Bacterial Community Responses to Soil-injected Liquid Ammonium Nutrition and Effect of Temperature on Barley (*Hordeum vulgare* L.) Grain Yield Formation

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BACTERIAL COMMUNITY RESPONSES TO SOIL-INJECTED LIQUID AMMONIUM NUTRITION AND EFFECT OF TEMPERATURE ON BARLEY (*Hordeum vulgare* L.) GRAIN YIELD FORMATION

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1.0 INTRODUCTION

1.1 Need for improved fertilization methods

Nitrogen (N) is one of the nutrients required for plant growth since it forms an integral part of various vital compounds. Upon uptake, it is may be assimilated into plant tissues and organs. Normally, nitrogen accounts for about 2-5% of the total plant dry matter. For this reason, it is usually required in appreciably larger amounts than the other nutrients (Marschner, 1995). The use of commercial fertilizers, especially nitrogen aims at crop growth and yield improvement. However, losses associated with the currently employed conventional fertilization methods calls for the adoption of alternative methods or improvement of the existing ones. The application of liquid ammonium fertilizer is emerging as a noble fertilization technique because it mitigates nitrate associated losses.

1.2 Nitrogen forms taken up by crops

In soil, nitrogen occurs both as organic and inorganic compounds, with 95% or more being organic (Miller and Cramer, 2004). The apparent inadequacy of nitrogen availability for uptake by crops necessitates the application of commercial fertilizers and has made it a common agricultural practice. Generally, nitrate and ammonium are the main inorganic nitrogen sources taken up by roots of higher plants. Upon uptake, NO_3^- can be reduced either within the root or shoot and excess amounts can be stored in different plant tissues like vacuoles (Marschner, 1995). Since NH_4^+ is usually toxic to plants when taken up in large amounts, it is thus a prerequisite that crops to which it is exposed must be well adapted to either prefer or tolerate its effects (Gerendas et al., 1997). Crops fertilized by NH_4^+ should be capable of regulating both internal and external acidic pH conditions to avoid 'ammonia syndrome' resulting from ionic imbalances (Mehrer and Mohr, 1998). Because of its toxicity effects on crops, NH_4^+ is normally assimilated within the roots (Britto and Kronzucker, 2002). The application of urea based fertilizer which upon hydrolysis confers NH_4^+ related characteristics has recently been gaining popularity (Miller and Cramer, 2004). However, mixed N nutrition occurring in the presence of both NH_4^+ and NO_3^- is capable of supporting better crop growth than sole application of either N form (Cramer and Lewis, 1993).

1.3 Limitations of nitrate based fertilizers

Nitrate availability in soil is greatly constrained by its high mobility rate which facilitates its rapid loss. Its loss mainly occurs through leaching and denitrification which reduces its availability within the rhizosphere. In nature, nitrification process whose end product is NO_3^- thus leads to appreciable N losses from agricultural land. Besides reducing nutrient availability for uptake, NO_3^- leaching potentially pollutes underground water bodies and also cause eutrophication of above ground water bodies through run-off. It is for these reasons that the current farming practices emphasize improved crop yields in a benign environment through a sustainable production system. The main purpose of improved farming method is to enhance food security to feed the ever increasing human population (Mangelsdorf, 1966; Vasil, 1998). This undertaking has opened up a window of research opportunity for the re-evaluation of previous agricultural practices. This is crucial since cereal production which provides the bulk of edible fibre consumes more than 60% of the total N fertilizers worldwide annually (Rao and Popham, 1999).

1.4 Agronomic requirements and economic importance of barley

Barley is a member of the grass family, Poaceae. It is a grain crop that is currently grown in more than 100 countries worldwide and is ranked fourth both in terms of quantity and area of production covered by cereals. It grows best on light soils rich in minerals with moderate water requirements. It is tolerant to cold stress and resistant to soil salinity than wheat. It exists either as winter or spring crop and its cultivars largely occur as six or two row grained ears. It serves as human food such as bread and other cereal products besides its usage as livestock fodder while green or hay when dry and silage in the conserved form. Dry straw can be used as animal bedding while grains can also be processed into animal feed concentrates. Several industrial brews such as beer, whiskey and malt syrup are among important alcoholic drinks manufactured from barley which are consumed by a large human population. The crop generates a lot of income to economies of countries such as USA, China, Canada, United Kingdom, Germany and Netherlands among others that produce and process its products (Foster, 1981).

1.5 Principle of CULTAN fertilization technique

The new fertilization method is based upon the injection of concentrated liquid NH_4^+ into soil and its subsequent adsorption onto clay particles and soil organic matter which enhance its long-term availability for crop uptake (Sommer, 1995). The fertilization method is developed within the tenets of emerging crop production paradigm, which emphasize improved crop yields in a sustainable manner. The adoption of such agricultural practice does not compromise ecological and environmental quality and it plausibly sustains and improves crop yields. When adsorbed onto clay soil matrix, NH_4^+ forms a sorption-complex which serves to regulate its uptake by inhibiting root growth and penetration into the concentrated zone (Zhang and Rengel, 2000). In Germany, the fertilization technique is very popular and is referred to as CULTAN. The acronym 'CULTAN' is an abbreviation of Controlled Uptake Long Term Ammonium Nutrition (Sommer, 1993; 2000; 2003). Similar methods are being practised elsewhere, though they are known by different names. For example, it is referred to as point injection in Canada and America (Janzen et al., 1991) while in Asia, more specifically Japan, sulphur-coated nitrogen are applied as slow release fertilizers and are commonly referred to as Controlled Nitrogen Release Fertilizers (CNRF) (Wakimoto, 2004). Adoption of the CULTAN fertilization method promotes localized NH_4^+ deposits within the rooting zone whose toxicity causes root inhibition into the injection-point whereas the less toxic peripheral zones promote intensive root growth network (Sommer, 2000).

1.6 Dilemma of inadequate and excess nitrogen nutrition

In most tropical soils, N is insufficient for satisfactory crop yield outputs. On one hand, the use of fertilizer inputs in developing countries, especially in sub-Saharan Africa, is quite low with essential nutrients often inadequately supplied due to high input costs, unavailability and poor marketing infrastructure (Dakora and Keya, 1997). On the other hand, developed countries are faced with environmental pollution resulting from excessive amounts of NO_3^- fertilizer applied in an effort to improve crop yields (Jeuffroy *et al*., 2002). Whereas developing countries are mainly faced with fertilizer inadequacy, developed countries are experiencing excessive fertilizer application problems with the potential of causing environmental pollution. A middle ground for both the developed and developing countries may be struck through improved fertilization techniques such as

CULTAN technique which has the ability of improving N availability for crop uptake during the cropping period. When fine-tuned, the fertilization technique may alleviate constraints associated with fertilizer input inadequacies of developing and excesses experienced in the developed worlds.

1.7 Potential of nitrification inhibitor incorporation into CULTAN

A potential N loss mitigation method that would be compatible with CULTAN fertilization technique is the incorporation of nitrification inhibitors into soil injected ammonium. Nitrification inhibitor (NI) incorporation into ammonium fertilizer can temporarily improve nitrogen retained as NH_4^+ through the suppression of the nitrification process (Crawford and Chalk, 1993). Application of NI influences not only fertilizer efficiency by reducing leaching or denitrification losses, but also the ratio of available inorganic N forms (Vanneli and Hooper, 1992). Occasionally, immobilization of N increases in response to NI-incorporation into soil thereby reducing N available for crop uptake (Vanneli and Hooper, 1993). However, the potential benefits accruing from NI-incorporation into NH_4^+ fertilizer through the suppression of nitrification supersedes the corollary effect of N losses when no nitrification inhibitor is incorporated. One of the commercially available nitrification inhibitors reported to successfully reduce nitrogen losses through less gas emissions is Nitrapyrin® (McCarty, 1999).

