi praecox*	280	NA	Vogel-Mikus et al. (2006)
Calcareous soil	Red clover	0∼1200	*Glomus mosseae*	Chen et al. (2003)
Uncontaminated & contaminated soil	*Cynodon dactylon*	0.08, 438	*Acaulospora* spp., *Glomus* spp.	Wu et al. (2010)
Field soil	Tomato	0.65∼75	NA	Cavagnaro et al. (2010)
Sandy soil	Maize	0∼900	*Glomus mosseae* BEG167	Shen et al. (2006)
Loamy soil	White clover	30∼270	*Glomus* spp.	Vivas et al. (2006)
Sandy clay loam	Red clover	0∼1000	*Glomus mosseae*	Li and Christie (2001)
Sandy clay loam	White clover	0∼400	NA	Zhu et al. (2001)
Farm soil	Rice	5	*Glomus etunicatum*	Purakayastha and Chhonkar (2001)
Raman type brown forest soil	Maize	250	*Glomus intraradices*	Seres et al. (2006)
Soil from industrial site	Sorghum	20μ Zn	*Glomus intraradices*, *G. spurcum*	Toler et al. (2005)
Contaminated agricultural soil	Barrel medics	627	*Glomus intraradices*, *G. intraradices* BEG 141, *G. mosseae* BEG 69, *G. mosseae* BEG 12, *Glomus* spp.	Redon et al. (2009)
Contaminated silt loam soil	*Andropogon gerardii*	0∼1000	*Glomus constrictum*, *Scutellospora pellucida*, *G. ambisporum*, *G. ambisporum*, *S. pellucida*, *G. constriticum*, *G. mosseae*, *G. constrictum*, *S. calospora*	Shetty et al. (1995)

[1] The unit of Zn concentration is mg Kg^{-1} soil, if not specified in the column. The soil is either contaminated or Zn is supplied as experimental treatment.

[2] The mycorrhizal fungi species are either naturally found or inoculated.

[NA] Data not available

6.2.2 Experimental set-up

The experiment was conducted in the form of pot cultures of *M. sacchariflorus* in a greenhouse at Saitama University, Japan. The pots were laid out in complete randomized design (CRD) with four replicates per treatment. The treatments were composed of the inoculation or no inoculation of the AM fungus, *Gigaspora margarita*,and the addition of three Zn concentrations in the soil (0, 100 and 1,000 mg kg-1). Zn was added as an aqueous solution of $ZnSO_4.7H_2O$. The Zn addition treatments were selected on the basis of the reported existence of soil zinc (Table 6.1).

In each pot (diameter 20.96 cm, vol. 2.7 L), 2 kg (natural) of soil was used. Initial moisture content and bulk density of the soil were estimated. To inoculate soils of respective treatments, AM fungi spores were applied in the form of a commercial inoculant namely 'Serakinkon' powder (The Central Glass Company, Tokyo, Japan). The inoculant was composed of 50 *Gigaspora margarita* Becker & Hall spores per gram powder (Higo et al., 2010; Kaneko and Tanimoto, 2009; Tawaraya et al., 1996) as *Gigaspora* was the most commonly isolated AMF on river bank sand with higher spore densities than those of other species; previous studies revealed the same result (Koske and Gemma, 1997; Kowalchuk et al., 2002; Rose, 1988; Sarkar et al., 2015a,b,c). 20 g serakinkon powder/kg soil was evenly mixed with soil before being used in a pot. Rhizomes with three nodes (∼2 cm) were employed as propagating materials. A temperature of 25 ± 3° C and 75% relative humidity were maintained (day and night) in the greenhouse throughout the experimental period. Pots were watered as necessary. The greenhouse culture lasted for 110 days.

6.2.3 Chlorophyll content and chlorophyll fluorescence

At harvest, chlorophyll was extracted from the leaves of *M. sacchariflorus* using N,N dimethylformamide (DMF). The leaves were cut, and fresh weight (∼5 mg) was measured. Pigments were then extracted into 5 ml DMF by incubating leaf cuttings 24 h in a refrigerator at 4° C. The chlorophyll and carotenoid concentration was determined via the method described by (Porra et al., 1989). Absorbance at 646.8 nm and 663.8 nm was measured with a UV-mini 1200-spectrophotometer (Shimadzu, Japan).Chlorophyll concentrations were calculated as μg/mg fresh weight (FW) basis. The plants were incubated in dark for 15 min before the measurements were taken in order to allow complete oxidation of the photosystem II (PSII) reaction centers. Maximum photochemical efficiency of PSII (Fv/Fm) was determined.

6.2.4 Hormone and enzyme analysis

IAA concentration in the tissues was measured using Salowski reagent (Gordon and Weber, 1951). For the analysis of endogenous H_2O_2 concentration, samples were extracted with cold acetone; and the method was followed as described by Cervilla et al. (2007) and Zaman and Asaeda (2013). Phosphate buffer (0.1 mol/L) at pH 6 was used to make extracts suitable for the measurements of POD and IAAO activities. IAA destruction was measured to determine IAAO activity (Zhang et al., 2009). POD was determined according to Goel et al. (2003) and the absorbance differences at 420 nm were plotted in every 30 s for 3 min (Chanjirakul et al., 2006).

6.2.5 Harvesting and processing

Plants were harvested at 110 days after transplanting at their senescence stage. Soil particles were carefully removed from plant roots using forceps. The whole stems, leaves, and roots were then divided into two parts: one part for AM colonization (roots) observation, hormone and enzyme analysis whereas the other part for estimating dry mass and analyzing nutrients. Stems, leaves, and roots (fraction) were cut into small pieces and dried to a constant weight at 80° C for 3 days, and final weights (dry mass) were recorded. Since not the whole roots, stems and leaves were dried, the fraction of oven-dried roots, stems and leaves were taken into consideration for estimating belowground (roots) and aboveground (including stem and leaf) dry mass. Ground plant tissues were kept in air-tight containers for further analysis.

6.2.6 AM colonization determination and nutrient analyses from soil and plant samples

To estimate the extent of AM colonization, roots were cleaned, cut into small segments (2 cm/segment), rinsed and cleared for at least 12 h in 10% KOH solution at room temperature, and then stained with 0.05% trypan blue solution (Koske and Gemma, 1989). Ten randomly selected root fragments from each treatment were mounted on slides, and intersecting vertical gridlines were observed at 100× magnification to determine the presence and absence of AM colonization (internal hyphae, vesicles, or arbuscules) (McGonigle et al., 1990). Total nitrogen (TN) concentrations plant tissues was determined with a CHN analyzer (CHN Corder MT-5, Yanoco, and Kyoto, Japan). Total phosphorus in plant samples was determined by perchloric acid digestion. The concentrations of Zn in plants were analyzed after oven drying to a constant weight at 72° C followed by digestion in a perchloric acid mixture, as described by (Tandon, 1993). Nutrient concentrations

in the tissue digest were quantified using an atomic absorption spectrophotometer (AA-6300; Shimadzu, Japan) using the method described in standard methods for the examination of water and wastewater by Gilcreas (1966).

