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Characterisation of natural and synthetic nitrification inhibitors and their potential use in tomato cultivation

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ABA	Abscesic acid
AMO	Ammoniamonooxygenase
AS	Ammonium sulphate
BH	Brachiaria humidicola
CEC	Cation exchange capacity
CIAT	International Centre for Tropical Agriculture
DCD	Dicyandiamide
D_{min}	Demineralized water
DMPP	3,4-Dimethylpyrazole phosphate
DMSO	Dimethylsulfoxide
ENTEC	DMPP containing ammonium fertilizer
h	Hour
HAO	Hydroxylaminoxidoreductase
HNO3	Nitric acid
HPLC	High Pressure Liquid Chromatography
IPCC	Intergavernmental panel on climate change
MES	Morpholinoethanesulfonic acid
mM	Milimolar
µM	Micromolar
MMO	Methane monooxygenase
N_2O	Nitrous oxide
NI	Nitrification inhibitor
NIs	Nitrification inhibitor
NNI	Natural nitrification inhibitor
NO	Nitric oxide
N-serve	A ammonium stabilizer (nitrapyrin)
PM	Plasma membrane
R	Root
s	Second
S	Shoot

Prolog

Nitrogen (N) was one of the main limiting mineral nutrients for plant growth before the Haber-Bosch synthesis for mineral N fertilizer production was industrialized. Therefore, during evolution various plant species may have developed adaptation mechanisms to minimize N losses by nitrate leaching or N_2O emissions as for example by biosynthesis of nitrification inhibitors such as some subtropical grasses.

Nowadays with a general high application rate of N fertilizers (urea, ammonium and nitrate fertilizers) in crop and vegetable fields a rapid nitrification with high storage of nitrate in the soil profiles, nitrate leaching and N_2O emission result in increasing environmental problems. Application of natural (e.g. crop residues of *Brachiaria* grass) or synthetic nitrification inhibitors (e.g. DMPP, N-serve and DCD) could inhibit this rapid nitrification and thus N losses via nitrate leaching and N_2O emissions. In vegetable production systems this may be associated with a better mineral nutrient acquisition (P, and micronutrients) due to rhizosphere acidification and improved root growth, and finally improved nutritional value of these main players of human health.

Thus the main goal of this PhD research was to evaluate and compare the potential of natural nitrification inhibiting compounds of Brachiaria grasses in comparision to DMPP (ENTEC). Finally these natural nitrification inhibitors (NNI) should be tested for application in a vegetable (tomato) production system.

Chapter 1: Introduction

During the last century particularly the recent decades, human activity toward modern industry has led to severe environmental problems, such as deforestation, salinity and soil acidification in the main agricultural areas, reduction in plant and animal diversity particularly in wild life, and global warming. Various attempts which have been undertaken to improve life standards, simultaneously created side effects which in part result in environmental problems, and however it seems to be a big challenge to balance both aspects in a better way, now and in future.

Without any doubt nitrogen plays an important role in feeding world population on one hand, and on the other hand agriculture production needs to be sustainable over time, particularly in developing countries, where there is no control on the effect of (N) fertilizer application on land degradation. Environmental pollutions and arable land degradation are the major constrains for humans, as direct and indirect consequences of inappropriate agricultural activities. By far nitrogen is the major pollutant in modern agriculture, and nitrate as a result of nitrification plays a central role in N losses and environmental pollutions. At the same time N is the most needed mineral nutrient for crops, which generally limits plant productivity in agricultural ecosystems. Therefore, a precise or adapted application and management of this essential element (N) is very important for plant production and global safety.

1.1. Generals on nitrogen

Nitrogen constitutes 78% of earth atmosphere volume. From the total N found in nature, 99.96% is present in the atmosphere, and biosphere contains only 0.005%. Nitrogen has an electronegativity of 3 and five electrons in its outer shell, therefore it is trivalent in most of compounds. Oxidation states of N range from +5 for NO_3^- to -3 for NH_3 or NH_4^+. In the oxidized (+) state, the outer electrons of N serve to complete the electron shells of other atoms, and in the reduced (-) state, the electrons required to fill the outer shell of N are supplied by other atoms (Stevenson 1982).

The triple bond in molecular nitrogen (N_2) is one of the strongest bonds between two atoms. Therefore, converting N_2 into other compounds requires too much energy, and in nature only some specific microorganisms such as rhizobium bacteria which possess nitrogenase enzymes can utilize N_2 biologically. On the other hand, over converting nitrogen compounds into elemental N_2, a high release of energy occurs which has important implication in nitrogen cycle in biosphere, atmosphere and lithosphere.

It is only over industrial or microbial conversion of N_2 to NH_3 that plants can easily absorb nitrogen. In the process of mineralization and decay of plant and animal residues, N_2 through nitrification and denitrification processes is also released into the atmosphere (Majumdar, 2002; Abbasi and Adams 2000; Bateman and Baggs 2005; Ishikawa et al., 2003).

1.2. Forms of nitrogen:

In nature N can be found in different forms. The main neutral hydride of nitrogen is ammonia (NH_3), and compare to water is 6 times more basic. When ammonia is dissolved in water, it forms ammonium ion (NH_4^+). Other products of nitrogen such as N_2O and NO are the main contributors of greenhouse effect and global warming. NO in human physiology, and recently in plants involves in signalling processes (Klessig et al. 2000, Besson-Bard et al 2008). NO_2^- or nitrogen dioxide has an unpaired electron (highly reactive) and is an important component of smog. Nitric acid (HNO_3), another nitrogen compound, is one of the strongest acids and oxidizing agent that generally is used for digestion and analysis of plant materials. Nitrogen is a main part of all living tissues in form of amino acids, amines, amides, nitro groups, imines, nucleic acids, proteins, chlorophyll, vitamins and too many other molecules like alkaloids, as secondary metabolites, which generally have a defensive role in plant biology.

1.3. Nitrogen in the soil

All forms of N, including inorganic and organic N, can exist simultaneously inside the soil. Organic waste and organic matters with animal or plant origin contain a large portion of nitrogenous compound which over mineralization can deliver nitrogen to medium. Nitrate has the advantage of immediate availability for plant and microbes, but it has disadvantages of high solubility and mobility in the soil. In contrast to nitrate, ammonium is not subjected to losses, because it can be held by soil clay minerals. Ammonium pools are always larger in the top 10 cm of soil, but NO_3^- fluctuates throughout the year and soil profile, and always depleted during periods of rapid plant growth (Jackson et al., 1988). However, volatilisation of ammonium in dry soils specially with high pH can be significant.

Nitrate may act as a potential for eutrofication when nitrogen is the limiting factor for the growth of certain bacteria and algae in free waters. Application of nitrogen fertilizers in many parts of the world, in order to increase the yield, ends in ground water or atmosphere. However under ideal cultivation systems the utilization rate of applied N fertilizer is only 50–70%, and in most of cases more than 50% of those fertilizers enters into air or ground water (Velthof et al., 1998; Ishikawa et al., 2003).

In soil, plant roots compete with different types of microbes for available nitrogen, particularly in an N-limited ecosystem (Neumann and Römheld, 2000; Neumann 2007). This plant-microbe competition for available N, however, is a short-term phenomenon (Jackson et al., 1989). Therefore, for investigating nitrogen uptake and partitioning in soil-plant-microbe system, short term experiments should be carried out. Soil microbes absorb substantially more NH_4^+ and NO_3^- than plants, and the rate of uptake (immobilization) for NH_4^+ is much higher than NO_3^- (Azam and Ifzal, 2006; Herrmann et al., 2005).

In a short term partitioning incubation, Jackson et al., and (1989) showed that ammonium is the dominant source of N to both plants and microbes. Microbes are better competents for NH_4^+ and NO_3^- uptake than plants, so microbial uptake is a major factor controlling NO_3^- availability to plants. Plants however compete better for NO_3^- than for NH_4^+ (Jackson et al., 1989).

Ammonium occurs in the soil as free NH_4^+ ions or bounded to mineral or organic colloids in the soil. Ammonia volatilization may occur specially with the surface application of NH_4^+ containing fertilizers, and normally increases with pH and temperature (Stevenson 1982). Incorporation of NH_4^+ fertilizers inside the soil, and application of other ammonium fertilizers than urea can reduce the extent of this volatilization. Plants are considered both as a sink and a source for ammonia, however ammonia emission under agricultural systems is high, but on the other hand, semiarid and natural ecosystems act as sinks for ammonia (Sommer et al., 2004).

Ammonia and nitrite (NO_2^-), produced over microbial decomposition of nitrogenous organic compounds, are capable of undergoing chemical reaction with organic matter, producing metallo-organic and organo-clay complexes which protect N-compounds against attack by microorganisms (Stevenson 1982). Oxidation and reduction reactions, however, are the basis of biological N transformation, which some of them take place in the cells of microorganisms and some in the tissues of higher plants. The largest N pool in soil is organic matter, which is mostly unavailable to plants. Over decomposition and mineralization it becomes available to plant roots. Decomposition of organic matter is mediated by microorganisms, therefore this microbial biomass is very important in soil physical and biochemical properties. Living microbes are as a source of enzymes for decomposition, as well as nutrient mobilization and acquisition in soil. On the other hand, microbial dead biomass represents an important soil N pool. With mineralization of organic matter, N is released as available form to plants. Furthermore organic matter decomposition is a continue process with different extents, so plant residues in soil always are in different decomposition strength. Mineralization-

immobilization turnover is very important in the availability of N for plants and microbes in soil. N mineralization (amonification) in soil is a result of organic matter decomposition by non-specific heterotrophic soil microorganisms under aerobic and anaerobic conditions. The major N mineralization occurs in the soil surface which has higher biological activity as a result of higher amount of recourses. Mineralization is always acompanied by immobilization of inorganic N specially NH_4^+, by microbes. Moreover, many microbes can utilize ammonium or nitrate as a source of energy. Microbial biomass is also able to incorporate low molecular weight N-containing organic compounds such as amino acids from the soil organic matter (Barraclough 1997). Soil ammonium may be driven directly from the mineralization of organic matter and the addition of ammonium-containing fertilisers.

1.4. Nitrification

Nitrification is oxidation processes of organic or ammonium forms of nitrogen to nitrate by nitrifying organisms, mainly species of *Nitrosomonas,* which convert ammonia to hydroxylamine by ammonia monooxygenase (AMO), and then to nitrite by hydroxylamine oxidoreductase. The later reaction is mediated by bacteria species of *Nitrobacter*. However, many other chemoheterotroph bacteria and microorganisms such as *Archaea* (Leininger et al., 2006; Adair and Schwartz 2008) are involved in process of nitrification. Nitrate as outcome of this process is quite vulnerable to leaching and denitrification. Nitrification is one of the main reasons of soil acidification particularly in tropical region of the world. Most of the applied N-fertilizer to soil in the form of NH_4^+ is usually oxidized quite rapidly to NO_3^- by nitrifying microorganisms (Subbarao et al., 2006b; Ishikawa et al., 2003).

Nitrification in soil has mainly chemoautotrophic origin, specially in agricultural soils, but sometimes heterotrophic nitrification (by fungi and many other heterotrophic organisms) may have a high contribution to ammonium oxidation. This is important specially in some forest soils (Verstraete and Alexander, 1972; Duggin et al., 1991) or acid soils (Brierley and Wood, 2001; De Boer et al., 1991), where autotrophic nitrification can be inhibited. This heterotrophic nitrification may also occur in agricultural soils under fertilization (Bateman and Baggs, 2005). Acetylene as a specific inhibitor of autotrophic nitrification can be used in study of heterotrophic nitrification. Therefore in nitrification incubation of soil, ammonium oxidation and NO_2^- oxidation can be blocked by acetylene and chlorate, respectively (Sahrawat et a., 1987; Herrmann et a., 2007).

Incubation experiment can be useful tools to study nitrogen transformation in soil. A soil water content of about 45% water holding capacity is the best for maximum nitrification. Moreover, this optimum soil water content differs with various temperatures (Grundmann et

al., 1995). Mosier (1998) mentioned that a water content of 60% of WHC is considered optimum for nitrification. Normally nitrite because of its high reactiveability is not accumulating in significant amounts in soil. Nitrite oxidizers (nitrobacters) are more sensitive to adverse soil conditions than NH_4^+ oxidizers. Therefore under calcareous soils or application of NH_4^+ fertilizers, nitrite may accumulate in detectable quantities. Nitrite is much more sensitive and simple for detection as an indicator of nitrification, and requires a smaller sample size (de Boer and Kowalchuk 2001). In soil incubation, chlorate is used for blocking nitrite oxidizing bacteria, however chlorate has relatively little inhibitory effect on ammonium oxidation. On the other hand, background nitrate in soils can not interfere with measurements. In incubation tests or field experiments accumulation of nitrite in the presence of chlorate is the reason for the presence of autotrophic nitrite oxidation (de Boer and Kowalchuk 2001).

Nitrate is soluble and negatively charged and generally is not held by the soil colloids. It is therefore subject to leaching under the appropriate conditions. In contrast, ammonium is positively charged and retained as a cation by the soil cation exchange capacity (CEC). Nitrate plays important role in both nitrification and denitrification processes. Denitrification is the opposite process to nitrification, in which NO_3^- under anaerobic conditions through acting as terminal electron acceptor in metabolic reactions for denitrifying bacteria, can be reduced to N_2 in optimal condition. However many other intermediates such as N_2O and NO can be released to atmosphere. Nitrate in both nitrification and denitrification processes is in close relation to NO and N_2O emissions. So, controlling the processes of nitrification is a potential tool to restrict N leaching, NO_x emissions and even ammonia volatilisation from soils in one hand, and increasing N use efficiency on the other hand. Denitrification is done mainly by facultative anaerobic bacteria (both autotrophic and heterotrophic), and this process acts as a balance to biological N_2 fixation. It is well known that in soil both nitrification and denitrification can occur at the same time and near to each other (Abbasi and Adams, 2000; Robertson et al. 1988; Bateman and Baggs 2005). The heterogeneity in soil moisture is mentioned as the main reason for this simultaneously emission (Abbasi and Adams, 2000a and 200b). Produced nitrate can simply diffuse to adjacent micro sites of denitrification, so maybe it causes a significant underestimation of mineralization and nitrate production in such condition (Abbasi and Adams, 2000a and 200b). For these reasons nowadays general idea is that nitrification more than denittrification participates in NOx emissions. Moreover, under such conditions, nitrous oxide (N_2O) rather than dinitrogen (N_2) is produced under aerobic conditions (Bateman and Baggs 2005; Bremner 1997; Mosier 1998), which has more negative and destructive properties.

1.5. Nitrification inhibitors

The main N losses in soil through nitrification, leaching and denitrification involve nitrate as central point, so limiting nitrate in the soil through application of nitrification inhibitors can alleviate the economic and environmental cost of nitrogen fertilizers. Nitrification inhibitors are natural or synthetic compounds that delay the bacterial oxidation of NH_4^+ to NO_2^- (first step of nitrification) for a limited period of time. They generally reduce the *Nitrosomas* bacteria activity in soil. These NIs normally have no effect on *Nitrobacters* which oxidize NO_2^- to NO_3^-. Despite huge work and great interest in nitrification inhibitors, only a few compounds are allowed for agricultural and environmental usage. This is because development, production and subsequent use of NIs are quite expensive, and even they need to be environmental friendly as much as possible. During last decades different synthetic nitrification inhibitors have been developed which block enzymatic pathway, mainly ammonium monooxygenase (AMO) of bacteria responsible for ammonium oxidation. AMO has wide range of substrate for catalytic oxidation, and the inhibitory effects of many NI compounds are due to competition for the active site of enzyme (McCarty, 1999). Oxidation of compounds such as acetylene by AMO generates highly reactive products which bind to enzyme and causing irreversible inhibition (Herrman et al., 2007). Many of these compounds, specially those having thiono-S can bind to Cu in the active site of AMO enzyme and inactivate it, while others such as heterocyclic N compounds inhibit AMO activity by their N ring (McCarty, 1999). However, using chemicals in production processes of agriculture products is going to be more restricted in close future. Natural products to some what can be suitable alternatives.

As an integral and important part of biological cycle in the soil, nitrification seems to be beneficial at a natural rate. Disturbance of natural habitants by human activity has lead, direct and indirectly, to such changes which favour nitrification, in term of temperature, land management, and soil organic matter. Thus adopting some techniques and approaches in order to reduce nitrification, and consequently its negative effects specially soil acidification are necessary.

Many chemicals can have inhibitory effect to specific N transformation processes. In practice it is critical to increase use efficiency of N fertilizers in one hand and reduce negative impacts of applied N and nitrification on the other hand. Heterocyclic N compounds have NI effect, and unsubstituted heterocyclic N compounds such as pyrazole, 1,2,4-triazole, pyridazine, benzotriazole, and indazole, which have two or three adjacent ring N atoms are potent

inhibitors of nitrification (McCarty and Bremner, 1989; McCarty 1999). By far Nitrapyrin, Dicyandiamide (DCD), and 3,4-Dimethylpyrazole phosphate (DMPP), are three major commercial NIs (Subbarao et al., 2006b; McCarty 1999). DCD and nitrapyrin have some disadvantages compare to DMPP. For example, DCD is very soluble in water resulting in spatial separation of NI and NH_4^+, and for large scale usage is too expensive, as well as its efficiency is not high. In addition, under certain conditions, it may cause phytotoxic problems. In contrast, nitrapyrin is adsorbed to soil minerals more strongly than NH_4^+, which also causes spatial separation from NH_4^+. Moreover this compound is corrosive, explosive and makes toxicities to plants (Sahrawat et al., 1987; Subbarao et al., 2006b). The effects of NIs, depend on soil conditions, are too complicated and are most likely to be greater on soils rich in nitrogen with high risk of leaching and denitrification. Inhibitory effect of NIs and related plant growth is largely depends on soil N status. Meanwhile, efficiency of these compounds largely depends on soil N status, soil physiochemical factors (texture, temperature, moisture, organic matter, pH) and climatic factors (temperature, rainfall intensity and frequency) which on one side, determine the size of these losses, and on the other side, influence dynamics of NIs in the soil (Adair and Schwartz 2008). High efficiency at lowest possible concentration and minimum side effect is very important in selection of a NI. It needs to persist in soil for longer time, and resist against being washed out from the soil profile, and be environmental friendly (McCarty and Bremner 1989).

Limiting nitrate production as a result of NIs, may also significantly decrease N_2O emissions (Mosier 1998; Zerulla et al., 2001; Müller et al., 2002; Hatch et al., 2005). In contrast, Weiske et al., (2001) showed no significant direct effects of the DMPP or DCD treated plots on NH_4^+ concentrations, in comparison to the controls. However plant uptake and higher affinity of NH_4^+ for uptake by plant roots could be the possible reasons.

Most of organisms which oxidize NH_4^+, also can use methane (CH_4) as an energy source **(**Bedard and Knowels, 1989; Bronson and Mosier, 1994; Chaves et al., 2006), so over inhibition of those bacteria, an increase in CH_4 emission might occur, which is potentially an important greenhouse gas. Normally higher dosage of NIs increase NH_4 concentration in soil (Chaves et al., 2006). Furthermore, most of the compounds which have NI activity, also may affect both AMO and methane monooxygenase (MMO), because structure of these two enzymes are very similar to each other (Hooper et al., 1997; Bedard and Knowles 1989; Bronson and Mosier 1994). It is necessary to consider that global warming potential of CH_4 and N_2O are 21 and 310 times of CO_2, respectively (IPCC 1996). Agriculture represents the

main contributes of global CH_4, N_2O and CO_2 emissions, 40%, 70% and 40%, respectively (Mosier 1998).

With incorporation of NIs into N-fertilizers, depending on soil physical and chemical properties, NH_4^+ would persist for longer time in soil. This ammonium in soil in part, is adsorbed to clay minerals and consequently with gradual release plants can benefit better in less application rates and numbers. Finally it would lead to increased use efficiency and better plant growth.

1.6. 3,4-Dimethylpyrazole phosphate (DMPP)

3,4-Dimethylpyrazole phosphate (DMPP) is a nitrification inhibitor, highly specific in inhibiting nitrification at low concentrations of 0.5–1.0 kg active compound ha^{-1} over a period of 4–10 weeks (Zerulla et al., 2001). ENTEC on the market is a combination of an N-NH_4 fertilizer with DMPP (1% of total N) in form of granules. However, compare to other nitrification inhibitors such as DCD, and N-Serve, it has no toxicity or other side effects, but instead, it offer potential advantages for cropping systems (Zerulla et al., 2001; Barth et al., 2001; Pasda et al., 2001). However tobacco and grape farmers in south Germany complained about negative effects of ENTEC on plants growth (personal communication), in which plants showing some N deficiency symptoms, and winter injury. This is probably because of its strong effect specially in light soils and early in spring which prolongate presence of nitrogen and late season uptake which consequently leads to winter damage. However, it is in particular importance that the duration of NIs effects depends on climatic conditions, site characteristics and probably the cultivated crop (Zerulla et al., 2001).

Application of NIs in general, and DMPP in particular, has important consequences including reduced leaching of nitrate and emission of N_2O (Weiske et al., 2001; Zerulla et al., 2001; Müller et al., 2002; Hatch et al., 2005), reduced emission of CO_2 (Weiske et al., 2001), and increasing yield (Zerulla et al., 2001; Pasda et al., 2001; Linzmeier et al., 2001). However the decrease of N_2O emission by application of DMPP could be related to an inhibitory effect on the enzymes of denitrification as the non-target process (Müller et al., 2002). This results in less application of mineral N fertilizer, saving financially and probably higher crop yields. Plants receiving ENTEC (DMPP containing fertilizer) show darker green leaf colour, which is typical symptom of ammonium nutrition in nutrient solution, however plants (leaves) may have a reduced nitrate content (Hähndel and Zerulla, 1999) which is quite important in fresh consuming of vegetable crops.

In most cases efficiency of inhibitors depends on their own chemical properties and several other factors, including soil texture and temperature. For example, effectiveness of both DCD

and DMPP was progressively decreased when soil temperatures increased (Irigoyen et al., 2003). So specially in warm climates with application of these inhibitors, it is necessary that soil temperature must be considered. With DMPP and probably other NIs the oxidation of the NH_4^+, in sandy soils compare to loamy soils, is more inhibited. The binding behaviour of DMPP is influenced markedly by soil textural properties (Barth et al., 2001), which in short term experiments is an important factor in inhibition property. Moreover, DMPP may have no effect on the N mineralization of the crop residues (Chaves et al., 2006).

1.7. Brachiaria as a source of natural nitrification inhibitor (NNI):

Ecological production as an adaptive measure to biological cycles in the soil requires limited application of chemicals including synthetic nitrification inhibitors. Depends on conditions, natural products could be suitable alternatives. Plants due to their phenotypic plasticity and adaptation properties are able to change their biochemical, physiological and morphological characteristics in response to environmental variation (Schlichting, 1986). In contrast to animals, plants through secretion and emission of chemicals respond to any change in their surroundings. Plants produce secondary metabolites which might be a potential nitrification inhibitor (Lodhi**,** 1978; Howard et al., 1991), for example grasslands through production and accumulation of phenolic acids and flavonoids that inhibit nitrification, display low nitrate content in the soil (Lodhi**,** 1978; Rice and Pancholy**,** 1973; Ellis and Pennington**,** 1989; Subarrao et al., 2006 and 2007). Olson and Reiners (1983) suggested that higher phenolic and terpenoid concentrations are responsible for inhibition of nitrification in the forest soils. However Stienstra et al., (1994) and McCarty et al., (1991) are not agree with this hypothesis**.**
Different plant-based substances have been shown to have biological activity specially against insects, weeds and bacteria. Different parts of tropical tree (*Azadirachta indica*) known as neem, have biological activity against bacteria, pests and diseases (Melathopoulos et al., 2000; Gahukar, 2000), medicinal usage (Schmutterer 1990), and specially for improving nitrogen use efficiency in agriculture (Kumar et al, 2007; Joseph and Parasad 1993; Sharma and Parasad, 1995). Neem and karanj seed cakes (Pongamia glabra Vent) (Majumdar, 2002) as well as plant polyphenols, vegetable tannins and waste tea products (Krishnapillai, 1979), terpenes and essential oils of ment (Patra et al., 2002; Patra et al. 2006), essential oils from Mentha spicata, *Artemisia annua*, and mustard (*Brassica juncea* L) (Kiran and Patra, 2003; Patra et al., 2001), and different types of quinines (Mishra et al. 1980) have been shown to inhibit nitrification in the soil. Carbon-rich secondary chemicals have been found to inhibit nitrifiers directly (Horner et al., 1988). Monoterpenes can inhibit nitrification but not denitrification, however, increased respiration activity indicating indirect inhibition of

nitrification by monoterpenes, due to immobilization of mineral N (Paavolainen et al., 1998). It is worth mentioning that litter quantity as well as quality controls mineralization rate in soil in most of natural ecosystems.

Most of these natural products also retard soil urease activity as a urease inhibitor. On the other hand, intensity of inhibition normally increases with the level of application (Patra et al., 2006). These natural compounds could decrease *Nitrosomonas, Nitrobacter*, and total bacterial and actinomycete populations in soil (Patra et al. 2006). Moreover, in contrast to synthetic nitrification inhibitors, natural products are less persistent, more biodegradable, economic and environmental friendly (Patra et al. 2006). Therefore, nitrification inhibitory properties of plant based substances offer better advantages for agricultural production, which can keep environment more healthy, too. However, there are not enough studies on this topic, so the biological inhibition of nitrification by crop plants or grasses still is not well understood.

Brachiaria spicies are C_4 grasses grow in tropical and subtropical regions of South America, Africa and Asia. They are waterlogged tolerant and mostly adapted to infertile and acidic soils of these tropical region mainly as pastures (CIAT, 1983). These plants are particularly very susceptible to saline conditions, getting a succulent effect specially in their root systems (Mergulhão et al 2002). During the field works with *B. humidicola* it has been found that soils under cultivation of this plant have low level of nitrate (CIAT, 1985; Sylvester-Bradley et al., 1988). It has been shown that root exudates and soil extracts of B. humidicola accession 26159 (BH) suppress ammonium oxidizing bacteria populations, and consequently nitrification and N_2O emissions, which was not observed for other grasses or *Brachiaria* species (Ishikawa et al., 1999, and 2003; Subbarao et al., 2005, 2006a, 2006c, 2007a and 2007c). Compounds released from the roots of BH are mainly responsible for its inhibitory effect on soil nitrification (Ishikawa et al., 2003; *Subarrao* et al., 2006a). Moreover, root tissue extracts of BH have substantial inhibitory effects on nitrification (Subarrao et al., 2006c, 2007a, 2007c). Roots of BH produce two methylated phenolic acids, methyl-*p*-coumarate and methyl ferulate, that have inhibitory effects on nitrification (Gopalakrishnan et al., 2007). There is an estimation of nearly 30% of the root mass turnover annually in BH pastures, equals to one ton root dry matter per ha (Fisher et al 1994), which could contain significant amounts of nitrification inhibitors added annually to the soil. This might be one of the main reasons for the observed low nitrification rates in soils, and this inhibiting effect appears to be stable as long as the grass stays in the soil (Ishikawa et al., 2003).

1.8. Nitrogen uptake by plants

In nutritional point of view nitrogen is on the top among all elements which are necessary for optimum plant growth. In agriculture and other ecosystems, nitrogen is the most limiting factor for plant growth. However agricultural crops have normally higher demand for it compare to non-cultivated crops. Nitrogen (10-30 g/kg) after carbon and oxygen is the next most abundant element in plant dry matter (McNeill and Unkovich, 2007). Despite extensive fertilization specially for horticultural crops, N deficiency is very common phenomenon, mainly because of climatic and edaphic conditions, as well as the dynamics of N inside the soil. This deficiency could appears as yellow colour due to chlorophyll degradation in older leaves (which may have early senescence), smaller leaves, early flowering, reduced fruit set, and reduction in growth rate and plant biomass and consequently yield.

Nitrate and ammonium are two major forms of nitrogen which plants can take up by their root systems in the soil. However plants are able to take up various forms of nitrogen compounds including, nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), ammonia (NH_3), Urea ($CO(NH_2)_2$), amino acids, peptides and low molecular weight organic compounds (Marschner 1995, von Wiren and Merrick, 2004; Chapin 1995). Nitrogen concentration varies with plant species, developmental stages and plant tissues. Woody plants typically have ≤5 g/kg for woody tissue and ≤20 g/kg for leaves, but N concentration is typically 10-20 g/kg dry matter for grasses and 20-30 g/kg for legumes, and tends to be higher in younger tissues (Schjoerring et al., 2002). Shoots usually are stronger sink for N than roots and therefore shoots has higher N concentration than roots (Schjoerring et al., 2002). Through mass flow via soil water, diffusion or root extension, ammonium and nitrate can reach to the root surface. However ammonium movement by these phenomena is limited by NH_4^+ fixation in clay minerals. Energetically point of view NH_4^+ is beneficial for plants, however data from growth characteristics suggest that over NH_4^+ nutrition plant needs higher amount of energy to exert H^+ efflux (Britto et al., 2001a and 2001b; Kronzucker et al., 2001). Plant uptake of NH_4^+ and NO_3^- is a function of their concentration in soil solution, root distribution, soil water content and plant growth rate (Schjoerring et al. 2002). Ammonium after absorption must be assimilated into amino acids, or translocated to other parts than roots, otherwise it makes NH_4^+ toxicity on plants (Schjoerring et al. 2002; Loque and von Wiren 2004), and however in field condition ammonium toxicity is not common. Glutamine as the first and main assimilatory products, may has very important role in alleviating NH_4^+ toxicity (Schjoerring et al., 2002). However glutamine synthetase is relatively sensitive to NH_4^+.

Nitrate on other hand, after uptake can be reduced and assimilated in root or shoot by nitrate reductase enzyme which is synthesised in response to NO_3^- uptake. Assimilation of NO_3^- by plants involves the reduction of NO_3^- to NO_2^- , and reduction of NO_2^- to NH_4^+ by nitrite reductase (another inducible enzyme), which exist in cytoplasm and chloroplasts, respectively. Then ammonium generally is assimilated into glutamine. Nitrite is a transitory intermediate in plants with a short life, similar to soil condition. Nevertheless, it is highly toxic to plants and microorganisms.

Many investigations indicate that ammonium as a sole nitrogen form, or as the main form in combination with nitrate has inhibitory effects on plant growth (Gerendas et al., 1997; Siddiqi et al., 2002; Roosta and Schjoerring, 2007; Zhang and Rengel, 1999, and 2003). Reduced growth due to NH_4^+ nutrition has often several justifications such as decreased net photosynthesis (Neumann and Römheld 2000; Gerendas et al., 1997), acidification of external culture medium (Brito and Kronzucker, 2002; Britto et al., 2001; Claussen, 2002), a lack of osmolites such as cations, nitrate and sucrose, which contribute to the rate of leaf expansion (Rahaab and Terry, 1995) or hormonal imbalance of plants (Barker and Ready 1994; Gweyi, 2006). Symptoms of NH_4^+ toxicity including marginal necrosis and interval chlorosis on leaves, wilting, and inhibition of root growth are very common. However by moderate supply and buffering nutrient solution, still there is a rapid inhibition of leaf growth (Walch-Liu et al., 2000; Rahayu, 2003; Britto and Kronzucker, 2002). This could be mainly due to a 70% reduction in zeatin + zeatin riboside (the active component of cytokenin) concentrations in the xylem sap within 24 h of exudation from tobacco under NH_4^+ nutrition. Nitrate, on the other hand can return this inhibitory effect of NH_4^+ on leaf growth (Walch-Liu et al., 2000; Rahayu, 2003; Britto and Kronzucker, 2002; Siddiqi et al., 2002; Britto and Kronzucker, 2001), similar to cytokenin application effects. NH_4^+ supply to plants increases the level of ABA in roots xylem sap and leaf tissues (Rahayu, 2003), so by its interaction with cytokenins, they regulate leaf growth, and plant development. Disturbance of hormonal balance of plant may is an important feature of NH_4^+ toxicity. Sensing of NO_3^- triggers the expression of a series of genes involved in utilization of nitrate (Forde and Clarkson 1999). However nitrate inside the plant may has several function as a nutrient, as an osmolite (Blom-Zandstra and Lampe 2007), or as a signaling molecule (Wang et al., 2000; Forde and Clarkson, 1999). Then it has been suggested that growth inhibition by NH_4^+ is related rather to the absence of NO_3^- as a signal than to the presence of NH_4^+ (Rahayu et al., 2005; Onyango Gweyi 2006).

Ammonium toxicity can be reduced by rapid conversion of NH_4^+ to amino acids in the roots or by sequestering NH_4^+ in a vacuolar reservoir (Marschner et al., 1995, Forde and Clarkson

1999, Schjoerring et al., 2002). Immediate assimilation of NH_4^+ into amino acids is catalyzed by the enzymes glutamine synthetase and glutamate synthase. So glutamine synthetase has a very important role in reducing NH_4^+ toxicity (Schjoerring et al., 2002; Britto et al., 2001). Young leaves tended to have higher apoplastic NH_4^+ concentrations than older non-senescing leaves. Aarnes showed high concentrations of NH_4^+ in all parts of spruce seedlings indicating metabolic control of NH_4^+ concentrations in tissues and that NH_4^+ can be stored in acidic compartments (Aarnes et al., 2007; Aarnes et al., 1995). Conifers and ericaceous plants adapted to acid soils with low or no nitrification have a preference for NH_4^+ as the main nitrogen source. These plants can tolerate high concentrations of NH_4^+ and have been found to possess a reduced capacity to use NO_3^- (Schjoerring et al., 2002, Britto and Kronzucker 2002, Forde and Clarkson 1999).