1.8 Merits of CULTAN fertilization technique

The popularity of CULTAN fertilization technique is due to the merits associated with its application. The method bases upon NH_4^+ as the dominant N form, which inhibits root penetration through the injection depot. This occurs because of its toxicity to roots. Proliferation of intensive root network around the injection zone is thought to trap diffusing ions, while the root growth inhibition by free ions within the injection-point offers a self-regulatory mechanism for N uptake as opposed to conventional nitrate fertilizer application that is made in splits to coincide with crop growth stages deemed by the farmer to require increased nutrient supply, which may, however, not be the case based upon the internal crop nutrient status (Sommer, 2000). Since NH_4^+ assimilation within the crop roots requires translocation of carbon skeletons from shoot to root, it offers a further

regulatory measure because the uptake has to be determined by the proportion of C-skeleton translocation to the roots (Cramer and Lewis, 1993). The resulting organic nitrogenous compounds are thus incorporated into root system or channelled to other parts including the shoot. The supply of C-skeletons through photosynthesis and translocation of photosynthate to the root in conjunction with the redistribution of root assimilated nitrogenous compounds creates a counter-current trafficking of the growth essentials in opposite directions which result in a source-sink relationship for different products that are highly dependent upon each other. Along side these physiological merits, the advantages attained through the use of mechanized farm equipments which facilitate the wide adoption of CULTAN fertilization technique in large scale and in different crops are some of the factors that have contributed to its popularity.

1.9 Objectives of the study

The study focused on generating information to bridge the knowledge gap between crop and microbial responses in CULTAN-fertilized soils. In addition, crop growth and yield responses resulting from the interaction effects of CULTAN fertilization and different growth temperatures have not been evaluated to date. To be able to exploit the full potential of CULTAN-fertilization method, its interaction with abiotic and biotic factors need to be addressed. Currently, various mechanisms and processes relating to CULTAN-fertilization are not well understood and some are at best only assumed. Though it is known that NH_4^+ adsorbs onto clay particles and soil organic matter to form sorption-complex within the injection-point (Sommer, 2000), it is however, not known how stable the injected NH_4^+ is within the soil. In case the injected NH_4^+ is stable to any extent, it is unknown for how long it would support crop growth. Such information on NH_4^+ stability can help in decision making whether a single injection at a certain concentration would be sufficient to support crop growth throughout the entire season or whether there would be need for multiple injections to be performed at intervals coinciding with particular crop growth stages. Therefore, the first objective of this study was to evaluate the stability of soil-injected liquid ammonium and crop growth responses.

No data is available to support the view that highly concentrated NH_4^+ is toxic to soil microbes, especially bacteria. This presumption seems to stem from previous reports highlighting root growth inhibition by concentrated NH_4^+ fertilization (Sommer, 2000). Chemolithoautotrophic bacteria are known to use ammonia as an energy source during oxidation when they transform ammonia to nitrate via nitrite (Purkhold et al., 2000). There is however, no experimental data to illustrate bacterial community responses to soil-injected liquid NH_4^+. In light of this information gap, the second objective of the study focused on the assessment of the occurrence and response of bacterial communities associated with soil-injected liquid NH_4^+ analyzed by targeting 16S rRNA gene. Attempts to detect occurrence and abundance of ammonia oxidizing bacteria (AOB) while targeting ammonia monooxygenase subunit A (*amoA*), functional gene, were also made.

The direct influence of temperature on plant growth caused by effects on root mineral nutrient and water uptake as well as translocation is well known (Macduff and Jackson, 1991). In addition, the effect of temperature on microbial activity, particularly ammonia oxidation rates has been reported (Avrahami et al., 2003). However, no study has been conducted to elucidate the interaction of the two factors under a cropping system fertilized through the CULTAN method, which is predominated by NH_4^+ over NO_3^-. There is a high possibility of growth temperatures and N form available for absorption by crops interacting with each other, especially in the presence of mixed N nutrition resulting from the nitrification of soil-injected NH_4^+ to cause a suite of growth responses with strong implications on grain yield and yield forming factors. In this regard, the last objective of this study was to evaluate the impact of liquid NH_4^+ on barley yield and yield forming factors under different growth temperatures. In summary, the following specific objectives were addressed by the study:-

i) To evaluate stability of CULTAN-injected NH_4^+ and its effect on barley crop growth.
ii) To assess occurrence and characterize bacterial community responses to CULTAN fertilized soils.
iii) To investigate occurrence and abundance of ammonia oxidizing bacteria (AOB) in CULTAN-fertilized soils.
iv) To elucidate the interaction effects of soil-injected NH_4^+ and growth temperatures on barley grain yield and yield-forming factors.

2 MATERIALS AND METHODS

2.1 Determination of soil-injected liquid NH_4^+ stability

2.1.1 Experimental site location and crop growth conditions

The experiment was performed at Federal Agricultural Research Centre (FAL), Braunschweig, Germany. This was a pot experiment performed in three solar-irradiated growth chambers supplemented with an artificial lighting system. Each chamber measured 10 m long, 8 m wide and 10 m high with a glass roof slanting at 45^o. Illumination was mainly solar, but artificial light from lamps provided irradiation at 400 µmol $m^{-2}s^{-1}$ after the first month of sowing. Spring barley (*Hordeum vulgare* L.) cv. Maresi procured from Lochow-Petkus GmbH was chosen as a model cereal crop for the study. The containers were black polyvinyl with a capacity of 90 L and filled with 80 L soil. Each container had a diameter of 54 cm and was subdivided into four equal segments (quadrants) with a sowing area of 0.0572 m^2 to support 22 seedlings sown at a depth of 2.5 cm within an inter- and intra-row spacing of 5 cm. The containers were placed in wooden troughs covered with black plastic sheet holding irrigation water (Fig. 9a).

The containers had five 1 cm diameter irrigation holes on the lower side of each quadrant for irrigation water percolation into the soil. The base of each quadrant was filled with 2 cm layer of pebbles for easy water entry. Crops were irrigated from the bottom once weekly with 5 L for 2 h in the first month and 10 L were administered for 2 h twice a week during the rest of the growth period. Excess irrigation water was drained by hand-held pump and reutilized in subsequent irrigations. Fortnightly, 1 L of the drained water was sprinkled onto the soil surface to improve the moisture content. Irrigation water was contained in wooden troughs measuring 1.2 m long, 1.2 m wide and 0.08 m high and placed on mobile trolleys adjusted to 0.8 m high. Photoperiod was 14/10 h, day/night, respectively. Irradiation lamps were switched off 1 month after sowing. Relative humidity ranged between 55 – 75% while growth temperatures were 20/15 ^{o}C, day/night, respectively, throughout the season.

2.1.2 Experimental design

The experimental arrangement was a Randomized Complete Block Design (RCBD), comprising six nitrogen treatments. Each container represented a treatment while each

quadrant was a replication. Each treatment had four replications (quadrants) (Fig. 1). The six nitrogen treatments comprised four NH_4^+-based treatments while the other two were comparison checks fertilized with nitrate or non-fertilized control. Crops were sampled destructively at eight different growth stages as described in the next section.

2.1.3 Nitrogen treatments and sampling intervals

Crop and soil samples from each treatment were collected at eight different intervals. Out of the six nitrogen treatments included, four were NH_4^+-based in addition to nitrate and non-fertilized control. Two of the four NH_4^+ treatments were incorporated with nitrification inhibitors {NI}, (Nitrapyrin®) at a rate of 5 and 20%, designated as (NH_4^+ + 5%NI + Crop) and (NH_4^+ + 20%NI + Crop), respectively. The other two NH_4^+ treatments without NI incorporation comprised cropped, (NH_4^+ - NI + Crop) and uncropped treatment (NH_4^+ - NI - Crop). Each of the six treatments was sampled at eight different intervals with each coinciding with a specific crop growth phase. The first was at seedling growth stage (ZS 15) coinciding with 11 days after sowing (DAS), while the next three stages occurred at tillering stage, which was sub-divided into three distinct stages (ZS 21, 25 and 29) which coincided with 25, 30 and 39 DAS. The fifth sampling was carried out at stem elongation (ZS 36), which coincided with 60 DAS, whereas the sixth sampling stage performed at booting stage (ZS 45) coincided with 66 DAS. Seventh sampling was done at medium milk kernel stage (ZS 75) coinciding with 80 DAS and eighth sampling interval was performed at crop maturity (ZS 99), which coincided with 109 DAS. Developmental stages were based on Zadoks' growth scale (ZS) (Zadoks et al., 1974). After the sixth sampling, treatments awaiting the next two sampling schedules were rearranged into a single growth chamber. Season two sampling was performed similarly to those at season one. A summarized list of the six treatments is hereunder:-