6.2.7 Statistical analyses

Prior to commencing statistical analyses, all data were checked for normality, and equal variance was checked using the Levene' s test. All data are presented as mean ± SE (n = 3). The effects of mycorrhizal treatment on chlorophyll concentration in the leaves, fluorescence, root colonization, dry weight, nutrients (N, P) and Zn concentration of each plant component, were analyzed by two-way ANOVA followed by Tukey' s posthoc test at 0.05 significant levels. Correlations between parameters were judged by Pearson' s correlation coefficient . All statistical analyses were performed using SPSS for Windows (Version 13.0, SPSS, Inc., Chicago, IL, USA) and R package (Team, 2010).

6.3 Results

6.3.1 Root colonization

Microscopic observations revealed that almost all root samples of *M. sacchariflorus* in AM treatments were colonized by AM fungi both in treated and non-treated soil with Pb. Colonization level of roots was significantly decreased with the increase of Pb concentration in soil (P=0.00) (Table 2).The average colonization level of *M. sacchariflorus* was 18–28% in different treatments (Figure 6.1).

6.3.2 Chlorophyll content and fluorescence in leaves

Plants inoculated with*Gigaspora margarita* had higher leaf chlorophyll concentrations and Fv/Fm ratio than non-inoculated plants though it was not statistically significant (Table 2). At different levels of Pb application, chlorophyll concentrations decreased significantly (P=0.001) (Table 2) with increase of Pb concentration whereas Fv/Fm ratio also decreased but not significant (Figure 6.2).

6.3.3 IAA concentration and IAA catabolism

The major growth hormone IAA concentration was significantly decreased in plant' s roots, and leaves exposed to lead application. Inoculation of AMF increased the IAA concentration in plants leaves, roots and stems compare to non-inoculated plant (Figure6.3). On the other hand, IAAO activity was significantly (P<0.05)

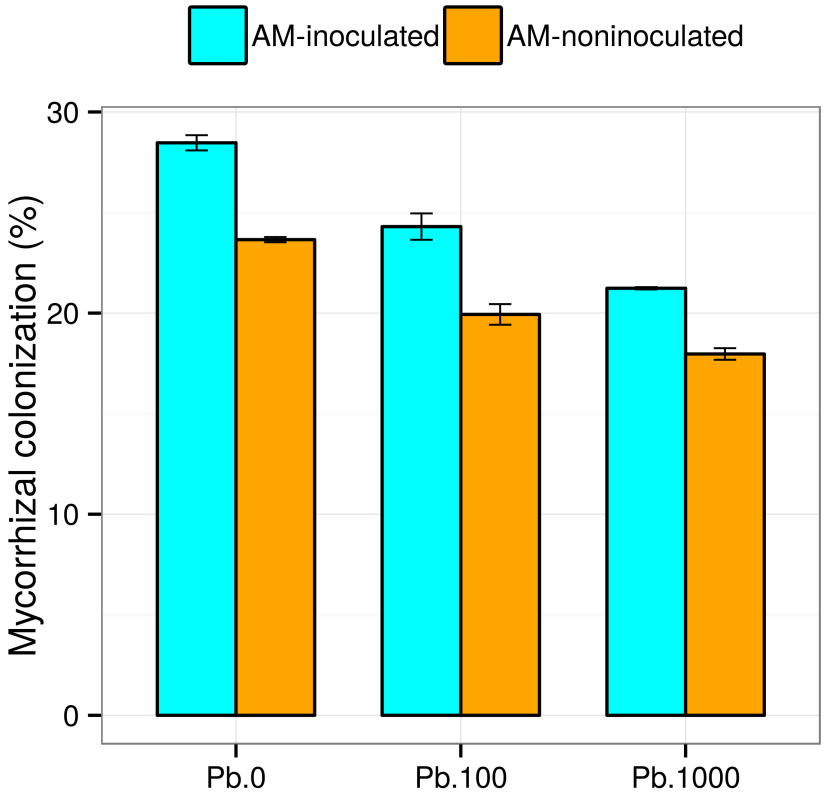

Figure 6.1: Arbuscular mycorrhizal (AM) colonization at harvest of herbaceous specie (*M. sacchariflorus*) plants grown in various doses of Pb with inoculation of AMF (*Gigaspora margarita*) and natural soil condition. Values represent means of three replicates, and 10 root segments per replicate were selected for observation. Error bars indicate ± standard error (SE) of mean.

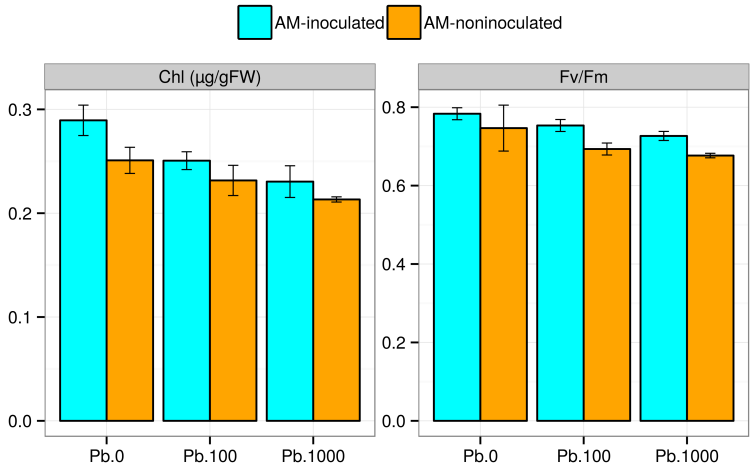

Figure 6.2: Chlorophyll content and chlorophyll fluorescence in leaves from different treatments where various doses of PB was added with AMF (*Gigaspora margarita*) and natural soil. Error bars indicate ± SE of mean.

elevated in plants under AMF inoculated conditions (Figure6.3). Moreover, the interaction effect of AMF and Pb doses were significant in root IAA (P=0.00) (Table 2); stem and root IAAO destruction (P=0.00) (Table 2).