1.9. Why ammonium?

Ammonium energetically and economically point of view, is a preferential form of nitrogen for plant uptake and assimilation. Under laboratory experiments most of plants show toxicity symptoms with ammonium nutrition, however these symptoms are not common on field crops. Ammonium is taken up by plant roots through NH_4^+ transporters across the plasma membrane (Lauter et al., 1996; Loque and von Wiren 2004). There are two high-affinity transporter systems for nitrate and one for ammonium in roots of higher plants (Glass et al., 2002). Low affinity NH_4^+ transport occurs through non-selective cation channels or K channels (Howitt and Udvardi, 2000; Kronzucker et al., 2001; Loque and von Wiren, 2004). Inside the plant ammonium also is generated as a key intermediate in processes such as nitrate reduction, photorespiration, phenyl propanoid metabolism, degradation of transported amides, and protein catabolism (Joy, 1988; Schjoerring et al., 2002). So excess uptake of ammonium might happen which together with the ammonium released from catabolic processes within the cell, can cause toxicity. Measurement of xylem ammonium is not an easy task, because interference from other metabolites such as amino acids and amines may cause big uncertainties about the magnitude of xylem NH_4^+ concentrations (Schjoerring et al., 2002). Electroastatically, uptake and assimilation of ammonium is a proton generating process. In order to make an electrical-balanced charge, NH_4^+ uptake leads to acidification of extracellular pH through H^+ exclusion (Joy, 1988). On the other hand, NO_3^- is taken up by an H^+-cotransport system in the plasma membrane so nitrate uptake and assimilation is a proton consuming process, leading to alkalination of extracellular pH (Marschner, 1995). These changes in extracellular pH have important implication for plant nutrition and management particularly where nutrient deficiency is a major problem such as calcareous soils.

There are different NH_4^+ transporters which differ in their biochemical properties, localization, and in regulation at the transcriptional level. Nitrogen status of a local root portion as well as of the whole plant can control ammonium transportation (Loque and von Wiren 2004). However toxicity symptoms are very common under NH_4^+ which normally is coupled with a reduction in plant dry weights and root:shoot ratios compare to NO_3^- fed plants (Roosta and Schjoerring, 2007; Siddiqi et al., 2002; Claussen, 2002). In tomato there is a strong accumulation of ammonium in leaves, stem, and roots at a growth medium concentration above 1 mM. The increase in tissue NH_4^+ coincided with saturation of glutamine synthetase activity and accumulation of glutamine and arginine. Low tissue levels of calcium and magnesium in the NH_4^+ fed plants constituted part of the NH_4^+ toxicity syndrome (Schjoerringet al., 2002; Roosta and Schjoerring, 2007). Glutamine synthetase incorporates NH_4^+ into glutamine, but root glutamine synthetase activity and expression are repressed when high levels of NH_4^+ is supplied (Schjoerring et al., 2002). However, in tolerant species like rice ammonium uptake may have different regulatory control than nitrate adapted species (Tobin and Yamaya, 2001). Rice has the same number of ammonium transporter homologs as have been isolated from tomato (von Wirén et al., 2000). Roots of rice have two ammonium-inducible transporters compared to only one, (AMT1;2) in tomato, showing a comparable transcriptional regulation in tomato (Sonoda et al., 2003).

There are several explanation for toxic effect of NH_4^+, including: acidification of soil, acidification of the cytosol (Britto et al., 2001; Britto and Kronzucker, 2002), NH_4^+-induced cation deficiency (specially Ca) and cation versus anion imbalance (Redinbaugh and Campbell, 1993) deficiency of carbon sources in the root zone, stimulated nitrogen assimilatory capacity, and disturbed phytohormone and polyamine status, ((Rahayu et al., 2005; Onyango Gweyi 2006; Gerendas et al., 1997; Zhang and Rengel, 1999; Britto and Kronzucker, 2002), or high cost of energy for H^+ or NH_4^+ efflux in order to keep low NH_4^+ concentrations in cytoplasm (Britto et al., 2001). Until now there is no distinct specific transporter for ammonium efflux movement. However plants activate NH_4^+ efflux to cope with NH_4^+ influx under high external concentrations of ammonium (Babourina et al., 2001 and 2007**;** Britto et al., 2001). This efflux was suggested to have a cytosolic origin (Britto and Kronzucker 2003). Nitrate with an unknown mechanism can alleviate NH_4^+ toxicity. On toxicity extent of ammonium, NO_3^-/NH_4^+ ratios are quite important. Nitrate primarily may affect the NH_4^+ low-affinity influx system, and NH_4^+ transport is inversely linked to Ca^{2+} net flux (Babourina et al., 2007). Protection against a low pH at the surface of root (Imsande, 1986), enhancing xylem loading of NH_4^+ (Babourina et al., 2007; Kronzucker et al., 1999), or

through increasing the expression of enzymes that remove NH_4^+ from the cytoplasm (Redinbaugh and Campbell, 1993; Kronzucker et al., 1999), or alleviating the NH_4^+ induced plasma membrane depolarization of cells (Wang et al., 1993) and reduction the internal ratio of cations to anions in plants (Britto and Kronzucker, 2002), or affecting low-affinity NH_4^+ transporters (Babourina et al., 2007) maybe also are the mechanisms involved in this alleviation. When NH_4^+-induced low pH, potassium releases into external medium (Babourina et al., 2001), that means NH_4^+ can compete with K^+ for absorption through some K^+ inward channels and non-selective cation channels (Howitt and Udvardi, 2000; Kronzucker et al., 2001; Loque and von Wiren, 2004). There are enough evidence showing better plant growth and development even in nutrient solution with a combination of ammonium and nitrate. In soils also following NH_4-fertilizer application, even with NI applications, similar situation (ammonium and nitrate nutrition) for plants uptake could happen.

1.10 Objectives of the research

Despite toxicity symptoms which are mainly under laboratory conditions and nutrient solution, ammonium nutrition of plants specially in vegetable production systems could have very important positive implications, from economic, environmental and nutritional point of view. This could lead to great changes in plant growth and production, specially in $CaCO_3$ containing soils which have a relatively high pH. Imbalanced nutrition frequently limits plant production under such conditions. Increasing new insights into ‘’ecological food production’’ and ‘’organic farming’’ need to stabilize ammonium in a soil on one hand, and to use natural plant-based substances such as NNIs on the other hand, as an environmental friendly step. There is high a potential for detection of plant based chemicals not only for NI, but also for fungicides and insecticides, as well. Therefore in the present research project, improving nitrogen management for vegetable production systems in calcareous soils through first: stabilization of ammonium in soil, and second: finding natural alternatives for synthetic nitrification inhibitors (e.g. from Brachiaria humidicola) are the main objectives. Therefore, first the effectiveness of chloride as an routine chemical compared to DMPP should get evaluated as an NI, then the production, release and efficiency of *Brachiaria* root exudates as well as root and shoot extracts as the main source of NNI should be investigated in the present work, and as a further step the role of ammonium and nitrate nutrition on tomato growth, and the effects of calcium on ammonium toxicity of tomato plants should be investigated.

Chapter 2: General Materials and Methods

In this chapter, a general description of plant materials, and plant cultivations as well as the general methods which were routinely used for this study in following chapters are provided. Other methods related to a particular experiment are described in the respective chapter.

2.1 Plant cultivation

In these series of experiments *Brachiaria humidicola* (Rendle) Schweick, accession 26159 (as a possible source of NI), and tomato as a target plant for investigation of NH_4^+ and NO_3^- nutrition effects, have been used.

2.1.1. Cultivation of *Brachiaria humidicola*

2.1.1.1 Cultivation from seeds

Seeds from Brachiaria humidicola (Rendle) Schweick (accession 26159) (BH) were rinsed in 10 mM $CaSO_4$ for 5h and germinated in fine quartz sands (0.2-0.5 mm) in growth chamber under 25 ˚C, and regularly watered with distilled water. Two weeks old seedlings were transferred to aerated hydroponic culture system with a half strength nutrient solution for four days.

2.1.1.2 Vegetative propagation

Due to difficulties in seed germination of BH plants (a long process with very low germination rate, about 14%), some of germinated seedlings from the first experiment were transferred to big soil pots (a mixture consist of TDK, soil, sand, 2:4:1) in greenhouse condition (20-26 ºC). These were kept as a source of new plants which they produce as new shoot or tiller. For the next experiments generally two weeks before starting any experiment, some of these plants removed from the soil, and using a sterilized blade separated to several single plants containing roots. In optimum condition 4-5 new plants can be produced and separated from one single plant. They transferred to NO_3^- or NH_4^+ nutrition medium, which new shoots (tiller) were produced at the crown of plants (junction section of root to shoot). After about 1 week under these conditions, with a size of about 10 cm, uniform seedlings again were selected and transformed as new plant for experimental purposes.

2.1.1.3 Hydroponic culture system

Plants after germination and four days in half strength nutrient solution transferred to full strength nutrient solution consisting of 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.1 µM $CuSO_4$, 0.01 µM $Mo_7O_{24}(NH_4)_6$, tomato15 and grass 83 µM Fe-EDTA, 0.7 mM K_2SO_4, 0.5 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2mM KCl, 2mM N as $(NH_4)_2SO_4$ or $Ca(NO_3)_2$, and for ammonium treatments 1 mM $CaCl_2$ was supplied to each pot.

The nutrient solution was replaced every 3-4 days and if it was necessary pH was adjusted to pH 6, using MES, H_2SO_4 0.5 M, KOH 0.5 M, or a suspension of 0.5 M $Ca(OH)_2$. Plants were grown under controlled conditions, in a climate chamber with a 16/8 h light regime, and 200 µmole/cm^2/s with a light/dark temperature regime 28/25 ºC, and 60% relative humidity. Plants had different ages at the time of harvest in each experiment.

2.1.2. Cultivation of tomato plants

2.1.2.1.Preculture

Tomato seeds (*Lycopersicon esculentum* var money maker) were rinsed in 10 mM $CaSO_4$ solution for 4 h, and germinated in quartz sand (0.2-0.5 mm), at 25 ºC in growth chamber. After germination two weeks old seedlings were transferred to half strength nutrient solution for 4 days, and later to full nutrient solution.

2.1.2.2.Hydroponic culture system

After transferring to nutrient solution, plants were grown under controlled conditions, in a climate chamber with a 16/8 h light regime, and 200 µmol/cm^2/s light intensity. Temperature regime was 28/26 ºC, with 60% relative humidity in growth chamber. The nutrient solution was replaced every 3-4 days.

Table 2.1. Concentrations of macro and micronutrients for hydroponic culture of tomato and *Brachiaria* plants (according to Walch-Liu et al., 2000).

Macronutrients	mM
N	2
K	2.5
Mg	1.2
P	0.5
Ca	2
Cl	1.2-3.2
S	3.2
Micronutrients	µM
B	10 (for Tomato), and 1 (for Brachiaria)
Fe	15 (for Tomato), and 83 (for Brachiaria)
Cu	0.1
Zn	0.5
Mn	0.5
Mo	0.01

2.2. Collection of root exudates:

Root exudates always were collected at 2 h after starting light period. Plants root first rinsed and washed with distilled water for at least 2 minutes, then they were placed in a small plastic beaker containing 500 ml distilled water for collecting root exudates, under continues aeration. Root exudates were collected for 6 (from 10am to 16pm) or 24 hours (from 10am to 10 am in the next day). Then they were transferred to 0.5 liter plastic bottles and kept in deep freezer (-20 ºC).

2.3. Collection of xylem exudates

At harvest for tomato plants, in the morning shoots were cut using a sterilized blade at 2 cm above the pot surface. Xylem sap collected using plastic tubes which have been fixed with Para film. Collection was conducted for 8 h during the light period. Regularly collected xylem sap removed to ependorf tubes using syringe and kept in -20 ºC for further analysis.

2.4. Plant harvest and determination of growth parameters

Quantitative characteristics of plants were measured during growth period, including chlorophyll content via SPAD meter, transpiration or water consumption, root and shoot overall length, and pH of nutrient solution as an indicator of root activity under nitrate or ammonium nutrition. At harvest plant separated in different parts such as shoot, root and leaves of different developmental stages (young leaves and old leaves). Fresh weight of shoots and roots were determined directly after harvest. Dry weight measured after plant material was dried at 60 ºC. Aliquot of these dry weights were used for mineral analysis.

Further, for Brachiaria plants, fresh materials were cut to small pieces by scissors and immediately were frozen in liquid nitrogen, grinded to a homogenates form with mortar and stored in –20 ºC for further application in nitrification bioassay.

2.5. Plant mineral analysis:

Harvested samples of roots and shoot (leaves) were washed and gently pressed between fine sorption paper to remove extra water, and then fresh weight was recorded. The samples were placed in the oven at a temperature of 60 ºC for 5 days. Dried samples were grinded using grinding machine into fine powder. Analysis of mineral nutrients in shoot and root tissues was performed after dry digestion of 100 mg dry weight at 500 ºC for 5 h in a muffle furnace. After cooling, the samples were extracted 2 times using 2 ml of 1/3 HNO_3 (v/v) and heated to dryness. The ash was dissolved in 2 ml of 1/3 HCl (v/v), and then diluted to 10 ml with hot deionised water. After addition 0.1 ml of Cs/La buffer to 4.9 ml ash solution Mn and Fe were measured. Other mineral elements such as Zn, Cu, and Mg were determined in original or diluted ash solution by atomic absorption spectrometry. While K and Ca by flame photometry, and C and N by Elemental analyser (Erb, Model NCS 2500) were measured.

2.6. Preparation of root exudates and tissue homogenates for characterization of nitrification inhibiting compounds

Frozen root exudates (500 ml) were melted at room temperature, filtered and concentrated using a rotary evaporator at 40 ºC and a relative pressure of 20-30 mb to 15 ml. Then 2.5 ml aliquots of this concentrated root exudates was used for nitrification bioassay.

Also freeze dried samples of root exudates were dissolved in 20 ml of methanol which later methanol was removed via rotary evaporator. It was finally extracted with DMSO as a final concentration of 0.8% in the samples.

Incubation of unextracted plant materials were conducted using 0.5 gram of frozen root or shoot homogenates, which was applied per sample soil (2.5 g soil) for determination of their potential nitrification inhibition effect.

For extraction, 4.0 g of root or shoot homogenates was extracted sequentially in mortar with hexane, ethyl acetate, ethanol and water, respectively, each one of two times extraction with 10 ml solvent. Distilled water (30 ml) was then added to each fraction and vacuum filtered, the whole mixture concentrated using rotary evaporator to 12.5 ml (for 5 samples). DMSO was added to a final concentration of 0.8% in sample. 2.5 ml aliquots of this concentrated applied per sample in nitrification bioassay.

2.7. Bioassay for nitrification potentials

The bioassay consists of rapid determination of nitrification inhibitory (NI) potential of single compounds or mixtures of compound via inhibition of nitrite formation in soils.

2.7.2. Principle

- Incubation of soils with ammonium sulphate and unknown test substances (NI) with potential inhibitory effects on nitrification (Kandeler 1993; Berg and Rosswall , 1985).
- Determination of nitrite formation after standardized incubation times in comparison with a control treatment without NI addition.
- Reduced formation of nitrite by inhibition of ammonium oxidation indicates NI activity. Nitrite oxidation during incubation is inhibited by addition of sodium chlorate (Belser and Mays, 1980).

2.7.3. Application

Testing of single substances or substance-mixtures for NI activity

2.7.4. Chemicals

- Sodium chlorate-solution (1.5 M)

  15.97 g $NaClO_3$ in 100 mL H_2O_{dist}. (final volume)
- Ammonium sulphate stock (13.3 mM)

  1.762 g $(NH_4)_2SO_4$ 1 L H_2O_{dist} (final volume)
- Ammonium sulphate solution (1.33 mM)

  100 mL stock solution + 900 mL H_2O_{dist}
- Potassium chloride solution (2 M)

  149.12 g KCl in 1 L H_2O_{dist} (final volume)
- Sodium nitrite stock (1000 mg NO_2-N/L)

  4.9257 g $NaNO_2$ in 1 L H_2O_{dist} as blank (final volume)

2.7.5. Laboratory equipments

- Biologically active soil with ammonium-oxidizing potential (standard soil, *Limburger Hof*, BASF).
- Horizontal shaker and balance
- Filtration unit (Kandeler, 1993), or lab centrifuge

2.7.6. Procedure

- Sieving dry soil ≤ 1 mm mesh, adjusted to 50 % of water holding capacity (1 kg dry soil + 145 mL H_2O_{dist}); again sieving ≤1 mm mesh
- Pre-incubation at room temperature 10-14 d to stimulate microbial activity

2.7.7. Test

- Transferring 2.5 g moist soil (see above) into a 50 mL erlenmeyer or plastic beaker
- + 2.5 mL NI containing solution
- + 7.5 mL ammonium sulphate solution (1.33 mM)
- + 0.05 mL sodium chlorate solution (1.5 M).
- Replicates, 4 samples is shaken for 5 h (or 24 h) with 200 rpm at 25°C for 24 or 50 h,
- One sample stored at –20 °C as blank.
- Control: Replacement of NI solution by H_2O_{dist}

- After 24 h (or 50 h), add 15 mL KCl solution, mix, filtration with Blue-Ribbon filters or centrifugation.
- Photometric nitrite determination via spectrophotometer (Kandeler, 1993), autoanalyser or HPLC (Vilsmeier, 1984)
- Calibration standards: 0 – 0.1 – 0.2 – 0.4 mg NO_2-N/L

2.7.8. Spectrophotometric determination of nitrite

- Ammonium chloride buffer (0.19 M, pH 8.5)
- 1 g NH_4Cl + 90 mL H_2O_{dist}
- adjust to pH 8.5 with NH_4OH_{conc}
- adjust volume to 100 mL
- A calibration curve with 0; 0.2; 0.4; 0.6; 0.8; 1.0 mg NO_2-N/mL) each 1 mL
- Colour reagent: Sulfanilamide: 4 mg + N-(1-naphtyl)-ethylendiaminhydrochloride (10 mg), dissolve in 10 mL H_2Odist, add 2 mL o-phosphoric acid, adjust volume to 20 ml

- Measurement: mix 0.5 ml soil extract + 0.3 ml NH_4Cl buffer + 0.2ml colour reagent
- Measure absorption after 15 min at 520 nm

2.7.9. Calculation

- Nitrite concentration without NI = 100 % (Mean of control samples minus blank) and nitrite concentration with NI (Mean of samples minus blank) = X %

2.8. Statistical analysis

Excel and SPSS softwares were used for analysis of data, and comparisons of means were conducted using one way ANOVA and Duncan test at level of ($p<0.05$). Results in the tables, text and figures are given as means ± SD.

Chapter 3: Efficiency of DMPP and chloride as microbial nitrification inhibitors in soil

3. 1 Abstract

Microbial oxidation of ammonium to nitrate has important implication for ecosystem functioning. Many chemicals including chloride can inhibit nitrification under soil and laboratory conditions. This study was conducted to evaluate the effects of chloride in comparison to DMPP on nitrification in the soil. Despite mixing with fresh soil extracts and 2 weeks pre-incubation, net nitrification started after 3 weeks incubation. DMPP specially with double concentrations inhibits nitrification for longer time until end of incubation period. Chloride as NH_4Cl or KCl also significantly inhibited nitrification compared to control until end of 7 weeks incubation. Specific ion and osmotic effects of chloride seems to be the reason of this inhibitory effect. The results suggest that nitrification process and responsible microorganisms are very sensitive to a wide range of chemicals and salts in the soil. For a new environment or following dry-wet cycles, population establishment of nitrifiers may not be achieved very easily. Adverse climatic and soil factors, as well as salt concentration have additive inhibiting effects on nitrification. Under such conditions many chemicals which typically are not inhibitors, can function as potent nitrification inhibitor. Slightly acidic condition, low organic matter and ammonium content, long dryness, all contribute to slow down population establishment of ammonium oxidizing bacteria in soil, leading to enhancing inhibitory effect of chemicals such as DMPP or chloride and for a longer time.

3.2 Introduction

Nitrogen fertilizers are one of the major destructive pollutants on the globe. Nitrification is the process in which a relatively immobile cationic form of nitrogen (NH_4^+) is converted into a more mobile anionic form (NO_3^-). This conversion has important implications for physical, chemical and biological functioning of soil and ecosystems, so it may also affect ecosystem productivity. In addition, low nitrate availability could influence composition of plant communities by favouring species that have preferential ammonium nutrition. Nitrate as the result of nitrification is in centre of almost the all N losses from the soil. Nitrogen as well as cation losses can occur due to nitrification, which finally leads to soil acidification as a consequence of higher H^+ concentrations in soil. In cultivation systems, control of nitrification for a limited time may help to apply the exact amounts of N-fertilizer which plants actually need (Bronson and Mosier, 1994; Patra et al., 2001; Subbarao et al., 2006b). Consequently, this leads to an increase in plant N use efficiency through reduced N application rates and numbers.

On the other hand, ammonium nutrition of crops under field condition might have positive implications for plant growth and development. Depending on soil conditions and more importantly plant species, these beneficial effects could be increasing N use efficiency, reduction in N losses and consequently environmental and health risk, a better and more balanced nutrition of micronutrients which specially are not present in available forms in soil (calcareous or high pH conditions), better utilization of unavailable phosphorus forms such as Ca-P or rock phosphate (Hinsinger et al., 2003; Onyango Gweyi, 2006), and more effective bioremediation of contaminated soils. However, NH_4^+-fertilizers after application to the field are oxidized to nitrate within few days (Abbasi and Adams, 2000; Azam et al., 2002). Blocking this oxidation in order to have stabilized ammonium in bulk soil, is achieved through application of synthetic nitrification inhibitors such as N-Serve, Dicyandiamide (DCD) and 3,4-Dimethylpyrazole phosphate (DMPP). To large extent, the efficiency of these compounds depends on their chemical structure, mode of action, dynamics inside the soil, and their resistance to biological and chemical degradation. DMPP however is one of the most effective and widely used nitrification inhibitor with high efficiency and no side effect (Weiske et al., 2001; Pasda et al., 2001; Irigoyen et al., 2003). Its typical dosage is 1% N-NH_4^+ which is too much lower than other inhibitors such as Dicyandiamide (DCD) (Zerulla et al., 2001).

Potential distinct advantages such as significant and more effective inhibition of nitrification and reduction of N_2O emission (Weiske et al., 2001; Zerulla et al., 2001; Hatch et al., 2005), and CO_2 emission (Weiske et al., 2001), increasing yield (Zerulla et al., 2001; Pasda et al., 2001; Linzmeier et al., 2001) and consequently increasing N use efficiency are attributed to DMPP, compare to other NIs. However because of simultaneous absorption of ammonium and DMPP to clay minerals (Pasda et al., 2001; Azam et al., 2002), the difference between kinetics of adsorption for NH_4^+ and DMPP regulates the effectiveness of inhibition. Therefore, it has the highest efficiency in light soils (Barth et al., 2001; Pasda et al., 2001).

Apart from environmental and economic point of view, stabilized ammonium in the soil also has other beneficial effects, mainly in vegetable production, where normally the amount of N uptake by crops exceeds its assimilation rate, therefore N accumulates as nitrate in leaves and other vegetative tissues. Nitrate reduction capacity of plants which is mainly a function of nitrate reductase activity is the main limiting step leading to nitrate accumulation. Upon consumption of these vegetable materials by human, serious problems regarding health specially with vegetable crops such as lettuce and spinach can occur. Therefore under

application of nitrification inhibitors, plants in their root medium have a higher ratio of NH_4^+:NO_3^-, as well as preferential uptake of NH_4^+ would lead to less nitrate accumulation.

Beside synthetic nitrification inhibitors, many chemicals can inhibit nitrification. Chloride, for instance with a concentration of 7-50 mM has been found to reduce nitrification rate in laboratory and field condition (Golden et al., 1980; Wickramasinghe et al., 1985; Darrah et al., 1987; Wade, 1997; McGuire et al., 1999; Chen and Wong, 2004). Despite chloride ions, salt concentration of soil and its osmotic pressure, also can suppress nitrification. Generally, there is a reduction in microorganisms activity with increasing salt concentrations (Darrah et al., 1985; Monaghan, and Barraclough, 1991). Darrah et al., (1985) with application of 7.3 mM kg^{-1} soil chloride found that ammonium chloride but not ammonium sulphate inhibits nitrification, because with ammonium sulphate the salt concentration in the soil solution was restricted by the precipitation of calcium sulphate. With different chloride salts, KCl, $CaCl_2$, and $MgCl_2$ and NaCl, Wade, (1997) suggested that chloride alone controls *Rhizoctonia* disease and chloride might be used for management of *Rhizoctonia* root and crown rot of sugar beet production, indicating antibiotic effect of Cl in the soil. Moreover, take all root rot (*Gaeumannomyces graminis*) in wheat is decreasing with applying chloride fertilizers (Christensen et al., 1981), suggesting a reduction in plant water potential could be the inhibiting effect of Cl on take all disease, because the rate of this disease decreases with reduction in plant water potential. This could be due to a reduction of nitrification and higher rate of NH_4^+/NO_3^- under application of chloride fertilizers (Christensen and Marcia 1985). In contrast Löffler et al. (1986) suggested that nitrite rather than ammonia may is responsible for the declining effect of ammonia-generating compounds on populations of *Fusarium oxysporum* sp. dianthi in soil. So, following these antimicrobial properties of chloride the present experiment was conducted to evaluate the effectiveness of DMPP and chloride on inhibition of microbial nitrification.

3.3 Materials and Methods

This experiment was conducted with a loamy soil which has been kept in dry condition for more than 20 years. This soil was mixed with fresh soil extract (0.66 l extract of 1 kg fresh loamy soil) from a long term experiment investigation (with a pH 6.3, P_2O_5; 161 mg /100g soil, K_2O;18 mg /100g soil, Mg; 12 mg/ 100 g soil, C; 0.2%, N: 0.23%), which was homogenously mixed to 4.5 kg dry soil, and pre-incubated at 50% of water holding capacity at constant temperature of 20 ^oC for more than 2 weeks to induce and increase its microbial activity. Water holding capacity of soil has been measured before starting, and the final moisture of soil in pots, has been adjusted to about 60% of its water holding capacity (WHC),

which is equal to 18% of the soil weight. After sieving the soil through 0.2 mm mesh, fertilizers were homogeneously distributed into the soil, and again several times passed through mesh. Added N was on the basis of 120 mg N-NH_4 as $(NH_4)_2SO_4$, or NH_4Cl.

Treatments were ammonium sulphate without DMPP (AS-D as control), ammonium sulphate with normal concentration of DMPP (1% of N-NH_4^+) (as AS+D), and with double concentration of DMPP, 2% of N-NH_4^+ (as AS+DD), ammonium sulphate + chloride (30.5 mg/100 g dry soil) as KCl (a solution of ~ 48mM Cl^-), and ammonium Chloride (NH_4Cl) (amount of chloride was ~ 2.5 times of N-NH_4).

DMPP were dissolved together with ammonium sulphate in distilled water and mixed homogeneously into the soil. The same procedure was carried out for chloride treatments. An amount of 59 g fresh soil (on basis of 50 g dry soil) was placed into the small 50 g plastic pots. The pots transferred to trays which were included a wet cover beneath the pots to maintain water status of pots more stable. Then the pots were closed firmly with bleu plastic cover for protection against water loss during whole incubation period, and they placed in growth chamber with 23/20 °C for exact period of time based on experiment condition.

However the water content of pots has been checked every 4-5 days and kept stable. The time course for sampling (0, 1, 2, 3, 5, 7 weeks) were chosen based on the expectation and experiment condition (23 ºC), as well as literatures results (Abbasi and Adams, 2000; Azam et al., 2002; Williams et al., 1998).

3.3.1 Analysis

Pots representing a defined sampling date were removed from growth chamber and immediately transferred to –20 ºC until further measurement. After weighing 30 g of fresh soil of each pot and transferring into a 100 ml plastic bottle, 30 ml of calcium chloride solution (0.025 M) was added to each bottle, (soil: solution of 1:1), and shacked for one hour with 200 rpm. Following this shaking period pH of each bottle was measured with pH meter (model MPC227, Mettler Toledo) while the whole soil solution was mixing using a magnet. Then the whole solution was filtrated using Schleicher and Schell (Dassel) blue ribbon filters (No. 110), with discarding the first drops of filtrate. From this filtrate, separate samples for determination of nitrate and ammonium were pipetted into ependorf tubes, (1 ml). Nitrate was immediately measured with nitrate quick test reflectometrically (three times for each sample) using the Merck-Reflectoquant Quicktest (Merck, Darmstadt, Germany). For determination of ammonium the samples first received 10 µl of (10 mM) formic acid for its stabilization, then stored at –20 ^oC and finally measured with HPLC. Excel and SPSS softwares were used for

analysis of data, and comparisons of means were conducted using one way ANOVA and Duncan test at level of ($p<0.05$). Results in the tables, text and figures are given as means ± SD.

3.4 Results

In present experiment a soil which for a long time has been stored in dry condition (more than 20 years) was used, however, before starting it mixed with fresh soil extract and incubated for more than two weeks. Two weeks incubation generally is enough for induction of bacteria activity inside the soil (Trevors, 1983), specially when it is combined with fresh soil extract. Original soil nitrate concentration was about 95 mg NO_3^- per kg dry soil, (21 mg N/ kg dry soil), with a pH of 5.8 (extracted with 0.025 M $CaCl_2$). Although the native concentration of NH_4^+ was not measured in the soil, however due to very low organic matter and long storage history of soil, its ammonium must not be in detectable size.

In contrast to expectations, net nitrification (measurable nitrate) started after three weeks (Fig. 3.1 and Fig. 3.2). There was no significant difference between treatments for soil pH, nitrate, and ammonium concentration at beginning of experiment. After one week incubation, treatments did not show any difference in soil pH, nitrate and ammonium concentrations, however pH and ammonium show some fluctuations between treatments. These fluctuations become more significant with progress of incubation time. At two and three weeks after incubation still there was no significant difference in terms of produced nitrate (net nitrification). Significant differences between double concentration of DMPP (2% of N-NH_4) and other treatments occurred after three weeks for ammonium concentrations in samples. It was only inside weeks four and five that net nitrification became visible. At this sampling date, while the concentration of nitrate in control (AS-D) was 238.1 mg kg^{-1} soil, it was about 15.4 and 101.8 for one and double application of DMPP, respectively, which was very similar to concentrations at beginning of experiment. There was also significant differences between chloride treatments compare to control, and these differences continued to the end of experiment. After four and five weeks of incubation, a significant negative correlation between pH and nitrate in soil samples was observed (Fig. 3.1). Ammonium concentration was highest for double application of DMPP, while it measured at lowest concentration for control (AS-D) at all sampling dates. When net nitrification was visible and detectable, always there was a positive correlation between pH and ammonium concentrations inside soil samples. Nitrate production negatively correlated with pH, and after weeks five and seven still there were significant differences between DMPP treatments and others, and also between chloride treatments and control. These differences expected to continue even after 8

weeks until the end and complete nitrification of applied fertilizer. However, at beginning we expected that full nitrification would be achieved in 4-6 weeks. Because of changes in ammonium as well as pH changes during first 3 weeks of incubation, despite undetectable net nitrification, gross nitrification has occurred, which became measurable after 3 weeks. Nevertheless, the trend of nitrification inhibition of treatments was in the order of: Control < ASKCl < NH_4Cl < DMPP≤ double DMPP.

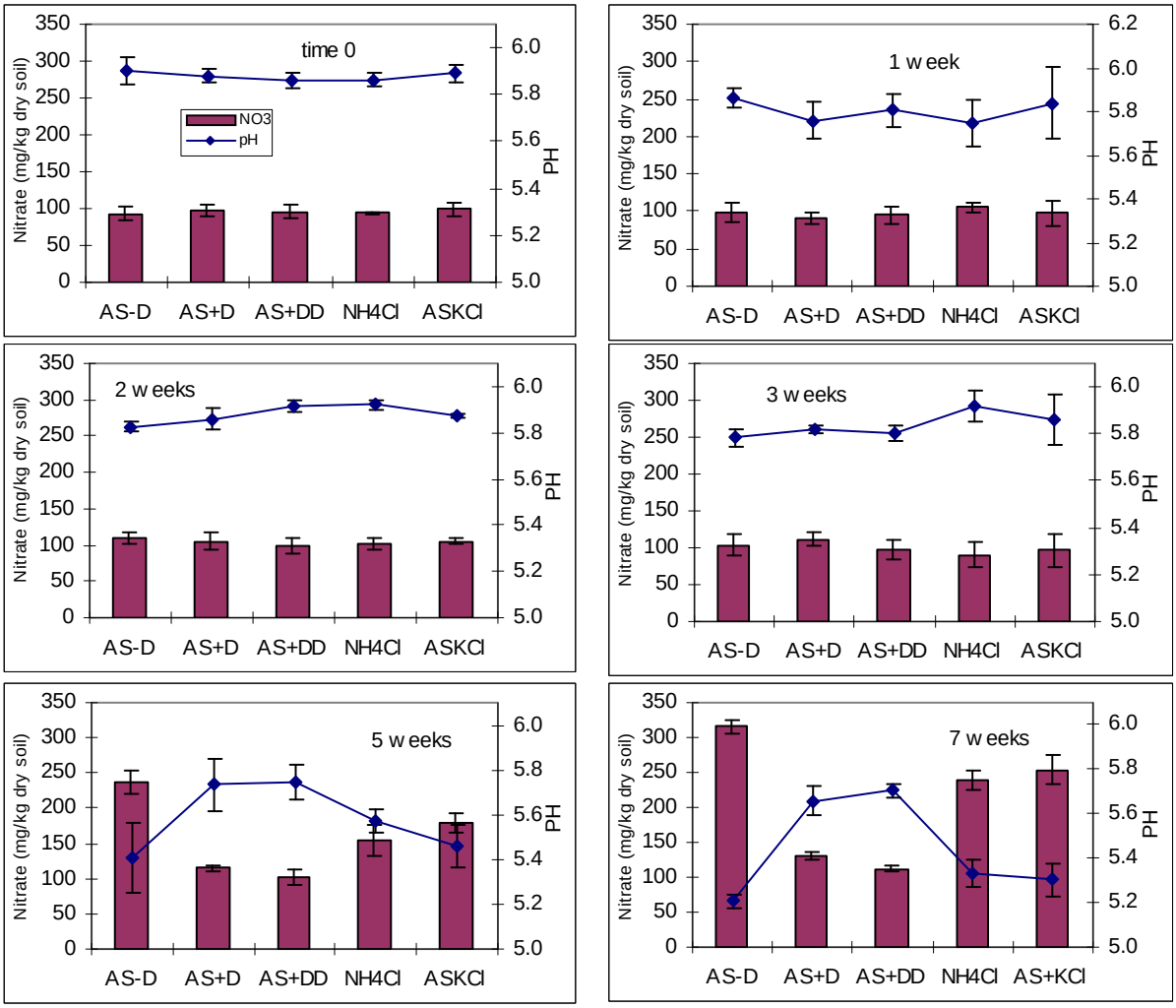

Fig. 3. 1. Nitrification or nitrate concentration and pH in soil samples of different treatments, from zero-time to seven weeks after incubation. Treatments are as AS-D= ammonium sulphate minus DMPP as control; AS+D= ammonium sulphate + DMPP; AS+DD= ammonium sulphate + double concentration of DMPP; NH_4Cl= ammonium chloride; ASKCl= AS + KCl.