(i) NO_3^- =N-form; $Ca(NO_3^-)_2$ applied at 4g N container as a single application **[T_1]**
(ii) NH_4^+ = NH_4^+ without Nitrapyrin®, but cropped, (NH_4^+ - NI + Crop); **[T_2]**
(iii) NH_4^+ = NH_4^+ with 5% Nitrapyrin® and cropped, (NH_4^+ +5% NI + Crop); **[T_3]**
(iv) NH_4^+ = NH_4^+ with 20% Nitrapyrin® and cropped, (NH_4^+ +20% NI + Crop); **[T_4]**
(v) NH_4^+ = NH_4^+ without Nitrapyrin® and uncropped, (NH_4^+ - NI - Crop); **[T_5]**
(vi) Non-fertilized control treatment (Without any N-form application); **[T_6]**

2.1.4 Growth nutrient application and nitrification inhibitor incorporation

Soil used in the experimentation was from three portions constituted by sub- and two separate top- soils mixed in ratios of 1:1:1, respectively. Nitrate was provided as $Ca(NO_3^-)_2$ while NH_4^+ was applied as diammonium phosphate (DAP), $(NH_4^+)_2HPO_4$. Each treatment received 4 g N of either NO_3^- or NH_4^+. Nitrate was sprayed whereas NH_4^+ was injected at the centre of each quadrant. Injection was performed at a depth of 7 cm using 1 cm diameter aluminium rod. The holes were fitted with 20 ml Eppendorf tubes to simulate spoke wheel injectors used for CULTAN injection under field conditions (Fig. 2a and b). After injection, the holes were refilled with soil and marked with thin wooden pegs. Additional 18 g P in the form of $Ca(H_2PO_4)_2.H_2O$ was applied in nitrate and non-fertilized treatments to balance out the P contributed by DAP fertilizer. Essential nutrients whose effects were not being evaluated were adequately supplied by mixing into soil so as to ensure availability of sufficient mineral nutrition. Macro nutrients were provided as P = 5 g, K = 13 g, Mg = 27 g, Ca = 6 g per treatment, whereas micro-nutrients too were applied as Fe = 25 mg, Mn = 10 mg, Cu = 5 mg, B = 3 mg, Zn = 5 mg and Mo = 0.5 mg per treatment. Incorporation of the two Nitrapyrin® levels, 5 and 20% into two of the ammonium treatments was applied after its dissolution into toluene and acetone in seasons one and two, respectively.

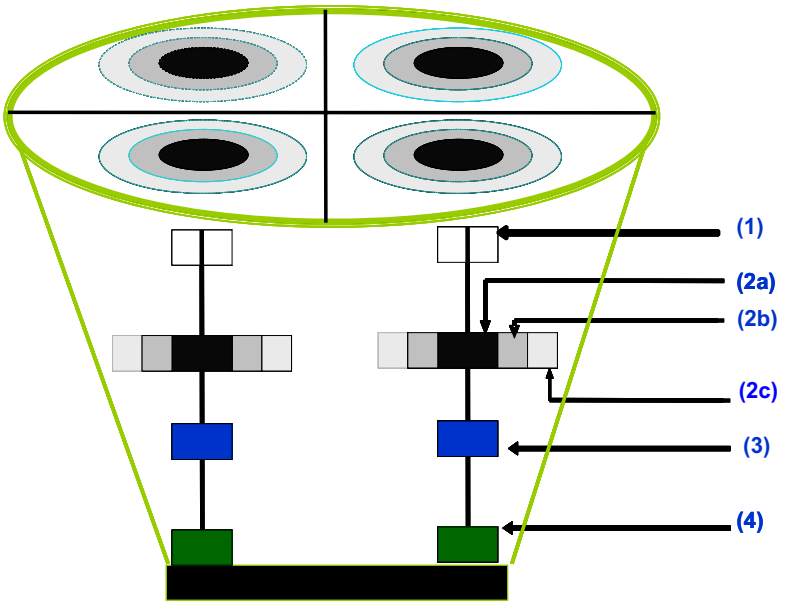

Fig. 1: A diagram showing both vertical and lateral soil sampling zones in two of the quadrants. Zone 1 is situated 2 cm below the surface while 2a was at 7 cm depth and was coplanar with 2b and 2c though at different circumferences. Zone 3 was 15 cm below the surface or 8 cm below the injection point and finally zone 4 was at a depth of 27 cm.

Fig. 2a: Spoked injection wheel with valves

Fig. 2b: Injection shares with valves

Fig. 2a and b: A diagram showing spoke wheeled liquid NH_4^+ injection equipment (a) and ammonium injection shares (b) with pressurized valves to release the liquid fertilizer during field operation

2.1.5 Crop sample categories at different intervals

Crop stand of 22 was sustained in each quadrant, out of which seven were edge plants and the remaining 15 were used for growth measurements and analyses. The first harvest performed at 11 DAS comprised 15 seedling shoots used for biomass and ion determination. Second sampling performed at 25 DAS was sub-divided into two, with the first category comprising 10 plants processed for biomass, carbohydrate, total nitrogen and nitrate concentration analyses, whereas the second category of 5 plants, which were fractionated into main and minor tillers had each of the five main tillers further fractionated into stem and different leaves. First fully open leaf of the main tiller was harvested, but not used for any analysis. The two successive leaves below uppermost open leaf were pooled together and used to determine the concentration of organic acids. Fourth leaf from the five main stems were pooled and used for chlorophyll concentration determination. Leaf samples were packed into aluminium containers filled with liquid nitrogen and transferred into -80 ^{o}C freezers. Frozen leaf samples were ground in cooled pestle and mortar filled with liquid nitrogen. Samples from 25, 30, 39 and 60 DAS were used for chlorophyll measurements in season one, whereas season two had an extra interval sampled at 66 DAS. The organic acid concentrations were determined from leaf samples harvested at 30, 39 and 60 DAS. Whole plants were used to determine biomass and relative growth rates of samples dried in the oven at 105 ^{o}C for 48 h. Water soluble carbohydrates were analyzed from 30, 39, 60 and 66 DAS in both seasons. Sub samples were used to determine ion concentration and total ash. The crop samples were ground using Brabender laboratory mill with 1 mm grid.

2.1.6 Growth parameters, grain yield and yield components

Barley crop growth and yield responses to different N-forms were compared. The samples harvested at the eighth stage (109 DAS) were used for grain yield estimates. Out of the possible 22 barley plants per quadrant, seven were edge plants and the remaining 15 were considered for yield estimates. Crop growth duration until maturity was recorded. Shoot heights were estimated while tiller numbers were counted also. Surviving plant population at harvest was counted. Total number of tillers was counted and the average number of tillers calculated. Fertile ear numbers were counted and the number of grains per ear averaged. Ear and shoot samples were oven dried at 60 ^{o}C for 48 h and ground for chemical analysis. The

dried grains were threshed, winnowed, weighed and counted. Thousand grain weights were determined by multiplying average weights of three replicates of 250 grain weights by four.

2.1.7 Crop analyses

Crop samples were analyzed for a number of parameters as described below. Biomass was determined from sub-samples of five non-fractionated whole plants that were oven dried at 105 ^{o}C for 48 h. Total ash was determined by combusting the crop samples at two phases, initially at 185 ^{o}C for 90 min and then secondly at 320 ^{o}C for a similar period.

2.1.7.1 Chlorophyll analysis

Chlorophyll concentration was determined from the fourth leaf harvested from the main tiller. The leaf samples were preserved in liquid nitrogen immediately after harvest then transferred to -80 ^{o}C freezer. They were later powdered in frozen pestle and mortar filled with liquid nitrogen during grinding. The pigments were extracted in 80% acetone as detailed in (Schittenhelm and Menge, 2006) after which chlorophyll a and b absorbance were spectrophotometrically determined at 647 nm and 663 nm, respectively, while that of carotenoids were determined at 470 nm. Pigment concentrations were calculated using the formula developed by Lichtenhaler (1987).