6.3.4 Reactive oxygen species (ROS) production and peroxidase (POD) activity

A significantly higher H_2O_2 concentration (P=0.00) was found in roots, stems and leaves of plants under high level Pb application which was reduced with inoculation of AMF. The activity of POD also showed similar trend with exposure of Pb and inoculation of AMF (Figure 6.4. The interaction effect of AMF and Pb doses were significant in root H_2O_2 and POD (P=0.00) (Table 2).

6.3.5 Above and below ground dry mass (DM) production

AMF showed a significant effect on dry mass production of the *M. sacchariflorus* plants with exposure of Pb doses. Below ground dry mass (BDM) significantly increased with the inoculation of AMF compared to non-inoculated plant (P=0.00) (Table 2) and above ground dry mass (ADM) also increased but not statistically identical (Figure 6.5). Exposure of lead significantly decreased BDM (P=0.00) but ADM was statistically similar (Table 2).

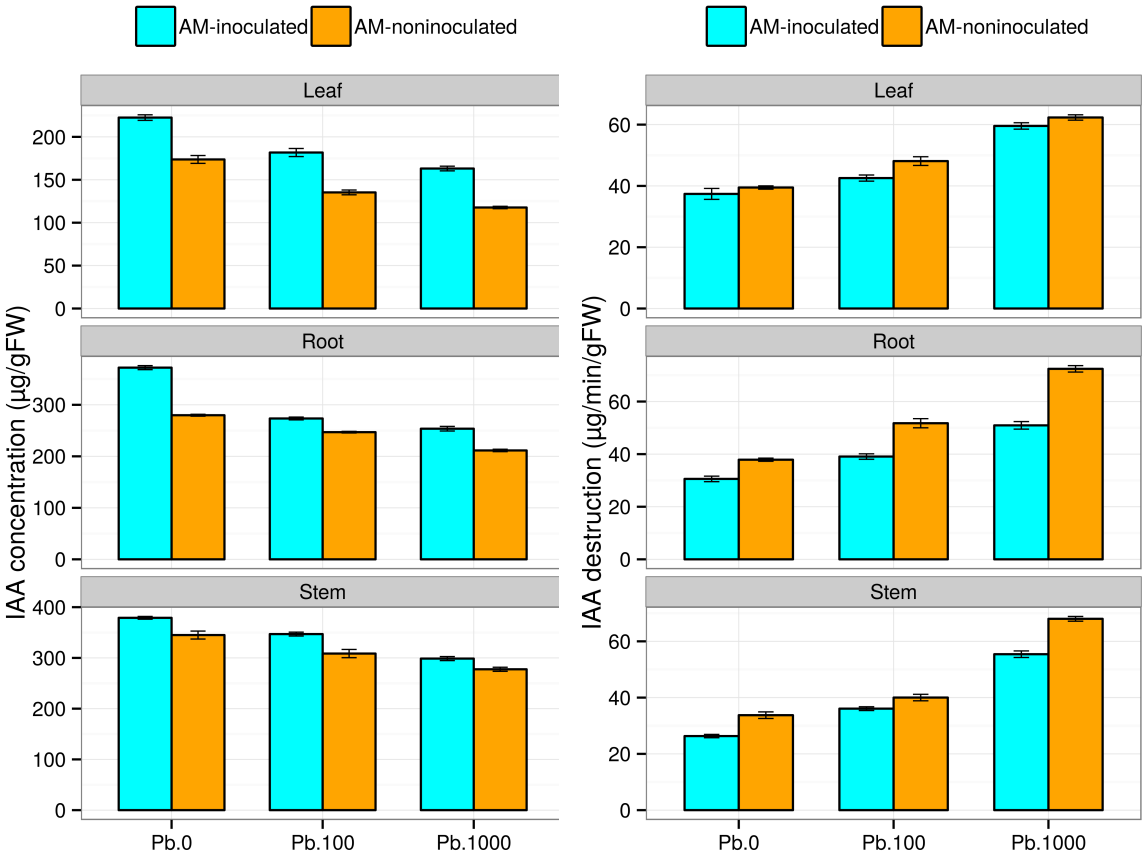

Figure 6.3: IAA concentration (left) and IAAO activity (right) of *M. sacchariflorus* grown under different doses of Pb with AMF (*Gigaspora margarita*) and natural soil. Error bars indicate ± SE of mean.