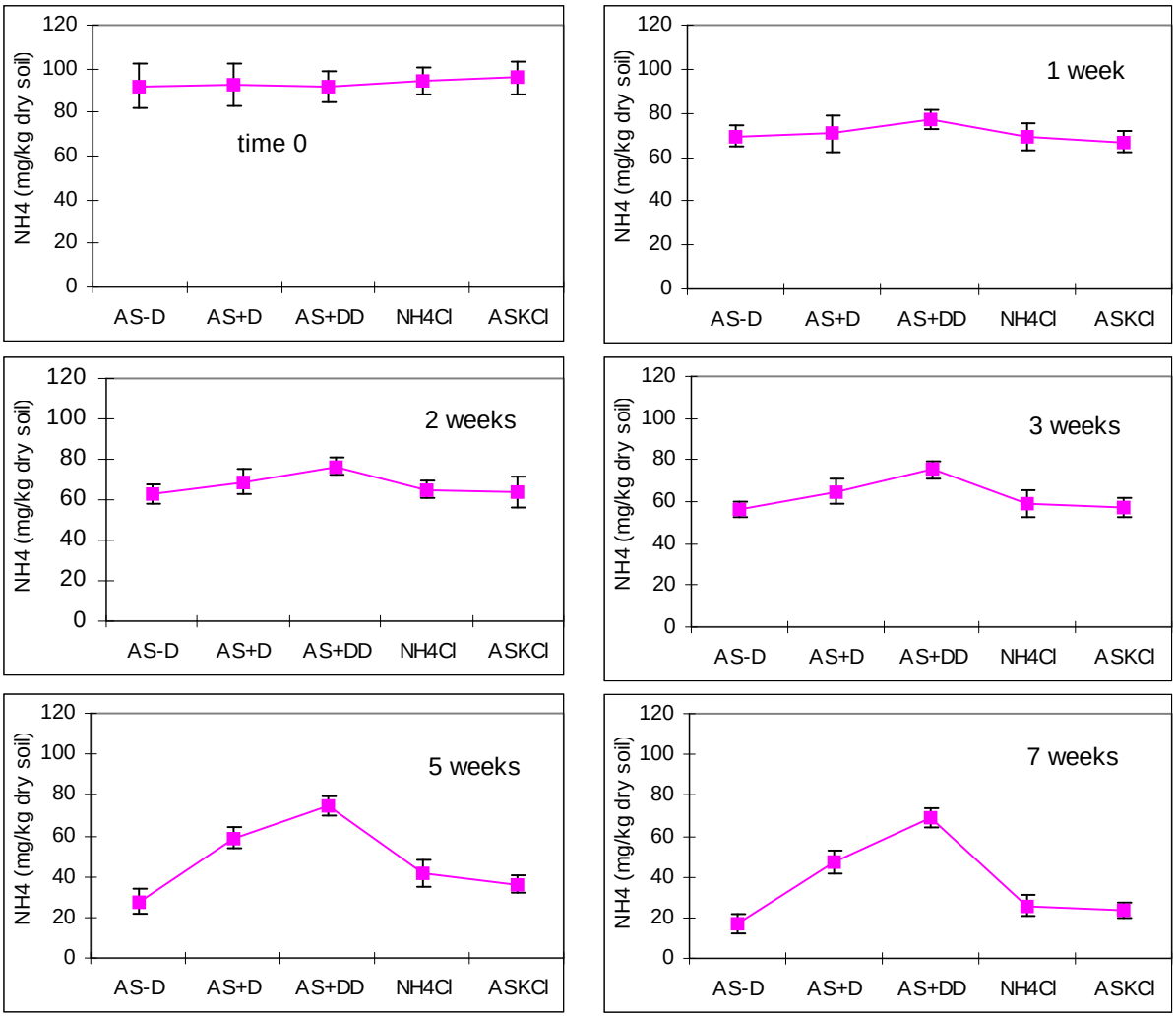

Fig. 3. 2. Ammonium concentration in soil samples measured from zero-time to seven weeks after starting incubation, extracted with 0.025 M $CaCl_2$. Treatments are as AS-D= ammonium sulphate minus DMPP as control; AS+D= ammonium sulphate + DMPP; AS+DD= ammonium sulphate + double concentration of DMPP; NH_4Cl= ammonium chloride; ASKCl= AS + KCl.

3.5 Discussion

Nitrification rate in soil is a function of vegetation and soil types and controlled by several factors, including temperature, moisture, pH, substrate availability, nutrient availability, and allelopathic effect, however, nitrification is highly heterogeneous in space and time even under a homogeneous vegetation cover (Ste-Marie and Paré, 1999; Abbasi et al., 2000b; Carlyle et al., 1990; Baldwin et al., 1983). The effects of these factors on nitrification are variable and it is difficult to predict the production of nitrate for a given soil under a certain situation. Moreover, biological processes in the field may be quite different from those in laboratory conditions. There is always a lag in net nitrification under laboratory soil

incubation. This is mainly because of low carbon availability, which could cause a relative change in nitrification and nitrate immobilization (Hart et al., 1994). In present experiment net measurable nitrate occurred after 3 weeks of incubation (Fig. 3.1). However, others indicate that active nitrification starts with a lag period of 4–10 days (Mulvaney et al., 1997; Williams et al., 1998) or even more than two weeks (Vitousek et al., 1982; Killham, 1994; Avrahami et al., 2003) following rewetting of soils. Also after soil rewetting and applying NH_4 fertilizers, ammonium preferably assimilated by microorganisms (Azam and Ifzal, 2006; Herrmann et al., 2005; Ste-Marie and Paré, 1999), while nitrate immobilization would occur in microsites where ammonium is not available (Davidson et al., 1992). However in presence of both nitrate and ammonium, NH_4^+ immobilization is faster than NO_3^-, and occurs in 12 h after adding fertilizer, instead of 48 h for NO_3^- (Azam and Ifzal, 2006). Moreover, remineralization of immobilized N starts in 48 h and is faster for NH_4^+ than NO_3^- (Azam and Ifzal, 2006). Despite adding 120 mg N-NH_4 per kg soil, only about 90 mg of that amount was detectable as ammonium at zero time, just after application of treatments (Fig.3.2). This can be explained due to NH_4^+ fixation by clay minerals, volatilisation as NH_3, or inefficient extraction of ammonium, which has been done with 0.025 M $CaCl_2$.

In this experiment ammonium limitation to nitrifiers could not play any role, because a 120 mg N-NH_4 per kg soil is enough to always have a free portion of NH_4^+ in soil solution. However, under field condition these nitrifiers have little chance due to their normally poor ability to compete with roots, mycorrhizae and decomposers, for ammonium (Neumann, 2007). Despite specific inhibiting effect of DMPP on enzymology of bacteria, molecule of DMPP, itself, maybe absorb N due to CH, CH_3, CN chemical bounds, however the applied amount of DMPP was not high enough to fix NH_4^+, NO_2^- or NO_3^- significantly. All these direct and indirect effects (including inhibition and fixation) could be possible explanation for low nitrification in present experiment or under field condition. A lag in starting nitrification is not avoidable until the ammonium oxidizer populations become well established (Vitousek et al., 1982), this is mainly because these organisms have a long generation time (Killham, 1994). Similarly in this experiment net detectable nitrification started very late, after 3 to 5 weeks incubation.

Soil drying and rewetting which frequently happening in surface soils, is a common stress for the microorganisms (Fierer et al., 2003). Long drying history of the soil in this experiment as well as other factors including low organic matter content might be the main reason of this delay. Drying even for a short time may induce lysis and losses of microbial biomass and consequently, this also may influence microbial community composition. This change in

microbial composition, on the other hand, is generally depends on plant species (Fierer et al., 2003). It is well known that in soils with low organic matter content, normally there is a lag of starting nitrification which might be more than two weeks (Vitousek et al., 1982; Killham, 1994; Avrahami et al., 2003). Furthermore, generally there is positive correlation between soil organic matter and microbial biomass (Chander et al., 1997). So nitrogen mineralization and nitrification appear to be limited by soil low organic matter, as well as microbial establishment. Similarly in this experiment, low organic matter content, as well as low ammonium content of soil could partly explain the lag of three weeks net nitrification. Therefore, time consuming adaptation of nitrifying bacteria to new soil conditions is important factor to be considered.

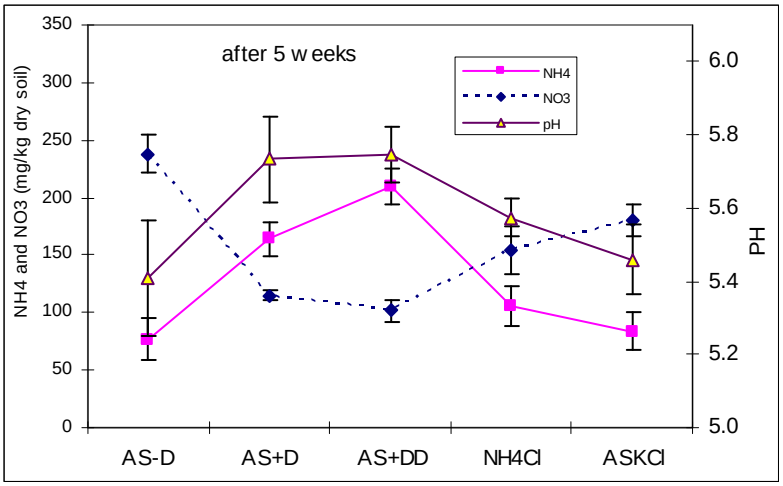

Fig. 3. 3. PH, nitrate and ammonium concentrations of soil samples after five weeks incubation. Nitrogen was 120 mg N-NH_4^+ per kg soil.

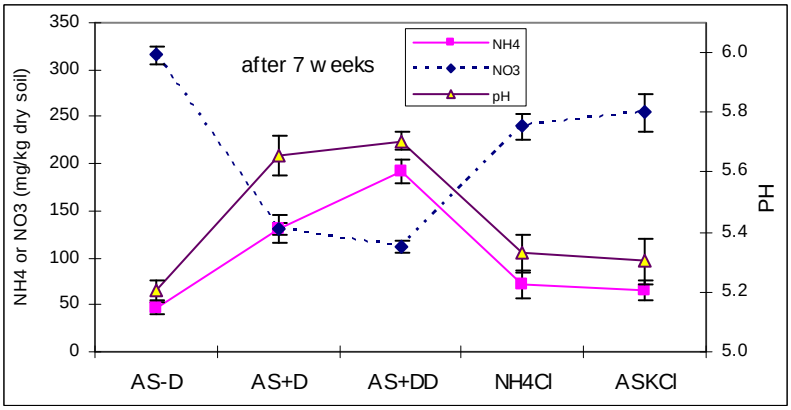

Fig. 3. 4. PH, nitrate and ammonium concentrations of soil samples after seven weeks incubation. Nitrogen was 120 mg N-NH_4^+ per kg soil.

Nitrification has been inhibited in all treatments specially in first 3 weeks, however ammonium concentration showed a steady reduction with incubation time. This may be partly due to ammonium fixation in soil clay minerals, and microbial immobilization. Uptake and immobilization of NH_4^+-N is reported to be more than NO_3^--N, while remineralization of immobilized N is slower in NH_4^+- in soil (Azam et al., 1986; Herrmann et al., 2005; Azam and Ifzal, 2006). Long dryness, slightly acidic pH, low organic matter and ammonium content of soil leads to very slow establishment of nitrifying population which can result in low net nitrification. Moreover, population shifts can simply occur over any change on soil or medium condition (Avrahami et al., 2003; Horz et al., 2004). So depend on condition, ammonia oxidizers may need several weeks to adapt to a new soil condition through changes in the community structure (Avrahami et al., 2003; Vitousek et al., 1982; Killham, 1994). Adaptation of microbes to new soil condition is a time consuming phase, therefore it takes longer time for bacteria to reach a population resulting in net nitrification. DMPP strongly inhibited nitrification and after 7 weeks of incubation, still there were significant differences between DMPP applications and other treatments. Normally higher dosage of nitrification inhibitors increases NH_4^+ concentration in soil (Chaves et al., 2006). DMPP specially with double concentration extended the presence of ammonium in soil more strongly, this effect also confirmed by other authors (Weiske et al., 2001; Zerulla et al., 2001; Hatch et al., 2005, Pasda et al., 2001). Nevertheless, in South Germany, tobacco and grape farmers have complain about effects of ENTEC (a DMPP containing NH_4^+ fertilizer) on plants, with symptoms of yellowing in tobacco farms and winter damage to grape orchards. This could be due to DMPP dynamics in the soil, its adsorption in heavier soil and its strong effect in light soils. On the other hand, applied concentration of chloride (30.5 mg/ 100g soil) in soil also significantly decreased nitrification activity compare to control, indicating efficiency of chloride in inhibiting nitrification. Nitrate concentration and pH presented a better indication of nitrification. These parameters, however showed high correlation with proceed in nitrification time, and pH always correlate negatively with amount of produced nitrate (Fig. 3.1, Fig. 3.2 and Fig. 3.3). This is always the reason of acidification of soils in tropical and subtropical regions, which through washing nitrate and basic cations out of soil, soil conditions getting worse.

Original pH of the soil was about 5.8, a relatively acidic pH for soil nitrifying bacteria, which over nitrification it reduced lower in control to near 5.40 and 5.30 after five and seven weeks, respectively. Soil pH can significantly affect microbial activities (Trevors, 1983), with a full inhibiting effect on nitrification at pH lower than 4.5 (Ste-Marie and Paré, 1999). This may

has an additive effect to low organic matter content of soil regarding limitation of bacterial establishment, and potentially could limit nitrification more strongly. Nitrification inhibition under low pH and acid soil condition is well known (Wickramasinghe et al., 1985; Golden et al., 1980; Ste-Marie and Paré, 1999; Curtin et al., 1998). When pH is the limiting factor for microbial activity, other factors can not improve nitrification. Therefore, pH appears as an important regulator of net nitrification (Ste-Marie and Paré, 1999; Carlyle et al., 1990). Soil pH, apart from its direct effect on bacterial activity can also indirectly affect nitrification rate by influencing soil physical, chemical and biological properties. Most of chemical reactions in soil are specially well known to be influenced by pH (Ste-Marie and Paré, 1999; Curtin et al., 1998). Normally, under field condition there is an increase in N mineralization and net nitrification after adding lime to acid soils. This is always coupled with an increase in CO_2 evolution, which is an indicator of soil biological activity (Curtin et al., 1998; Ste-Marie and Paré, 1999). If other factors remain stable, in semiarid areas of the world high calcium content could stimulate nitrification through increasing soil pH. Presence of high concentration of chloride ions (>20 mM) may cause acidity and pH reduction even in neutral soils (Wickramasinghe et al., 1985), however, in present study chloride had no effects on soil pH (Fig. 3.1). The role of pH in slightly acidic soils may be more important, because nitrification finally results in more acidification of soil. These additional H^+ ions which produced over nitrification can limit the process of ammonium oxidation, at least by (nitrozomonas) bacteria. Most of toxins and heavy metals become more available in low pH (exactly the points which bacteria start to show less activity), compare to neutral or basic pH. Generally microbial biomass and enzyme activities inside the soil decrease with increasing heavy metal pollution, but the amount of reduction differ among the enzymes. Enzymes involved in the carbon cycling were least affected, whereas enzyme activities related to N, P and S cycling, showed a considerable decrease in activity (Kandeler et al., 1996). Soil nitrifying bacteria are very sensitive to a high range of contaminants (Kandeler et al., 1996; Hu et al., 2002), so lower pH also indirectly via more availability of heavy metals, can also suppress nitrification.

In this experiment chloride significantly retarded nitrification in soil samples compare to control. Chloride form of fertilizers can suppress nitrification, particularly when fertilizer is locally applied in precision farming, and this effect has been long recognized (Golden et al., 1980; Darrah et al., 1987; McGuire et al., 1999). Chloride is believe to increase osmotic pressure of the soil more than other onions such as sulphate (Darrah et al., 1987; Golden et al., 1980), so it has been suggested that increasing solute concentration and osmotic pressure of soil solution with application of chloride could be the inhibiting effect of this chemical on

nitrification. With increasing osmotic pressure the rate of nitrification decreases (Darrah et al., 1987; Low et al., 1997). The extent of inhibition is a function of osmotic pressure of the soil solution and the osmolite properties of fertilizer used. However in some experiments even low concentration of chloride inhibited nitrification, which does not seem to be an osmotic effect (Darrah et al., 1987; Golden et al., 1980). Our results support the specific role of chloride ions on nitrification rather than a mere role of osmotic properties, so the role of osmotic pressure is second to it. Similarly in studying ammonium chloride, ammonium sulphate and sorbitol Golden et al., (1980) concluded that specific ion effect together with solute concentration (osmotic pressure) can inhibit nitrification more effectively. Chloride and its different derivatives can act as strong oxidizers and potent biocides (Chen and Wong, 2004), and in nitrification test chlorate is used at very low concentration (7mM) to inhibit nitrite oxidation (Kandeler, 1993). Undergoing some (chemical or biological) changes in soil, may chloride (Cl) produces other compounds which similar to chlorate or chlorite are toxic for nitrifying bacteria!. Even low concentration of Chlorite (ClO_2^-, 0.05mg/L) can inactivate ammonia-oxidizing bacteria in several hours, and with a higher concentrations, it inactivate all ammonium oxidizing bacteria just in 30 minutes (McGuire et al., 1999). All these factors including: low soil pH and organic matter, chloride specific and osmotic effects, and adaptation period for colonizing bacteria, and even competition between nitrifying bacteria and other microorganisms, and immobilization of small amount of nitrate which may produced in first weeks of incubation, could explain the lag period in starting net nitrification in this experiment. Furthermore, high concentration of NH_3 or NH_4^+ in soil solution can inhibit nitrification and it has been shown that NH_4^+ injection into the soil inhibits nitrification very strongly (Pang et al., 1973). So ammonium chloride may be an effective fertilizer for application in agricultural systems, where chloride is not a problem, and regarding nitrogen use efficiency it may has higher recovery rate compare to sulphate forms. This equals to 390 kg Cl/Kg^{-1} soil in 10 cm soil surface, however on basis of Cl/N similar to our experimental case, an amount of 150-250 kg per hectare is enough to have significant nitrification inhibition. In arid, semi-arid or Mediterranean-type ecosystems, particularly soil surface which is the main place of microbial activity, may undergo long periods of dryness (Fierer et al., 2003). These soils might have low nitrification rate, and nitrification probably will not start immediately just after raining. The results of present study suggest that following frequent wetting-drying cycles, or long drying periods of soil, biological activity may not be established very simply. Therefore, in many soils in arid and semi-arid regions around the world, where raining is not distributed equally around the year or irrigation rates are not

enough, similar situation (limited nitrification) may exist. All soils normally have some amount of chloride as original concentration, which nitrifying bacteria are adapted to it. Could be a critical level above which the activity of these bacteria may be affected severely. Therefore in various soil, nitrifiesrs may differently respond to chloride concentrations. However it seems that even the effect of slight concentration of chloride at the beginning of population establishment could have significant inhibitory effect. This should be the case also for other chemicals such as heavy metals through their specific and osmotic effect (Kandeler et al., 1996).

3.6 Conclusion

There was a delayed nitrification (2-3 weeks lag phase). Chloride at applied rate (30.5 mg/100 g soil) had a distinct lower inhibiting effect than DMPP at rate 1 or 2. However, no difference between KCl and NH_4Cl was observed. Double amount of DMPP as suggested by BASF had a stronger inhibition. In whole incubation period there was a close correlation between nitrification and decrease of pH.

Under optimal conditions ammonium fertilizers after application into the soil oxidize to nitrate within few days. Nitrate as the outcome of reaction represents a challenging global pollutant, however this process and responsible microorganisms are very sensitive to a wide range of chemicals and salts in the soil. Adverse climatic and soil factors, as well as salt concentration have accumulative or additive inhibiting effects on nitrification. Therefore, under unfavourable conditions besides potential inhibitors such as DMPP, DCD or Cl, chemicals which typically are not inhibitors, can function as potent nitrification inhibitors. Slightly acidic condition, low organic matter and ammonium content, long soil dryness, all contribute to slow down population establishment of ammonium oxidizing bacteria in soil, leading to enhancement of inhibitory effect of chemicals such as DMPP or chloride and for a longer time. Finally, while the mechanism of nitrification inhibition by chloride is not clear, it could be mentioned that reactivity and highly oxidizing properties of chloride and its different compounds which would produced in soil over their application and their biochemical changes, might be a possible reason.

Chapter 4: Effects of root exudates from *Brachiaria humidicola* on nitrification

4.1. Abstract

Nitrification inhibitory (NI) of climax ecosystems has been suggested for decades. However this inhibitory effect seems to be a feature of wild genotypes rather than commercial cultivars. Many plants particularly grasses have been shown to have this NI activity, and recently Brachiaria genotypes specially B. humidicola has been suggested and attracted for its role in inhibiting nitrification, through root exudates. In this chapter during a series of experiments it has been shown that B. humidicola root exudates when collected in distilled water, independent of light intensity, plant age, N-form, and N-concentration, had no inhibitory effect on nitrification. However, when root exudates collected in a medium containing 1 mM NH_4Cl, it shows significant nitrification inhibition. A passive secretion, due to root cell membrane damage as consequence of low pH of collecting medium, possibly is involved. This is more supported by the fact that this inhibitory effect is a function of longer collection period (24h instead of 6h).

4.2. Introduction

Precise nitrogen management requires enough knowledge regarding spatial distribution of mineral nitrogen. If plants themselves could precisely manage nitrification, it could offer very important economic and environmental implications. Finding such plants and related physiological and molecular characteristics can help to introduce such highly valuable properties to farming crops. This consequently leads to less application rate of fertilizers and higher N recovery rates. Plants can not move from their place, but instead they can use different strategies to coupe with unfavourable conditions surrounding them and to change these unfriendly conditions optimal for their growth and development. Despite physiological and morphological changes, exudation of primary and secondary metabolites by roots as well as emission of chemicals through leaves is well known phenomena in this case. Plant roots with their activity can change the physical, chemical, and biological conditions of rhizosphere. These changes have important implications for plants in terms of nutrients acquisition and toxins degradation which occur in root medium (Neumann 2007). Plants can allocate 20-60% of their photosyntesis fixed carbon to roots (Neumann 2007). Nevertheless, root exudates normally comprise 5-10% of net fixed carbon in plants (Jones et al. 2004). Similar to any other plant-related phenomena, root exudates quantity and quality could be influenced by climatic and soil factors, as well as plant physiological status. Moreover, root exudation patterns are controlled by plant, microbial and soil factors (Meharg and Killham,

1995). Plant age and developmental stage are very important in terms of root exudates quantity, and normally root exudates decreases with increasing plant age, specially after flowering which is coincidence with a reduction in microbial number (Liljeroth and Baath, 1988). Root exudation has positive correlation with root growth, however, after flowering generally root growth stops, therefore no exudation after flowering and senescence may occur. This indicates that actively growing root systems is very critical for a considerable root exudation. Any rapid and great change, particularly in root medium, could also through leakage of ions and metabolites or change in permeability of membranes modify root exudates composition. It is quite important also to notice that during procedures of collection, concentration and extraction of root exudates, degradation or inactivation of NNIs may happen.

The N-form (nitrate or ammonium) and their ratios in rhizosphere can have a nutritional important effect through changes in pH, which generally is the main factor controlling nutrients availability and uptake in most of the soils. External medium pH may change the ionic states of compounds released from the roots (Subbarao et al., 2006a, 2006b and 2007a), and therefore these compounds may be, absorbed again by plant roots, fixed to organic and inorganic soil particles, deactivated by microbial activity (Jones and Darrah, 1996), or they can occur biologically very reactive inside soil solution. Nutrient deficiencies such as P and Fe deficiencies always increase the rate of exudations. This is through release of organic compounds such as organic acids, phenolics or phytosiderophores, aiming at increasing availability of limiting nutrients (Neumann, 2007). Furthermore, soil physical and chemical characteristics are one of the main determinants regarding effectiveness of root exudates specially on microbial activity. Temperature, soil moisture content, pH and oxygen availability directly and indirectly through influencing microbial activity in soil, can affect the dynamics of root exudates in rhizosphere. Under field conditions, these factors can also influence plant growth and consequently root exudation, in turn.

Brachiaria plants are C_4 species and among the most important pastures widely adapted to grow in tropical and subtropical parts of South America, Africa and Asia (CIAT, 1983). Different Brachiaria species consist of 85% of total planted pasture area in South America (Nakamura et al., 2005), so commercially they are very important economic player in the tropics, particularly in Brazil. The genus Brachiaria contains a wide range of species, which have been adapted to poor acidic soils and even are tolerant to drought and harsh environments. Huge diversity in Brachiaria plants could be a main reason of their adaptation capacity to different edaphic and climatic conditions. Under such conditions, compare to any

other plants, they have relatively higher biomass production, and this is mainly because of their ability to uptake and use nutrients very efficiently in these poor soils (Nakamura et al., 2005; Cazetta and Villela, 2004). Meanwhile throughout tropical America B. *brizantha* Stapf cv Marandu, B. *humidicola* Schweick and B. *decumbens* Stapf are the most important of these species (Mergulhão et al., 2002; Bernardino, 2002). In addition, the *Brachiaria* improvement programs focus on developing commercial cultivars that combine high level of resistance to biotic and abiotic stresses such as adaptation to low soil fertility and high aluminium, and tolerance to drought.

It has been observed during field surveys that soils of BH generally have low levels of N-NO_3^- (CIAT, 1985; Sylvester-Bradley et al., 1988). Later in recent years it has been shown that root exudates of *Brachiaria* plants specially BH (accession 26159) can efficiently suppress nitrification process in laboratory, soil and field conditions (Ishikawa et al., 2003; Wang et al., 2005; Subbarao et al., 2005; 2006a, 2007a, 2007b). During a series of publications these authors indicated that only BH suppressed nitrification in soil, and this inhibition occurs only under NH_4^+ rather than NO_3^- nutrition. They suggested that nitrification inhibition abilities are a plant specific reaction to stress conditions, specially under low N level in the soil (Ishikawa et al., 2003; Subbarao et al., 2007). The differential plant effects on potential nitrification may not necessarily influence the gross microbiology of the soil, but may affect physiologically distinct sub-components of the microbial biomass (Wheatley et al., 1990).

Similar to other plant species, BH plants can use both forms of nitrate and ammonium. However, this plant is one of the most adopted species to ammonium nutrition with special ability for ammonium uptake. It has also high efficiency in terms of nitrogen uptake under low N condition (Nakamura et al., 2005; Rao et al., 2001). However Rao et al., (2001) showed that with increasing NH_4^+ concentration in medium, growth of BH increases, whereas growth of B. brizantha and B. decumbens inhibited. Under N limited condition, in natural or agricultural soils nitrate absorption by plants is more important. Since long time it has been known that plant based substances could have nitrification inhibitory effects (Moore and Waid 1971; Sahrawat et al., 1987; Lodhi 1978; AlSaadawi 1988; White 1988; Sylvester-Bradley et al., 1988; White 1994; Subbarao et al. 2005, 2006b, 2007d). Also allelopathic effects of plants and microbes have been well known, particularly under laboratory conditions. Many of compounds originated from plants are potential allelopatic to other organisms or even neighbouring plants (Inderjit, and Keating 1999; White, 1988). Terpenoids in leaf litter of pine trees (White, 1988), phenolics, alkaloids, and fatty acids (Gopalakrishnan

et al., 2007; Subbarao et al., 2007c) has been reported for most of these biocide activities. Most of the effects observed, are mainly from water extract phase of plant materials tested on specific plants (for example germination inhibition of lettuce seeds, as a model), or microbe. However there is not enough publication on effects of plants root exudates on nitrification. Subbarao et al., (2006, and 2007) showed strong inhibition of collected root exudates of BH on nitrification which this inhibitory effect was lasting up to 70 days, more efficient than synthetic NIs such as N-Serve and DMPP. DMPP, N-serve, and DCD are three main commercial nitrification inhibitors with highest efficiency on inhibiting nitrification and N_2O emission. However the effect of these inhibitors under standard condition would not be more than 4-6 weeks (Zerulla et al., 2001; Pasada et al., 2001). However, the biological inhibition of nitrification by crop plants or pasture species is not well known, and still there are too many questions need to be answered. To test whether the reported release of NIs is a passive or active phenomena, in this study nitrification inhibitory effects of root exudates by BH, which have been grown under different pre-treatments and growing conditions such as N-form and N-concentrations, light intensity and plant age , have been investigated.

4.3. Materials and Methods:

Seeds from *Brachiaria humidicola* accession 26159 germinated at 25 ºC in fine sands (0.2-0.5 mm diameter). Germination period was very long (4 weeks), with low rate of heterogeneous germinated seedlings (~15%). After reaching a size of about 10 cm (2-3 leaves) they transferred to treatment conditions in nutrient solution, containing NH_4^+ as $(NH_4)_2SO_4$ or NO_3^- as $Ca(NO_3)_2$, with 1, 2, or 4 mM N, depends on experiment. However normal N concentration was 2 mM N. Ammonium treatment conducted since the beginning of seedlings growing or when plants supposed to receive short-time (1-2 weeks) NH_4^+. In this case, first they received 2 mM N-NO_3^- as pre-culture for 2 weeks, after which they transferred to NH_4^+ (2mM N). Different variable factors such as light intensity or plant age on production and release of NIs were tested. In treatments with pH control, a solution of 3 mM MES and two times checking pH in the morning and in afternoon and adjusting with KOH, $Ca(OH)_2$ and H_2SO_4 were performed.

Different light intensity situations were applied using the same growth chamber, in which for high light intensity (400 μmol/cm^2s^{-1}), plants were placed at centre of table in growth chamber, and in positions which light intensity was constant. For intermediate light intensity plants placed at the corner of growth chamber, where light intensity was 240 μmol/cm^2s^{-1}. Low light intensity was achieved in same growth chamber (same growth conditions except light intensity) by locating plants under different layers of plastic inside a box, where they

received 180 µmol/cm^2s^{-1} light intensity. Plants were grown under a light/dark regime of 16/8, and a temperature of 28/25 ºC in nutrient solution culture. Nutrient solutions were changed every 3 days.

Root exudates were collected 2 h after starting light period, for 6 hours from 10 am to 4 pm, or for 24 hours from 10 am to 10 am in next day. Collecting medium generally was 500 ml distilled water, or in one case similar to procedure done by Subbarao et al., (2006a and 2007a) it was 1mM NH_4Cl, however before collection, plant roots were washed in distilled water for at least 1-2 min. After collection root exudates were concentrated at 35 ºC using rotary evaporator to a volume of 15 ml, which 2.5 ml of it was applied per replicate in our Bioassay test (chapter 2). Four sample (replicates) for incubation and two samples which were kept at freeze condition for original NO_2^- concentration inside the soil and samples, were used.

4.3.1. Soil incubation

- 2.5 g of an activated fresh standard soil were placed in a plastic bottle,
- adding 7.5 ml of 1.33 mM $(NH_4)_2SO_4$ sollution to each bottle
- adding 50 µl of $NaClO_3$ 1.5 M for blocking NO_2^- oxidation by Nitrobacters
- adding 2.5 ml distilled water in the case of control or 2.5 ml of concentrated or extracted root exudates for treatments
- The mixture then was shaked at 200 rpm for 24 h, which later extracted with 7.5 ml of 2M KCl, filtered and measured photometrically.

4.4. Results

The results presented in this chapter are extracted from a series of different experiments which were conducted in growth chamber. In the first experiment which plants were originated from seeds, germination rate was nearly 15% and very heterogeneous in terms of germination time (1-4 weeks) and form of plants. So in the next experiments plants grown as new cuttings, separated from pot plants, were used for propagation. The same way was used by Subbarao et el., (2006a and 2006c). Effects of various treatments and growth factor on release of NNIs through root exudates of BH are presented as follow.

4.4.1. Effects of DMPP concentrations

DMPP (3,4-Dimethylpyrazole phosphate) was used in all incubation experiments as a standard control. At beginning (Fig. 4.1.) a concentrations of 1, 10, 50, 100, 250, and 500 times more than prescribed dosage (1% of N-NH_4) were tested. Concentration of 1% of N-NH_4 is not effective in our bioassay (data not shown).