2.1.7.2 Water-soluble carbohydrate (WSC) analysis

Water soluble carbohydrates (WSC) were determined using high performance liquid chromatography (HPLC) as described by Schittenhelm (1999). In brief, about 0.02 g of dry plant material was mixed into 1 mL distilled water vortexed and incubated for 7.5 min in a shaker set at 80 ^{o}C warm water bath. After the initial incubation, the tubes were removed, mixed by inverting and once again reincubated for 7.5 min in 80 ^{o} C water bath. The samples were cooled at room temperature, centrifuged and then pipetted into 2 mL tube. A repeat extraction performed and extracts were pooled, filled into 1.2 mL HPLC glasses and compared with standards using a computer integrated with a differential refractometer.

2.1.7.3 Total carbon and nitrogen concentrations

Total nitrogen and carbon were analyzed through the DUMAS method from 0.2 g using an automated FP-2000 LECO analyser (LECO Corporation, St. Joseph, MI, USA). The equipment comprises an automatic sampler, combustion oven (1049 ^{o}C) and analyzer. Nitrogen was measured by the warm conductivity detector (WCD) whereas carbon was measured by an infrared detector (IRD).

2.1.7.4 Nitrate analysis

Crop sample nitrate concentration was analyzed using colorimetric SKALAR method (Skalar-Analytik GmbH, Erkelenz, Germany). In summary, about 0.5 g of dried crop samples or 0.2 g of frozen material was extracted in warm water and vortexed. The supernatant was used for nitrate and ammonium analysis using Skalar analyzer.

2.1.7.5 Cation and anion analysis

About 3 g of ground plant material was extracted for 90 min in 150 ml calcium-lactate solution, filtered and used for potassium, calcium, magnesium and sulphur using atom absorption spectrophotometer (AASP) set on omission mode. Phosphorus was analyzed from the same extract using spectrophotometer, Zeiss-Braun-Analysenstrasse model, Germany at an extinction of 720 nm. Chloride was analyzed using ion chromatography (761 compact IC – Metrohm Ionanalytik, AG CH-9101 Herisaue, Sweden), following the method described by Small et al. (1975).

2.1.7.6 Organic acid analysis

Frozen leaf samples (0.2 g) were extracted twice for 30 min in 1 mL boiling distilled water, then centrifuged for 10 min at 20,000 x g. The supernatant was pooled and analyzed for organic acids using high performance liquid chromatography (HPLC) by adopting a Rezex ROH organic acid H^{+} column run on 0.005 M sulphuric acid in 30 ^{o}C at a flow rate of 0.5 ml min^{-1}. The detection of the organic acid was achieved using a Shodex RI-TI refractive index detector (Showna Denko, K. K. Tokyo, Japan).

2.1.8 Soil analyses

At each sampling schedule, soil samples were collected from six different zones consisting of four vertical zones 1, 2a, 3 and 4 and two lateral zones comprising 2b and 2c per replication in each NH_4^+ treatment. Only the four vertical zones were considered in nitrate and non-fertilized treatments (Fig. 1). Zone 1 was 2 cm below the soil surface while zone 2a with a radius of 2.5 cm was located at a depth of 7 cm. Zones 3 and 4 were at depths of 15 and 27 cm, respectively, below the soil surface along the vertical column. Zone 2b formed a ring around zone 2a with a breadth of 2.5 cm while the outermost ring surrounding 2b was 2c and was 2.5 cm wide (Fig. 1). Each zone was sampled up to a depth of 3 cm. Six of the eight soil sampling intervals were analyzed. They included 11, 25, 30, 39, 60 and 109 days after fertilization (DAF) in the first season. In the second season, only five of the six sampling intervals were analyzed. Interval 39 DAF was omitted. Root development around the injection-points were scored on a scale of 0 to 3, depicting; 0 = none depot existence, 1 = slight depot formation, 2 = moderate and 3 = well formed depots.

2.1.8.1 Ammonium and nitrate determination

Soil sub samples were homogenized by sieving through a 5 mm mesh from which 25 g was weighed into a 250 mL conical flask and mixed with 100 mL of 0.0125 M $CaCl_2$. The soil samples were then placed on rotary shaker to extract for 1 h then paper-filtered. The filtrate was used for the determination of NO_3^- and NH_4^+ concentrations by employing colorimetric SKALAR method (Skalar Analytik GmbH Erkelenz, Germany).

2.1.8.2 Potassium and phosphorus determination

Extractable P was analyzed using spectrophotometer at an extinction of 720 nm, whereas K was determined through emission mode of atomic absorption spectrophotometer (AASP). The procedure was similar to that described for crop samples in section 2.1.7.5.

2.1.8.3 Cation exchange capacity

Cation exchange capacity (CEC), soil structure and texture were determined at the Agricultural and Environmental Research Centre (LUFA), Hannover, Germany. The cations were analyzed using spectrophotometer while sand, clay and humus were also determined.

2.1.8.4 Soil pH

Soil pH was determined through the adoption of VDLUFA (1991) method where 10 g of air dried soil was sieved through a 2 mm mesh and weighed into 100 ml conical flask to which 50 mL 0.01 M $CaCl_2$ solution was added, stirred to mix using a rotary glass rod then left to extract for 1 h. The extract was used to measure soil pH using a pH meter.

2.1.9 Data analysis

Statistical analysis was performed using statistical analysis system (SAS) for windows program version 9.1 based upon the general linear model (GLM). Multivariate analysis of variance (ANOVA) was performed to determine and compare treatment differences between the two seasons and among the treatments. The data presented in this section are treatment averages for each separate season. Where applicable, zonal differences were analyzed and differences between sampling intervals were compared among soil samples of the six treatments. The uncropped NH_4^+ treatment had no crop information since it was not sown. Nitrate and non-fertilized treatments had only soil samples along the vertical section since zones 2b and 2c were not considered. Similarly, crop biomass estimates at different growth stages, chlorophyll, total nitrogen, NH_4^+ and NO_3^- concentrations as well as the water soluble carbohydrate concentrations were subjected to multivariate analysis to compare the differences among subsequent harvest intervals in each season and the overall of the two seasons. One way ANOVA was also performed to compare the mean yield differences among the nitrogen treatments. The means were considered significant if $P < 0.05$ and post-ANOVA was performed using Tukey test to determine the level of statistical significance. Where appropriate, correlation analyses were performed. The inclusion of non-fertilized control enabled the assessment of N fertilization effect, while the effect of NH_4^+-based fertilizers on crops was assessed by comparing cropped and uncropped treatments. Additionally, nitrate treatment provided a basis for the comparison of the two nitrogen forms whereas the influence of nitrification inhibitor (NI) incorporation of two different concentrations were also included for comparison against treatments without NI incorporation and incorporation of low and high NI concentrations.

2.2 Characterization of bacterial community responses to CULTAN fertilization

Soil samples from which bacterial DNA was extracted were five of the six treatments described under ammonium persistence ev aluation. Ammonium treatment incorporated with 20% NI was omitted. Details of the treatments are described in section 2.1.3.

2.2.1 Soil samples analyzed for bacterial occurrence

Soil samples harvested in the first season were used for analysis. The soil samples used for molecular microbiological investigations were sampled at 30, 60 and 109 DAF. Briefly, three of the treatments were NH_4^+ -based out of which, two were not incorporated with NI. The treatments were cropped NH_4^+ without NI presented as (NH_4^+ - NI + Crop), uncropped designated as (NH_4^+ - NI -Crop) and cropped NH_4^+ treatment incorporated with 5% NI was designated as (NH_4^+ + 5%NI + Crop). Nitrate and non-fertilized control treatments were included as comparison checks. To assess ammonium nutrition spatial diffusion effects on bacterial communities, both lateral and vertical zones were considered. Only two vertical zones were chosen in nitrate and non-fertilized control treatments and no lateral zones were considered. Zones along the vertical section included 2a and 3 located at depths 7 and 15 cm below soil surface, respectively (Fig. 1). Zone 2c also located at a depth of 7 cm was coplanar to zone 2a across the lateral section though separated from it by 2.5 cm radius distance. The breadth of each zone was 2.5 cm and sampling depth was 3 cm. Three replications were considered for each sample collected at 30, 60 and 109 DAF for each treatment and zone (Fig. 1).