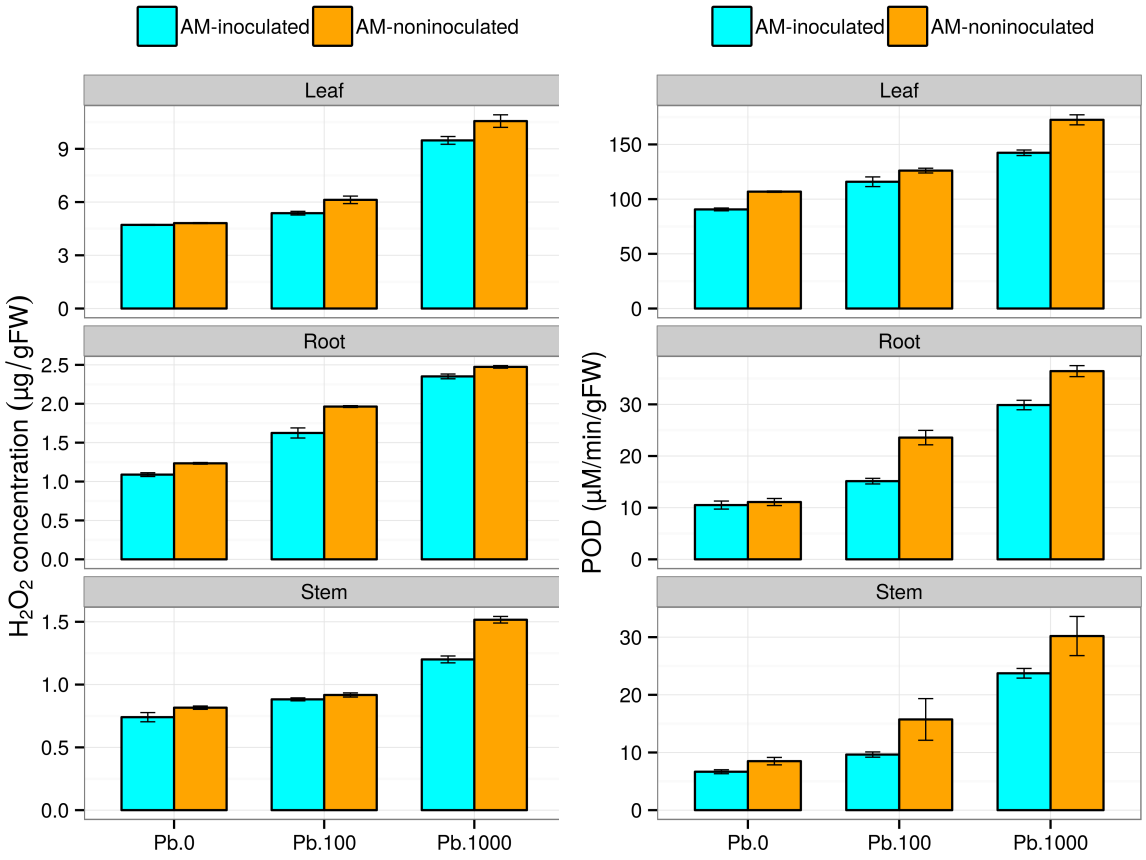

Figure 6.4: H_2O_2 concentration (left) and POD activity (right) of *M. sacchariflorus* grown under different doses of Pb with AMF (*Gigaspora margarita*) and natural soil. Error bars indicate ± SE of mean.

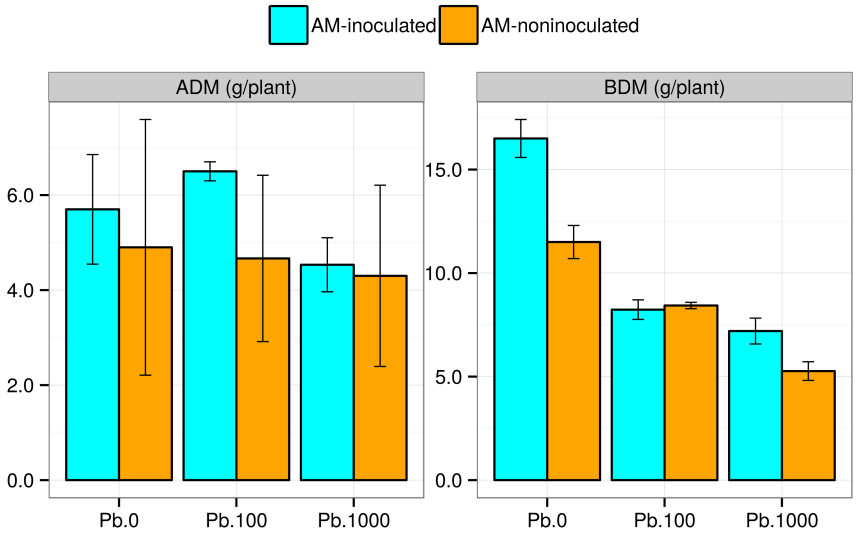

Figure 6.5: Above and below ground dry mass production of *M. sacchariflorus* where various doses of Pb was added with AMF (*Gigaspora margarita*) and natural soil. Values represent the total dry mass ± SE of mean.

6.3.6 Total phosphorus (TP) and total nitrogen (TN) concentration in plants

Inoculation by AM fungi showed a positive effect on P concentration of plants whereas exposure of Pb significantly decreased P concentration in plant (Figure 6.6). P concentration in plants (roots, stems, and leaves) was the highest in AM fungi inoculated with control (no lead exposure) treatment; whereas it was the lowest in the of lead (Pb1000) treatment of non-inoculated plant (absence of AM fungi) (Figure 6.6). The interaction effect of AMF and Pb doses were significant in stem and leaf TP ($P<0.05$) (Table 2).TN concentration in plants varied with the AM fungi application as well. Nitrogen concentrations in the roots, stems, and leaves were maximum in control (Pb0) with AMF treatment. Exposure of Pb did not have significant effect on N concentration in plants (Figure 6.6) (Table 2).

6.3.7 Lead (Pb) content in plants

Lead content in plants (above and below ground) was significantly increased ($P=0.00$) (Table 2) with the application of higher doses of lead. Inoculation of AMF with different doses of Pb also showed remarkable effects. Lead content of inoculated plant was higher both in above and below ground dry mass than non-inoculated