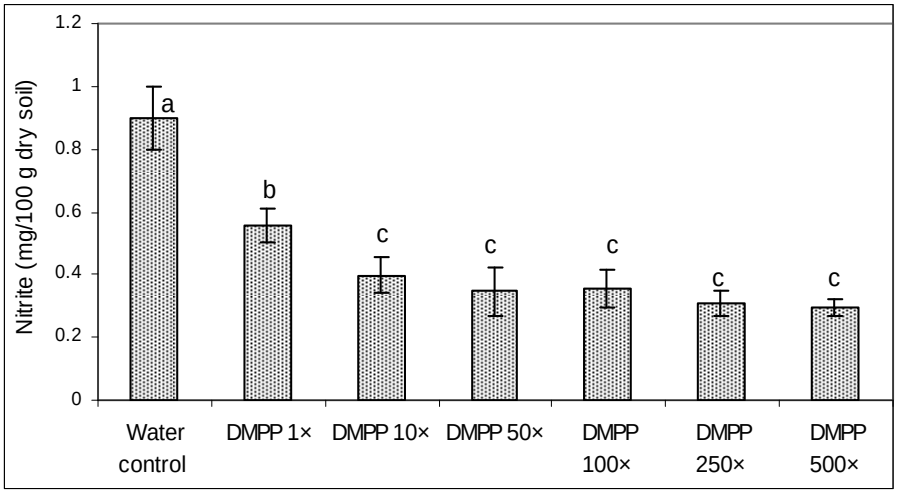

Fig. 4. 1. Nitrification inhibitory effect of different concentrations of DMPP shown as, magnitude of the normal concentration (1% N-NH_4^+), compare to water control (in 50 h incubation). Data are average of 4 replicates ± SD.

4.4.2. Effects of N forms and pH of pre-culture solution

Root exudates of plants which have been grown in nutrient solution under NO_3^- and NH_4^+ or under NH_4^+ with buffered pH of 6 (Fig. 4.2. A), and have been collected for 24 h, showed some inhibiting effect on nitrification (lower nitrite production), but only in tendency. Plants grown with buffered-NH_4^+ showed higher inhibition. However, compare to water control or nitrate grown plants this inhibition was not significant.

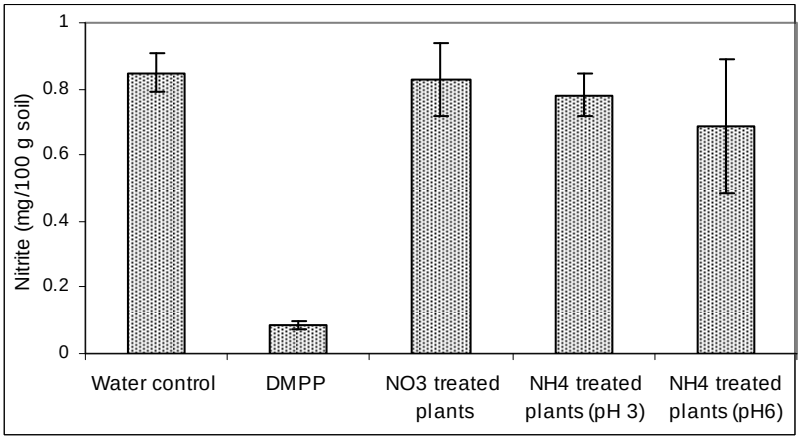

Fig. 4. 2. Nitrification inhibitory effect of root exudates of plants treated with NH_4^+, NO_3^-, or buffered-NH_4^+, collected in 500 ml d-water for 24 h (24 h incubation). Data are the average of 4 replicates ± SD. DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+. PH of medium after collection was ~ 3.5 for NH_4^+ and ~ 7 for NO_3^- plants.

4.4.3. Effects of plant age

The effects of root exudates of young plants (three weeks old) compare to old plants (seven weeks old) are presented in (Fig. 4.3). NH_4^+ grown plants particularly for young plants showed inhibitory effects despite this inhibition is not significant (Fig. 4.3.A and Fig 4.3.B).

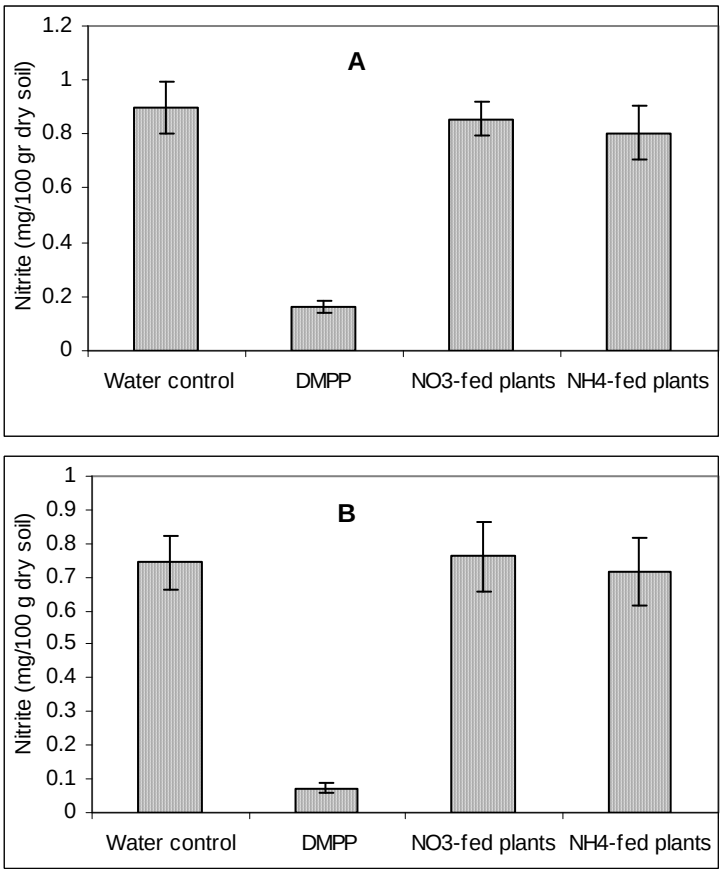

Fig. 4. 3. Effects of root exudates of 3-weeks young (A) and 7-weeks old (B) plants grown under 2 mM N-NO_3^- or NH_4^+, the pH of nutrient solution for both adjusted to 5 using MES and H_2SO_4 or KOH. Data are the average of 4 replicates ± SD. DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+. PH of medium after collection was ~ 3.5 for NH_4^+ and ~ 7 for NO_3^- plants. Root exudates collected in 500 ml d-water.

4.4.4. Effects of collecting period

In Fig (4.4) the effect of root exudates collected for 6 or 24 h on nitrification is shown in comparison with DMPP as standard nitrification inhibitor. From this figure it gets obvious that independent of N-form during pre-culture and collection time, no nitrification could be achieved.

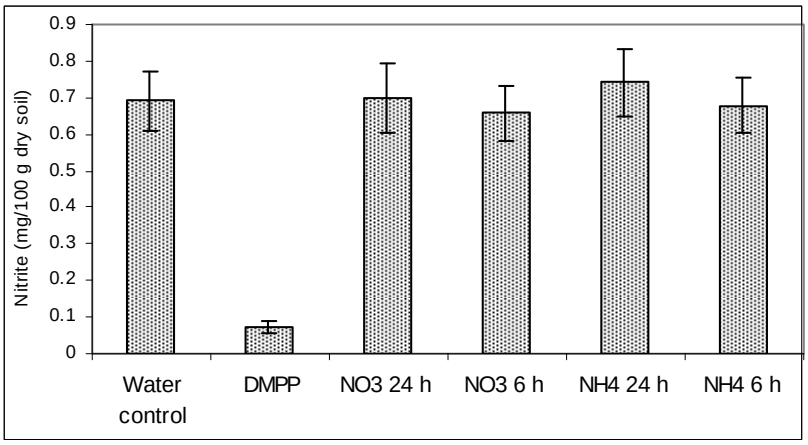

Fig. 4. 4. Effects of root exudates collected in d-water for 6 or 24 h, from plants pre-cultured with ammonium or nitrate (without pH adjustment during both pre-culture and collection). Data are the average of 4 replicates ± SD. DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+. PH of medium after collection was ~ 3.5 for NH_4^+ and ~ 7 for NO_3^- plants.

4.4.5. Effects of N concentrations

When plants were grown under unbuffered medium containing different N concentrations (Fig. 4.5.), their collected root exudates showed no significant NI inhibiting activity.

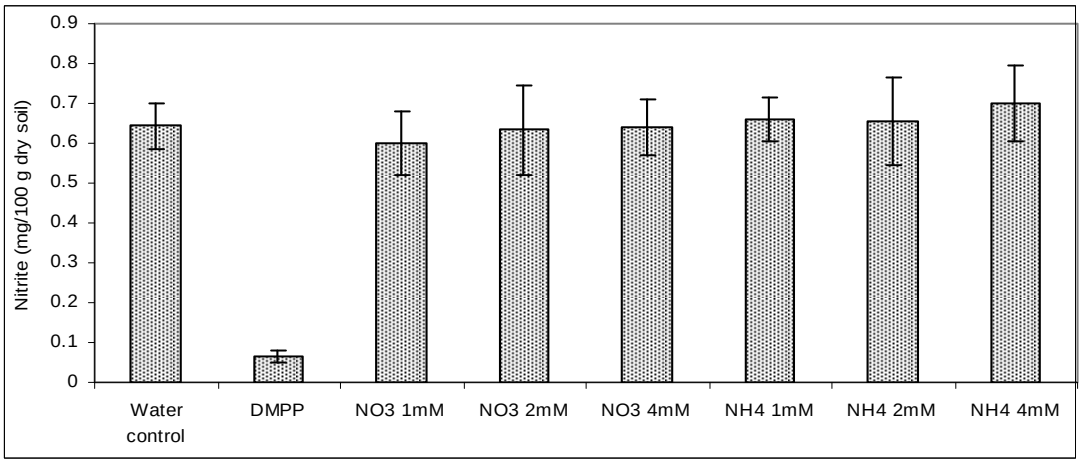

Fig. 4. 5. Effects of root exudates of plants pre-cultured with different N concentrations as NO_3^- or NH_4^+. Root exudates collected in 500 ml d-water. Data are the average of 4 replicates ± SD. DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+. PH of medium after collection was ~ 3.5 for NH_4^+ and ~ 7 for NO_3^- plants.

4.4.6. Effects of different light intensity

The effects of root exudates of plants under high, middle and low light intensity are presented in (Figs 4.6). Plants under low light intensity showed insignificant NI effect. The order of nitrification is NH_4^+-HL>NH_4^+-ML>NO_3^--HL>NH_4^+-LL When root exudates were collected for 6 h, almost the same trend exists among treatments. However in 6 h collection (Fig. 4.6A), nitrification rates for all treatments were less than 24 h collection period (Fig. 4.6.B).

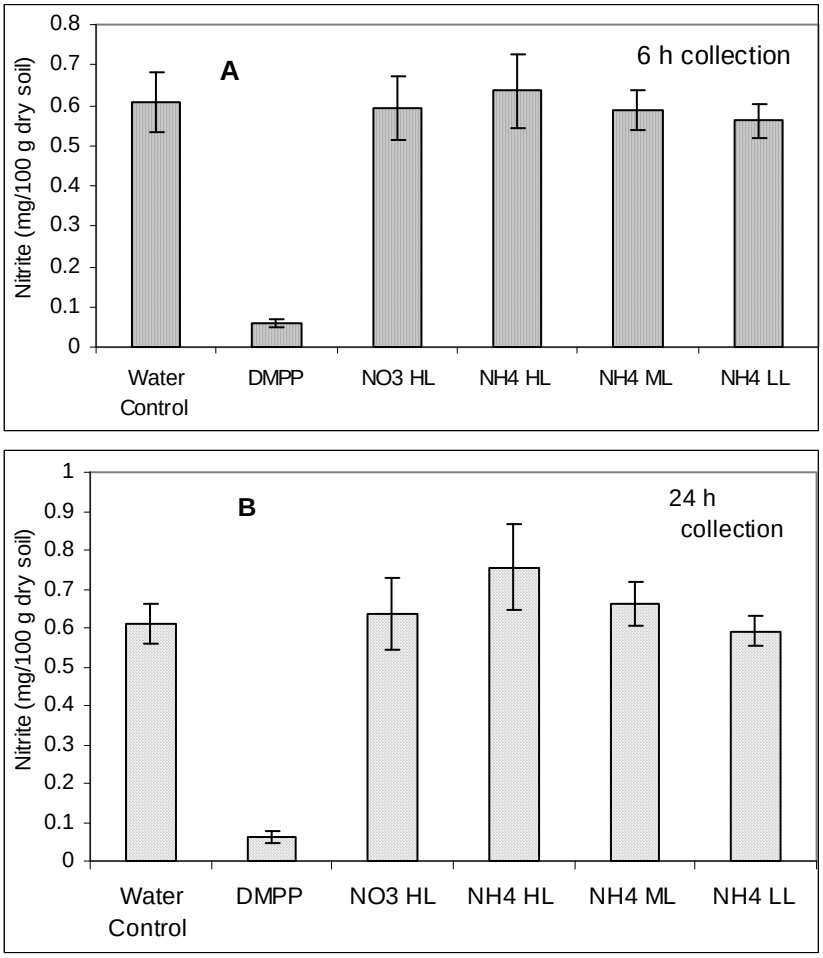

Fig. 4. 6. Effects of different light intensities, plants grown with NH_4^+ under low, middle and high light intensity compare to NO_3^--high light intensity (both 2 mM N); (A) for 6 h collection, and (B) for 24 h collection. Root exudates collected in 500 ml d-water. DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+.

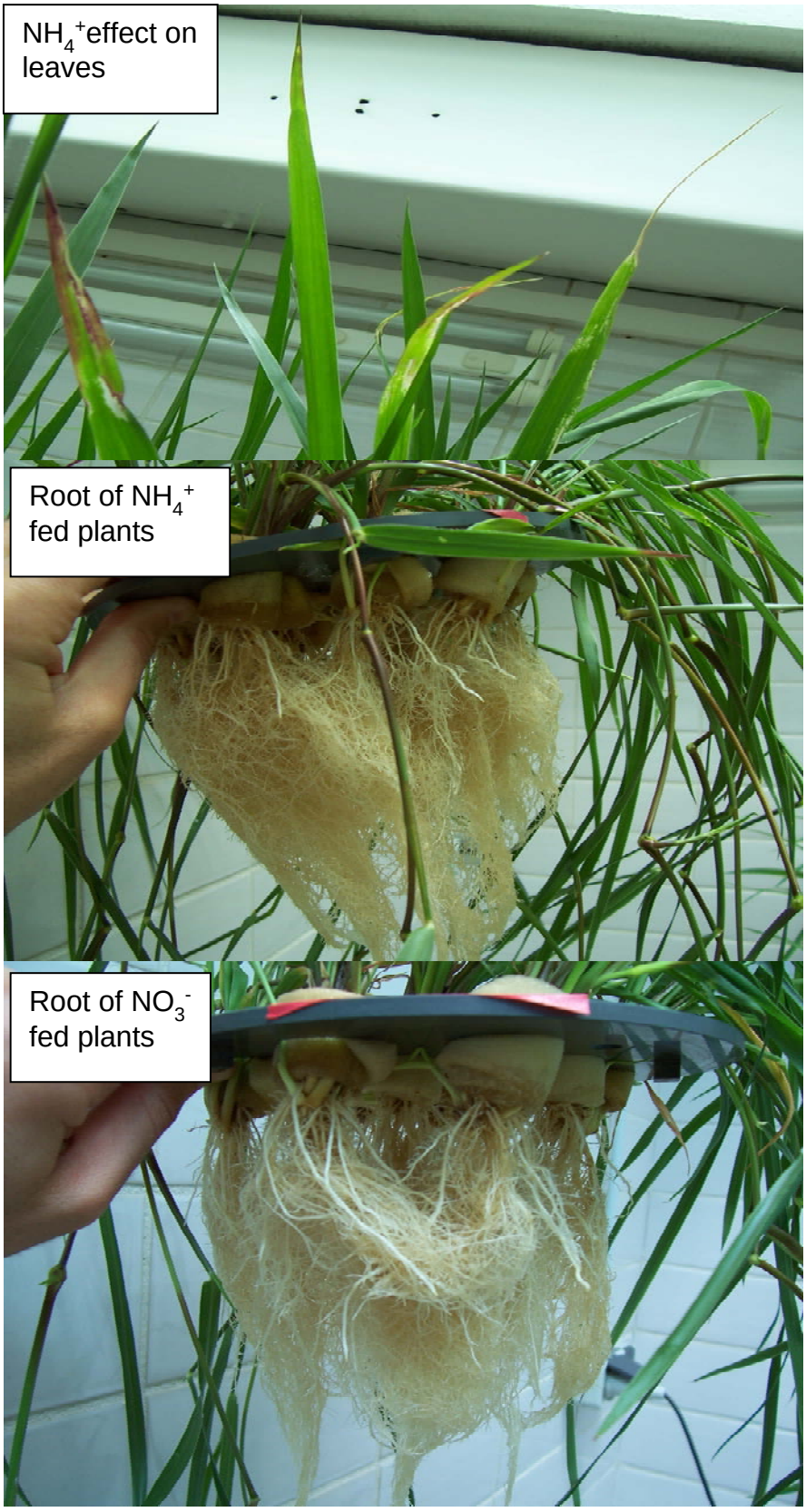

Fig. 4. 7. Typical ammonium effect on leaf tips, and root growth of ammonium and nitrate fed plants, respectively, in 2 mM N as $Ca(NO_3)_2$ or $(NH_4)_2SO_4$ with 10 plants per pot.

4.4.7. Effect of freeze drying of root exudates

The effect of freeze drying of root exudates was presented in Fig. (4.8). Collected root exudates were freeze dried, and extracted with methanol, and further with DMSO. Data showed significant NI activity of root exudates of plants grown in NH_4^+ (independent of light intensity). Furthermore nitrate grown plants showed no significant NI activity (Fig. 4.8).

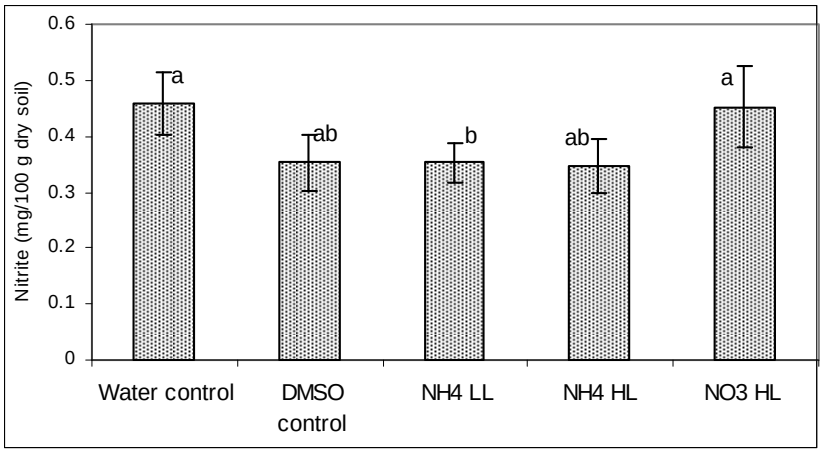

Fig. 4. 8. Nitrification inhibitory effects of freeze dried root exudates (collected in d-water) of plants pre-cultured with NH_4^+ or NO_3^- in high or low light intensity, which have been extracted finally with 0.6% DMSO (on basis of final volume). Data are average of 4 replicates ±SD.

4.4.8. Effect of NH_4Cl in collection medium

Effects of collected root exudates in 1 mM NH_4Cl for plants pre-treated in NH_4^+ or NO_3^- under different light intensities are presented in (Fig. 4.9). Significant inhibition of nitrification compared to control was shown for ammonium, but in 24 h rather than 6 h collection period. There is no significant inhibitory effect for nitrate. However, root exudates of nitrate grown plants show insignificant inhibition after 24 h collection.

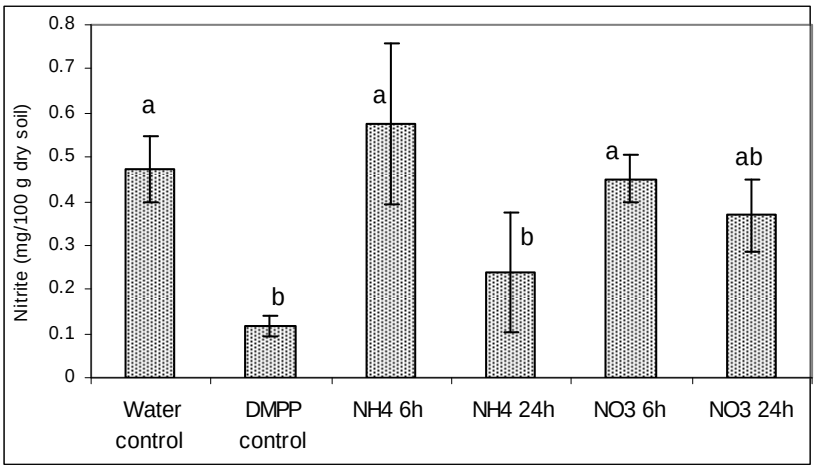

Fig. 4. 9. Nitrification inhibitory effect of root exudates of NH_4^+ or NO_3^- pre-treated plants collected in distilled water containing 1 mM NH_4Cl (6 h versus 24 h collection). Plants pre-cultured with NH_4^+ or NO_3^-. The pH value for ammonium was ~4 and ~3 for 6 and 24 h, and pH value for nitrate was ~5 and ~4 respectively. DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+.

4.5. Discussion

Despite no significant differences among various concentrations of DMPP regarding nitrification in a 50 h incubation test, a concentration of 50 times more than normal (1% of N-NH_4^+) was used in all further incubation experiments (Fig. 4.1.). This was mainly to avoid big differences among various incubations conducted during 3 years experiments. However, even very high dosage (50 times more concentrated) of DMPP can not inhibit nitrification completely (Figs. 4.1, 4.2, 4.3, and 4.4).

It is almost three decades that the roles of phytosidrophores have been well established mainly in iron uptake of plants. Their collection procedure still is used for collecting root exudates even for other purposes. Collecting for 4-6 h, two h after starting light period, phytosidrophores are highest in root exudation (Römheld and Marschner 1986). However, in these experiments also plant roots were transferred 2 h after light period to distilled water medium for collection root exudates. Meanwhile, Subbarao et al., (2006a and 2007a and 2007b) used a collecting medium of distilled water containing 1 mM NH_4Cl or KNO_3, and they indicated that only plants under NH_4Cl in collection medium show significant nitrification inhibitory effect, which this inhibition is several times higher for plants grown (pre-cultured) with NH_4^+ rather than those pre-cultures with NO_3^-. When plants grown in buffered-NH_4^+, root exudates showed insignificant inhibition (Fig.4.2). This might be due to phenolic compounds or terpenoids which have been reported to have NI activity (White 1988;

Gopalakrishnan et al., 2007). Younger plants rather than old plants represent better inhibition. This may be due to decomposition of root cells as separated debris in older plants. Similarly, 6 h collection of root exudates represent better rather than 24 h under distilled water as collection medium. This could probably be due to osmotic effect of distilled water medium and degradation of root cells for longer period. On the other hand, exposure of plant roots to external solutions of very low ionic strength is also likely to increase exudation rates due to an increased transmembrane concentration gradient of solutes (Neumann and Römheld 2000). Therefore collecting in distilled water can not prevent or reduce the root exudation, but instead even it can increase it through osmotic conditions (root medium change from nutrient solution to d-water). Promising inhibition also occurred with freeze dried root exudates of plant grown in various light intensities (4.8). This might be due to avoiding some degradation of NI compounds during routine concentration via rotary evaporator. However, the inhibitory effect of applied concentration of DMSO must not be ignored. The highest inhibitory effect (Fig. 4.9) occurs when 1 mM NH_4Cl was used in collecting medium. This inhibition is a function of collection period and N-form. Similar results were obtained by others (Subbarao et al., 2005; Subbarao et al., 2006a, 2006c,; Subbarao et al., 2007a and 2007c; Ishikawa et al., 1999; 2003).

Our results are not in line with works done by Subbarao et al., (2005; 2006a, 2007a and 2007c) where emphasis was on NH_4^+ rather than NO_3^- nutrition, regarding effective production and exudation of NNIs from roots of BH. Root exudates can contain a wide range of plant compounds from low molecular weight, including gases such as ethylene, CO_2, organic acid anions, phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides, inorganic ions, to high molecular weight compounds such as proteins and mucilage, which is composed of polysaccharides and polygalacturonic acid (Marschner 1995). Therefore, in BH root exudates, which might be a mixture of various compounds, the NI effect of specific compounds may be covered by other compounds. All plant species have a specific compound as root exudates, which is a unique biochemical fingerprint for a given species (Neumann, 2007). Gopalakrishnan et al., (2007) identified two main compounds in BH as ingredient of NI activity: methyl (E)-3-(4-hydroxyphenyl)-prop-2-enoate [methyl-p-coumarate (methyl- p-coumarate) and methyl (E)-3-(4-hydroxy-3-methoxyphenyl)- prop-2-enoate (methyl ferulate). Different alkyl esters of p-coumarate and ferulate exhibited NI activity, with ethyl and propyl esters showing the highest NI activity, but their free acids are not active (Gopalakrishnan et al. 2007). Furthermore, Subbarao et al., (2006c and 2007a) showed that the BNI-activity released from roots is composed of at least three types of active components which have

different pH stability. The main portion of the BNI-activity released in the presence of NH_4^+ is of Type-I, which is stable to pH changes from 3.0 to 10. This compound inhibits the function of *Nitrosomonas europaea* through the blocking of both AMO (ammoniamonooxygenase) and HAO (hydroxylaminooxidoreductase) pathways.

On one hand plants particularly in their tissue under stress produce phytoalexins (antimicrobial compounds) and low molecular weight secondary metabolites, with high implication in natural ecosystems (Hammerschmidt 1999; Gutierrez-Mellado et al., 1996), and on the other hand the diversity of microorganisms inside rhizosphere strongly influenced by root exudates (Neumann, 2007). It is well known that flavonoids secreted from roots have various functions in protecting plants against pests and diseases, as well as they are a chemoatractant for beneficial microorganisms (Neumann 2007). Except flavonoids, however for any effective functioning, root exudates need to be produced and released in large amounts from the root tips which is the main place of almost all exudations (Neumann and Römheld 2000; Neumann 2007).

In our experiments, in root and shoot extracts (chapter 5) and to less extend in root exudates, there was always a negative correlation between N concentration in nutrient solution and NI activity of plant materials independent of N-forms. However Subbarao et al., (2006a, 2006c and 2007a) showed that amount of BNI production and release is a function of N status of plant as well as NH_4^+ nutrition rather than NO_3^-, in which higher N content of plants leads to more NI compounds production. They also indicated that natural inhibition of nitrification is an adaptive mechanism in order to conserve and use N more efficiently under N limiting conditions, therefore N stress could be the main factor behind the evolution of NNIs as an adaptive mechanism (Ishikawa et al., 2003; Lata et al., 2004; Subbarao et al., 2007a).

Subbarao et al., (2007c) released a patent indicating fatty acids particularly some isomers of unsaturated linolenic fatty acid haveing strong NI activity. It is clear that active exudation of fatty acids specially unsaturated long chain isomers of linolenic acid is not possible (Neumann, personal communication). This could be possible only as a result of damage or passive exclusion of root debris. Despite the works which have been done (Subbarao et al., 2006a, 2006c, 2007 a, 2007b, 2007c) still there is not strong evidence supporting the effect of low pH or ammonium signal on NNI release from BH.

Microbial and physiochemical degradation of root exudates (Neumann and Römheld 2000), might inactivate NNIs during the collection and concentrating procedures, specially when temperature is above 10-15 ^oC (Puttanna et al., 1999). Fixing to organic and inorganic colloid materials (Neumann and Römheld, 2000), reabsorbtion by plant roots, and chemical

inactivation might be some explanation for no significant inhibitory effects of root exudates under some experimental conditions.

The inhibitory effect of exudates when collected in 1mM NH_4Cl, could be seen as a secondary response of BH to salt or osmotic conditions in collection medium. Mergulhao et al., (2002) showed that BH is relatively sensitive to salinity specially to chloride in growth medium, with a succulent effect on leaves and roots which is more pronounced on roots (Mergulhao et al., 2002; Cazetta and Villela, 2004). The same succulent effect was observed in our pot plants in greenhouse (data not presented). So the presence of Cl in collecting medium may trigger release of phytoalexins, which could have an inhibitory effect on nitrification.

Finally no significant NI activity under distilled water, but significant inhibition effect in collecting medium containing ammonium chloride, indicates no active release of NNIs. This is further supported by low pH as an indirect effect of NH_4^+ uptake in collecting medium, which can damage the root cell membranes, and leaching of NNIs could occur as a passive phenomena.

Based on their capabilities plants can change their biochemical, physiological and morphological characteristics in response to environmental variations, and the nature of these changes usually determines a species ability to succeed under temporary or permanent environmental stress. It is quite important that interactions among stress factors that occur parallel in infertile acid soils must always be considered (Wenzl et al., 2003). So the observed low nitrate status under BH, also might be due to abilities and high affinity of BH plants to uptake NO_3^-, specially under limited N condition (Sylvester-Bradley et al., 1988; Nakamura et al., 2005; Rao et al., 2001). However the ability of plants to inhibit nitrification is also presented in this work (Fig. 4.9). However, our findings can not support the idea that BH plants release controlled root exudates which strongly and efficiently suppress nitrification. Nevertheless as our results showed, under specific conditions plants can have NI activity in their root exudates, however this inhibitory effect is a passive phenomena rather than controlled and active release.

4.6. Conclusion

There were no detection of NI in root washings, independent of plant age, light intensity, collection time, pre-culture conditions (N-form), treatments (H_2O, N-form, and PH) except under extreme low pH (PM damage) in collecting medium. Therefore, under proper collection conditions for root exudates, (avoiding membrane damage and potential degradation or chemical modification of exudate compounds due to extended collection times in distilled water), there was not any evidence for a controlled release of NI compounds from *Brachiaria* roots, independent of plant age, the amount and form of N supply (NO_3^- or NH_4^+), the pH of the growth medium and the light intensity during the pre-culture period.

However, NI activity was detectable in root washings when the plants were exposed to extended collection times (24 h) in combination with NH_4^+ supply (Fig.4.9), but not with NO_3^- in the collection solution or after short-term collection (6 h). This observation is consistent with the findings of Subbarao et al., (2005, 2006a and 2007a) but it also strongly suggests that the observed release of NI compounds was rather a consequence of membrane damage due to inadequate collection conditions, than mediated by controlled exudation from undamaged roots. Supplying only ammonium (1 mM) in distilled water as root washing medium over extended time periods (24 h) will lead to rapid ammonium uptake and medium acidification associated with the risk of Ca^{2+}-leaching, an important element required for membrane stabilisation. Accordingly, Cakmak and Marschner, (1988) reported detrimental effects on membrane stability in roots of cotton seedlings due to the lack of Ca^{2+} in the root washing medium, which was detectable already after an incubation period of only six hours.

Chpater 5: Effect of shoot and root homogenates and extracts from *Brachiaria humidicola* on nitrification

5.1 Abstract

Following no active release of NNI compounds in root exudates of BH (chapter 4), plant shoot and root materials, with or without extraction, were applied in bioassay test for potential nitrification inhibitory and more characterization of NI compounds. Shoot but not root materials when applied (without extraction) in a soil incubation test, showed high nitrification inhibitory effect, however the variation of data were high. On the other hand, under application of root materials, significant increase in nitrification could occur. In contrast further and sequential extractions of shoot materials, independent of N-form, shows the ethanol extract fraction would contains the possible inhibitory compounds.

5.2 Introduction

Since long time it has been suggested that grass species have the ability to inhibit soil nitrification specially in natural ecosystems (Rice 1974; Ishikawa et al., 2003; Lata et al., 2004; Subbarao et al., 2006b), but this idea has been challenged by other scientists (Bremner and Mc-Carty 1988; White, 1988; Miranda et al., 1994). This inhibitory effect may be caused by compounds released as root exudates (mainly water soluble) or from decaying plant residues (water soluble and insoluble fractions). It seems that NI production and activity of root exudates and plant materials such as leaf litter and root extracts is under plant control (Rice 1974; Lata et al., 2004; Subbarao et al., 2006b, 2007a).

Nitrifier microorganisms through oxidation of ammonium to nitrate play a critical role in natural and agricultural ecosystems. A controlled nitrification or higher NH_4:NO_3 ratios seems to be beneficial for ecosystem functioning, however human activities generally led to huge changes in ecosystem, favouring nitrification and denitrification and finally environmental and health problems. If plant can control nitrification, many problems related to N fertilizers application, including their pollution potential could be reduced. Inhibitory effects of root exudates on nitrification have been discussed in chapter (4). Depending on growth conditions and plant growth characteristics, root exudates of *Brachiaria humidicola* accession 26159 (BH) may have suppressing effect on nitrification, when it applied in a rapid bioassay for detection of nitrite inside the soil. Plants have different mechanisms to cope with stress conditions in their close environment. This could be through release of compounds such as alkaloids, phenolics, amino acids and flavonoids into rhizosphere. Each plant species has a specific dominant chemical compound(s) as its own fingerprint. Upon release of those

compounds in rhizosphere, they can have a suppression effect on nitrification, directly through enzymatic inhibition of nitrifiers or indirectly through attracting competent microorganisms which can out-compete nitrifiers. Phenolics, alkaloids, flavonoids and fatty acids have been suggested to have significant NI activity. So any stressful conditions may help plant to produce and release significant amount of NI compounds into rhizosphere. Despite insignificant inhibitory effects of collected root exudates in distilled water on nitrification, which have been done under various treatments (chapter 4), over collection in 1 mM NH_4Cl, root exudates showed significant NI activity. This is in line with the published results of Subbarao et al., (2006a, 2006c and 2007a). On the other hand, under NH_4Cl containing collection medium, inhibitory effect of root exudates is a function of collection period. When root exudates collected for 24 h, NI activity was much higher compare to 6 h collection (Fig. 4.9). This inhibitory effect of 24 h rather than 6 h collection period in NH_4Cl containing medium indicates a passive secretion of NI compounds into collecting medium through root cells membrane damage. Therefore in previous works done by Subbarao et al., (2006a, 2006c, 2007a) inadequate and sub-optimal conditions for collection of root exudates may lead to root damage and consequently leaching of NI compounds, which would not be released as root exudates from undamaged roots. Long term (24 h) collection of root exudates in presence of 1 mM NH_4Cl, and using 60 days old plants as well as more inhibition of root exudates of NH_4^+ pre-treated plants than NO_3^- pre-treated plants, all support this idea. This damage could be mainly due to H^+ release as a result of NH_4^+ uptake from the collecting medium, which finally this acidification could have a digestive and/or corrosive effects on root cell wall and membranes. However, plant tissues can contain NI compounds, and extracts of several plant species have been shown to have NI activity (Mishra et al., 1980; Rice 1974; Sahrawat 2003; Kiran and Patra, 2003a and 2003b; Lata et al., 2004; Ishikawa et al., 2003; Patra et al., 2006). Our results in chapter 4 (Fig. 4.9), when we used the similar methods of root exudates collection as Subbarao et al., (2006, 2007), showed NI activity which is consistent with their findings. However linoleic and linolenic acid (Subbarao et al., 2007c, and 2007b), and alkaloids and phenolics (Gopalakrishnan et al., 2007) has been reported as the major NI compounds in root exudates and root tissues.