2.2.2 Bacterial DNA extraction from environmental soils

Genomic bacterial DNA was extracted from soil samples described above using Bio101 systems, FastDNA® spin kit for soil (QBiogene, USA). In summary, 0.5 g soil was weighed into screw-cap tubes with ceramic and silica-beads (Roth Karlsruhe, Germany). Buffer (122 MT) was added to the soil to facilitate cell disruption with the help of FastPrep® instrument (model FP120, QBiogene, USA) operated at a speed of 5.5 for 30 s after securing the screw cap tubes during processing. The tubes were centrifuged at 14,000 x g for 5 min after which the extract was transferred into 1.5 ml tube. The resulting pellet was resuspended in 950 µL sodium phosphate buffer to which 250 protein precipitating solution (PPS) was added and mixed by hand shaking then centrifuged at 14,000 x g for 5 min. About 1 mL

binding matrix suspension solution was added and the tubes were inverted by hand for two minutes to enhance binding of DNA to matrix and placed on a rack to settle for 3 min. About 700 µL of the upper supernatant portion was carefully discarded and the remaining 600 µL pippeted into a SPIN Filter and centrifuged at 14,000 x g for 1 min. The catch tube was emptied and the remaining supernatant was refilled and the process repeated. The DNA was washed with 500 µL of salt-ethanol-wash-solution (SEWS-M) and centrifuged at 14,000 x g for 1 min. The flow-through was discarded and filter replaced in the same catch tube and centrifuged once again at 14,000 x g for 2 min. The washing step was repeated to remove co-extracted contaminants. Finally, the filters were removed and replaced in fresh catch tubes, air dried for 5 min at room temperature and eluted with 60 µL of (TE) Tris-EDTA-buffer. About 40 µL DNA aliquots were stored at -20 ^{o}C while 20 µL was used for immediate analysis. The purity of extracted DNA was analyzed by electrophoresis on 1.0% agarose gel and photographed using BioDocAnalyze (Biometra Goettingen, Germany). DNA quantification was achieved using a fluorescent dye, PicoGreen® (MoBiTec., Goettingen, Germany) and the measurements were performed on Labsystems Fluoroskan II (GMI, Albertsville, Minnesota, USA) at a wavelength of 485 nm emitted at 530 nm.

2.2.3 DNA amplification, purification and quantification

Amplification of genomic bacterial DNA was achieved through adoption of polymerase chain reaction (PCR) procedure, an *in vitro* process that employs a heat-stable polymerase enzyme. Forward and reverse universal bacterial primers targeting 16S rRNA genes were used. Forward primer (Com1), (5'-CAGCAGCCGCGGTAATAC-3') targeted positions 519 to 536 while reverse primer (Com2+Ph), (5'-CCGTCAATTCCTTTGAGTTT-3') targeted positions between 907 and 926. Cycling was performed on a thermocycler (model Primus 96^{plus}, MWG Biotech, Ebersberg, Germany) in 50 µL micro-tubes (Flat Cap Micro Tubes). The reaction mixture comprised 1× PCR buffer containing 1.5 mM $MgCl_2$, deoxynucleoside triphosphate solution (dNTPs) of 0.2 mM per tube. Forward and reverse primers were added at a rate of 0.5 µM each in addition to 2.5U/100 µL of DNA polymerase (Hot Star Taq. Qiagen). Ten-fold diluted DNA template was added to the reaction mix at a volume of 1 µL per tube. The cycling program comprised an initial denaturation step of 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C lasting 60 s each. The annealing temperature was 50 °C for 60 s, followed by elongation at 72 °C for 70 s and primer extension lasted 5 min at 72 °C. The amplified PCR products were analyzed on 1%

agarose gel. The DNA products were compared to a standard of 1 kb PlusLadder™ (Invitrogen, Eggenstein, Germany) loaded at a volume of 5 µL per well. Electrophoresis was facilitated by an electric current of 100 V for 60 min. The DNA products were digitally photographed using a transilluminating camera, BioDocAnalyze (Biometra Goettingen, Germany). Amplified PCR products were cleaned using QIAquick kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The DNA was finally eluted in 30 µL buffer and quantified using fluorescent dye, PicoGreen® (MoBiTec., Goettingen, Germany) at a wavelength of 485 nm emitted by fluorometer at 530 nm, Labsystems Fluoroskan II (GMI, Albertville, Minnesota, USA).

2.2.4 Single-strand conformation polymorphism (SSCP) technique

The genetic band profiles were analyzed through the deployment of polymerase chain reaction based single-strand conformation polymorphism (PCR-SSCP) technique. Separation of the single-stranded DNA fragments was achieved on non-denaturing polyacrylamide-gel.

2.2.4.1 Reagents and equipments used in SSCP technique

A 25 mL polyacrylamid-gel was prepared by mixing 7.813 mL of 2 x MDE solutions to 2.5 mL of 10x TBE and 14.69 mL of double distilled water in a 50 mL beaker. The mixture was filtered through a polyether sulfone-membrane of 0.45 µm pore sizes fitted to a syringe. The mixture was degassed under a vacuum chamber for 2 min after which 10 µL of TEMED ($C_6H_{16}N_2$) and 25 µL of 40% APS were added under constant stirring. The notched glass and thermostatic plates were each lined with 1 mL bind-silane and repel silane, respectively, (Pharmacia Amersham Biotech, Freiburg, Germany). The two plates were clamped onto each other leaving 1 mL space between them into which the gel was casted and a 26 tooth-comb fixed to create wells for loading DNA after polymerization. The polymerized gel was built onto the macrophore and run for 17 h at 400 V, 20 mA, 10 W. (Schweiger and Tebba, 1998; 2000).

2.2.4.2 Generation of the single-stranded DNA

Single-stranded DNA was generated from purified amplified PCR products digested by lambda-exonuclease (Pharmacia Amersham Biotech, Freiburg, Germany). The reaction mix was incubated at 37 °C for 45 min once again purified using MiniElute kit (Pharmacia Amersham Biotech, Freiburg, Germany) following the manufacturer's protocol. About 9 µL

of SSCP loading buffer was added to the purified single stranded DNA and denatured at 95 °C for 2 min then cooled for at least 3 min on ice. About 5 µL of denatured single strand DNA was loaded into each well and run under conditions described above. (Bäckman et al., 2003).

2.2.4.3 SSCP gel silver staining and development

After running the gel for 17 h, the notched glass plate was unclamped, separated and placed into a basin filled with 500 mL of 10% acetic acid and swivelled at a speed of 20 rpm for 30 min during fixation. The plate was washed thrice in deionised water for 5 min each time after which the gel was stained for 30 min in silver nitrate solution (500 mL double distilled water, 0.5 g $AgNO_3$ and 0.75 mL of 37% formaldehyde). The gel was developed in cold sodium carbonate deca-hydrate solution (600 mL double distilled water, 33.75 g sodium carbonate deca-hydrate, 0.6 mL of 0.2% sodium thiosulphate and 1.2 mL of 37% formaldehyde solution). Gel development was carefully performed and as soon as the bands became visible the reaction was stopped by removing the notched glass plate from developer solution and placing it once again into acetic acid. After 2 min, it was removed from acetic acid and placed in a water-filled basin for 1 h and finally air-dried under a fume-hood and later scanned. The scanned gel photos were then transformed using Corel Draw.

2.2.4.4 Band selection, excision and soaking

The developed bands were carefully identified and representatives chosen for cloning and sequencing. Band dominance (strongly staining), uniqueness (specific to treatments/zones) and commonness (uniformly found across treatments/zones) were the criteria used for their selection. The selected bands were excised and covered with a drop of PCR water to ease peeling off from the gel plate. Excised band slices were placed in PCR tubes then transferred into 50 µL crush and soak buffer comprising 0.5 M ammonium acetate (10 mM Mg^{2+}-acetate, 1 mM EDTA [pH 8.0], and 0.1% sodium dodecyl sulphate). Band slices were soaked in buffer and incubated at 37 °C for 3 h while spinning at 550 $Umin^{-1}$ on a thermomixer. About 40 µL of the solution was pipetted into a two-fold volume (80 µL) ethanol, precipitated at -20 °C over night and centrifuged at 130,000 x g for 25 min at 4 °C and the resulting supernatant was discarded whereas the pellet was dried at 37 °C for 15 min in a thermomixer before dissolving in previously incubated 12 µL of 10 mM Tris-EDTA buffer for 1 h. The extract was used to perform 50 µL PCR to amplify the DNA. The PCR

product was checked on 1% agarose gel, purified by 'Nucleospin Extract II' (Macherey & Nagel, Dueren, Germany) following manufacturer's protocol and ligated and transformed.