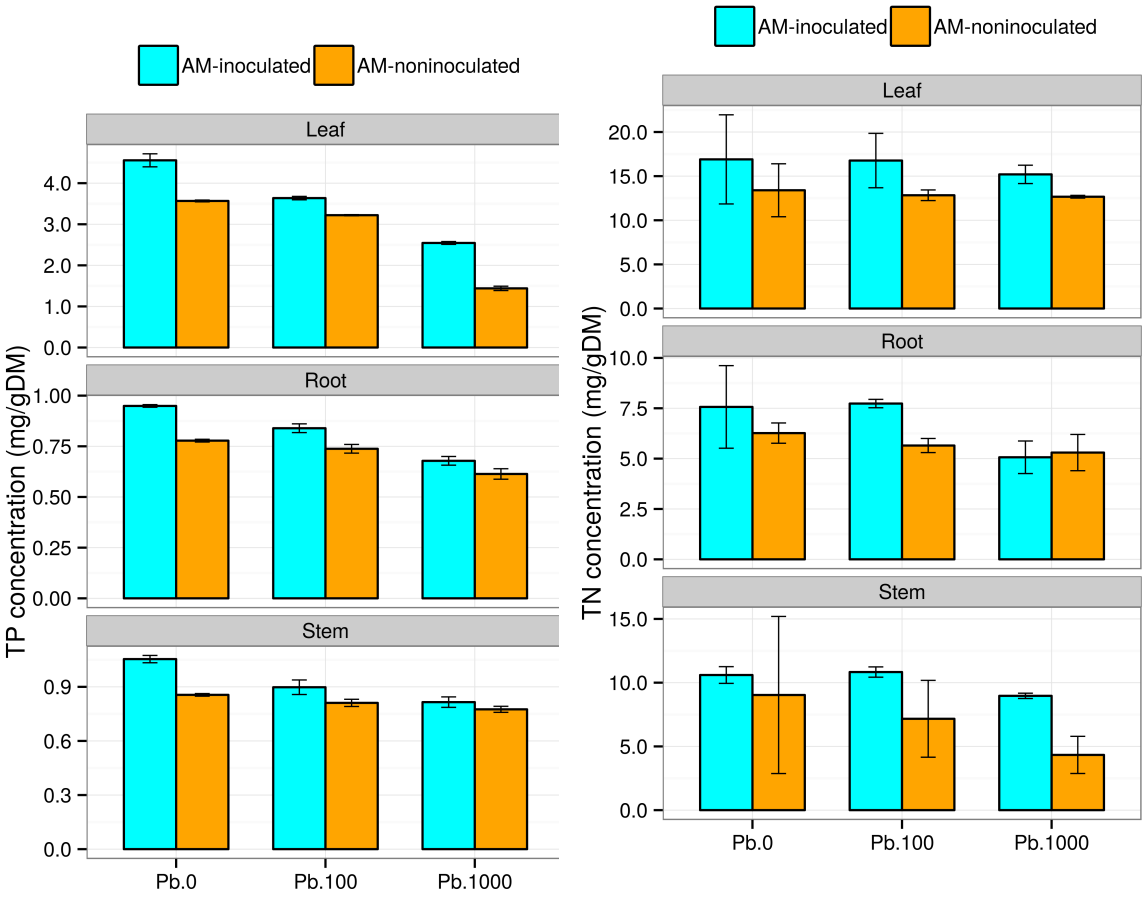

Figure 6.6: TP (mg g^{-1} DM) (left) and TN (mg g^{-1} DM) (right) concentration of *M. sacchariflorus* in different treatments where various doses of Pb was added with AMF and natural soil. Error bars indicate ± SE of mean.

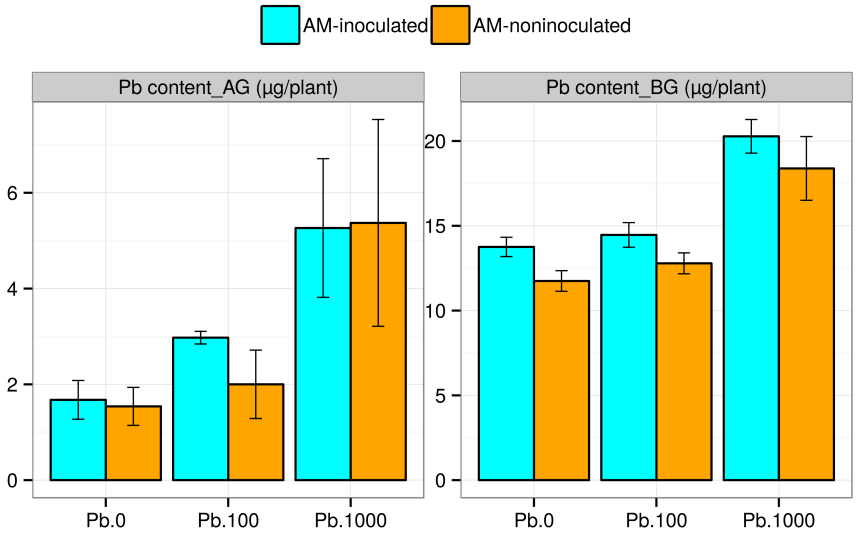

Figure 6.7: Lead (Pb) content of plants in different treatments where various doses of Pb was added with AMF (*Gigaspora margarita*) and natural soil. Error bars indicate ± SE of mean.

plant whereas below ground Pb content increased but above ground Pb content decreased compared to non-inoculated plant in high dose of lead (Pb1000) treatment (Figure 6.7).

6.4 Discussion

6.4.1 Root colonization, growth, hormone and enzyme activity of plants

Performance of floodplain *M. sacchariflorus* to Pb contamination at the presence of AM fungal strain were evaluated in the present study. When the Pb addition level was high, the mycorrhizal infection rate significantly decreased compared to the control receiving no Pb (Andrade et al., 2004; Arriagada et al., 2005). de Souza et al. (2012) also showed that comparing the control with the highest Pb concentration in the soil, the mycorrhizal colonization were reduced by approximately 30% in *Calopogonium mucunoides* though Chen et al. (2005) and Sudova and Vosatka (2007) reported that mycorrhizal colonization rate varied with plant species, mycorrhizal species and the concentration of applied or existing Pb in soil. This result was found as because the population of soil microorganisms and their activities

was reduced due to high concentrations of Pb in soils Baath (1989). In addition, the symbiotic association between plants and beneficial microorganisms can also be disturbed if an excessive concentration of free HMs present in the soil Giller and Malmqvist (1998). In this study, we observed that the inoculation of *G. margarita* promoted the growth of *M. sacchariflorus*, especially under conditions of Pb-contaminated soil, which is indicative of the high mycorrhizal dependency of this plant species. Inoculation of AMF also increased the chlorophyll concentration, Fv/Fm and IAA concentration in plants under different level of Pb stress. For all of the determined growth parameters, the inoculated plants showed higher values than the non-inoculated plants, including all of the treatments with the highest Pb concentrations. This has also been observed in studies with others plant species that were grown in HM-contaminated soils de Souza et al. (2012); Gaur and Adholeya (2004). Mycorrhizal infection consistently improved plant growth. This can easily be explained by enhanced nutrients uptake via the extrametrical mycelium Sarkar et al. (2015b,a,c). The mycorrhizal plants showed a more than two fold increase in P uptake compared with corresponding non-mycorrhizal plants Sarkar et al. (2015c). The improved P nutrition of host plants might be one of the major mechanisms involved in the alleviation of metal toxicity as a result of mycorrhizal colonization.

The high concentration of cellularH_2O_2 and the elevated POD activity in our experimental plants suggest that the ROS scavenging system was activated there under the Pb stress conditions. Previous research results also suggest that AM are able to enhance production of antioxidant enzymes, which can alleviate the stress of heavy metals (Avery, 2001; Ruiz-Lozano, 2003) and our result is the reprint of previous results. The alleviating potential of AM on heavy metal stress is determined by different factors: type and concentration of heavy metal, plant specification and growth conditions (Hildebrandt et al., 2007). Molecular analyses have indicated mechanisms involved in heavy metal tolerance of AM. Root AM colonisation of plants under heavy metal stress results in expression of specific genes responsible for production of proteins (including metallothioneins) that increase the tolerance of plants to stress (Rivera-Becerril et al., 2005). There are many AM and plant genes involved in this tolerance to heavy metal stress, including metal transporter genes, which are expressed at different levels, and AM symbiosis can regulate the transcription of such genes (Gonzalez-Guerrero et al., 2005; Hildebrandt et al., 2007; Lanfranco et al., 2002).