The hypothesis here is that BH plants produces NI compounds which are not released actively as root exudates. To pursue more characterization and identification of NI compounds in BH, as was the overall aim of this research, application of plant shoot and root materials to the bioassay test was considered as next step. Therefore unextracted and extracted plant materials (fractionation of root and shoot extracts), as the aim of this present chapter, has been

suggested to lead in more characterization of NIs compounds. On the other hand, in practice if the inhibitory compounds are synthesized throughout the plant, then substantial amounts of these compounds can be added to soil through leaf litter and roots specially in natural ecosystems (Fisher et al., 1994).

5.3 Materials and Methods

5.3.1 Plant culture

Brachiaria humidicola (BH) plants germinated from the seeds or they propagated vegetatively from new shoots (young tillers) which produced at crown of plants. Plants have been grown in nutrient solution under controlled conditions in growth chamber. Four replicates per treatment and 10 plants per pot were used in all studies. Details of various experiments have been presented in (Fig. 5.1).

Experiments	**Details of conditions**
N-forms	2 mM N as $Ca(NO_3)_2$ or $SO_4(NH_4)_2$
N-concentrations	1, 2 and 4 mM N as $Ca(NO_3)_2$ or $SO_4(NH_4)_2$
PH of plants growing medium	Buffered pH using 3 mM MES, as well as two times per days pH adjustment with KOH, $Ca(OH)_2$ or H_2SO_4
Light intensity	Low light intensity 180 µmol $m^{-2}s^{-1}$, middle light intensity 240 µmol $m^{-2}s^{-1}$, and high light intensity 400 µmol $m^{-2}s^{-1}$
Plant age	Young seedlings (3 weeks old) compare to old plants (7 weeks old)
Periods and methods of root exudates collection	- 6 h versus 24 h collection of -Collecting in distilled water or in NH_4Cl containing medium

Fig. 5. 1. Details of various experiments conducted in present chapter for physiological characterizations (production, release and effectiveness) of NNIs in BH.

When pH was to be controlled, it was adjusted (to a pH 5) using 3 mM Morpholinoethanesulfonic acid (MES) buffer in nutrient solution. The maximum daily variation of pH in nutrient medium ranged between 4.5 and 5.2. which have been recorded and adjusted (to pH 5 using KOH and H_2SO_4) 3 times a day.

5.3.2 Plant homogenates

Before and after harvesting, growth characteristics of plants for each experiment and related treatments have been recorded. Plant shoots and roots where separated and weighed, then the middle part leaves and the whole root system of plants were homogenised to a fine powder

using liquid nitrogen. The homogenates were stored at –20 ºC until further analysis and application in bioassay for NI detection test.

5.3.3 Sequential extraction of plant material

Four grams of frozen shoot or root homogenates where extracted sequentially with solvents of increasing polarity (hexane, ethyl acetate, methanol or ethanol, and water), to achieve a separation of substance classes with different polarity. Plant material was sequentially extracted twice with 10 ml of each solvent using mortar and pestle, just before starting extraction with the next solvent. Subsequently 50 ml of distilled water was added to each fraction and concentrated to 10 ml at 35 ºC using a rotary evaporator. Dimethylsulfoxide (DMSO) was added to a final concentration of 0.8% (v/v), and the final volume was adjusted to 15 ml for 5 samples (4 replicates + 1 freeze sample, for original nitrite concentration), each one 2.5 ml.

5.3.4 Biotest for NI potential

The bioassay consists of rapid determination of nitrification inhibitory (NI) potential of single compounds or mixtures of compound via inhibition of nitrite formation in soils (see chapter 2). An amount of 2.5 g of a pre-incubated standard sandy soil + 7.5 ml of $(NH_4)_2SO_4$ 1.33 mM (as energy source for ammonium oxidizing bacteria) + 50 µl of $NaClO_3$ 1.5 M for blocking NO_2^- oxidation by Nitrobacters + 2.5 ml distilled water + 0.5 g fresh shoot or root homogenates or 80 µl of DMSO containing hexane, ethylacetate, ethanol or water extracted fractions. The mixture then was shaked with 200 rtf for 24 or 50 h, extracted with 7.5 ml of 2 M KCl, filtered using blue ribbon filter paper, and the reaction of nitrite inside the samples with acid sulfanilic (4 mg) was measured photometrically.

5.4 Results

5.4.1 Plant growth and nutritional status

Fig (5.2) shows pH changes in nutrient solution (Fig. 5.2A) and plant fresh biomass (Fig. 5.2B) under NH_4^+ or NO_3^- nutrition. There is no difference between 1 mM and 2 mM NH_4^+. There is identical negative trend of changes (2 units) for both NO_3^- and NH_4^+ from the starting pH 5. However this changes is for relatively young plants, because the pH changes with bigger plants, as well as higher light intensity is much faster (data not presented). Plants root and shoot fresh weight (Fig. 5.2B) showed significant difference among NH_4^+-HL compare to NH_4^+-LL intensity and also compare to NO_3^-. On the other hand with increasing light intensity, plant fresh weight increases.

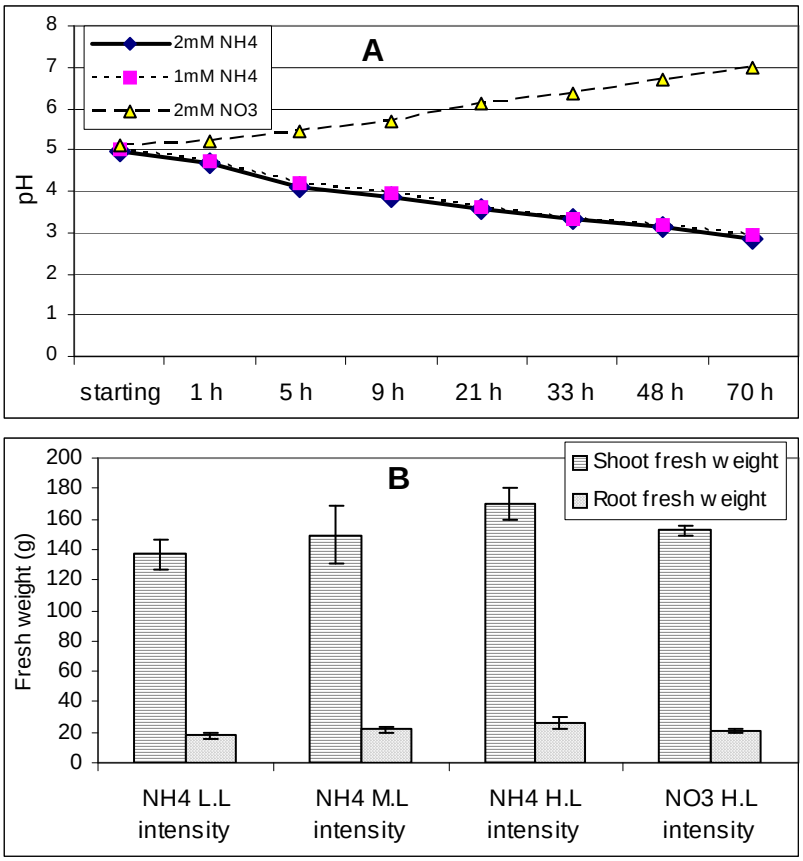

Fig. 5. 2. (A) PH changes in nutrient solution of young BH plants under NH_4^+ and NO_3^- nutrition (10 plants per pot); and (B) biomass production as fresh weight root and shoot under different light intensities (low, intermediate and high light intensity).

Nutrients analysis for plants which grown in NH_4^+ under low and high light intensity compare to NO_3^- under high light intensity is presented in (Fig. 5.3). It tried to brought only nutrients which were affected the most under treatments, such as N, K, Ca, and Zn. Calcium concentration in shoots for plants grown in nitrate showed highest data. There was a positive correlation between nitrate and Ca concentration in the shoots (Fig. 5.3B). Surprisingly, shoot K concentration is significantly higher for plants grown in NH_4^+ under low light intensity than higher light intensity of plants pre-treated with NH_4^+ or NO_3^- (Fig. 5.3C). Similar trends exist for Zn (Fig. 5.3D), and to less extent for total nitrogen (Fig. 5.3A). Plants grown with nitrate have significant less concentration of K and Zn in their shoot compared to ammonium grown plants. Zinc concentration, regardless of N-form, seems to be influenced by light intensity, so plants grown with NH_4^+ under low light intensity show highest Zn concentration in their shoot (Fig. 5.3D).

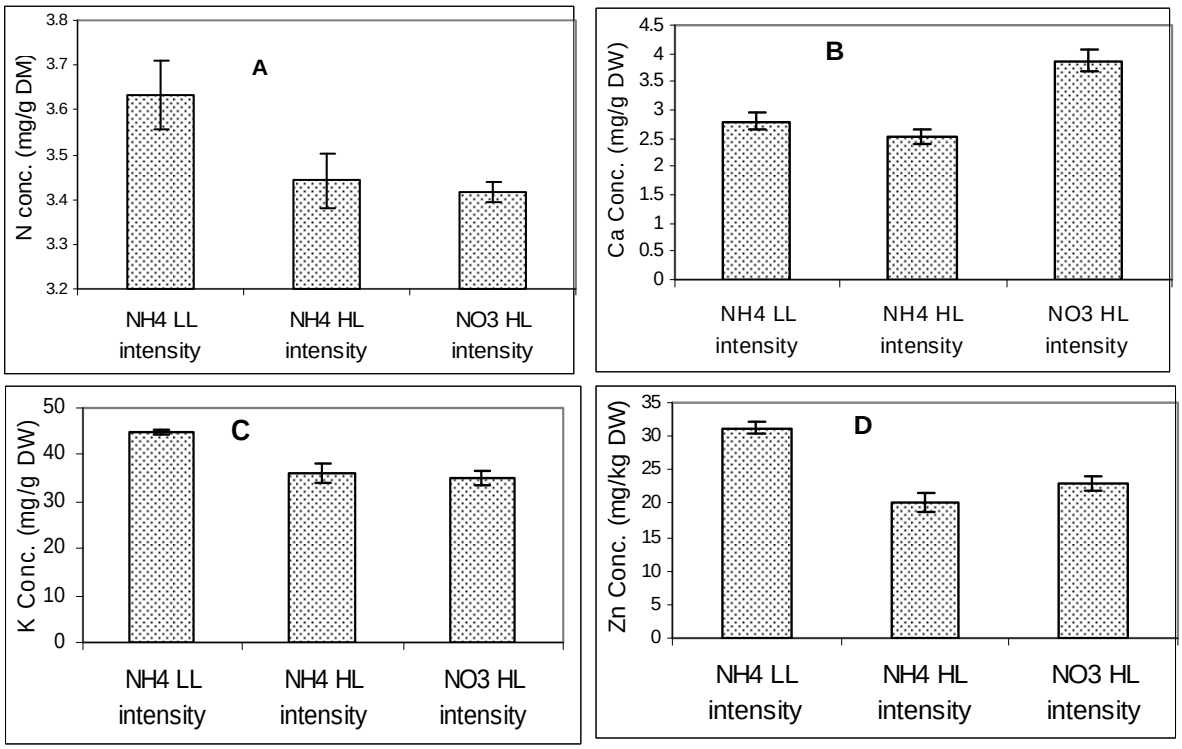

Fig. 5. 3. Nutrient concentrations in plants pre-cultured with 2 mM N as NH_4^+ (low and high light intensity) compare to NO_3^- under high light intensity (LL: low light: HL: high light); A: nitrogen, B: calcium, C: potassium, and D: zinc

5.4.2 Effects of shoot homogenates (without extraction)

Application of shoot and root homogenates without extraction showed significant nitrification inhibition compared to water control (Fig. 5.4, 5.5, and 5.6). In our first experiment (not presented data) there was strong inhibition of shoot and root homogenates regarding nitrification (Souri et al., 2006 abstract). Root homogenates, on the other hand had no effect on nitrification or even sometimes stimulated nitrification (Fig.5.4, 5.6A, and 5.7). Generally root homogenates had no effect on nitrification, however longer incubation of root homogenates promoted nitrification (Fig.5.7). It seems that in short term incubations, sometimes root homogenates may have inhibitory (Fig.5.4) effect (maybe because of NH_4^+ or NO_2^- fixation). When unextracted fresh plant materials incubated for 2, 4, 6, and 8 days, which only in their 2 last days they received NH_4^+ (in bioassay test), root homogenates showed constant positive nitrification correlation with progress in incubation time (Fig.5.7). Furthermore, shoot material until 6 days incubation showed constant positive NI correlation, in which they significantly inhibited nitrification, and the trend of inhibition was increasing. Root homogenates which incubated for 8 days and only at the end of day 6 they received NH_4^+, had high nitrite production rate (Fig. 5.7) as well as high concentration of nitrate (NO_3^-)

was observed in samples (Fig. 5.8). This rates of nitrite (nitrification) is almost double amount of that in control 8-days which received NH_4^+ at the beginning. There are similarities for amount of nitrate in these two treatments. However with double times application of $NaClO_3$ (one at the beginning and another 6 days later) production of nitrate is quite mysterious. In this inhibitory effects of root and shoot homogenates, there was no difference between plants grown in NO_3^- and those grown in NH_4^+. Ammonium concentrations in samples (Fig. 5.6B) showed that free ammonium in adequate concentration occur inside the samples, although some fixation to plant materials may partially happen.

Buffering nutrient solution pH improved NI activity of plant materials (Fig. 5.5). It is particularly significant in plants which grown in NH_4^+ under light intensity, which normally under unbuffered condition didn't show any NI activity (data not presented).

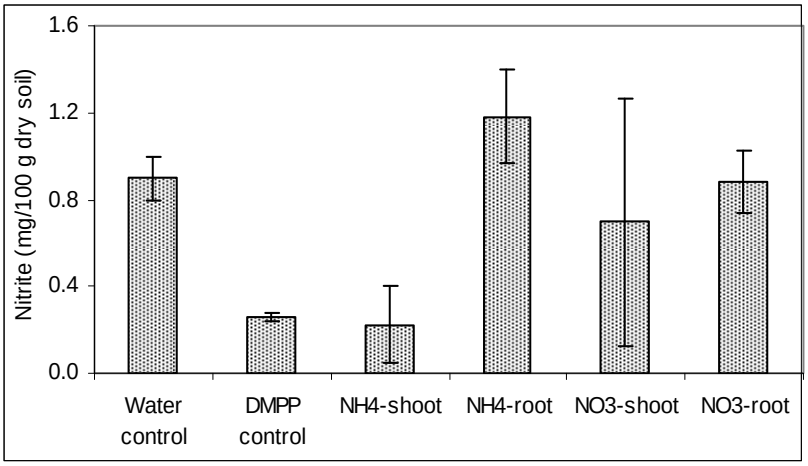

Fig. 5. 4. Effects of unextracted fresh shoot and root homogenates of plants pre-cultured with 2 mM N as NH_4^+ or NO_3^- (50 h incubation). DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+.

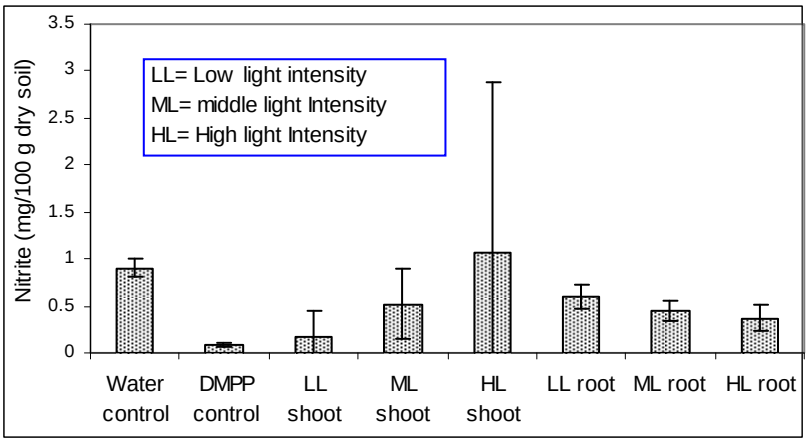

Fig. 5. 5. Effects of unextracted fresh shoot and root homogenates of plants pre-cultured with NH_4^+ under different (low, middle and high) light intensity (50 h incubation). Plants growth medium was adjusted to pH 5 using MES. DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+.

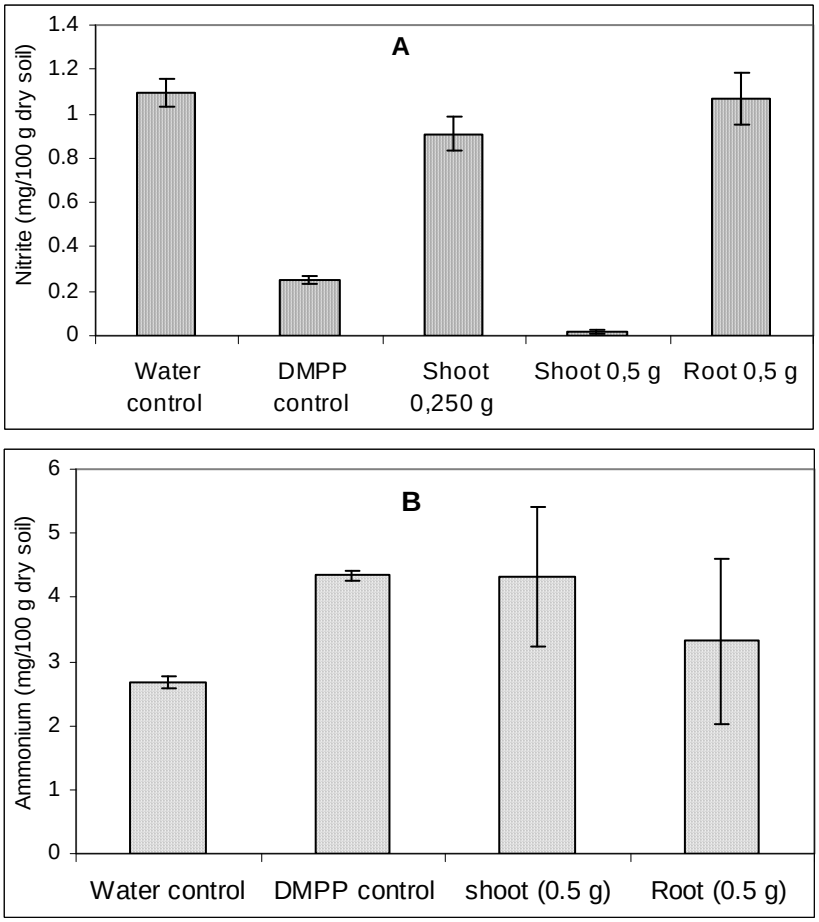

Fig. 5. 6. (A) Effect of unextracted fresh shoot (0.25 versus 0.5 g) or root homogenates of plants grown in 1mM N-NO_3^-. (B) Ammonium concentration of samples. DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+.

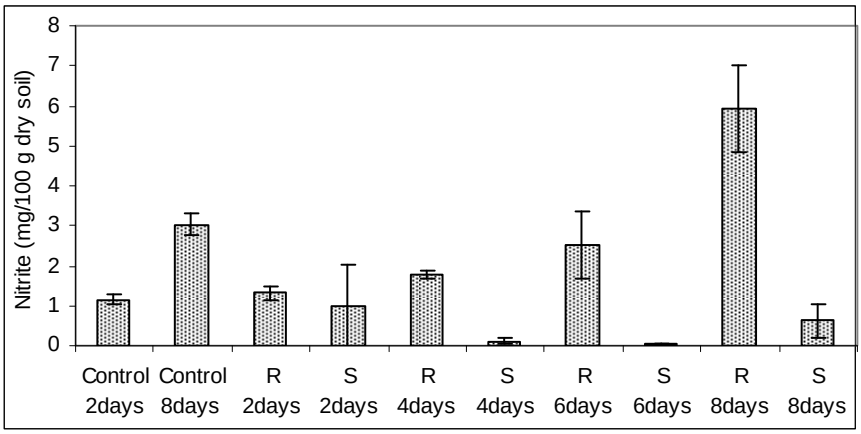

Fig. 5. 7. Effects of different incubation periods of plant root (R) and shoot (S) homogenates which received NH_4^+ only at two last days. All samples received 2.5 g fresh soil + 50 µl $NaClO_3$ + 7.5 ml d-water at the beginning. Samples for 2-days incubation received NH_4^+ at the beginning, 2 days later samples for 4-days incubation received NH_4^+ + 50 µl $NaClO_3$, and so on. Controls received NH_4^+ at the beginning. Control 8-days at the middle (day-4) received 50 µl $NaClO_3$. Plants precultured with NH_4^+ nutrition.

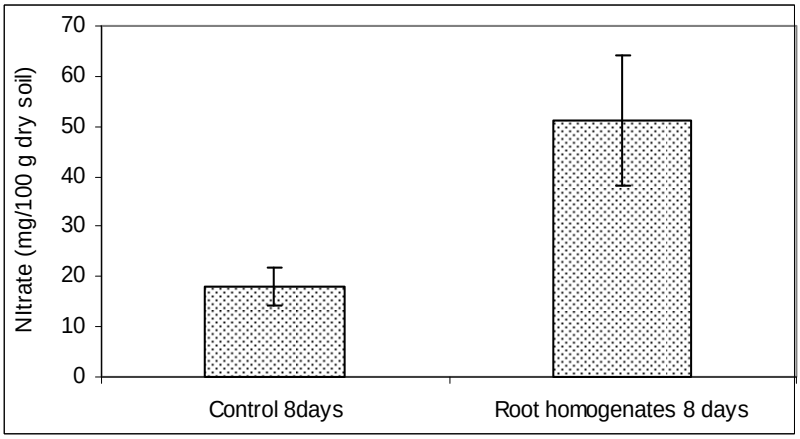

Fig. 5. 8. Amount of nitrate produced after 8 days incubation of root homogenates, (which received NH_4^+ only in two last days), and control 8-days (which received NH_4^+ at the beginning). Chlorate for inhibition of nitrite oxidation was added at beginning and also at the time of adding NH_4^+.

5.4.3 Improvement of methods

The procedure for extracting plant materials involves application of different solvents. These solvents at different time of extraction may have influence on specific target factor which already is under studying. For these reasons the effects of different concentrations of these compounds on nitrification, which have been used in different parts of experiments, were

determined as a percentage of content of samples medium. So, a range of concentrations of linoleic acid (Fig. 5.9), DMPP (see chapter 4), ethanol (Fig. 5.10) and dimethylsolfoxides (DMSO) (Fig. 5.11) were tested on nitrification. This may help to avoid interruption and interference of these compounds which actually may occur in incubation test. The results showed that even low concentration of these compounds can inhibit nitrification. However, DMSO showed the lowest inhibitory effect, meanwhile it is one of the most important laboratory solvents.

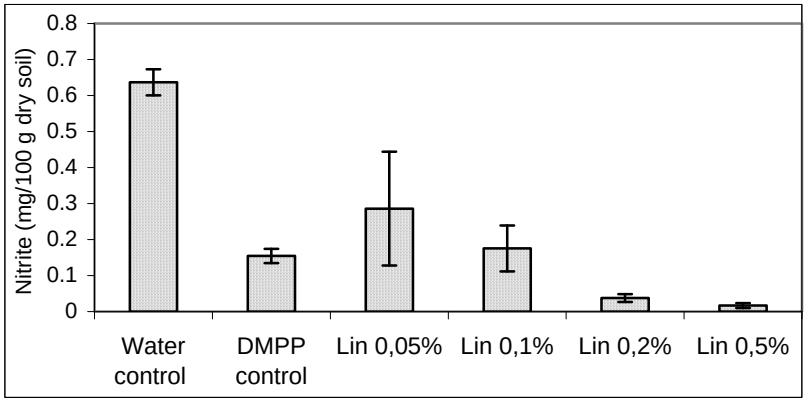

Fig. 5. 9. Effects of different concentrations of linoleic acid on nitrification (24 h incubation), as a percentage of solution in incubating medium. DMPP was used at a concentration of 50 times more than normal concentration of 1% $N\text{-}NH_4^+$.

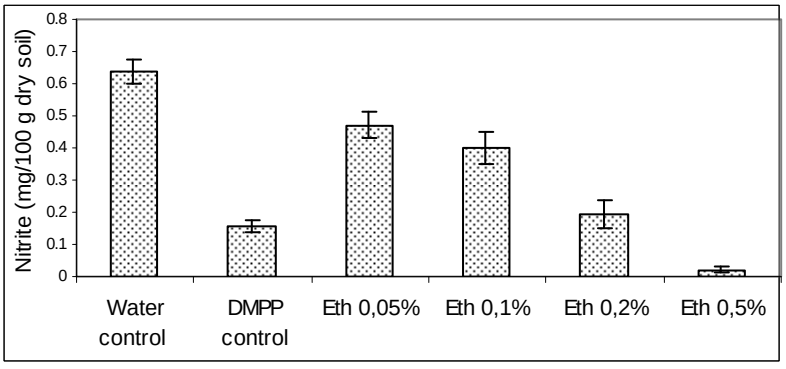

Fig. 5. 10. Effects of different concentrations of ethanol 95% on nitrification (24 h incubation), as a percentage of solution in incubating medium. DMPP was used at a concentration of 50 times more than normal concentration of 1% $N\text{-}NH_4^+$.

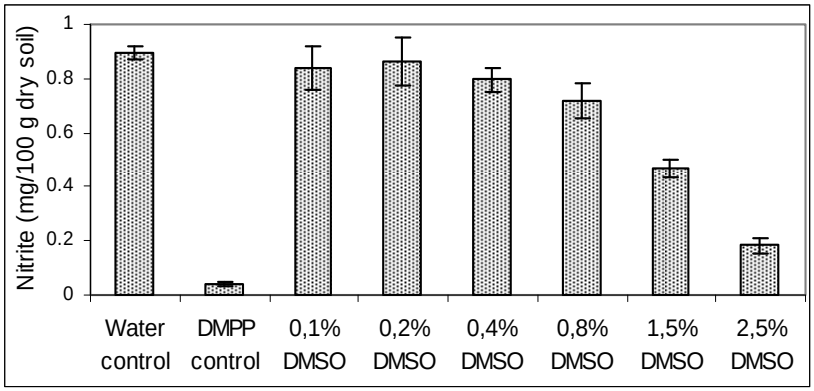

Fig. 5. 11. Effects of different concentrations of dimethylsolfoxides (DMSO) on nitrification (24 h incubation), as a percentage of solution in incubating medium. DMPP was used at a concentration of 50 times more than normal concentration of 1% $N\text{-}NH_4^+$.

5.4.4 Effects of extracted shoot homogenates

Application of plant homogenates specially shoot homogenates, always led to high variation of data. This makes explanations and interpretation rather difficult. However, for further identification of NI compounds in plant materials, extraction in different solvent was proposed as the next step. Since root homogenates showed no NI activity, only shoot (leaves material) of NH_4^+ and NO_3^- fed plants were used for this step. Four grams shoot homogenates was extracted sequentially from unpolar to polar solvents, with the order of hexane – ethylacetate – ethanol - water, each one 2 times with 10 ml. Results of the extraction is presented in (Fig. 5.12), showing NI activity of unextracted shoot homogenates (original shoot) together with five other fractions of extraction. However even hexane, and ethylacetate fractions (Fig. 5.12, and Fig.5.13) show some inhibitory effects on nitrification, which may be due to too many different compounds inside those fractions. Nevertheless, the fraction of ethanol extraction shows the expected NI activity, which is in line also to nitrification inhibitory effects of unextracted shoot homogenates (as partially water soluble). Fig. (5.12) is the sequential extraction of ammonium pre-treated plants which compare to nitrate pre-treated plants shows no significant differences, except for water and ethyl acetate extraction which may be due to experimental error and the solvent in usage.

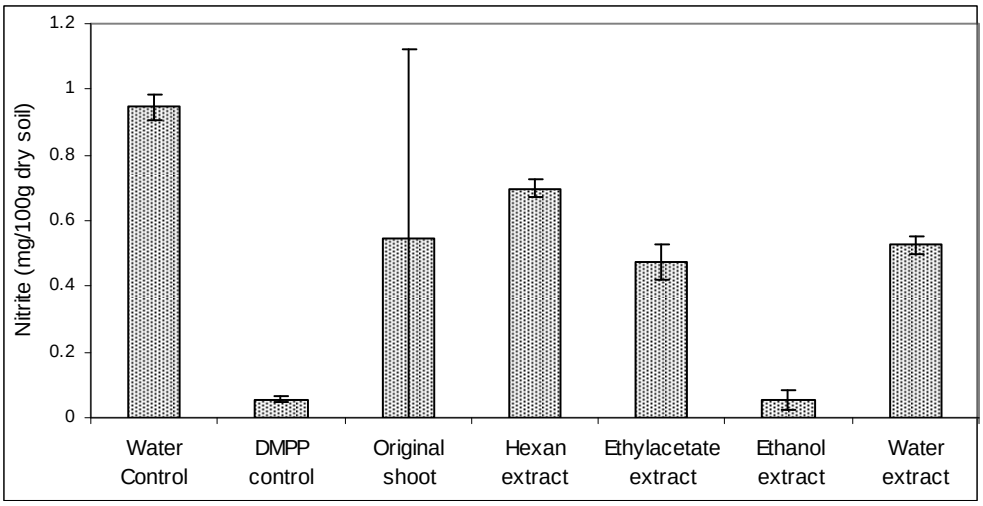

Fig. 5. 12. Effects of shoot homogenates of NH_4^+ pre-cultured plants, extracted sequentially with different solvents, respectively, (each one two times 10 ml), evaporating and finally extracting with DMSO (0.8% final concentration in samples). DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+.

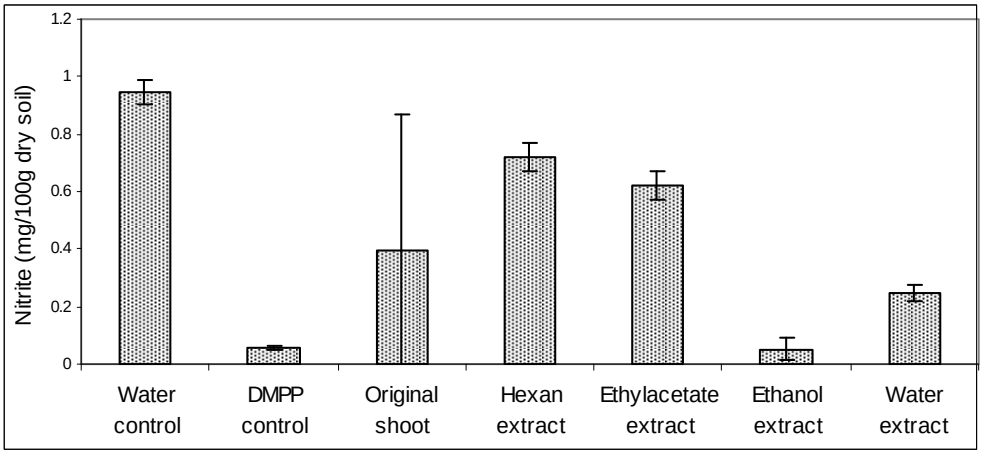

Fig. 5. 13. Effects of shoot homogenates of NO_3^- pre-cultured plants, extracted sequentially with different solvents, evaporated and finally extracted with DMSO (0.8% final concentration in samples). DMPP was used at a concentration of 50 times more than normal concentration of 1% N-NH_4^+.

5.5 Discussion

Relatively low concentration of calcium and potassium under ammonium particularly with high light intensity indicates that root cells also might potentially damaged by low pH induced by NH_4^+ uptake (Fig. 5.3B). On the other hand, the idea that in BH higher light intensities induce much higher N uptake, as ready compounds to interfere with nitrite measurements, could not be acceptable (Fig. 5.3A). Therefore, high variation of data can not be justified by

interfering higher N concentration of samples. Nevertheless, spatial distribution of absorbed N in plant is under question and still might be a reason of variation.