2.2.5 DNA cloning (ligation and transformation)

Gene inserts of reference clones were amplified with vector-specific primers. The amplicons were cloned into pGEM®-T-Easy vector (Promega, Mannheim, Germany) using TA cloning kit. Ligation was performed in 0.5 µL T 4 DNA-Ligase, 0.5 µL pGEM-T-Easy Vector and 2.5 µL buffer of 3.5 µL reaction mix per 1.5 µL DNA template. The mixture was hand shaken and incubated overnight at 4 °C. Two controls, positive (with insert) and negative control (distilled water) were included to help assess success or contamination of the ligated products. Transformation was achieved by incubating 2 µL of the ligated DNA and 36 µL of competent SM 109 cells for 20 min. Competent cells were heat-shocked for 45 s at 42 °C and immediately cooled for 2 min on ice. A ratio of 1:20 dilution of room temperature SOC medium was added then spinned at 600 rpm for 90 min while incubating at 37 °C. About 100 µL of the transformant was plated twice onto LB/ampicillin/X-Gal/IPTG/agar (LAXI) using sterile plating-rod on a clean bench and incubated at 37 °C overnight.

Nutrient agar was prepared from 2.5 g NaCl, 2.5 g yeast extract, 5 g trypton and 7.5 g agar and poured into Petri-dishes on a clean-bench. The plates were incubated overnight for 16-24 h at 37 °C. The resulting bacterial white cell colonies (with vector inserts) and blue cell colonies (without inserts) were assessed. Blue cell colonies were ignored during the selection of three separate white cell colonies for plating onto agar nutrient plates for further multiplication. A portion of the cell colonies on master plate were picked and lyzed in 50 µL buffer prepared from 0.05 M NaOH and 0.025% SDS. The cells were incubated for 15 min at 95 °C at a speed of 600 min^{-1} on a thermomixer. 450 µL solution of sterile double distilled water was added, vortexed and centrifuged for 4 min at 10,000 rpm. The product was used for 25 µL PCR amplification. Upon amplification, 5 µL of amplified PCR product was checked on 0.8% agarose gel while the remaining was purified using Qiagen kit and eluted with 30 µL EB buffer. About 14 µL of the DNA was digested in 16 µL lamda-exonuclease at 37 °C for 45 min. The digested product was purified using Qiagen mini Elute kit and finally eluted with 10 µL EB buffer. About 9 µL bromophenol blue loading dye was added to the eluted DNA at a ratio of 1:1 (vol/vol). Clone and genomic DNA were concurrently run

on the same SSCP gel plates for easy comparison. Clone bands of similar migration positions to the excised genomic bands were chosen for sequencing.

2.2.6 DNA Sequencing

Selected clone bands were sequenced in MWG Licor sequencer (Biotech) using Epicentre-Kit.

2.2.6.1 Sequencing reagents and equipment

A 41 cm sequence gel was prepared by mixing 16.8 g urea, 4 mL of 10x TBE long run and 6 mL of 40% Rapid Gel then filled up with distilled water up to 40 mL mark, dissolved and filtered into a syringe fitted with 0.45 µm perforated membrane then degassed for 2 min. Upon degassing, 40 µL of TEMED and 72 µL APS were added while constantly stirring then casted into a sloppy screw-clamped gel plate. A fine toothed comb was fixed to create wells during the 2 h polymerization. The plate was fitted onto a sequencer and both upper and lower chambers were filled up with 1x TBE long run solution. The cloned DNA material was loaded and run for 17 h.

2.2.6.2 Sequencing procedure

Sequencing was performed in MWG Licor sequencer (Biotech) using Epicentre-Kit. Master mixes were prepared from 7.2 µL buffer, 4.8 µL distilled water, 1.0 µL polymerase and infrared labelled 1.0 µL forward/ reverse primers. Aliquots of 14 µL prepared in 0.5 mL tubes using Sequitherm Excel^{TM} II DNA sequencing kit-LC, Epicentre Biotechnologies. Each of the 14 µL master mixes and 3 µL DNA templates were spinned. Aliquots of 4 µL of forward /reverse master mixes were transferred into each of the four termination mixes then spinned shortly. 2 µL termination mixes of adenine, cytosine, guanine and thyamine (A, C, G, T) were pipetted into 96 well-plate and shortly spinned. A drop of mineral oil (Sigma) was added to both reaction mixes and run for 90 min on M-13 Forward thermocycling programme. After amplification, 2 µL of stop-loading buffer was added, spined and denatured for 2 min at 95 °C and cooled for 3 min at 4 °C. 1 µL of the denatured product was loaded for sequencing and run for 17 h. The sequences were retrieved, processed and blasted in NCBI (Altschul et al., 1990). They were aligned in Mega-Program (Kumar et al., 2004) and imported into ARB-program for analysis and treeing (Ludwig et al., 2004).

2.2.7 Statistical Analyses

The SSCP gels were scanned and images adopted to analyze banding profiles in GelCompar® II programme package, version 4.5 of 2005 (Applied Maths BVBA, Belgium).

2.2.7.1 Digital image analysis

Each of the scanned SSCP gel images was transformed in Corel Draw. The banding lanes on each gel were automatically searched. Banding strips in each lane were evaluated on the basis of the corresponding densitometric tone curve values. Mobility of the band profiles were normalized with four external bacterial standards comprising *Bacillus licheniformis, Rhizobium trifolii, Flavobacterium johnsoniae* and *Rhizobium radiobacte.* They appeared in the same order in which they have been listed here above and were represented by I, II, III and IV on the marker lane upon migration on the gel. Band and reference positions were automatically aligned. In case the reference positions were not properly matched during the automatic alignment, internal standards were introduced to reorganize band positioning. The edited digital image was saved and desired gel strips were highlighted to calculate similarity matrices of the constructed dendrogram based on unweighted pair group method with arithmetic averages (UPGMA) and pearson correlation coefficients. Algorithms of the pearson's correlation coefficients were utilized to compare nitrogen treatment effects of the corresponding banding profiles. Permutation test was performed to determine the differences among gel and treatment profiles whereas significant differences among band profiles were determined as described by Kropf et al. (2004) in SAS program.

2.2.7.2 Sequence analyses

Nucleotide sequences were determined by the Sanger method (Sanger *et al*., 1977). Clone sequences were processed as described by Ewing and Green, (1998) and Ewing *et al*. (1998). The sequences were compared with complete EMBL database nucleotide sequences by blasting as described by Altschul et al. (1990). Sequences with best BLAST scores were imported into ARB-program for treeing and analysis. Phylogenetic trees were constructed in ARB program for analysis (http:/www.arb-home.de) (Ludwig et al., 2004). Nearly full-lengths of sequences (>1000 bases) obtained were aligned using Mega-software then imported to ARB. Imported sequences were automatically aligned with existing 16S rRNA

sequences in ARB alignment tool, ARB_EDIT, visually inspected and manually edited where necessary. Phylogenetic placement was achieved by comparing reference and obtained sequences in the domain bacteria. Ambiguous base positions were excluded during sequence similarity calculations. Overall phylogenetic analyses were determined by distance matrix, maximum parsimony and maximum likelihood methods as described by Ludwig and Schleifer (1999) and Ludwig et al. (1998). Statistical significance levels of interior nodes were determined by performing bootstrap analyses of neighbour joining method combined with Jukes-Cantor correction to infer distance matrix trees. Variability of individual alignment positions were determined in ARB_SAI tool based on remove or include criterion.

2.3 Evaluation of the biodiversity of ammonia oxidizing bacteria

2.3.1 Detection of ammonia oxidizing bacteria (AOB)

Soil samples for ammonia oxidizing bacteria (AOB) analysis were similar to those used to investigate bacterial community biodiversity and responses to CULTAN fertilization (section 2.2). Only vertical zones, 2a and 3 of the five nitrogen treatments were considered. No lateral zone was included. Treatment and sampling zone details as well as DNA extraction were as described in sections 2.2.1 and 2.2.2.