6.4.2 Nutrients and Pb uptake

Inoculation of AMF increased the TP concentration in plants. Some previous research results prove that plants produce organic root exudates such as malic and citric acids and or acid phosphatase when P is deficient, resulting in enhance-

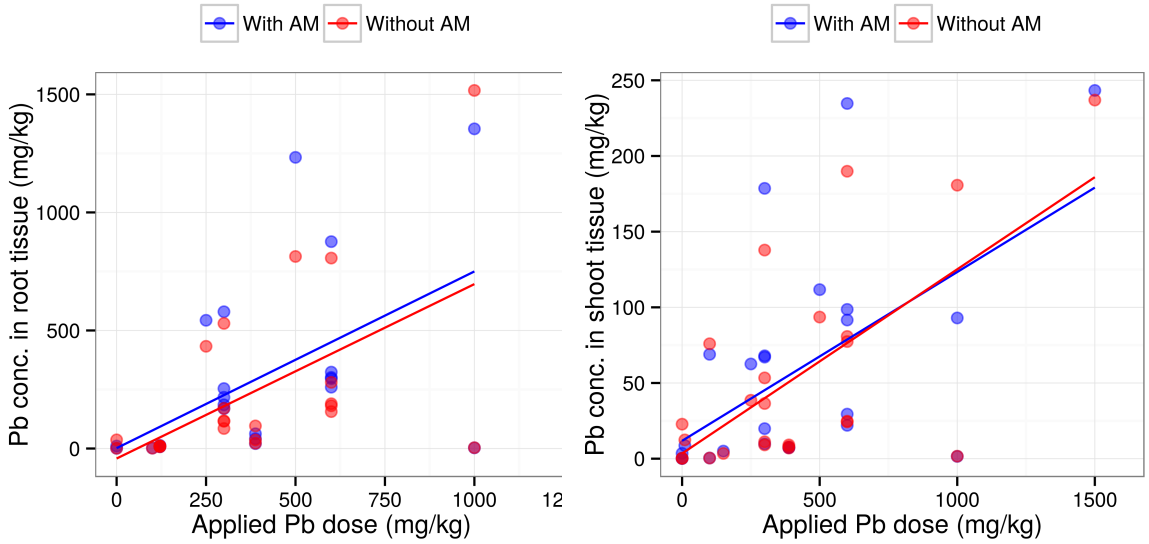

Figure 6.8: Lead distribution pattern in root (a) and shoot (b) of different plants with exposure of Pb and inoculation of arbuscular mycorrhizal (AM) fungi. The data points were reviewed from previous research (Andrade et al., 2004; Arriagada et al., 2005; Chen et al., 2005; de Souza et al., 2012; Diaz et al., 1996; Lin et al., 2007; Neagoe et al., 2013)

ment of nutrient uptake (Khan et al., 2000). In addition, the interactive effects of plant roots and microbial populations in the rhizosphere increase root exudation of organic products and hence activity of soil microorganisms, and eventual plant nutrients uptake including P, $NH4^{+}$, $NO3^{-}$, K, Mg, Fe, Cu and Zn (Cavagnaro et al., 2006; Khan et al., 2000; Sarkar et al., 2015b,c,a). There are many microorganisms in the soil that can enhance the solubility of different P sources (such as rock phosphate) by producing organic acids, including AM, *Aspergillus sp.*, *Bacillus sp.*, *Enterobacter sp.*, *Pseudomonas sp.*(Sarkar et al., 2012) though *Gigaspora margarita* Becker & Hall spores did not have any role for enhancing the solubility of P sources (Sarkar et al., 2015c). TN concentration in plants (roots, stems, and leaves) was the highest in AM fungi inoculated plant compared to non-inoculated plant (Sarkar et al., 2015c) but it was not significantly influenced by Pb concentration (Chen et al., 2005).

Enhanced uptake of Pb at different levels of Pb application was found in the present study, and the protective effects of AM fungi against Pb toxicity of *M. sacchariflorus* plants under Pb contaminated conditions were also observed. Not only metal concentration but also the total mass of metals transferred from soil to

plant is important to consider when evaluating heavy metal partitioning in soil-plant systems. The total shoot metal content depends on the plant biomass, and the ability of mycorrhizal inoculation to affect plant growth seems to be the most important parameter concerning metal accumulation in plant. Although plant Pb concentration increased with increasing Pb levels irrespective of inoculation, much Pb was retained in the mycorrhizal roots and translocation to the shoots was inhibited. In contrast, non-mycorrhizal plants experienced higher Pb concentrations in their shoots. We also consider the previous research results from different plant species with various doses of Pb to compare our results and found the similar trend (Figure 8). The large specific surface area of AM hyphae allows the fungus to absorb high levels of nutrients, even beyond the growing zone of the plant roots. This process is called phytostabilisation, by which AM increase plant ability to immobilise heavy metals in the soil through absorbing such metals in their hyphae and consequently decreasing translocation from plant roots to shoots (Chen et al., 2003). Accordingly, it can be stated that AM are able to keep heavy metals out of plants or reduce concentrations in plants (Hildebrandt et al., 2007). Though some previous research findings support our experimental results but according to our observation, this is the first report to evaluate the effects of *Gigaspora margarita* on specific host plant such as *M. sacchariflorus* under Pb stress as the effects varies on AMF species and host plant as well.

6.5 Conclusion

Contribution of AMF on stress tolerance to Pb of *M. sacchariflorus* were evaluated where AMF showed potential ability to minimize the stress of Pb and finally increased the plant growth. Accumulation of Pb was also remarkable and at high concentration accumulation in root was high but translocation to aerial part was hindered. *Miscanthus* spp. have drawn much attention for being used as biofuel and its commercial production has been initiated in many parts of the word. In addition, this species can be consider for the phytoremediation of Pb contaminated soil with combination of mycorrhizal fungus though commercial trial is needed in the filed condition for the sustainable exploitation.

Chapter 7

Conclusion & scope of study

Mycorrhizal *Miscanthus sacchariflorus* and *Phragmites japonica* plants were more effective than non-mycorrhizal plants at increasing growth (both for above ground and below ground dry mass) and chlorophyll content of leaves. Mycorrhizal *Miscanthus sacchariflorus* plants (root, stem, and leaf) also had higher concentration of N, P, K, Mg, Fe, Cu, and Zn but Ca concentration decreased with increasing colonization level; Mn concentration decreased in the root, but were unaffected in the leaf and stem. Moreover, AM fungi had some potential effect on N loss minimization. Thus, AMF (*Gigaspora margarita*) had a significant effect on nutrient assimilation and vegetation growth of the *M. sacchariflorus* plant in nutrient-deficient sandbar soil. *Miscanthus* spp. has drawn much attention for being used as biofuel and its commercial production has been initiated in many parts of the world. Mycorrhizal association and behavior with this species in cultivated soils, where nutrients are usually high, should be thoroughly studied. In addition, the dry mass production and chlorophyll content of leaves were the highest in *Phragmites japonica* plants inoculated with AM fungi in natural soil, where the AM colonization level was also high; the lowest AM colonization level occurred in the sterilized soil (where AM fungi was absent). N, P, K, Mg, Fe, and Cu concentration in the plants (root, stem, and leaf) showed a similar trend to that of dry mass production, but Ca concentration decreased with increasing colonization level; Mn concentration decreased in the root but increased in the leaf. AM fungi also had a potential effect on N-loss minimization. More again, AMF had a significant effect on nutrient assimilation and vegetative growth of the *P. japonica* plant in nutrient-limited riverbank soil. But for *P. cuspidatum* did not show the significant differences among different treatments due to low level of AM colonization. From the above statements and results, it can be concluded that AMF have much effect on natural vegetation growth and nutrient assimilation but it differs from species to species. In the current study, we could not analyse the community structure of AM fungi in the experimental soil. Therefore, the results reflect the case of a