Despite adaptation and tolerance of BH to ammonium (Figs 5.2, 5.3), toxicity symptoms such as leaves tip necroses, especially in younger leaves of plant is not avoidable (Fig 4.7). This is independent of buffering the nutrient solution medium. The symptoms on roots, in severe condition are inhibition of root development specially root hairs, with burned tips which is typical proton effects. Buffering nutrient solution containing ammonium prevented these symptoms particularly root damages. Nevertheless necrosis of leave tips still exists, but to less extent. Regardless of N-form, nitrogen uptake of plants is quite fast, which has been shown as pH changes (Fig. 5.2). A pH of less than 2 also was not avoidable in root medium containing ammonium, particularly under higher light intensity. Regardless of N-forms and pH effect, NI activity was a character of shoot materials but not root materials. However, these NI effect normally due to high variation among replicates were not significant (Fig. 5.4, 5.5, and 5.7). Generally, there was not such variation for root homogenates. Sometimes small inhibitory effect of unextracted fresh root homogenates could be due to fixing properties of root surface exchange positions. Meanwhile, with increasing incubation time (Fig. 5.7 and 5.8), there was a positive correlation between nitrite production and incubation periods. Nevertheless, it seems that some of this nitrite production originates from plant materials, or in a better optimistic way it can be suggested that enzymes released and accumulated under root material decaying in soil could play a role (beside bacterial enzymes). Furthermore, nitrate concentration supports these ideas (5.8). In contrast, unextracted shoot homogenates improved NI activity with increasing incubation periods up to 6 days (Fig. 5.7). This indicates that NI compounds release gradually over decaying shoot residues. On the other hand, microbial degradation and inactivation of NI compounds, adsorption to soil colloids, reabsorbtion by plant roots may also be possible explanation for insignificant or high data variance. Subbarao et al., (2006a, 2006c and 2007a) showed that amount of NNI production and release is a function of N status of plant as well as NH_4^+ nutrition rather than NO_3^-, indicating that higher N content of plants produce more NI compounds. However, our data shows that NI effect is a function of less N concentration in plants (Fig. 5.6A), similar to natural brachiaria stands in tropical and subtropical South America.

To test the hypothesis that NI compounds are released from damaged plant root cells of Brachiaria, the NI potential of fresh root and shoot tissue (homogenised to a fine powder in liquid N_2) was measured after soil incorporation. Despite a high variability, the data presented evidence that:

- There was no indication for substrate (NH_4^+) limitation in the NI tests (e.g. by NH_4^+ immobilisation or inactivation), since the soluble NH_4^+ concentration in soil samples with incorporated plant material was always higher than in the control treatments with addition of water and NH_4^+ substrate solution alone.
- NI potential was detectable in shoot tissue but not in roots.
- The NI effect of soil-incorporated shoot tissue lasted for at least 8 d, while root tissue even stimulated nitrification with increasing incubation time.
- NI activity was detectable in shoot tissue from Brachiaria plants pre-cultured with NH_4^+ or NO_3^- supply, although the variability of the data tended to be higher in NO_3^--fed plants.
- NI activity of shoot tissues tended to increase with increasing light intensity during the pre-culture period, but the variability of the data also increased.

Subbarao et al., (2007c) identified linoleic acid as NI compound in root washings of Brachiaria. The NI potential of pure linoleic acid additions to soil samples has been confirmed also in the present study (Fig. 5.9), although its mechanism of NI inhibition is not clear. Since unsaturated fatty acids are rapidly oxidised it seems to be likely that not the unmodified linoleic acid molecule itself exerts long-lasting NI effects in soils reported by Subbarao et al., (2006a, 2007a and 2007c). The finding that oxidation products of linoleic acid, such as linoleic acid hydroxy-peroxides have a cytotoxic potential (Kaneko et al., 1994) may offer a possible explanation.

Although linoleic acid seems to be a NI compound released into root washings of Brachiaria (Subbarao et al. 2006a, and 2007a) as a consequence of unfavourable conditions, promoting membrane damage, surprisingly, soil incorporation of fresh homogenised root material did not show any NI activity. Possible explanations could be that:

The NI compounds in the root tissue homogenates were biochemically inactivated after decompartmentation of the homogenized tissue. This effect did not occur during leaching into root washings. Also Gopalakrishnan et al. (2007) couldn't detect linoleic acid as NI compound in root extracts of Brachiaria, although this compound was detected in root washings. Linoleic acid in free and esterified form is a constituent of plant cell membranes (Kaneko et al. 1994) and may be selectively liberated under conditions causing membrane damage (i.e. unfavourable conditions for collection of root washings). The concentration of NI compounds in the root tissue samples was too low to find any significant NI effects.

Attempts to characterise the NI compounds detected in shoot tissue of Brachiaria plants revealed a particularly high activity in the ethanol-soluble fraction, both in plants with NH_4^+

and NO_3^- preculture, after extraction with solvents of increasing polarity (hexane, ethylacetate, ethanol, water). This solubility pattern is in line with observations of Subbarao et al 2008 (unpublished) who isolated linoleic acid as NI compound from Brachiaria leaves by 80% methanol extraction.

5.6 Conclusion

The working hypothesis that Brachiaria plants exhibit a regulated NH_4^+-induced root exudation of NI compounds into the rhizosphere, as an adaptation to stabilize nitrogen in N-limited natural ecosystems in the tropics, could not be confirmed by the results of the present study. The data rather suggest release of NI compounds as an experimental artefact due to membrane damage, caused by unfavourable conditions for collection of root exudates. However, this finding does not exclude the concept of N stabilisation by plant-borne NI compounds in these ecosystems. Since NI activity was found also in the plant tissue, N stabilisation could be mediated by liberation of NI compounds from plant residues of Brachiaria as a very competitive and long lasting tropical pasture species.

Chapter 6: NH_4^+ and NO_3^- nutrition of tomato and effects of calcium on NH_4^+ toxicity

6.1. Abstract

Tomato is a typical sensitive plant to ammonium nutrition in solution culture. It also represents one of the main and most adopted vegetable crops to calcareous soils. The aim of this chapter is to represent the complementary effects of ammonium and calcium to each other under such condition in improving tomato plant performance, where acidification of rhizosphere induced by ammonium uptake compensate for micronutrients deficiencies induced by calcium. On the other hand, ammonium induce plant toxicity (mainly root damage) compensated by high concentration of calcium in medium. Therefore, stabilized ammonium through application of synthetic or natural nitrification inhibitors represent a very important nutritional player under such conditions. Improved growth characteristics particularly root growth when ammonium fed plants received supplemental calcium support our hypothesis. Meanwhile, compared to nitrate fed plants ammonium uptake reduces plant water uptake. This means under stabilized ammonium nutrition, significant reduction in water consumption could occur which is economically quite important in arid and semi arid regions. Improved root and shoot growth, leaf area and chlorophyll contents and consequently water relation of plants compared to sole ammonium fed plants, indicates magic role of calcium in lowering ammonium toxicity symptoms.

6.2. Introduction

Ammonium nutrition of plants could have positive environmental, economical and nutritional consequences. In root medium or inside the plants, ammonium represents an equilibrium between NH_4^+ and NH_3, which have different membrane permeabilities (Loque and von Wiren 2004). In addition low affinity NH_4^+ transport occurs through non-selective cation channels or K channels (Howitt and Udvardi, 2000; Kronzucker et al., 2001; Loque and von Wiren, 2004). So despite precise action of ammonium transporters over NH_4^+ concentrations, plant roots can not completely control NH_4^+ uptake, therefore excess uptake of ammonium mainly in nutrient solution may happen, which is out of tolerance for some plant species and causes toxicity. The mechanisms of toxicity still are not well known.

Tomato is one of the most sensitive cultivating plants to ammonium nutrition in hydroponic culture. Severe toxicity symptoms are common under NH_4^+ nutrition which normally is coupled with growth inhibition and a reduction in plant dry weights, leaf area and root: shoot ratios compare to NO_3^- fed plants (Ludewig et al., 2002; Roosta and Schjoerring, 2007; Siddiqi et al., 2002; Claussen 2002). When concentration of ammonium in growth medium is

above 1 mM, tomato plants have a strong accumulation of ammonium in leaves, stem, and roots (Schjoerring et al., 2002). The increase in tissue NH_4^+ coincides with saturation of glutamine synthetase activity and accumulation of glutamine and arginine. Glutamine synthetase incorporates NH_4^+ into glutamine, but root GS activity and expression are repressed when high levels of NH_4^+ is supplied (Schjoerring et al., 2002). Ammonium may simply replace calcium at membranes (Cramer et al., 1985), in addition low tissue levels of calcium and magnesium in the NH_4^+ fed plants constitute part of the NH_4^+ toxicity syndrome (Schjoerring et al., 2002; Roosta and Schjoerring, 2007).

It is generally believed that a series of mechanisms rather than a single mechanism cause these negative effects on tomato plants. Increasing free ammonia in apoplast, low pH damage, NH_4^+ induced cations deficiency, high cost of energy-derived H^+-efflux, (Ludewig et al., 2002; Redinbaugh and Campbell, 1993; Schjoerringet al., 2002; Babourina et al., 2007; Britto et al. 2001; Britto and Kronzucker, 2002), are among the possible reasons which perhaps together govern the toxicity symptoms. On the other hand, calcium is a structural important essential element with critical function in cell walls and membranes, and represents a counter cation for inorganic and organic anions in the vacuoles (osmoregulator), and as an intracellular messenger in cytosol (Marschner 1995). The movement of calcium through symplast or apoplast pathways via cytosolic Ca^{2+} concentrations must be finely balanced, to allow a control over rates of Ca^{2+} delivery to xylem and prevent toxic cations accumulation (White and Broadley 2003). The apoplastic pathway is relatively non selective between divalent cations (White 2001), and its presence and activity could result in the accumulation of toxic solutes in the shoots (White and Broadley 2003). However controlled xylem loading may also occur.

Calcium has distinct function in higher plants mainly in membranes, cell walls, enzymes and interaction with phytohormones, so it can have an important role in plant tolerance to various stresses (White and Broadley 2003). It is well known that in absence of calcium, ion selectivity is lost and deficiency and toxicity of specific nutrients may occur. Calcium ions tends to bond phosphate and carboxylate groups in phospholipids and protein structure, which results in membrane stability. The role of calcium ions also is well known as a ubiquitous intracellular second messenger in plants (Evans et al., 2001; Hepler, 2005). Any stimulus from biotic or abiotic stresses induces cytosolic Ca^{2+} oscillation through Ca^{2+} channels in organelle membranes or plasma membranes. On the other hand, the symplastic pathway allows the plant to control the rate and selectivity of Ca transport to the shoots (White 2001), therefore NH_4^+ primarily may damage symplastic pathway which regulates the selectivity of

ions. In addition shoot calcium has been correlated with cation exchange capacity (CEC) of plant roots, which is located in root apoplast, and is attributed to free carboxyl groups of galacturonic acids of cell wall pectins in the middle lamella (Sattelmacher 2001).

In the present experiment we have tried to consider most of mechanisms involved in ammonium toxicity symptoms, with special emphasis to the role of calcium on membrane stability and plant (root) cell wall hardening. In this experiment root damage, probably by membrane and cytosolic sensitivity, hypothesised to be the main cause of toxicity symptoms of NH_4^+ on tomato plants. Therefore, it has been suggested that high calcium levels can protect the cell wells and specially cell membranes from the adverse effects of ammonium toxicity, as it plays the same role in salt and sodium toxicity (Bush et al., 1995; Cramer 2004). In addition it might reduce xylem hydraulic resistance similar to plants grown under nitrate nutrition.

6.3. Materials and Methods

6.3.1. Plant culture

Seeds of tomato (*Lycopersicon esculentum* var Money Maker) were germinated in a mixture of fine sand (0.2-0.5 mm) and TKS1, a peat medium containing low level of nutrients, (1:1 Volume). Two weeks after germination homogeneous seedlings were transferred to nutrient solution containing 2 mM N-NO_3^- for 2 weeks (pre-culture period) in growth chamber under controlled conditions (30/26 ˚C, and 70% relative humidity with a 16/8 light regime). Nutrient solutions were changed every 4 days.

6.3.2. Water consumption related to nitrate uptake

Tomato seedlings (L*ycopersicon esculentum* var Money Maker) after germination were transferred to nutrient solution containing 2 mM N in form of $Ca(NO_3)_2$ as pre-culture conditions in growth chamber. They were grown for 2 weeks in such condition. Then plants transferred to nutrient solution containing 0.8, 2, and 5 mM N-NO_3, with 4 replicates and 4 plants per pot. Root length, root and shoot fresh and dry weight, and water consumption (transpiration) per pot was measured and compensated (resupplied) after 48, 72 and 96 h.

6.3.3. Nitrate and ammonium nutrition of tomato and role of calcium

The response of tomato plants to ammonium, nitrate, split application of ammonium, pH, and calcium concentration in nutrient solution was studied under controlled condition in growth chamber using various treatments as following:

Treatments	Details
Nitrate	2 mM N as $Ca(NO_3)_2$
AS (sole)	2 mM N as $(NH_4)_2SO_4$ (no buffering of pH)
AS 3S (in 3 splits)	2 mM N as $(NH_4)_2SO_4$, but by 3 split applications (adding each time 1,66 ml of 0.5 M $(NH_4)_2SO_4$ to nutrient solution equal to 3×0.66 mM), (no buffering of pH)
AS 6S (in 6 splits)	2 mM N as $(NH_4)_2SO_4$, but by 6 split applications (adding each time 0,83 ml of 0.5 M $(NH_4)_2SO_4$ to nutrient solution equal to 6×0.33 mM), (no buffering of pH)
AS+$CaSO_4$	2 mM N as $(NH_4)_2SO_4$ + 10 mM Ca^{2+} as $CaSO_4$ in root medium
AS+$CaCO_3$	2 mM N as $(NH_4)_2SO_4$ + a gradual 2-3 mM $CaCO_3$ in root medium to have a fixed pH ~6.7.

Fig. 6. 1 . Treatments and details of variables under study.

All treatments cinsisted of 4 pots (as replicates) each one with three plants. Nitrogen was applied as 2 mM N-NH_4^+ or N-NO_3^-, and all NH_4^+ treatments also received 1 mM $CaCl_2$ as original calcium concentration. Plants treated with AS+$CaSO_4$ received 10 mM Ca^{2+} during both pre-culture and treatment periods.

With split applications of the mM amount of N-NH_4^+ which was applied at starting of any nutrient solution change, we expected to have less ammonium toxicity due to enough time that plants have to assimilate absorbed NH_4^+. However, this experiment mainly focuses on hardening of root cells (membrane stability) through application of Ca^{2+} (in forms of $CaSO_4$, or $CaCO_3$). The nutrient solution containing ammonium and calcium sulphate (AS+$CaSO_4$) could not resist strongly against pH changes. On the other hand, for calcium carbonate treatment a stable and buffered pH of 6.6 was maintained using 0.5-1.0 g of $CaCO_3$ per pot which is equal to 2-3 mM Ca^{2+}. This would eliminate the effect of low pH.

6.3.4. Determination of growth characteristics

Before applying treatments, seedlings shoot and root lengths were recorded for later comparison to their lengths at harvest time. PH and water consumption of plants, which is an indicator of transpiration and stomata activity, was measured daily since the beginning of experiment. The water consumption of plants during last 4 days just before harvest has been

presented. Chlorophyll content as well as other related growth characters were determined at harvest. Nutrient concentrations were measured based on dry digestion and with atomic absorption and flame photometry. Root xylem sap was collected by plastic tubes after cutting plant stem 2 cm above the soil surface. Regularly the collected sap removed to ependorf tubes and kept in refrigerator.

6.4. Results

6.4.1. Water consumption related to nitrate uptake

Tomato plants which pre-cultured in 2 mM $Ca(NO_3)_2$ were transferred to different concentration of nitrate as 0.8, 2 and 5 mM N-NO_3 (Fig 6.1). Water consumption was measured regularly after 48, 72 and 96 h for two times nutrient solution change during 8 days (Fig. 6.1). At the beginning, after 48 h and even until 72 h there was no significant difference for transpiration among plants with different N concentrations. Plants start to show significant differences in transpiration after 72 h when they received 5 mM N rather than other concentrations, which showed significant higher water consumption compared to plants received 0.8 mM N. These differences continue get larger in the next 4 days when they received renewed nutrient solution (Fig. 6.1B). At the end of 8 days plants under different concentrations of N showed clear differences, indicating highest water consumption when plants received higher (5 mM) N-nitrate in nutrient solution (Fig. 6.1B). Despite no significant differences for shoot dry weights (Fig. 6.1D), their fresh weights (Fig. 6.1C) showed significant difference among plants which received 0.8, 2 and 5 mM N. There was no difference between root fresh weight and root length among plants, however root but not shoot, when plants received 5 mM N showed significant lower dry weight compared to plants which received 0.8 mM N.

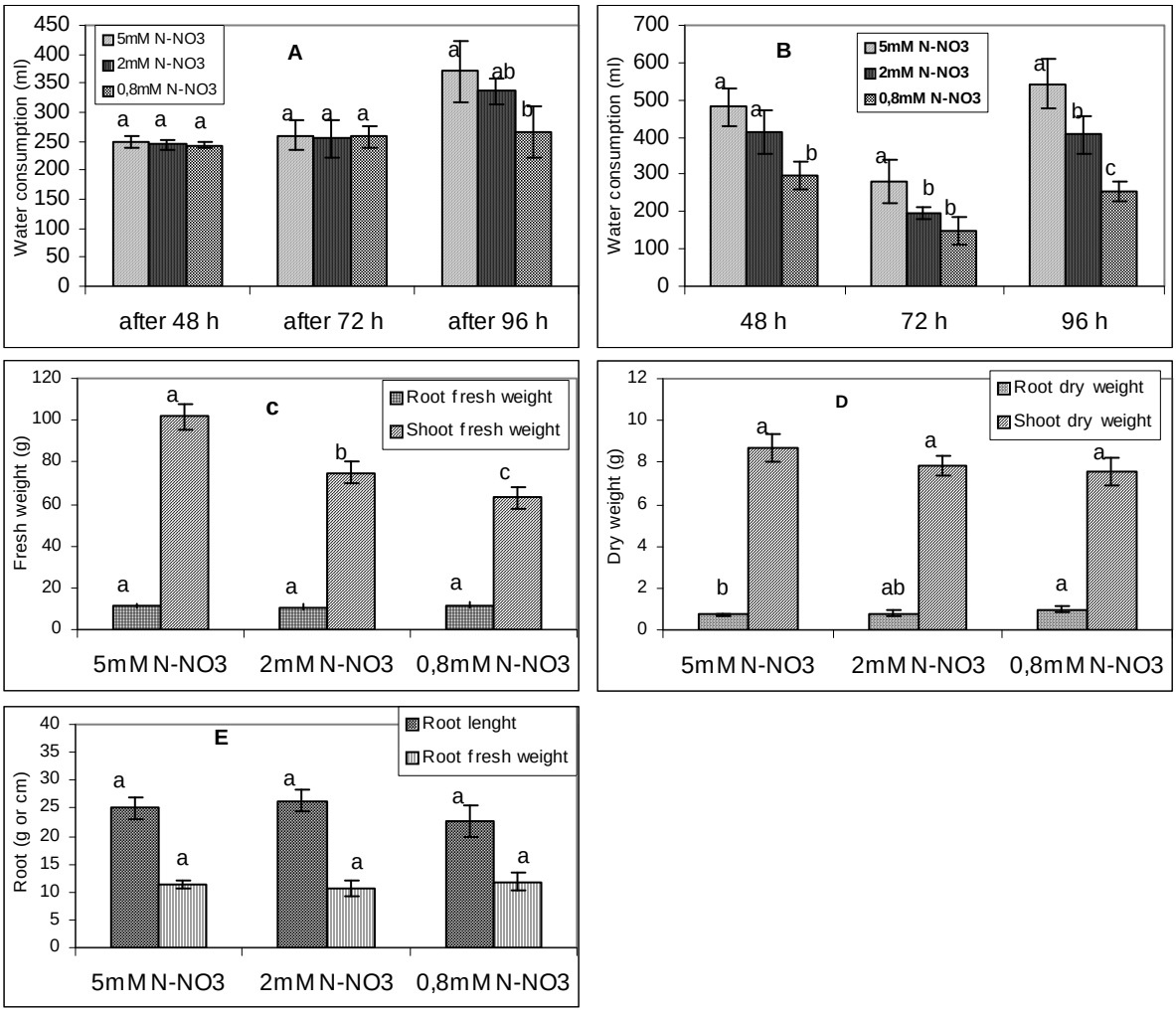

Fig. 6. 2. Water consumption of tomato plants during 8 days, grown with different concentrations of nitrate in nutrient solution; Water consumption during first 4 days in nutrient solution (A); Water consumption during second 4 days (B); Root and shoot fresh weight at harvest (C); Root and shoot dry weight (D); Root length and root fresh weight relation (E).

6.4.2. Effects of NO_3^-, NH_4^+, and Ca^{2+} on plant growth characters

6.4.2.1. Root length

When plants received different N treatments as nitrate, ammonium sulphate (AS control), ammonium sulphate with 3 split applications (AS 3S), ammonium sulphate with 6 split applications (AS 6S), ammonium sulphate + calcium sulphate (AS+ $CaSO_4$) and ammonium sulphate + calcium carbonate (AS+ $CaCO_3$), except for root length of $CaSO_4$ treated plants which received 10 mM Ca (during both pre-culture and treatment periods), root and shoot

lengths showed no significant difference before applying treatments (Fig. 6.1A). However, at harvest (Fig. 6.1B) plants showed significant differences in both shoot and overall root lengths with highest length under nitrate nutrition. Nitrate compared to ammonium represented a better N source for plant root and shoot growth. Plants received AS+$CaSO_4$ or AS+$CaCO_3$ also showed improved root length compared to AS sole (control) plants. Despite lower shoot and root lengths of Ca treated plants compare to nitrate, however they showed significant higher root and particularly shoot growth than control. Plants with split applications of ammonium, on other hand, showed no significance difference to each other and compared to control plants which received their ammonium at the beginning. In control and split applications, root growth inhibited immediately after transferring to NH_4^+ (Fig. 6.1A, 6.2B).

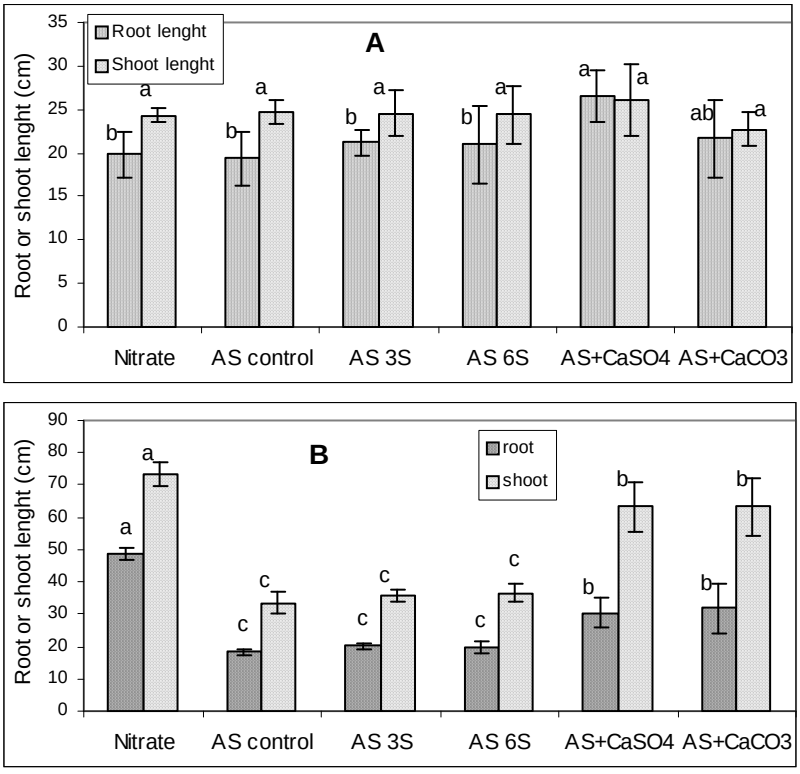

Fig. 6. 3. Shoot and overall root length before treatments (A); and at harvest (B). Ammonium sulphate (AS control), ammonium sulphate with 3 split applications (AS 3S), ammonium sulphate with 6 split applications (AS 6S), ammonium sulphate + calcium sulphate (AS+ $CaSO_4$) and ammonium sulphate + calcium carbonate (AS+ $CaCO_3$).

6.4.2.2. Number of lateral shoots

Number of lateral shoots was strongly influenced by N-forms and Ca concentrations in the nutrient solution (Fig.6.4A). Plants fed with nitrate had highest number of lateral shoots per pot (15 shoots). Under ammonium in nutrient solution, only when plants received higher and additional concentration of Ca (as $CaSO_4$), or pH and Ca effects (as $CaCO_3$), they showed more lateral shoot production which was quite significant compared to control. However there was no significant difference between sulphate or carbonate forms of calcium in this case.

6.4.2.3. Senescenced leaves and chlorophyll content

Fig. (6.4B) shows the number of leaves which plants lost until harvest. Ammonium treatments showed high leaf chlorosis, senescence, and abscission which started 1 week after receiving ammonium. Nitrate grown plants, as well as plants grown with ammonium which received supplement calcium as $CaSO_4$ or $CaCO_3$ show the lowest number of abscessed leaves. Chlorophyll content of upper part or younger leaves (Fig.6.4C) showed higher amounts for ammonium rather than nitrate grown plants, however there is not significant difference between control, split applications and those plants received Ca as AS+$CaSO_4$ or AS+$CaCO_3$. On the other hand, chlorophyll content of older or lower leaves showed significant differences among plants. In older or lower leaves of plants under control (AS sole) and split applications there was a significant reduction in chlorophyll content, however most of these plants lost more than half of their leaves (lower leaves) until harvest (Fig 6.4B). Chlorophyll content of whole plant showed the highest data when plants received $CaSO_4$ or $CaCO_3$, even better than nitrate nutrition.

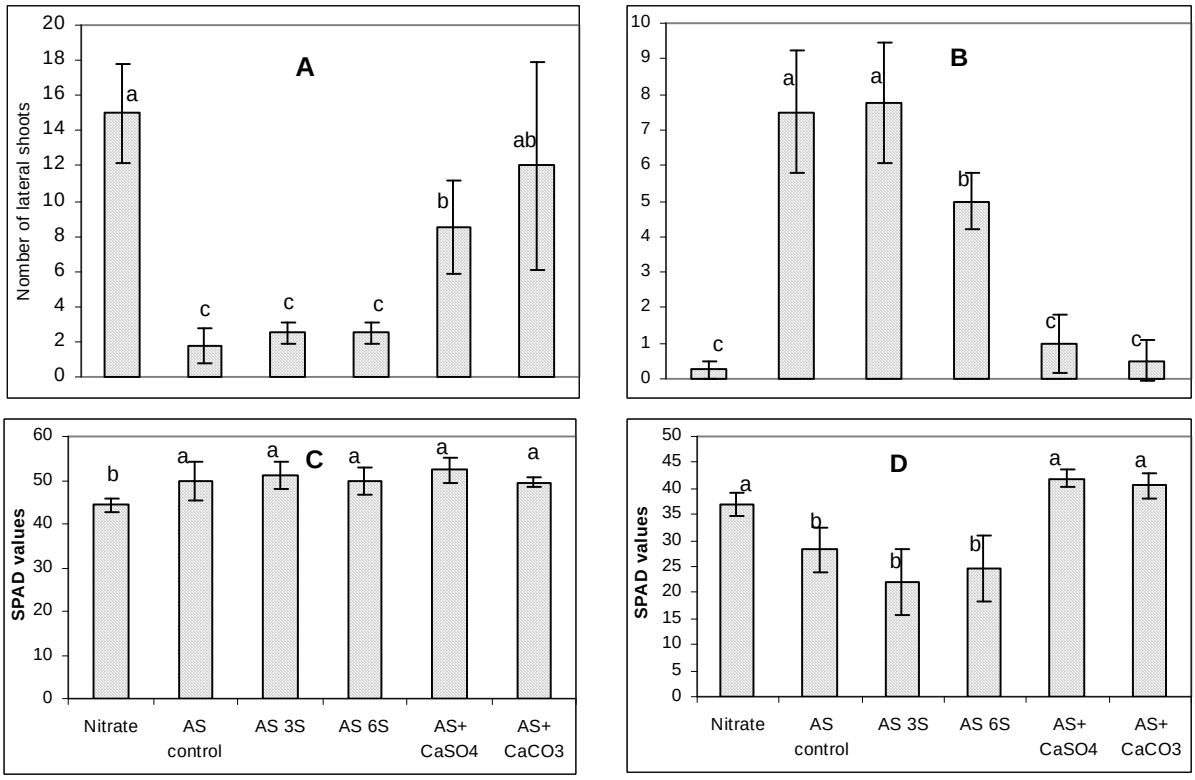

Fig. 6. 4. Number of lateral shoots per pot (shoots ≥ than 3 mm) at harvest (A); number of lost leaves (B); chlorophyll content of top leaves (C); chlorophyll content of lower leaves (D)

6.4.2.4. Root and shoot biomass

Nitrogen forms strongly affected plant shoot and root production (Fig. 6-4). Plants grown with nitrate as well as ammonium, when they received $CaSO_4$ or $CaCO_3$ had highest root fresh (Fig.6.5A) and dry matter (Fig.6.5C). Similarly, shoot fresh matter was highest for these plants compared to control or split applications (Fig.6.5B). Plants grown with nitrate showed significant higher fresh shoot production compared to all other treated plants (Fig. 6.5B), however for dry weight, nitrate grown plants did not show any significant difference compared to ammonium treated plants which received Ca^{2+} as $CaSO_4$ or $CaCO_3$ (Fig.6.5D).

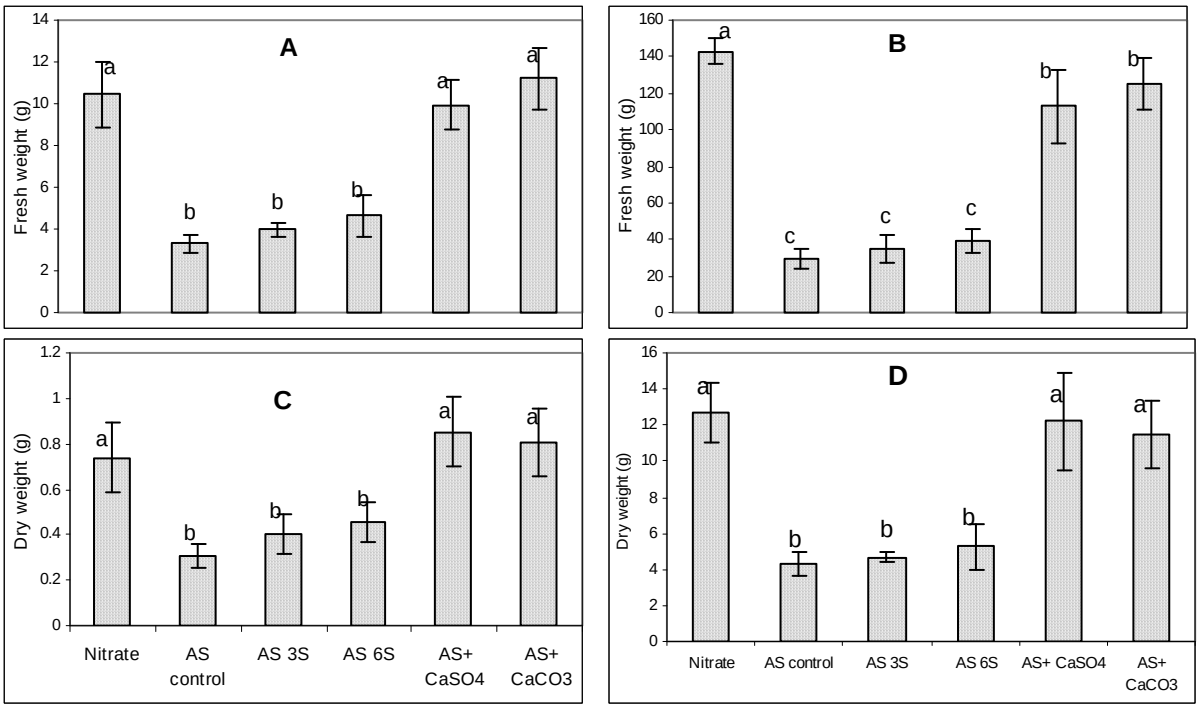

Fig. 6. 5. Root fresh weight (A), shoot fresh weight (B), Root dry weight (C), and shoot dry weight (D)

6.4.2.5. Water consumption and xylem sap flow

In nutrient solution, water consumption is strongly influenced by N forms as well as the presence of higher Ca^{2+} concentrations (Fig. 6.6). While ammonium inhibited water uptake in plants under control and split applications, Ca^{2+} ion in medium significantly improved water uptake of plants. There was no significance difference for water consumption between plant treated with $CaSO_4$ and those received nitrate, during the most period of experiment (not presented results), indeed for two first weeks $CaSO_4$ treated plants had the highest water consumption, however with growing plants and increasing plant size 10 mM Ca^{2+} as $CaSO_4$ seems to be not enough to keep growing conditions compatible with nitrate. Nevertheless, with progress in plants development and size, nitrate and AS+$CaCO_3$ seem to have higher water consumption. $CaSO_4$ and $CaCO_3$ in nutrient solution surprisingly improved transpiration which is a function of higher leaf area due to a better growth as consequence of healthy roots. Starting pH for $CaSO_4$ and $CaCO_3$ treatments was 6.4 and 6.8, respectively, and for other treatments it was about 5.4. In nitrate grown medium there was a significant increase of pH (5 to 7), however very rarely under nitrate nutrition pH exceeds 7. Moreover, even under micro molar daily concentrations of ammonium (in plants under 6 split applications)

there was a sharp reduction of pH (at the beginning when roots were not damaged). PH changes during 72 h in nutrient solution followed the same order which happenned at 24 h. Always the lowest pH was recorded for $CaSO_4$ treated plants, except at the beginning of experiment when control and split application of ammonium showed lower pH (data not presented).