2.3.2 Selection and optimization of *amoA* primers

A review of published primers was made to select *amoA* primer pairs. Primer pair developed by Rotthauwe et al. (1997) was chosen. Forward primers targeting positions 332 to 349, (amoA-F; 5'–GGGGTTTCTACTGGTGGT- 3') and reverse primer (amoA-R+Ph; 5' –CCCCTCKGSAAAGCCTTCTTC- 3') {K=G or T; S=G or C} targeted the stretch between 802 and 822 of the open reading frame of *amoA* gene sequence of *Nitrosomonas europaea*. The primers generated 491 bp length PCR products. Different concentrations were tested and 30 pmol μL^{-1} was adopted. Additional 1.2 µL of $MgCl_2$ was applied to the reaction mix.

2.3.3 Selection of AOB for use as positive control

Amplification efficiency of the selected *amoA* primer set was tested on *Nitrosospira multiformis*, ATCC acquired from Jena University. The obtained *amoA* band size was 491 base pair length. *N. multiformis* clones were reamplified and checked on 1% agarose and yielded a similar size to the original plamid DNA. One of the five clones was adopted as a comparison check for *amoA* bands.

2.3.4 PCR amplification of AOB with *amoA* primers

Amplification of *amoA* genes were performed on primus thermocycler (MWG Biotech, Ebersberg, Germany) in 50 µL tube (Flat Cap Micro Tubes). The reaction mixture comprised 1× PCR buffer containing 1.5 mM $MgCl_2$, to which 1.2 mM $MgCl_2$ was added, besides deoxynucleoside triphosphate (dNTPs) of 0.2 mM, forward and reverse primers of (0.3 µM each) as well as 2.5U/100 µL of DNA polymerase (Hot Star Taq, Qiagen). To this, 2 µL of 10-fold diluted template DNA was added in 50 µL reaction mix for amplification.

The cycling programme comprised an initial denaturation step of 95 °C for 15 min, followed by 36 cycles of denaturation at 94 °C lasting 90 s each. Primer annealing temperature was 60 °C for 90 s, followed by elongation at 72 °C for 90 s. The final extension step at 72 °C lasted 5 min. The PCR products were electrophorized and compared on 1% agarose to standards of 1 kb PlusLadder^{TM} (Invitrogen, Eggenstein, Germany). The PCR products were amplified, purified and quantified as described in section 2.2.3 and digested to generate single-stranded DNA following the procedure outlined in section 2.2.4.2.

2.3.5 Development of *amoA* SSCP gels

Single-strand conformation polymorphism gels were developed from *amoA* genes based on the same procedure as described in section 2.2.4.

2.3.6 Band selection, ligation, transformation and sequencing

Both unique and common bands were selected, excised, ligated, transformed and sequenced as described in sections 2.2.5 to 2.2.6.

2.3.7 Statistical analysis

Statistical analyses of digital gel images were performed using Gelcompar software programme as described in section 2.2.6.1 and the generated similarity matrices were used for permutation test. Phylogenetic tree was constructed in ARB program using databank published *amoA* sequences.

2.4 Ammonia oxidizing bacteria population abundance in CULTAN-fertilized soils

2.4.1 Quantification of *N. multiformis* gene copies

Three separate PCR assays were performed to amplify *N. multiformis amoA* genes. The three PCR product replications were purified using QIAquick kit (Qiagen, Hilden, Germany) and eluted in 30 µL buffer. The products were pooled, mixed and quantified using fluorescent dye, PicoGreen® (MoBiTec., Goettingen, Germany). Serial dilution ranges of 10^{-2} to 10^{-10} were performed and measured by fluorometer, Labsystems Fluoroskan II (GMI, Albertville, Minnesota, USA) at 485 nm wavelength emitted at 530 nm. The measured DNA concentration was employed to calculate gene copy numbers. The amplification curve was normalized and threshold cycle (C_t) determined and used to calibrate gene copy numbers. Equation 1 outlines the calculation of standard gene copy numbers while equation 2 gives the DNA weight. When equation 2 is substituted into 1, equation 3 is formed. The new equation can be adopted to estimate standard gene copy numbers. Two constants were used, 6.023×10^{23} was Avogadro's constant whereas 6.6×10^{11} was the fragment base pair length of DNA strand.

Equation 1:

$$\text{Standard copy numbers } \mu L^{-1} = \frac{\text{DNA concentration [ng . } \mu L^{-1}]}{\text{DNA weight [ng . copy}^{-1}\text{]}}$$

Equation 2:

$$\text{DNA weight [ng . copy}^{-1}\text{]} = \frac{\text{Fragment length [bp]} \times 6.6 \times 10^{11} \text{ ng.[mol}^{-1}\text{.bp]}^{-1})}{6.023 \times 10^{23} \text{ (Copies. mol}^{-1})}$$

Equation 3:

$$\text{Standard copy numbers } \mu L^{-1} = \frac{\text{DNA concentration [ng . } \mu L^{-1}] \times 6.023 \times 10^{23} \text{ (Copies. mol}^{-1})}{\text{Fragment length [bp]} \times 6.6 \times 10^{11} \text{ ng.[mol}^{-1}\text{.bp]}^{-1})}$$

2.4.2 Real-time PCR optimization

Real-time PCR assay was performed in 20 µL reaction mixture consisting of 1 ng template DNA, 10 µL of QauntiTech SYBR Green master mix (Qiagen) and 30 pmol μL^{-1} of

forward and reverse *amoA* primers. Non-coloured 50 µL flat-capped PCR micro tubes were used. The PCR cycling protocol adopted for *amoA* quantification was as follows: initial denaturation step at 95 °C for 15 min, followed by 50 cycles of denaturation at 94 °C lasting 60 s each. Primer annealing was at 60 °C for 60 s, elongation at 72 °C for 60 s and a final extension step at 72 °C for 5 min. Generated curves were analyzed to determine the threshold cycle for each assay.

2.4.3 Generation of *amoA* standard curves

Curves generated from selected ammonia oxidizing bacterial species, *N. multiformis* were used as standard AOB species to estimate the abundance of *amoA* genes in soil genomic DNA. The standard curves were developed through the performance of serial dilutions ranging between 10^{-3} and 10^{-7}. The diluted positive control DNA ranged between 10^{-3} and 10^{-7}. Along side the positive control were soil sample DNA template and non-template control (NTC). The amplified PCR products were electrophorisized and compared on 1% agarose to standards of 1 kb PlusLadder ^{TM} (Invitrogen, Eggenstein, Germany).

2.4.4 Threshold cycle determination

Threshold cycles of serially diluted *N. multiformis* were used to estimate *amoA* gene copy numbers of bacterial genomic DNA of the nitrogen treatments.

2.4.5 Melting point curves of *amoA* genes

The melting point (MP) of *amoA* genes was continuously recorded during the amplification. While some MP peaks of the soil extracted DNA corresponded to those of the standard AOB, a few did not. Given that melting point is a proof of purity, those with similar peaks indicated PCR product similarity, whereas dissimilar peaks suggested impurity or presence of a different product.

2.4.5 Statistical analysis

No statistical analysis was performed on the generated Real-time PCR data because replicate test sample assays and non-template control (NTC) curves overlapped. Because of this limitation, no replicate quantification assays were factored to calculate ammonia oxidizing bacteria population estimates.

2.5 Effect of temperature on CULTAN-fertilized barley

2.5.1 Experimental site and crop growth conditions

The experiment was performed in three separate growth chambers at Federal Agricultural Research Centre (FAL), Braunschweig, Germany. Spring barley, *Hordeum vulgare* L. cv. Maresi was used as a model cereal in the study. The seeds were sown singly within an inter- and intra- row spacing of 5 cm at a depth of 2.5 cm in 90 L soil filled container. The soil was a mixture of top-, sub- and sandy-soil proportions in the ratio of 1:1:1, respectively. It was free-draining sandy loam comprising 14.6% clay, 39.6% silt, 45.8% sand and 1.6% humus with a near neutral pH of 7.4. The total nitrogen and carbon were 0.03% and 0.43%, respectively. Growth chambers were cubes measuring 3 m (27 m^3) fitted with 16 lamps on two horizontally adjustable metallic frames. Half of the lamps comprised sodium bulbs while the other eight were fluorescent potassium tubes. The two lamp sets produced 600 µmol $m^{-2}s^{-1}$ of photosynthetic active radiation (PAR) during the 14/10 h day/night photoperiod. Chamber relative humidities ranged between 55 – 70% and irrigation was regularly provided by hand held sprinkler. Five 1 cm diameter holes at the bottom of container drained excess water to guard against logging. The draining percolate was collected and reutilized in subsequent irrigation schedules.