small subset of AMF interacting with the target plant species. Further study is needed to understand the behaviour of AMF community on the same. Pinpoint of the internal mechanism of AM fungi for nutrient uptake and their role in limiting nutrient loss also demand detailed investigation.

Exposure of Zn did not have remarkable effect on root colonization of *M. sacchariflorus* plant though Zn uptake of plant increased with increased doses of Zn. AMF (*Gigaspora margarit*a) inoculation showed significant effect for stress minimization, growth and growth enhancing parameters and finally Zn uptake in the present study. The rate of uptake is very much dependent on AM species, soil condition and plant genotype. Considering that the AM fungus is a non-host specific symbiont, and that the host plant always plays the dominant role in the symbiosis, much attention also should be paid to host plants and AM species. More systematic research on the mechanisms involved in arbuscular mycorrhizal growth, nutrients uptake, metal absorption and transportation processes in plants is necessary for the application of this symbiosis in future.

Addition of Pb significantly decreased mycorrhizal colonization. Inoculation of AMF with Pb increased chlorophyll content, Fv/Fm, total dry mass, IAA, TN, and TP whereas H_2O_2 level, IAAO activity, POD activity was low compare to non-inoculated treatments. Moreover, application of AMF with Pb doses induces concentrations of Pb in the plant where at higher dose Pb (1000mg $^{-1}$) induces lower content of Pb in the aerial part of the plant but higher content in root. AMF enhanced the tolerance of *M. sacchariflorus* against Pb toxic condition and accumulate Pb in plant root whereas translocation to the shoots was inhibited in higher dose Pb (1000mg kg^{-1}). Contribution of AMF on stress tolerance to Pb of *M. sacchariflorus* were evaluated where AMF showed potential ability to minimize the stress of Pb and finally increased the plant growth. Accumulation of Pb was also remarkable and at high concentration deposition in root was high but translocation to aerial part was hindered. *Miscanthus* spp. have drawn much attention for being used as biofuel and its commercial production has been initiated in many parts of the word. In addition, this species can be consider for the phytoremediation of Pb contaminated soil with combination of mycorrhizal fungus though commercial trial is needed in the filed condition for the sustainable exploitation.

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Appendices

Figure 7.1: Location map of soil and plant propagules collection. Red mark designates center of sampling points.

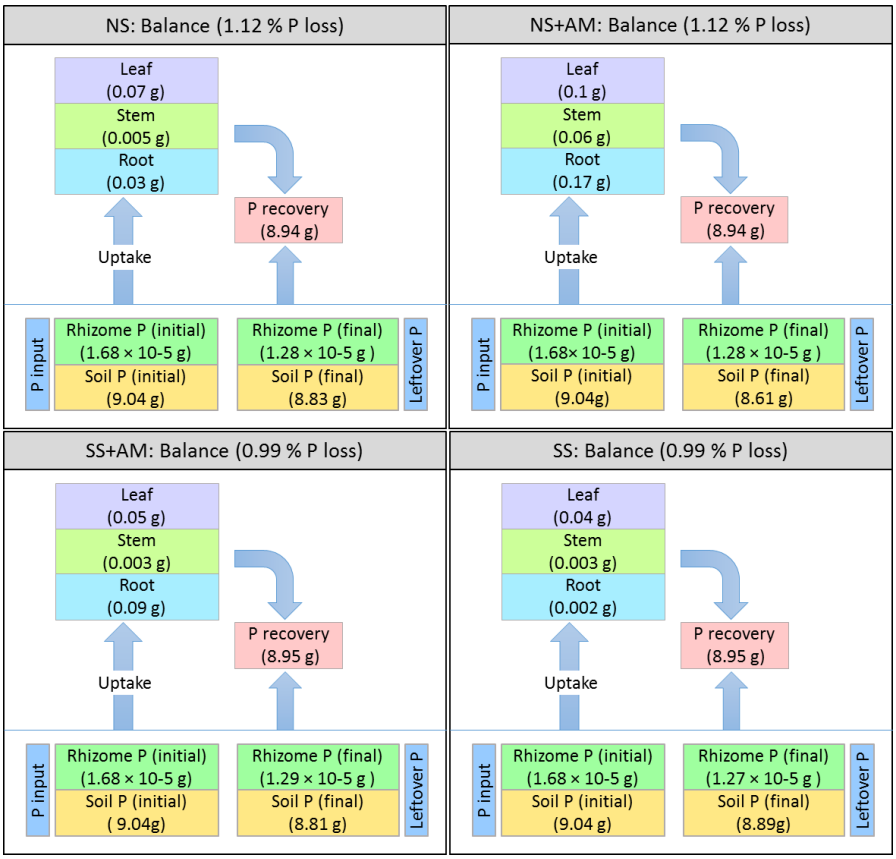

Figure 7.2: Phosphorus budgets of soil and plants as affected by native and inoculated AM fungi in *M. sacchariflorus*