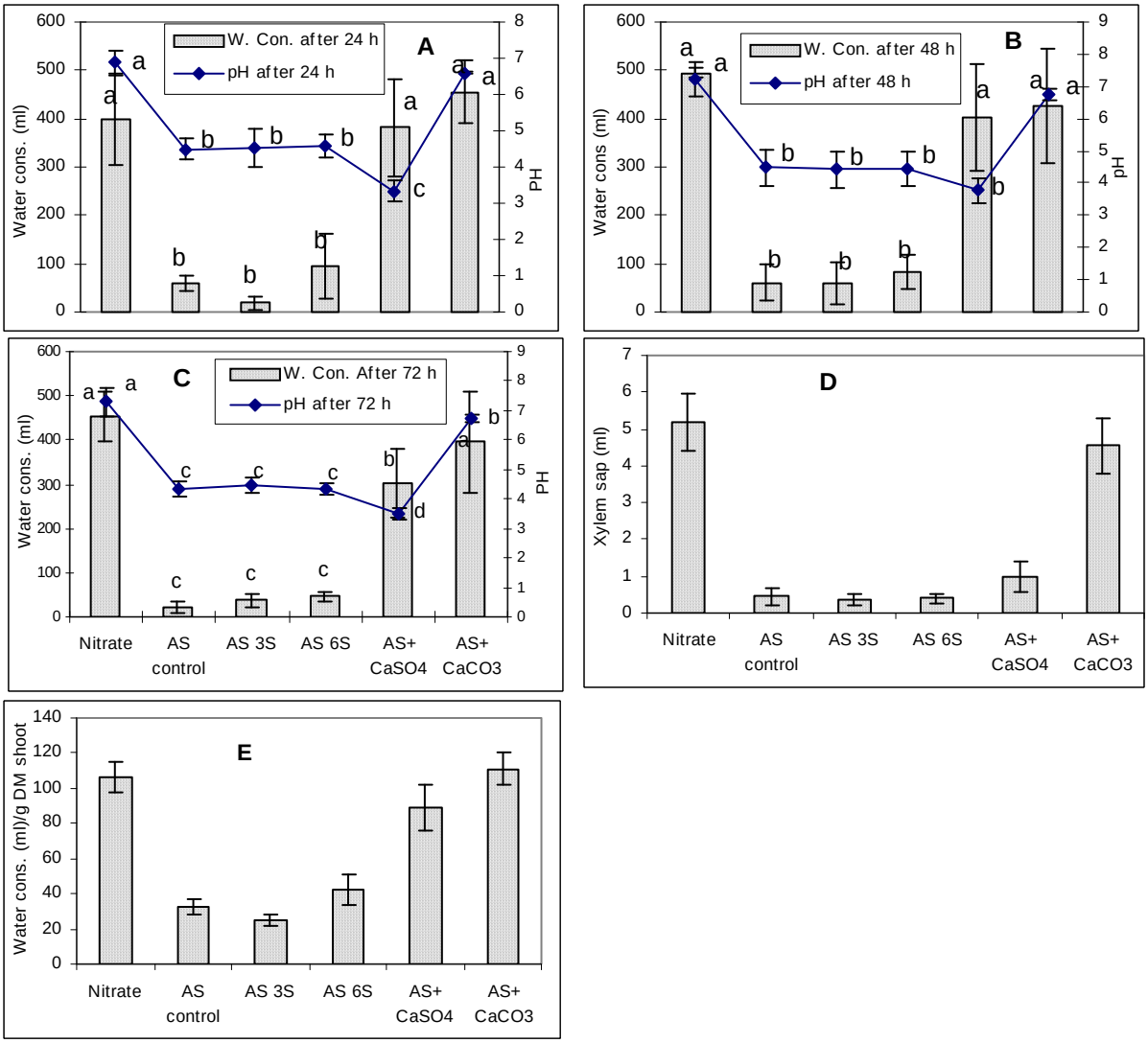

Fig. 6. 6. Water consumption and pH changes of plants after 24h (A), 48h (B), and 72h (C) of last nutrient solution change, just before harvest. Xylem sap exudation per plant which collected during 8 h (D). Whole water consumption during 72 h per g shoot dry weight (E).

Xylem sap which has been collected at harvest didn't completely follow water consumption trends among treatments. Nitrate and AS+$CaCO_3$ grown plants had the highest collected xylem sap, similar to their water consumption. Plants treated with $CaSO_4$ didn't follow their water consumption and they showed no significant difference compared to control or split applications. It is worth mentioning that except this xylem sap flow, for most other growth characteristics, AS+$CaSO_4$ treated plants show close similarities to plants grown under nitrate or AS+$CaCO_3$.

6.4.2.6. Nutrient concentrations

Total nitrogen (Fig. 6.7A) showed highest concentrations of N in AS+$CaSO_4$ and AS+$CaCO_3$ grown plants, even more than nitrate fed plants. Plants belong to AS sole (control) as well as split applications, despite lower N concentration in shoots, had highest total N concentration in roots. Nitrate grown plants had the significant lowest N in their root, which must be considered based on their root biomass.

Potassium concentrations in shoot (Fig 6.7B) showed significant higher amounts in plants grown in nitrate, AS+$CaSO_4$ and AS+$CaCO_3$ compare to AS sole and split applications. These differences would get even wider if the contents of nutrients would be considered. Potassium was particularly higher in shoots rather than in roots in AS+$CaSO_4$ treated plants more than any other treatments. Despite receiving additional Ca as $CaCO_3$ or 10 mM $CaSO_4$, nitrate grown plants had highest concentration of calcium in their shoot (Fig.6.7C). Control plants (AS sole) as well as plants with split applications of AS showed the lowest Ca concentration specially in their root. Similarly Mg concentration in shoot (Fig. 6.7D) is relatively high and not significant in all plants except AS+$CaSO_4$ treated plants, which show the lowest Mg concentrations. However roots of the latter plants in contrast to nitrate or AS+$CaCO_3$ treated plants showed the highest Mg concentration. Significant higher Mg concentration in shoots rather than roots occurred for all treatments, but not for AS+$CaSO_4$. Manganese concentration was quite higher in nitrate and AS+$CaCO_3$ treated plants compared to all other plants including AS+$CaSO_4$ grown plants (Fig.6.7E), both in root and shoot, although the concentrations were higher in root rather than shoot. Plants treated with AS+$CaSO_4$ showed trends in root and shoot Mn concentration similar to control and split applications.

In (Fig. 6.7F) all plants showed several fold higher Fe concentrations in root compared to shoot, however in shoot highest concentrations occurred for nitrate and AS+$CaCO_3$ plants, which were significantly higher than all other plants. AS+$CaSO_4$ as well as AS sole and split application plants showed insignificant similar concentrations. Zinc and cupper

concentrations (Fig 6.7G, 6.7H) in root and shoot showed very similar trends, but not with big differences among plants for both shoot and root. However upon conversion of these data as nutrient content per dry matter, significant differences must be exist.

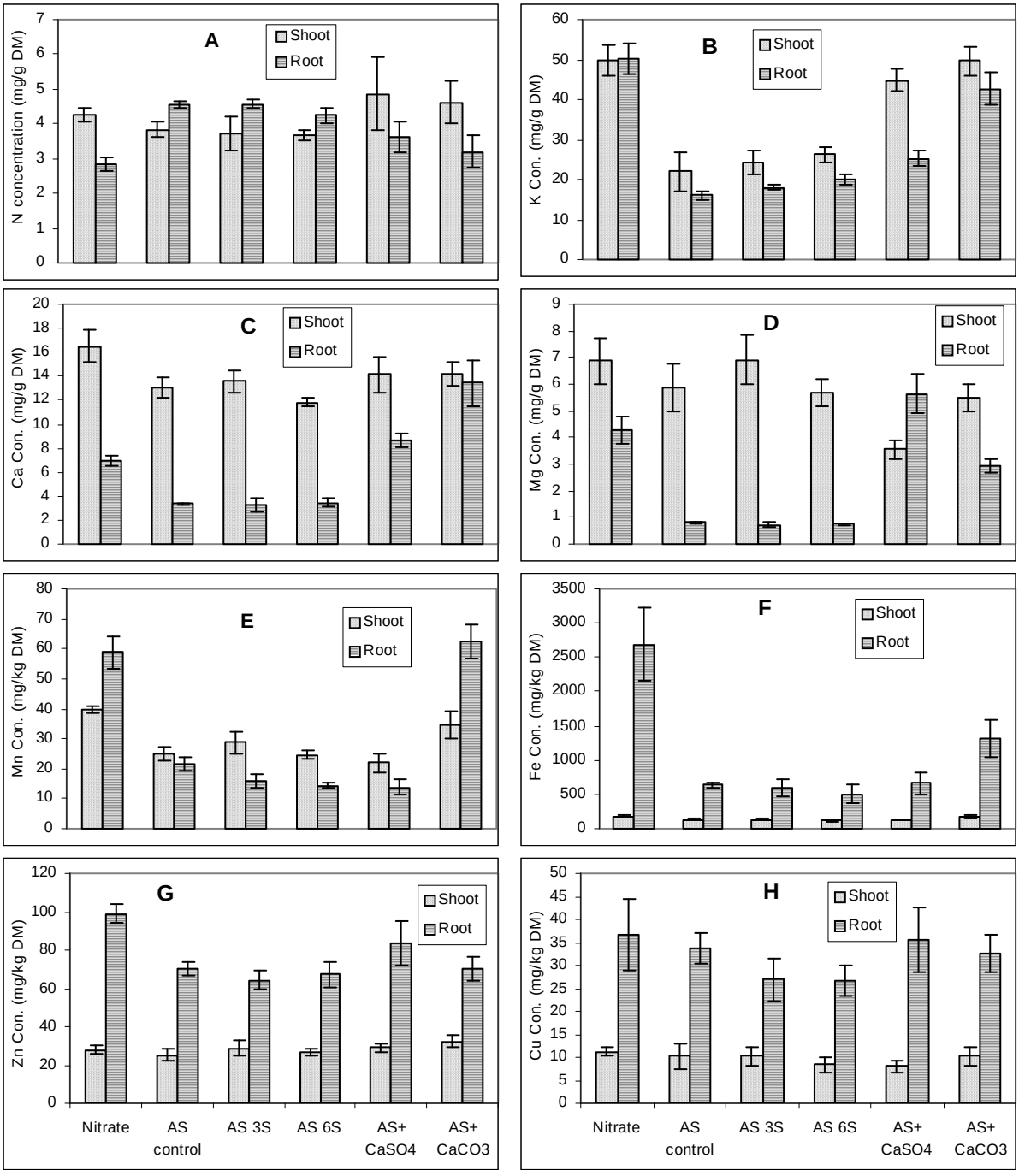

Fig. 6. 7. Nutrient concentrations in root and shoot of plants.

6.5.Discussion

Water consumption of crops and water preservation is quite important in semiarid and arid parts of the world. There is good indication that NH_4^+ improves drought and salinity stress compared with nitrate in soil. Our soil experiment also supports this fact (data not presented), where plants with stabilized ammonium use less water compared to nitrate fed plants. However, in nutrient solution to continue transpiration and water consumption, adequate nitrate concentration in root medium is necessary (6.2A and 6.2B) and sole ammonium impaire water uptake and transpiration due to root necrosis. No significant difference in water consumption at the beginning indicates that for plants, applied amounts of 0.8, 2 and 5 mM $N\text{-}NO_3$ is enough to maintain normal transpiration and water uptake. Increased water consumption after 96 h might be due to higher plant growth following 3 days growing in higher N concentrations (2 and 5 versus 0.8 mM). This increased water consumption continued more strongly in the next 4 days. Interestingly, plants had no significant difference in terms of their shoot dry matter. There was, however, positive correlation between water consumption and shoot fresh weight. It is well known that nitrate functions differently in plant physiology, including osmotic adjustment and as a signalling molecule (Blom-Zandstra and Lampe 2007; Wang et al., 2000). Probably here both mechanisms play role in observed higher water consumption. This might be due to higher uptake rates of NO_3^- and water than their assimilation when plants receive higher NO_3^- concentrations. This consequently leads to higher turgidity of plant cells and watery tissues. Also it is possible that a photosynthesis limiting steps (such as enzyme activity) might be involved as a limiting factor for assimilation, particularly at the immediate transfer of plants to higher NO_3^- concentrations (5 mM). Van Ieperen et al., (2003) indicated that xylem hydraulic resistance decreases with increasing nitrate concentrations, which leads to higher water uptake, watery tissue and finally significant higher fresh weight, but not dry weight (Fig. 6.2C and D). With progress in tomato plant development, there is a high hydraulic resistance between plant and tomato fruit tissue (van Iperen et al, 2003), which could lead to some disorders such as blossom end rot. This highlights the role of nitrate in reduction of xylem hydraulic resistance. Therefore theoretically it must be possible that adequate nitrate improves the nutrients (uptake) and movements inside the xylem and phloem vessels, which is quite important in the case of Ca related to Blossom end rot. This is nicely presented for calcium and other nutrients in Fig. (6.7). Uptake and distribution of Ca is generally believed to be the main factor controlling blossom end rot, which is a common problem during the production of tomato fruits (DeKock

et al., 1982). Any limitation in transpiration and water import, could also limit Ca^{2+} content and distribution in plant.

An amount of 10 mM Ca^{2+} as $CaSO_4$ kept root length growth and root biomass (even in low pH of nutrient solution) comparable to nitrate or AS+$CaCO_3$ grown plants. This indicating an effect of root growth stimulation due to Ca^{2+} ions, which is very important under NH_4^+ nutrition. Compare to control, in plants treated with AS+$CaSO_4$ or AS+$CaCO_3$, the ratio of increased growth for shoots were much higher than roots length (Fig. 6.2B). Also improved root dry weight (Fig. 6.5C and D) indicating lateral root growth improvement, or higher shoot dry weight indicates higher lateral shoots and leaf area production. In control and split applications, root growth (root biomass) inhibited completely just after application of ammonium, because root overall length changed from 21 cm to 20 cm (Fig. 6.2). This reduction in root length is mainly because of digestive effect of low pH in root growth medium (nutrient solution), which finally leads to root necrosis particularly on root tips. Furthermore, root hairs and root tips which are the main place for nutrients uptake, are the most sensitive to low pH and were damaged first, but for plants which received supplemental calcium this damage was not significant.

More lateral shoot production under nitrate or ammonium treatments which received additional $CaSO_4$ or $CaCO_3$, indicating a cytokinin-like behaviour of NO_3^- and Ca^{2+} ions. Both nitrate and calcium plays important role in plant cells signalling and cell division (Wang et al., 2000; Marschner 1995). On the other hand, generally there is a positive correlation between nitrate, cytokinin concentration in growth medium and lateral shoot production in tomato (Rahayu 2003; Gweyi 2006). In addition, cytokinin levels in plant are in close relation to NO_3^- status of plant. Ammonium as well as urea suppresses lateral shoot production (Gweyi 2006). In nutrient solution but not in soil system, highly leaf senescence and yellowing in NH_4^+ treated plants is probably due to water stress, N limitation which force plant to remobilise N from older leaves, ethylene production as a result of stress conditions, and a higher ratio of abscisic acid/cytokinin. Cell division, expansion and elongation all seem to reduce with ammonium, while they tended to increase with nitrate concentrations in root medium in our plants (Fig. 6.2B).

Higher concentration of chlorophyll due to ammonium nutrition is well known (Bligny et al., 1997; Forde and Clarkson 1999), but this is merely in younger leaves, and similarly in our plants in nutrient solution and soil culture (not presented data) has been observed. However, in severe toxicity conditions (nutrient solution), leaf yellowing and senescence also need to be considered. Less chlorophyll contents of older leaves compared to young leaves of NH_4^+ fed

plants may be due to ethylene production under NH_4^+ stress, nitrogen limitation to plants due to root necrosis, and finally water stress. In other word ammonium treated plants get higher concentration of chlorophyll in young parts, at the expense of abscission of most of their older leaves (Fig. 6.4B and C and D). For some replicates, plants lost more than 70% of their leaves even in split applications. Therefore chlorophyll degradation as a response to NH_4^+ nutrition starts at older leaves and progresses to younger leaves with times, and the severity of lose is a function of ammonium treatment duration. Apart from other physiological responses, NH_4^+ showed an effect on leaf and plant growth similar to growth inhibitors. In plants grown under $CaSO_4$ and $CaCO_3$, chlorophyll content for both top and lower leaves are higher, indicating a coping strategy of these plants with toxicity effects of NH_4^+ nutrition (Fig.6.4D) when they receive supplimental calcium. Plants seem to exert a highly control over NH_4^+ toxicity, when they received additional Ca^{2+} or adjusted pH. Root membrane integrity and cell wall stabilization due to Ca^{2+} effect, and improving ammonium movement by apoplastic pathways could be the possible explanations. It might be possible that other cations will pass trough symplastic pathways. Therefore there would not be significant interference with plasma membrane and cytoplasm biochemistry.

Significant low fresh and dry matter production of control and split application plants, are mainly due to growth inhibitory effect of NH_4^+ (Schjoerring et al., 2002; Siddiqi et al., 2002). Plants received nitrate, or ammonium plus $CaSO_4$ or $CaCO_3$ showed significant higher biomass production, indicating improving effects of Ca^{2+} or pH on growth characters. Shoot:Root dry matter ratio for nitrate grown plants was higher than ammonium grown plants (not presented results). Claussen, (2002) showed that under ammonium nutrition in hydroponic culture reduction in fruit dry matter is more than vegetative parts of tomato, while nitrate nitrogen rather supported an increase in dry matter accumulation in the reproductive organs.

Improvement of growth characteristics under AS+$CaSO_4$ or AS+$CaCO_3$ applications also could be because of antagonist effect of Ca^{2+} ions to NH_4^+ ions in nutrient solution and on membrane sites. Split applications of ammonium, which supposed to give enough time to plants to assimilate absorbed NH_4^+, could not improve growth conditions of tomato plants. For example plants which received 6 times applications of 330 µM NH_4^+ compared to AS sole (control) plants which received their 2 mM N once at the beginning, showed no difference except for the number of leaves that plant lost (because of NH_4^+ toxicity effect). This indicates that µM concentrations of ammonium in nutrient solution is also toxic and suppress tomato plant growth. However most of researches which have been done dealing

with this topic, indicate toxicity symptoms under high concentration of N-NH_4^+ in nutrient solution (Roosta and Schjoerring, 2007; Siddiqi et al., 2002; Claussen 2002).

Better and higher transpiration (water consumption) of AS+$CaCO_3$ and AS+$CaSO_4$ treated plants compared with AS sole or split applications of AS indicating the role of pH and Ca^{2+} ions in water uptake. Despite receiving only 1mM Ca^{2+}, nitrate treated plants showed the highest transpiration. Similar conditions (due to pH and Ca^{2+} effect) exists for AS+$CaCO_3$ treated plants which also showed high rate of transpiration. However, in AS+$CaSO_4$ grown plants only a Ca^{2+} effect was the only reason of higher water consumption compared to AS sole plants, because nutrient solution pH in AS+$CaSO_4$ treated plants decreased to near 3.3. For most of experiment duration, plants grown under AS+$CaSO_4$ had the lowest medium pH, and at the same time growth characteristics of these plants were comparable to nitrate or AS+$CaCO_3$ treated plants. This indicates that high concentration of Ca^{2+} can not completely remove negative effects of NH_4^+ toxicity (low pH effect). On the other hand, effect of external medium pH on water uptake seems to be quite significant, due to a trend of transpiration reduction in $CaSO_4$ treated plants with time. This might be due to sensitivity of aquaporine proteins (water uptake channels) to low pH. Claussen (2002) mentioned that ammonium nitrogen can lead to a decrease in water use efficiency, and in this case proline is a reliable indicator of the environmental stress imposed on hydroponically grown tomato plants. In our study a sharp reduction in pH under ammonium, and a more slowly increase in pH with nitrate indicating organic acid release (a compensation for OH^-) through plant roots. Nevertheless tomato is well known for its ability to release organic acids under different growth conditions (Neumann and Römheld, 1999). Plant growth and development (tolerance) in low pH of nutrient solution containing ammonium+10 mM $CaSO_4$, probably is a physiochemical effect of Ca^{2+} ions in exteracellular and intracellular medium. Calcium mechanically through binding to exchange positions on root cell surface, competition for non selective cation channels, increasing salt concentration and osmotic effect of external medium can limit NH_4^+ uptake, as it is observed in similar ways in salt stress plants (Cramer et al., 1985; Tuna et al., 2007). Cell internal mechanisms of Ca^{2+}-induced tolerance to ammonium involves precipitation of Ca^{2+} ions on cell wall (Marschner 1995; White and Broadley 2003), binding to phospholipids at outer side of membranes and increasing their integrity and selectivity (Sattelmacher, 2001), an highly activated Ca^{2+} influx/efflux protein systems which limit NH_4^+ influx, and the role of Ca^{2+} as secondary messenger and signalling molecule (Sanders et al., 2002 ; Knight et al., 1997; Blackford et al., 1990; White and Broadley, 2003).

Ammonium and water shortage (by limiting Ca partitioning to fruits) both could lead to blossom end rot in tomato (mainly in nutrient solution and in calcium deficient soils). A mild water shortage may improve Ca concentrations inside the plants. However a longer water shortage can induce calcium deficiency and change the availability of certain elements, and consequently mineral composition of plant (DeKock et al., 1982; van Ieperen et al., 2003).

In present experiment, ammonium damaged and suppressed water uptake systems in plant roots, while at the same condition presence of Ca^{2+} ions (even with low pH in root medium) improved water uptake almost similar to nitrate fed plants. Low water availability in the root environment causes higher hydraulic resistance of xylem elements (Van Ieperen et al., 2003). During tomato leaf or fruit development hydraulic resistance of xylem vessels decreases (Van Ieperen et al., 2003), which is coincident with high activity of phytohormones such as sytokinin in these developing organs. Changes in xylem hydraulic resistance are necessary to reduce the effects of diurnal water stress in the plant. Therefore in our experiment a higher pH as well as Ca^{2+} ions under $CaCO_3$ nutrition, or a higher Ca^{2+} concentration in nutrient solution under AS+$CaSO_4$, or high NO_3 concentration (Fig. 6.2) could reduce hydraulic resistance of xylem, finally leading to better water uptake and plant growth. In addition shorter or smaller diameter vessels which may occur directly under NH_4^+ or indirectly through NH_4^+-induced water stress, also significantly increase xylem hydraulic resistance (Van Ieperen et al., 2003). Similar phenomena exist for salt stress plants (Cramer 2004; Cramer et al., 1985; Navarro et al., 2000; Tuna et al., 2007), however in salt stressed plants water stress is an indirect effect, but in our plants NH_4^+ had a direct effect on water relations of plant because of increasing hydraulic resistance, through physiochemical damage and consequently necrosis of root systems.

To our surprise, xylem sap flows were not consistent with water consumption of plants (Fig. 6.6D). Similar to their water consumption (transpiration), nitrate grown plants had highest xylem sap amount collected during 8 h. The same trend exist for AS+$CaCO_3$ treated plants (pH 6.7). This might indicate the role of pH on xylem flow and transpiration, however in our study root health positively correlated with root medium pH. Plants treated with AS+$CaSO_4$ didn't have the same trend of xylem flow compared to transpiration amounts of plants. In contrast to all improved growth factors, in this case they were more similar to control (AS sole) plants. This indicates a shoot-originated controlling factor for transpiration. This controlling factor could be, cell signalling by cytosolic Ca^{2+} oscillation, micro molar cytosolic or apoplastic NO_3^- concentrations that may stored during pre-culture condition and is released over Ca signalling, or a hormonal-calcium dependent effect. Also it is possible that because

collection of xylem sap happened at harvest, 10 mM Ca in AS+$CaSO_4$ could not counteract a further pH decline and H^+ effect in plasma membrane stability.

Low K concentration (6.7B) in NH_4^+ treated plants may be because of competitive effect of NH_4^+ for uptake, or NH_4^+-induced K influx blocking through membrane depolarisation. Shoot: root K concentration ratios in AS+$CaSO_4$ treated plants was higher than all other plants including nitrate and AS+$CaCO_3$ grown plants. In addition a significant reduction in K and Mn concentrations under ammonium conditions compared to nitrate was not avoidable, and without any doubt potassium status of shoots improved under increasing Ca^{2+} concentration in nutrient solution.

For calcium (Fig. 6.7C), differences in root Ca concentrations were wider than differences among shoots. This may be due to higher availability of Ca in NO_3^- treated plants, or in ammonium treated plants which received $CaCO_3$ or $CaSO_4$. This can give an order of important factors improving Ca^{2+} uptake, as pH> NO_3^-> Ca^{2+} concentrations, respectively. It seems that root Ca concentration, to a great extent, is under pH control, so even 1 mM Ca^{2+} as $Ca(NO_3)_2$ is more effective than 11 mM Ca^{2+} in NH_4^+ nutrition (AS+$CaSO_4$). Calcium concentration in plant is also related to total salt in growth medium (Hoque et al., 2008). Supplemental calcium sulphate added to nutrient solution containing salt, significantly improved growth and physiological variables affected by salt stress (e.g. plant growth, fruit yield, and membrane permeability) and also increased leaf K, Ca^{2+}, and N in tomato plants (Tuna et al., 2007). Similarly Roosta and Schjoerring (2007) showed that low tissue levels of calcium and magnesium in NH_4^+ fed plants is common phenomena in tomato and cucumber plants.

Magnesium concentration of shoot material of AS+$CaSO_4$ treated plant was significantly lower than other plants including control and those with split applications (Fig. 6.7D). However root concentration showed significantly higher Mg compared to other treatments. Higher calcium in root medium under NH_4^+ nutrition may significantly reduced the movement and mobility of Mg in xylem sap. High Mg concentration in AS sole (control) or split applications may be due to remobilisation of Mg from leaves which become senescent, however Mg content of plants could not be similar when the dry matter of whole plants would be considered. Pei et al., (1999) showed that cytosolic Mg concentration has regulatory effect on vacuole ion channels, and it is in present of Mg that submicromolar concentration of Ca can do its physiological functions of vacuolar current. In all treatments except AS+$CaSO_4$ magnesium concentration was higher in shoot, specially for ammonium treatments which had significant less Mg in shoots compared to other treatments, although Mg deficiency

symptoms were not observed on plants. Here maybe the synergistic interaction effects (Pei et al., 1999) of both Mg and Ca must be considered, despite the same concentration of Mg which has been applied for all treatments.

Low concentrations of Mn in AS+$CaSO_4$ treated plants is mainly due to low pH in growth medium. Manganese uptake is severely inhibited by (low pH) proton (Marschner et al., 1987). While in two other groups, nitrate and AS+$CaCO_3$ grown plants, high concentration of both root and shoot compared to other treatments exist. From the data presented in the (Fig. 6.7) it can be concluded that Mn^{2+} uptake has a significant positive correlation with higher pH, for example in nitrate and AS+$CaCO_3$ treated plants. This might be due to cell membrane and cell wall integrity. Similarly Fe concentration of shoot material (Fig. 6.7) in nitrate and AS+$CaCO_3$ grown plants were significantly higher than AS sole and other ammonium treated plants. Zinc and cupper concentration did not change dramatically, in contrast to other nutrients. Micronutrients relatively are not mobile inside plants (Marschner 1995), therefore their remobilisation from older senescenced leaves could not play important role in this case. On other hand, particularly for Fe and Cu, however the role of NH_4^+ nutrition on their remobilisation has not been studied yet. Cytosolic pH or other related changes which could occur with ammonium nutrition may also have an effect on these nutrients remobilisation. These observations lead us to conclude that higher NH_3:NH_4^+ ratio in higher pH may reduce many negative effects of NH_4^+ uptake on micronutrient compositions of plants. Plants under cultivation of AS+$CaSO_4$ and AS+$CaCO_3$ had the highest total N concentration compared to AS sole and split applications of AS. This is more highlighted when we take into account higher performance and biomass production of these plants, which can lead us to conclude that ammonium uptake occurred at rather high rates without any toxicity effect. This resulted in plant growth characteristics compatible to nitrate nutrition (Fig 6.5). It is surprising that in plants grown under nitrate, Ca was the highest, while AS+$CaSO_4$ treated plants received 10 mM Ca (clearly a higher Ca concentration). On the other hand, N concentration of plants under AS+$CaSO_4$ and AS+$CaCO_3$ was highest, while general opinion is that Ca compete with NH_4^+ for uptake sites on root surface and ion channels. Therefore limiting NH_4^+ uptake by Ca^{2+} ions could not be a major reason for all improved growth characters observed in this research. Better assimilation of ammonium and preventing toxicity and damage effects of NH_4^+ by Ca or pH probably are the main reasons. Furthermore, higher Ca in nutrient solution did not increase Ca concentrations in plant shoots (Fig. 6.7B). However, upon conversion of N concentrations to content, significant differences could be observed compare to control plants. Similarly Zou et al., (2005) found that adding $CaCO_3$ improves the N concentration in

NH_4^+ treated tobacco plants. Nevertheless N concentrations of root in control or split application plants showed highest N. This may be mainly due to passive adsorption and penetration of NH_4^+ into the necrosis root cells.

6.6. Conclusion

Under ammonium nutrition tomato plants showed severe toxicity symptoms even with micromolar concentrations (in split applications). These symptoms are visualized as suppression of root and shoot growth, chlorophyll degradation in lower leaves extending to upper leaves with time, leave abscission and consequently growth inhibition. However, when supplemental calcium as calcium sulphate was added under ammonium nutrition, significant root and shoot growth of tomato plants occurred. This improved growth performance was mainly due to better root growth (root system) under supplemental calcium in nutrient solution, even under relatively a low pH. Supplemental calcium induced significant improvement in water consumption of ammonium treated plants. However, similarly nitrate as well as AS+$CaCO_3$ treated plants had high rate of transpiration. No significant difference between split applications of ammonium (3 and 6 splits) compared to sole ammonium (control) plants for all growth related factors was observed.

Chapter 7: General discussion

7.1. Inhibition of nitrification by chloride and DMPP

Agricultural activities in general and application of nitrogen fertilizers in particular, encourage nitrification and denitrification processes in soil. These two processes are in close relation with emission of contaminant trace gases such as NO and N_2O into atmosphere (Abbasi et al., 1997; Abbasi and Adams 2000a and 200b; Robertson et al., 1988). In cultivation systems more than 50-70 % of applied N is lost by different pathways from the soil (Velthof et al., 1998). Basicly plants can take up all forms of nitrogen except molecular nitrogen (N_2). However, some limitation may exist, for example, while plants can take up proteins, this uptake is limited to low molecular weight proteins. Nitrate and ammonium by far are the main N-forms for plant uptake. A mixed and balanced NH_4^+ and NO_3^- is probably the preferred form by most of plants to satisfy their nitrogen demands (Lauter et al., 1996). Meanwhile in nutrient solution but may not in soil, ammonium uptake suppress nitrate uptake in tomato plants (Loque and von Wiren 2004; Siddiqi et al., 2002). Many factors including environmental and soil conditions, plant species and their developmental stages control the form of N uptake. In acid soils of tropics or in cool climate of northern latitudes, however, amino acids may are the major N-forms for plant uptake (Kielland 1994).

Fixing properties of NH_4^+ to clay minerals offer potential advantages for plant nutrition as well as environmental friendly activities in agricultural, but also limitation due to slow access to plants at low root growth. This property keeps ammonium always in the upper soil horizons and prevents nitrogen (nitrate) leaching, thereby improves the access to plant roots. Application of nitrification inhibitors can prolongate the presence of NH_4^+ in soil and improve plant N use efficiency, chlorophyll content, protein content, yield and dry matter production (Zerulla et al., 2001; Pasda et al., 2001; Linzmeier et al., 2001; Subbarao et al., 2006b). Nevertheless in application of NIs particular attention must also be paid to rotation crops, which grow following main crop under NIs application. In all N transformation processes in soil microorganisms act as a central point. Despite important role of *Nitrosomonas* bacteria in oxidation of ammonium to nitrite, now general opinion is that many other bacteria and even microorganisms are involve in this process (Leininger et al., 2006; Adair and Schwartz 2008). For their energy requirements these microorganisms, may have evolved different pathways of ammonium oxidation. Therefore, for effective inhibition of nitrification, compounds with different and a wide range of action may be required. In present study, chloride similar to DMPP was shown to have significant NI activity (Figs 3.1; 3.2; 3.3; 3.4). Moreover nitrifier bacteria in soil seem to be sensitive to a wide range of chemicals including chloride. The

inhibitory effect of chemicals or sensitivity of nitrifiers depends on dosage and microbial population dynamics inside the soil. Chloride has been shown before to inhibit nitrification, in concentrations between 7-50 mM (Darrah et al., 1987; Golden et al., 1980; Chen and Wong 2004). Similarly in present study, the specific chloride ion effect as well as related osmotic or salt effects, as KCl or NH_4Cl forms, could be the main reasons of significant inhibitory effect of Cl on nitrification. Despite late starting of net nitrification (after tree weeks), however until 7 weeks, still there was strong NI activity in Cl treated soils. Nevertheless under laboratory soil incubations, always there is a lag of 1-2 weeks in net nitrification (Hart et al., 1994; Mulvaney et al. 1997; Williams et al. 1998). This mainly could be due to immobilization and population establishment of nitrifying bacteria in the soil. On the other hand, for soils which have been stored under dry condition (without microorganisms) for a long period, population establishment takes a longer time. It is particularly important that soil microorganisms absorb substantial higher amount of NH_4^+ and NO_3^- than plants. This immobilization for NH_4^+ is higher than NO_3^- (Azam and Ifzal, 2006; Herrmann et al., 2005). So the presence of other competitive microorganisms rather than nitrifiers may function as a nitrification inhibitor through immobilization of ammonium (Jackson et al., 1989).

Apart from chloride, NH_3 and NH_4^+ also have been shown to have significant NI activity (Pang et al., 1973). Ammonium and chloride in form of ammonium chloride may have additive effects on NI activity, e.g. the role of chloride in suppression of take all disease has been attributed to the role of Cl on slowing down nitrification rate. So ammonium chloride might be an effective fertilizer for application in agricultural systems. When and where salinity and chloride is not a problem in the soil, ammonium chloride may offer better advantages in terms of N recovery rate compare to sulphate forms of N fertilizer. Adverse climatic and soil factors, as well as salt concentration could have accumulative or additive effects on nitrification inhibition. Therefore, under unfavourable conditions, besides strong and commercial NIs such as DMPP, DCD or Cl, even chemicals which typically are not inhibitors such as heavy metals (Premy and Cornfield, 1969; Hu et al., 2002) or sodium (Azam and Ifzal 2006) can function as potent nitrification inhibitors. When nitrogen is a limiting factor, nitrifying bacteria can oxidize a wide range of chemicals, however different microorganisms may play role in the process of ammonium oxidation. Therefore, a clear prediction of nitrification in a given soil is not a simple task. Slightly acidic condition, low organic matter and ammonium contents, long dryness, all contribute to slow down population establishment of oxidizing bacteria in the soil, leading to enhancement and extension inhibitory effect of chemicals such as DMPP or chloride.