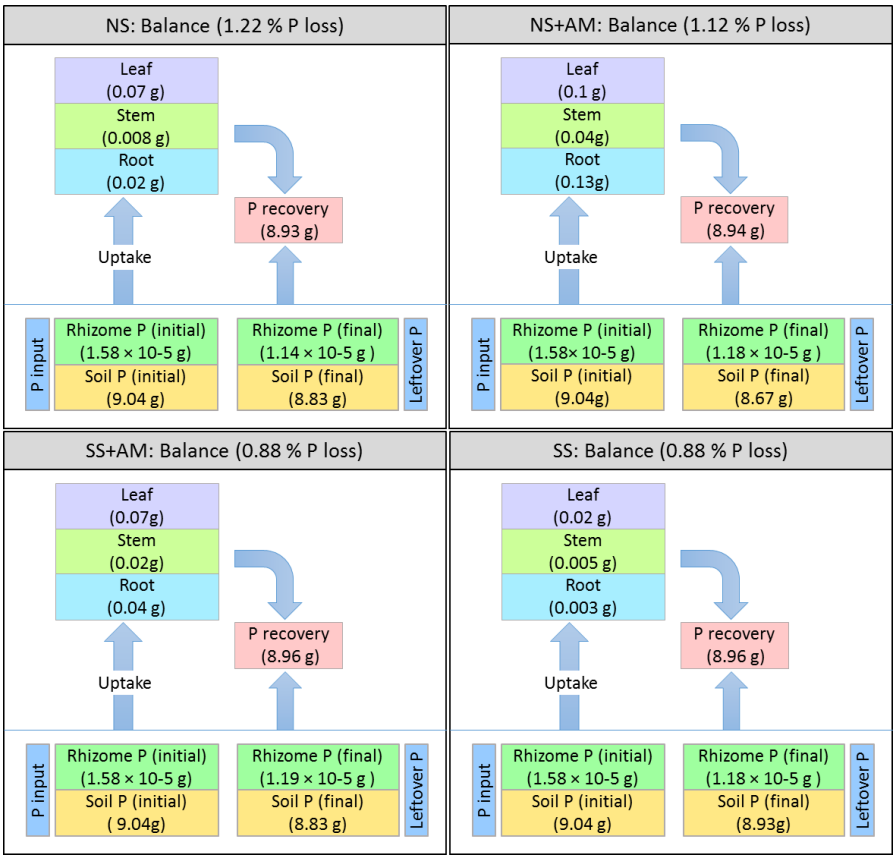

Figure 7.3: Phosphorus budgets of soil and plants as affected by native and inoculated AM fungi in *P. japonica*

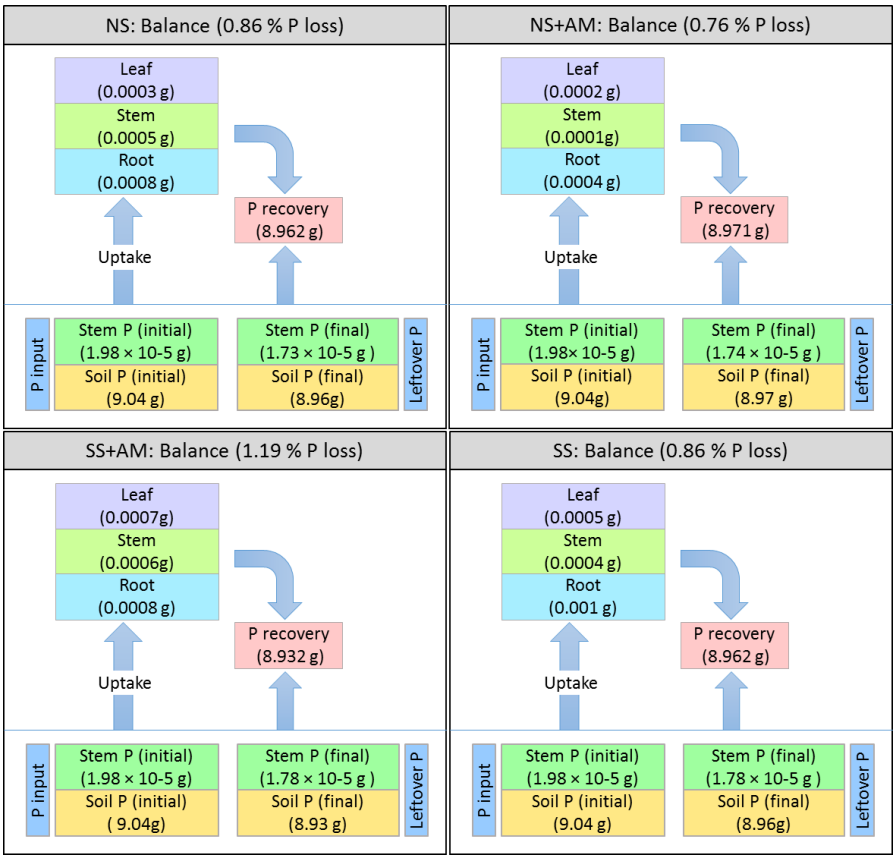

Figure 7.4: Phosphorus budgets of soil and plants as affected by native and inoculated AM fungi in *P. cuspidatum*

Table 7.1: ANOVA of effects of AM fungi and Zn treatments on growth and stress response of *M. sacchariflorus*.

		AM		Zn		AM×Zn	
		F-value	p-value	F-value	p-value	F-value	p-value
Fv/Fm		44.95	0.00	22.28	0.00	9.27	0.02
Chlorophyll		0.77	0.61	0.53	0.61	0.00	1.00
AGD		2.05	0.17	2.55	0.14	2.35	0.16
BGD		14.48	0.00	7.74	0.01	0.26	0.63
Colonization		164.55	0.00	17.47	0.00	0.19	0.68
IAA	Root	25.58	0.00	20.53	0.00	18.11	0.00
	Stem	1.65	0.25	0.14	0.87	0.16	0.70
	Leaf	0.85	0.57	2.28	0.16	0.50	0.50
IAAO	Leaf	0.91	0.53	0.87	0.45	0.40	0.54
	Stem	0.48	0.81	3.20	0.10	0.07	0.79
	Root	3.42	0.06	7.03	0.02	12.03	0.01
H_2O_2	Leaf	33.51	0.00	18.63	0.00	64.90	0.00
	Stem	3.58	0.05	32.04	0.00	0.48	0.51
	Root	21.21	0.00	125.08	0.00	2.58	0.15
POD	Leaf	337.58	0.00	135.48	0.00	109.78	0.00
	Stem	22.58	0.00	44.24	0.00	20.53	0.00
	Root	3.23	0.06	5.56	0.03	0.60	0.46
Zn	AG	4.43	0.03	20.46	0.00	0.49	0.50
	BG	5.38	0.02	24.20	0.00	2.17	0.18
TP	Root	2473.10	0.00	83.75	0.00	26.24	0.00
	Stem	12.09	0.00	17.60	0.00	9.11	0.02
	Leaf	14.24	0.00	6.58	0.02	13.47	0.01
TN	Root	2.09	0.16	1.05	0.39	1.49	0.26
	Stem	1.35	0.34	0.21	0.81	0.10	0.75
	Leaf	12.85	0.00	0.03	0.97	0.01	0.92

AGD, BGD, AG and BG represents above ground dry mass, below ground dry mass, above ground and below ground respectively.