7.2. NI activity of root exudates and shoot and root extracts of *Brachiaria humidicola*

Sustainable agriculture production is a necessary task in resource safety for future generations and needs precise control on long-term farming activities. High nitrification rate is not consistent with sustainable production. In addition, for economic and environmental reasons application of agrochemicals in production systems will be more restricted in future. Saving energy in farm, from fertilizers production and application to tillage operations could help to maintain our production systems more sustainable and productive. In such a sustainable system, a controlled release of mobile nutrients especially N according to plant requirements is highly required. Plants themselves through their root exudates have the ability to change the chemical, physical and biological conditions of their rhizosphere (Grayston et al., 1996; Marschner et al., 1987; Marschner, 1995; Phillips, and Tsai 1992; Neumann and Römheld 2000). These changes have important implications for plants in terms of nutrients acquisition and toxins reduction which frequently occur in the rhizosphere (Neumann 2007). If plants themselves can precisely manage nitrification in the soil, in economic and environmental point of view, it would have important practical implications. Finding such plants and related physiological and molecular characteristics can help to introduce such highly valuable properties to farming crops. Since long time nitrification inhibitory of plant based substances has been known (Sahrawat and Parmar 1975; Lodhi 1978; Santhi et al., 1986; AlSaadawi 1988; White 1988; Sylvester-Bradley et al., 1988). Recently *Brachiaria humidicola* has been considered as a plant with high potential for NNIs production and release as root exudates (Subbarao et al., 2005, 2006a, 2006c). To identify whether the reported release of NIs is a passive or actively regulated process, root exudates of plants grown in nutrient solution under different treatments were collected in distilled water. Root exudates of NH_4^+ and NO_3-precultured plants collected in distilled water didn't show significant NI activity (Figs 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.8) and the pH of collection medium declined to 3-4 for the plants which were pre-cultured with NH_4^+. However, NI activity was detectable in root washings when plants were exposed to extended collection times (24 h) in combination with NH_4^+ supply but not with NO_3^- in the collection solution. This observation is consistent with the findings of Subbarao et al., (2006a; 2006c: 2007a), who also reported release of NI compounds only in presence of NH_4^+ in the collection medium after a time of 24 h. However, it is well established that collection of root washings over extended time periods in media without Ca^{2+} supply has the risk of membrane damage due to leaching of Ca^{2+} from the plasma membranes (Cakmak and Marschner, 1987; Neumann and Römheld, 2007). This risk

is particularly expressed in acidic collection media, in present study, induced by sole NH_4^+ supply with a pH of less than 3. Accordingly, no NI activity was detected in the root washings when plant roots exposed to shorter periods (6 h) of the harmful conditions during exudates collection. These findings strongly suggest that release of NI compounds observed in this study and reported by Subbarao et al., (2006a, 2006c, 2007a, 2007c) was rather a consequence of root membrane damage due to acidic and protonic collection conditions, than mediated by controlled exudation from undamaged roots (extended exposure to NH_4^+ and consequently low pH and membrane damage). To test the hypothesis that NI compounds are released from damaged plant cells of Brachiaria, the NI potential of fresh root and shoot homogenates was measured after soil incorporation and incubation. Nitrification inhibition potential was detected in shoot but not in root tissues. The NI effect of soil-incorporated shoot tissues lasted for at least 8 d, while root tissue even stimulated nitrification with increasing incubation time. This NI effect is independent of N form, however, variability of data increases with increasing NO_3^- or in higher light intensity (Figs 4.5, 4.6, 5.4, 5.6). Meanwhile in our study inhibitory effects of unextracted shoot materials was a function of N concentrations or numbers of plants per pot. Concentrations more than 2 mM caused high variation of data. In contrast less concentration (1 mM N) prevented this data variation, showing significant nitrification inhibition (Fig. 5.6A) similar to soils under natural BH stands where N concentration insid the soil is not high.

Subbarao et al., (2007c) released a patent which identified several isomers of linoleic and linolenic acids as NI compounds in root washings of *Brachiaria*. The NI potential of pure linoleic acid additions to soil samples has been confirmed also in the present study (chapter 5), however the mechanism of the NI effect is still not clear. Since unsaturated fatty acids are rapidly oxidised, it is probably not the unmodified linoleic acid molecule itself, which exerts long-lasting NI effects in soils reported by Subbarao *et al*., (2007c). However, the oxidation products of linoleic acid, such as linoleic acid hydroxy-peroxides, which have a cytotoxic potential may offer a possible explanation (Kaneko, et al., 1994).

Although linoleic acid seems to be a NI compound released into root washings of BH (Subbarao *et al*., 2007a; 2007c) as a consequence of unfavourable conditions promoting membrane damage during the collection period, surprisingly soil incorporation of fresh homogenized root material did not show any NI activity (Figs 5.6 and 5.7). This might be due to (enzymatic) inactivation of NI compounds in root homogenates after decompartmentation of homogenized tissue. This effect may not occur during leaching into root washings. Linoleic acid in free and esterified form is a constituent of plant cell membranes (Kaneko et al. 1994)

and may be selectively liberated under conditions causing membrane damage (i.e. unfavourable conditions for collection of root washings).

Further fractionation and characterisation of NNI compounds in shoot tissue of *Brachiaria* plants revealed a particularly high activity in the ethanol-soluble fraction, both in plants with NH_4^+ and NO_3^- pre-culture, after extraction with solvents of increasing polarity (hexane, ethylacetate, ethanol, water). This solubility pattern is in line with observations of Subbarao et al., 2008 (unpublished) who isolated linoleic acid as NI compound from *Brachiaria* leaves by 80% methanol extraction.

Nevertheless, the results presented in this study can not support the hypothesis of a controlled release of NNI compounds in root exudates of BH in response to N- NH_4^+ as an adaptation to N-limited natural ecosystems in tropics. The data rather suggest that release of NI compounds as an effect of membrane damage, is caused by unfavourable conditions for collection of root exudates. However, this finding doesn't exclude the concept of N stabilisation by plant-borne NNI compounds in these ecosystems. Since NI activity was found also in the plant tissue (chapter 5), N stabilisation could be mediated by liberation of NNI compounds from plant residues of *Brachiaria* as a very competitive and long lasting tropical pasture species. Brachiaria humidicola are C_4 fast growing grasses, which produce enough shoot biomass which over its incorporation into the soil, significant inhibition of nitrification could occur. This is particularly important in vegetable production systems in developing country in tropic and subtropical regions of world, where intercropping and mixed culture is a highly valuable practice for soil perservation.

7.3. NO_3^- and NH_4^+ nutrition of tomato

Tomato represent one of the most sensitive species to NH_4^+ nutrition in nutrient solution (Loque and von Wiren 2004; Siddiqi et al., 2002; Claussen 2002). Severe toxicity symptoms can occur in few days after transferring to NH_4^+ medium. However, many factors including the intracellular and extracellular NO_3^-/ NH_4^+ ratios plays very important role in this ammonium toxicity on tomato plants (Britto et al., 2001; Kronzucker et al., 1999; Kronzucker et al., 2001). Based on preliminary experiments we hypothesised here that damages to root system is the main force behind NH_4^+ toxicity. The role of calcium in physiochemical and structural functions of plant cells is well known (Evans et al., 1991; Blackford et al., 1990; Marschner, 1995). Furthermore, in our study plants which grown in nitrate showed the highest Ca in their shoot, despite they received only 1 mM Ca, compare to 11 mM in AS+$CaSO_4$ and 3 mM in AS+$CaCO_3$ grown plants. Supplemental calcium as $CaSO_4$ was used to give a Ca ion

effect and $CaCO_3$ was used to give a pH effect (pH 6.6) Similar to other studies nitrate rather than ammonium represented preferable N-form for tomato plants. All growth related parameters improved under nitrate nutrition of plants compared to NH_4^+ grown plants (Figs 6.3, 6.5, 6.6, 6.7, 6.8). Healthy plants were observed in nitrate, AS+$CaSO_4$ and AS+$CaCO_3$. Increasing nitrate:ammonium ratios also has been reported to reduce ammonium toxicity. Despite we didn't use this, however calcium showed similar effct in this case to nitrate. Water uptake or transpiration represented an important indicator of root health and plant performance. Tomato plants under nitrate, or in NH_4^+ when supplied with supplemental Ca ions (as $CaSO_4$) or in a higher pH (as $CaCO_3$) showed a high transpiration rate compare to control plants (plants without additional Ca). This transpiration has positive correlation with plant root dry weight (Fig. 6.6, 6.7). On the other hand, ammonium treated plants showed an immediate inhibition of root growth after transferring to NH_4^+ medium (Fig. 6.3). However, when these plants received 10 mM Ca as AS+$CaSO_4$ or a constant pH of 6.6 by application of $CaCO_3$ (effects of pH and Ca^{2+}), they didn't show toxicity symptoms (Figs. 6.2, 6.5, 6.6, 6.7, 6.8). Their growth performance was more or less similar to nitrate-fed plants. When $CaSO_4$ or $CaCO_3$ were added to NH_4^+ nutrient solution, plant growth characteristics such as root and shoot biomass, root and shoot length, number of lateral shoots, chlorophyll content and mineral nutrients composition significantly improved. In control plants (in only ammonium (AS sole) or in plants which received split applications of ammonium, toxicity symptoms, including growth inhibition, reduced leaf area, yellowing and senescence of older leaves, root necrosis and water stress were common phenomena. This might be due to difficulties related to control of ammonium fluxes by plants (Loque and von Wiren 2004). Howitt and Udvardi, (2000) argued that the toxicity symptoms is mainly because of high concentration of NH_4^+ in apoplast, which normally needs to keep down. However Britto et al., (2001) didn't accept it and challenged such conclusion. Furthermore, the symplastic pathway allows the plant to control the rate and selectivity of cations transport to the shoot (White 2001), so NH_4^+ primarily may damage symplastic pathway which regulates the selectivity of ions. Therefore, positive effects of Ca is probably due to increasing plant cell membrane integrity and selectivity, which simply do not allow NH_4^+ ions to pass through. Moreover, NH_4^+ would pass mainly through apoplastic pathways to the shoot, where it can be assimilated to other compounds. Nevertheless, this apoplastic pathway also needs to be protected against adverse effects of ammonium, by calcium ions. Our findings are consistent with this hypothesis supporting the important role of Ca on protecting cell integrity and symplastic pathway. However, the role of other mechanism can not be ignored. It is in particular importance to

consider that there are different NH_4^+ transporters which differ in their biochemical properties, localization, and in regulation at the transcriptional level (Loque and von Wiren 2004). Root necrosis and consequently related water stress could be the main reason of toxicity symptoms in macro level. It is probably the cytosolic and membrane sensitivity which are expressed as NH_4^+ toxicity, in micro level. Physiochemical changes on membranes and cell walls as well as in cytoplasm would probably be the main protective mechanisms of Ca against negative effects of ammonium. Physical protection against a low pH at the surface of root, improving NH_4^+ compartmentation and assimilatory capacity through glutamine synthetase, increasing enzyme activity which can remove NH_4^+ from cytoplasm, increasing plasma membrane stabilization and integrity and cell wall hardening (Marschner et al., 1987), Cations (K, Mg, Ca) improvement (Redinbaugh and Campbell, 1993), preventing phytohormone imbalance (Gerendas et al., 1997; Zhang and Rengel, 1999; Britto and Kronzucker, 2002), are among the possible mechanisms which Ca also may induce better plant performance under NH_4^+ nutrition. Additionally, the growth improving effects of Ca might be due to, activation of an influx/efflux system of Ca at plasma membrane of root cells, stimulation of a symplastic (more selectivity for cations) rather than apoplastic pathways of ions transportation (White 2001), and reducing hydraulic resistance of xylem vessels (Van Ieperen et al., 2003). Under such conditions ammonium has less chance to pass through cell membranes. Calcium has an important role in structural and functional integrity of plant membranes and cell walls, regulation of ion transport and selectivity, and enzyme activities. Calcium may simply be displaced from its membrane binding sites by ammonium (Cramer et al., 1985; Cramer 2004). It is important to consider that Ca^{2+} ions function as a second messenger in mediating plant responses to external stimuli of biotic and abiotic origin. In plants despite precise control of ammonium transporters, NH_4^+ uptake may also occur through non-selective cation channels and K channels (Howitt and Udvardi, 2000; Kronzucker et al., 2001; Loque and von Wiren, 2004). Limiting NH_4^+ uptake by Ca ions could not be a reason for all improved growth characters observed in these plants. Better assimilation of ammonium and preventing toxicity and damage effects of NH_4^+ to membranes and sub cellular organelles by Ca or highr pH, probably are the main reasons, because AS+$CaSO_4$ and AS+$CaCO_3$ treated plants have the highest N concentration, similar to results obtained by (Zou et al., 2005). On the other hand, over application of $CaSO_4$ or $CaCO_3$, Ca concentration in plants shoots didn't increase (Fig. 6.7B). It seams that pH, NO_3^- and Ca concentrations in nutrient solution, respectively, are the most important factors improving Ca uptake. Signalling functions of these players (NO_3, internal and external pH, and Ca) could be

the main force behind fluctuation in Ca concentration inside plants. Similar to NO_3^-/ NH_4^+ ratios, intracellular and extracellular Ca^{2+}/ NH_4^+ ratios are likely play important role in growth improvement under ammonium nutrition. In many aspects of plant growth, Ca functions very similar to NO_3^- (Sanders *et al*., 2002; Rahayu, 2003). In different growth parameters, cytokinin-like effects are responses which have been observed in our study. Similarly, Rahayu (2003) stated that the inhibitory effects of NH_4^+ on leaf growth can rapidly (24 h) be reverted by re-application of NO_3^-, suggesting that hormonal signals rather than recovery from NH_4^+ toxicity were involved in this process (Rahayu 2003). However, immediate action of Ca as a hormonal signal could not justify the protection effects observed by application of calcium forms, despite the role of calcium in plant signal transduction is well established. Finally it can be concluded that with calcium a root-shoot protection system occurs against toxic effects of ammonium. In this system, physiochemical mechanisms seem to play the main role of root protection against NH_4^+. Direct and indirect effects of ammonium will produce stimuli which induce cytosolic calcium fluctuations. This response is in close relation with hormones and enzymes, totally as the intelligent system of plant, which keeps ammonium uptake and toxicity under control. Shoot-root signalling, however, can regulate the extent of plant responses and tolerance to NH_4^+ toxicity. For instance, in plants grown in AS+$CaSO_4$, xylem sap exudation was not consistent with transpiration, indicating a shoot-controlling system over physiological performance of plants under such conditions (high Ca concentration but low pH of root medium). Meanwhile, for plants grown under nitrate or AS+$CaCO_3$ xylem sap exudations were high and consistent with their transpiration (Fig. 6.7D). While xylem sap exudation is generally independent of plant transpiration, root pressure and metabolic activity of roots are generally believed to be driving force in xylem exudation.

Nevertheless, these findings indicate that calcium is necessary and critical in protection of particularly plant root cells. In semiarid regions, calcium is a major constitute of soil (as $CaCO_3$). Therefore, ammonium toxicity and growth reduction of tomato under application of NH_4^+ fertilizers may not occur in calcareous soils. On the other hand, under vegetable production systems, nitrification rates in these types of soil which predominated in arid and semiarid climates (see chapter 3), seems to be significantly higher than the other agroecosystems such as acid soils. This is mainly due to a better performance of nitrifiers at higher Ca concentrations as well as higher pH. Therefore ammonium can persist only for a short time inside the soil. Therefore, by application of synthetic or natural nitrification inhibitors stabilized ammonium could give better growth performance.

If grasses (such as *Brachiaria*) can be included in rotation systems of vegetable production, or as intercropping, beside other positive effects, their NI activity could keep mineralized or applied ammonium as the main N form in the soil. Moreover, low organic matter content of soil, imbalanced nutrition, and nutrients deficiency in plants are characteristics of arid zones. Under NH_4^+ uptake and consequently rhizosphere acidification, significant improvement of micronutrients deficiencies of plants, which frequently happening in these climatic zones, could occur. However, the potential of ammonium volatilisation of these soils should be considered, and appropriate measures must be under taken to keep it under control.

Summary

Nitrogen is one of the most limiting factor for crop production, thus agricultural production is highly dependent on N fertilizer supply. After application of ammonium fertilizers into the soil, microbe-mediated oxidation of ammonium to nitrate (nitrification) happens relatively fast. Nitrification, due to the mobility of the end product (nitrate), can result in spatial separation of nitrogen from root zone. Through inhibition of nitrification and stabilization of ammonium, long term preservation and accessibility of nitrogen can be improved, especially when plants themselves could produce and release nitrification inhibiting (NI) compounds. On the other hand, ammonium nutrition of plants can have important environmental, economic and nutritional implications for agricultural production. Micronutrient deficiencies are widespread phenomena particularly in calcareous soils, so rhizosphere acidification as a result of imbalanced cation/anion uptake caused by NH_4^+ uptake, could improve phosphorous and micronutrient status of plants.

Besides commercial NIs, many chemicals could also inhibit nitrification. In our study (Chapter 3) regarding efficiency of chloride compared to 3,4-Dimethylpyrazole phosphate (DMPP), it was found that chloride at applied concentration of 30.5 mg per 100g dry soil, could effectively inhibit nitrification. Despite a lag period of 3 weeks in detectable net nitrification, inhibitory effect of chloride continued to persist even after 7 weeks of soil incubation compared to control. Nevertheless, DMPP particularly with higher concentration (2 % of N-NH_4^+ instead of 1%) stabilized ammonium more strongly than Cl^{-1}. The extent of nitrification inhibition after 5 and 7 week of incubation was in order of: (2 % of N-NH_4^+) DMPP > (1 % of N-NH_4^+) DMPP> NH4Cl > KCl > control. The residue ammonium in the soil as well as the produced nitrate concentrations in samples showed a significant NI activity of chloride in both forms NH_4Cl and KCl. Nitrification-induced pH decrease, however, showed a better correlation with measured nitrate than ammonium in this experiment.

In a second series of experiments undertaken to identify whether the reported NI release by *Brachiaria humidicola* accession 26159 is an active or passive phenomena, root exudates of plants grown under various treatments, have been collected in distilled water or in 1 mM NH_4Cl. Under various pre-culture conditions such as N form (NH_4^+ versus NO_3^-), N concentrations (1, 2, 4 mM), light intensities (180, 240, 350 µmol m^{-2} s^{-1}), plant age (3-weeks old versus 7-weeks old) and collecting periods (24 versus 6 h), there was no significant NI activity when root exudates were collected in distilled water. However, NI activity was detectable in root washings when the plants were exposed to extended collection times (24 h) in combination with NH_4^+ supply, but not after short term collection (6 h) or with NO_3^- in the

collection solution. This observation is consistent with the results of Subbarao et al., (2006, 2007), but it also strongly suggests that the observed release of NI compounds was rather a consequence of membrane damage (passive phenomena) due to inadequate collection conditions, than mediated by controlled exudation from undamaged roots. It has been assumed that supplying only ammonium (1 mM) in distilled water as root washing medium over extended time periods (24 h) could lead to rapid ammonium uptake and medium acidification associated with the risk of Ca^{2+} desorption, which is an important element required for membrane stabilisation and integrity. To test the hypothesis that NI compounds are released from damaged plant cells of *Brachiaria*, the NI potential of fresh root and shoot homogenates was measured after soil incorporation and incubation. Surprisingly, NI potential was detected in shoot but not in root homogenates. The NI effect of soil-incorporated shoot tissues lasted for at least 8 d, while root tissue even stimulated nitrification with increasing incubation time. This NI effect was independent of the N form. However, the variability of data increased with NO_3^- form, higher light intensity or higher N concentrations during plant pre-culture. Independent of N forms, further extraction and characterisation of NI compounds in shoot tissue of *Brachiaria* plants revealed a particularly high activity in the ethanol-soluble fraction, both in plants with NH_4^+ and NO_3^- pre-culture.

In a third experiment, the role of Ca^{2+} ions on improvement of tomato growth under ammonium nutrition was investigated. In this experiment root damage, probably by membrane damage and cytosolic sensitivity were hypothesised to be the main cause of toxicity symptoms of NH_4^+ on tomato plants. At application of 2 mM N as NH_4^+, plant biomass, number of lateral shoots, and transpiration were strongly inhibited and an increased Ca^{2+} application into the nutrient solution counteracted these observed negative effects. Transpiration or water consumption was found to be a good indicator of plant performance under NH_4^+ nutrition. Plants grown under nitrate nutrition had the highest transpiration rates, as well as the best growth characteristics. There was a positive correlation between nitrate concentrations and transpiration rates. On the other hand, plants grown in ammonium (as control, or 3 and 6 split applications of NH_4^+ during 4 days) showed severe toxicity symptoms including growth inhibition and leaf abscission. However, when ammonium was applied together with 10 mM Ca^{2+} (as $CaSO_4$), or in a buffered solution of pH 6.6 with $CaCO_3$ (pH or/and Ca^{2+} effect), transpiration and other growth factors (e.g. root and shoot dry matter, number of lateral shoots), as well as the nutrients especially N concentrations in the biomass were significantly improved. In other words, shoot and particularly root growth were

inhibited when NH_4^+ treated plants (control and split applications) did not received $CaSO_4$ or $CaCO_3$. Micro molar concentrations of NH_4^+ in 6 split applications also could not prevent ammonium toxicity symptoms.

Improved growth and plant performance by increasing calcium concentration in the root medium could be achieved due to a better protection of root system from toxic effects of NH_4^+. These findings indicate that growth reduction of tomato culture by application of NH_4^+ fertilizers may not occur in calcareous soils. On the other hand, under regular irrigation, nitrification rates in these types of soil which predominated in arid and semiarid climates seems to be significantly higher than in soils widespread in subtropical agroecosystem (acid soils). Therefore ammonium will persist only for very short period, particularly when incorporated into the soil. However, after application of natural or synthetic nitrification inhibitors, through ammonium uptake and a consequent rhizosphere acidification, significant improvement of micronutrients deficiencies of plants, which frequently happen in these climatic zones, could occur. But possible ammonia emissions must be avoided on such calcareous soils by an adapted application technique for ammonium fertilizers stabilized by nitrification inhibitors e.g. in vegetable production systems.

Zusammenfassung

Stickstoff (N) ist einer der am meisten limitierenden Faktoren für die Pflanzenproduktion. Aus diesem Grund ist die landwirtschaftliche Pflanzenproduktion in hohem Maße abhängig von einer optimalen Stickstoffversorgung der Pflanzen. Nach der Applikation von ammoniumhaltigen Düngemitteln erfolgt in der Regel nach relativ kurzer Zeit eine mikrobiell vermittelte Oxidation von NH_4^+ zu NO_3^- (Nitrifikantion). Aufgrund der hohen Mobilität des Endprodukts dieser Reaktion (NO_3^-) in Böden, kann die Nitrifikation zu einer räumlichen Verlagerung von Stickstoff aus dem Wurzelraum führen.

Eine Hemmung der Nitrifikation und die Stabilisierung von NH_4^+ im Boden kann zu einer Verbesserung der Speicherung von Stickstoff in Böden und einer besseren Versorgung der Kulturpflanzen führen, vor allem wenn die Kulturpflanzen selbst Nitrifikationshemmstoffe (NI) produzieren und in die Rhizosphäre abgeben können.

Eine NH_4^+-Ernährung der Kulturpflanzen kann wesentliche Auswirkungen für die Umwelt, die Versorgung von pflanzlichen, tierischen Organismen und Menschen mit Nährstoffen und ökonomische Aspekte der landwirtschaftlichen Pflanzenproduktion haben. Beispielsweise ist Mikronährstoffmangel (Fe, Zn, Mn) ein weitverbreiteter limitierender Faktor für die Pflanzenproduktion, der vor allem auf kalkhaltigen Böden auftritt. Eine durch die Ernährung der Kulturpflanzen mit NH_4^+ induzierte Ansäuerung der Rhizosphäre, aufgrund einer unausgeglichene Aufnahme von Kationen und Anionen durch die Pflanzen, kann unter diesen Bedingungen zu einer erhöhten Verfügbarkeit von Mikronährstoffen für die Pflanzen führen.

Neben kommerziell hergestellten Nitrifikationshemmstoffen kann eine Reihe von weiteren chemischen Substanzen zu einer Hemmung der Nitrifikation führen.

In der hier vorliegenden Studie (Kapitel 3) in der die Effizienz von Chlorid mit dem kommerziellen Nitrifikationshemmstoff 3,4-Dimethylpyrazol-Phosphat (DMPP) verglichen wurde, zeigte sich, dass 30.5 mg Chlorid/ 100g Boden zu einer sehr effektiven Hemmung der Nitrifikation führte. Trotz eines Zeitraums von drei Wochen, in dem unabhängig von der Behandlung keine Netto-Nitrifikation beobachtet werden konnte, induzierte Chlorid sogar noch nach einer Inkubationszeit von sieben Wochen im Vergleich zur Kontrolle eine Hemmung der Nitrifikation. Trotz allem verursachte DMPP, vor allem in einer erhöhten Konzentration (2% von $N\text{-}NH_4^+$ statt 1% von $N\text{-}NH_4^+$), im Vergleich mit Cl^- eine stärkere Stabilisation von Ammonium. Die Effektivität der Hemmung der Nitrifikation nach fünf bzw. sieben Wochen war: (2% von $N\text{-}NH_4^+$)-DMPP > (1% von $N\text{-}NH_4^+$)-DMPP > NH_4Cl > KCL > Kontrolle. Residuales Ammonium im Boden, aber auch die produzierte

Nitratkonzentration in den Proben zeigten eine signifikante Nitrifikationshemmung durch beide Chloridformen, NH_4Cl und KCl. Die durch die Nitrifikation induzierte Verminderung des pH-Werts zeigte in diesem Experiment jedoch eine bessere Korrelation mit dem gemessenen Nitrat als dem gemessenen Ammonium.

In einer zweiten Serie von Modellversuchen sollte untersucht werden, ob es sich bei der in wissenschaftlichen Untersuchungen beobachtete Abgabe von Nitrifikationshemmstoffen durch *Brachiaria humidicola* Genotyp 26159 um einen aktiven oder um einen passiven Prozess handelt. Die Wurzelexsudation von *Brachiaria humidicola* 26159 Pflanzen wurden in destillierten Wasser oder 1mM NH_4Cl gesammelt und auf die Fähigkeit zur Nitrifikationsinhibition geprüft. Unter verschiedenen Wachstumsbedingungen, beispielsweise der Form der Stickstoffernährung (NH_4^+ oder NO_3^-), N-Konzentration (1, 2, 4mM), Lichtintensität (180, 240, 350 µmol m^{-2} s^{-1}), Pflanzenalter (drei Wochen oder sieben Wochen) und der Dauer der Wurzelexsudationssammlung (6h oder 24h) zeigte sich keine signifikante Hemmung der Nitrifikation, wenn die Wurzelexsuadate in destilliertem Wasser gesammelt wurden. Im Gegensatz dazu konnte eine Hemmung der Nitrifikation in Wurzelexsudaten von *Brachiaria humidicola* 26159 detektiert werden, wenn die Pflanzen mit NH_4^+ aber nicht mit NO_3^- ernährt wurden und Wurzelexsudate für einen Zeitraum von 24 Stunden gesammelt wurden. Diese Ergebnisse bestätigen die Beobachtungen von Subbarao et al. (2006, 2007), jedoch weisen sie ebenfalls darauf hin, dass die beobachtete Abgabe von Nitrifikationshemmstoffen eher die Konsequenz einer Membranschädigung (passiver Prozess), versucht durch eine inadäquate Bedingungen zur Sammlung von Wurzelexsudaten, war und nicht durch eine kontrollierte Wurzelexsudation aus intakten Wurzelzellen verursacht wurde. Es wird angenommen, dass die Zugabe von Ammonium (1mM) in destilliertes Wasser als Wurzelwasch-Medium über einen Zeitraum von 24 Stunden eine rapide Aufnahme von Ammonium und der Ansäuerung des Mediums verursachen kann, verbunden mit einer Desorption von Ca^{2+}, einem der wesentlichen Faktoren für die Membranstabilisierung und –integrität.

In einem Dritten Experiment, um die Hypothese zu testen, dass Nitrifikationshemmstoffe von beschädigten Pflanzenzellen von *Brachiaria humidicola* abgegeben werden können, wurde das Nitrifikationsinhibitionspotenzial von homogenisiertem Wurzel- und Sprossgewebe von *Brachiaria* nach einer Inkorporation und Inkubation in Bodenproben getestet. Erstaunlicherweise konnte ein Potenzial zur Nitrifikationshemmung im Spross- aber nicht in Wurzelhomogenaten nachgewiesen werden. Der die Nitrifikation inhibierende Effekt von in den Boden inkorporierten Sprossgewebe hielt etwa acht Tage an, während Wurzelgewebe im

Gegensatz dazu mit zunehmender Inkubationszeit zu einer Stimulation der Nitrifikation führte. Der Effekt einer Hemmung der Nitrifikation durch in den Boden inkorporiertes Sprossgewebe war unabhängig von der N-Form. Jedoch stieg die Variabilität der Daten mit N-Form (NO_3^-), höherer Lichtintensität, oder höherer N-Konzentrationen. Unabhängig von der N-Form zeigten weitere Extraktionen und Charakterisierung der Nitrifikationshemmstoffen aus dem Sprossgewebe von *Brachiaria humidicola* Pflanzen eine besonders starke Hemmung der Nitrifikation durch eine Ethanol-extrahierbare Fraktion, sowohl für Pflanzen, die mit NH_4^+ als auch mit NO_3^-vorkultiviert worden waren.

In einem Fierten Experiment wurde die Rolle von Ca^{2+}-Ionen für eine Verbesserung des Pflanzenwachstums von Tomaten bei einer Ammonium-Ernährung untersucht. Entsprechend der Hypothese wurde in diesem Modellversuch angenommen, dass Wurzelschäden durch Membran- und Cytosolsensitivität die Hauptursache von NH_4^+-Toxizitätssymptomen bei Tomate sein würde. Bei einer Applikation von 2mM als NH_4^+ waren die Biomasse der Pflanzen, laterale Sprossbildung und die Transpiration stark vermindert. Eine zunehmende Ca-Applikation in die Nährlösung konnte die beobachteten negativen Effekte kompensieren. Die Ergebnisse zeigten, dass die Transpiration bzw. der Wasserverbrauch gute Indikatoren für die Entwicklung von Pflanzen im Falle einer NH_4^+-Ernährung sind. Pflanzen mit Nitraternährung zeigten eine höhere Transpiration und eine Verbesserung anderer Wachstumscharakteristika. Es zeigte sich eine positive Korrelation zwischen Nitraternährung und Transpiration. Pflanzen, die mit Ammonium (als Kontrolle, oder als Split-Applikation von NH_4^+ über 4 Tage) kultiviert wurden, zeigten ausgeprägte Symptome einer NH_4^+-Toxizität, darunter Wachstumshemmung und Blattfall. Jedoch, wenn Ammonium zusammen mit 10mM Ca^{2+} (als $CaSO_4$), oder in eine mit $CaCO_3$ bei pH 6.5 gepufferte Lösung (pH und/oder Ca^{2+} Effekt) appliziert wurde, war die Transpiration und andere Wachstumsparameter (v.a. Wurzel- und Spross-Biomasse, Bestockung) aber auch die N-Konzentrationen in den Pflanzen verbessert. Mit anderen Worten, das Spross- und Wurzelwachstum von Pflanzen wurde gehemmt, wenn NH_4^+-ernährte Pflanzen (Kontrolle und Split-Applikationen) nicht mit $CaSO_4$ oder $CaCO_3$ behandelt wurden. Auch durch die Applikation mikromolarer Konzentrationen von NH_4^+ (in einer in 6 Applikationen aufgeteilten Split-Applikation) konnte die Ausbildung von NH_4^+-Toxizitätssymptomen nicht verhindert werden. Verbessertes Wachstum und eine bessere pflanzliche Entwicklung durch eine erhöhte Calciumkonzentration im Wurzelmedium konnten durch einen besseren Schutz des

Wurzelsystems vor den toxischen Effekten von NH_4^+ erreicht werden. Diese Ergebnisse deuten an, dass eine NH_4^+-induzierte Wachstumsbeeinträchtigung bei Tomaten möglicherweise auf kalkhaltigen Böden nicht auftritt. Auf der anderen Seite sind die Nitrifikationsraten in diesen Böden, die sehr häufig in ariden und semiariden Regionen der Erde anzutreffen sind, bei einer regelmäßigen Bewässerung signifikant höher als in anderen Agrarökosystemen, beispielsweise auf sauren Äckern. Aus diesem Grund ist die Verweildauer von Ammonium in diesen Böden sehr kurz, vor allem bei einer Einarbeitung in den Boden. Die Applikation von natürlichen oder synthetischen Nitrifikationshemmstoffen kann über eine Aufnahme von NH_4^+ und einer damit eng verbundenen Ansäuerung der Rhizosphäre zu einer signifikanten Verminderung von Mikronährstoffmangel bei Kulturpflanzen führen, der in diesen klimatischen Regionen häufig auftritt. Mögliche Ammonikemmisionen müssen jedoch auf solchen kalkhaltigen Standorten durch eine angepasste Applikationstechnik für mit Nitrifikantionshemmstoffen stabilisierten Ammoniumdüngern vermieden werden.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt, nur die angegebene Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene stellen als solche gekennzeichnet habe.

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