2.5.2 Experimental design

Temperature was the major treatment whereas nitrogen was minor. Three temperature regimes LTR, MTR and HTR were established and within each growth temperature comprised three nitrogen treatments, ammonium, nitrate and non-fertilized control (Table 1). Each of the nitrogen treatments were replicated twice. The nitrogen treatments were arranged in a randomized complete block design (RCBD) within each temperature regime. The experiment was performed in two seasons under similar conditions, but chambers were swapped in each season to balance out their effects on crop growth.

2.5.3 Temperature and nitrogen treatments

Three days prior to sowing, all the growth chambers were acclimatized to the same growth conditions of 13/9 ^{o}C for d/n, respectively. This growth condition was maintained

from sowing up to the onset of tillering stage. From tillering onset, each chamber was reset to correspond to LTR, MTR and HTR growth conditions (Table 1). Chamber temperatures were manually raised in a stepwise manner as the season progressed to coincide with the four distinct barley growth stages (Table 1). Temperatures were monitored by thermometers inserted into soil at 7 cm depths. The crops were considered to have attained subsequent growth stages when at least 50% exhibited descriptions given in Zadoks' growth scale (Zadoks et al., 1974). Each temperature regime had three nitrogen treatments (Table 1). Nitrate was applied as $Ca(NO_3^-)_2$ while NH_4^+ was applied as diammonium phosphate (DAP), $(NH_4^+)_2HPO_4$. Each treatment received 4 g N of either NO_3^- or NH_4^+. Nitrate was sprayed whereas NH_4^+ was injected into five different points per container (Fig. 3). The injection was performed at a depth of 7 cm using 1 cm diameter aluminium rod. The holes were fitted with 20 mL Eppendorf tubes to simulate spoke wheel injectors used for CULTAN injection under field conditions. After injection, the holes were refilled with soil and injection-point marked with thin wooden pegs. 18 g of P was added to nitrate and non-fertilized control treatments to balance P from DAP. It was applied as $Ca(H_2PO_4)_2$. Nutrients whose effects were not being evaluated were adequately supplied to ensure balanced mineral nutrition availability. Macro nutrients were divided into eight equal portions and spread out using a fine meshed sieve as P =5 g, K = 13 g, Mg = 27 g, Ca = 6 g per container, while micro-nutrients were applied as Fe = 25 mg, Mn = 10 mg, Cu = 5 mg, B = 3 mg, Zn = 5 mg and Mo = 0.5 mg per container.

2.5.4 Soil sampling zones

Crop and soil sampling were performed at the end of the season in correspondence with the crop growth duration. Aerial crop samples were categorized into edge and core plants. Soil was sampled from four vertical zones namely, 1, 2, 3 and 4 located at 2, 7, 15 and 27 cm depths in each of the five injection-points (Fig 3). Soil samples from similar zones were pooled, mixed and used for analyses. The two replicates were separately analyzed.

Table 1: Summary of temperature and nitrogen treatments in the three temperature regimes during the various barley growth stages

		Crop Growth Stages			
Treatments		**Tillering start to end**	**Tillering end of booting**	**Start to end of flowering**	**Flowering to maturity**
Temperature	LTR (d/n*); ^{o}C	6/2	11/7	14/10	16/12
	MTR (d/n*); ^{o}C	10/6	15/11	18/14	20/16
	HTR (d/n*); ^{o}C	14/10	19/15	22/18	24/20
Nitrogen Regimes	1. Non-fertilized control (no nitrogen fertilizer application) (T_1)				
	2. NO_3^- - N as $Ca(NO_3^-)_2$; as 4 g N per treatment as single application (T_2)				
	3. NH_4^+ - N as $(NH_4^+)_2HPO_4^-$ injected as 4 g N/container (T_3)				

* day/night temperatures within each growth temperature regime during the four stages

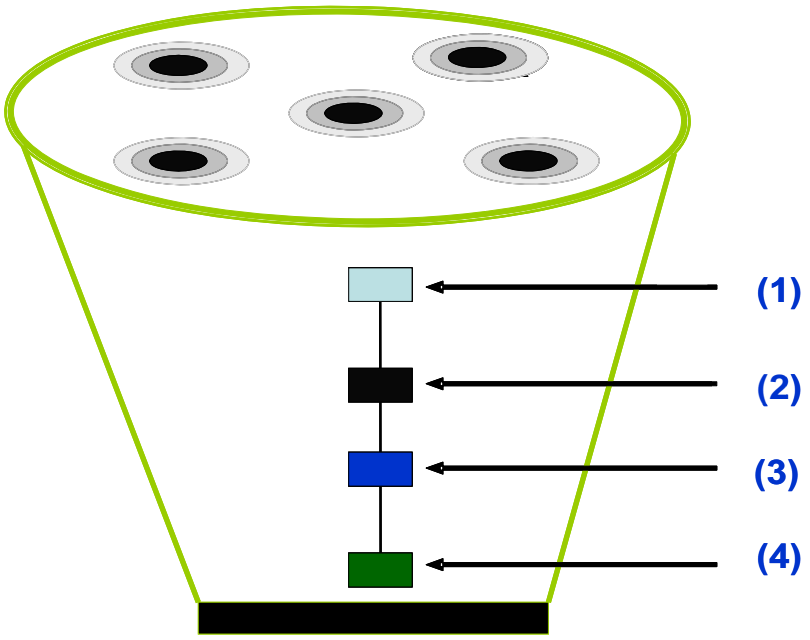

Fig. 3: Ammonium injection points shown on an aerial view of the soil surface in the container as well as the hypothetical diffusion zones of the five vertical sampling zones. Zone 1 was located 2 cm below the surface while 2a was at 7 cm depth. Zone 3 was located 15 cm below the surface or 8 cm below the injection point and zone 4 was at a depth of 27 cm below the surface.

2.5.5 Carbon Exchange Rates (CERs) and SPAD measurements

Gas exchange measurements were performed on the leaf immediately below flag-leaf (F-1) of main tillers. Four averagely similar looking barley crops in each replicate nitrogen treatment used. Since the leaves were too narrow to singly cover 2.5 cm^2 cuvet, two adjacent F-1 leaves of similar ages were joined in overlap for CER measurements. SPAD – 502 (Minolta Ltd., Japan), a handheld chlorophyll meter was used to estimate leaf N status on the same spots where CER was measured. CER measurements were performed at the onset of grain filling period using HCM-1000 differential infrared gas analyzer (IRGA) (Heinz Walz GmbH, 91090 Effeltrich, Germany) fitted with a leaf cuvet chamber. During measurements, carbon dioxide (CO_2) concentration was regulated at 350 μL L^{-1} while leaf cuvet air temperature was set at 25 °C. The saturating photosynthetic photon flux density (PPFD) was supplied by Walz lighting unit at 1800 μmol $m^{-2}s^{-1}$. Diurnal effects were accounted for by switching among nitrogen treatments in each round until the four replicate plants in each of the two nitrogen treatment replicates were covered. Estimates of maximum CER were calculated by HCM – 1000's operation soft ware based on methods of von Caemmerer and Fahquer (1981).

2.5.6 Shoot and root biomass estimates

The crops were harvested at physiological maturity and grains processed separately from stems. Edge plants totalling to 26 were harvested and separately processed from core plants 70 used for biomass and chemical analyses. Crop growth duration, stem length and total tiller numbers were recorded. Fresh and dry shoot weights were also recorded. Grain bearing ears were cleaved off from tiller stems and counted. The average number of tillers per plant as well as average number of grains per head bearing tiller was calculated. Thousand grain weights were determined. Roots were harvested from the entire container as described by Bloom *et al*. (1997) with slight modifications. Briefly, the roots were washed using tap water. Detachable sieves of varying mesh sizes, 2.5, 2 and 1 mm were used. Finer roots were recovered using a pair of forceps. After washing off the soil, shoot stumps were cleaved off from roots using pairs of scissors after which fresh weight was taken. Oven dried shoot and root weights carried out at 105 ^oC for 48 h were used for DM determination. Root and shoot samples to be used for chemical analyses were oven dried for 48 hrs at 60 ^oC. Agronomic nitrogen use efficiency (NUE) was estimated as described by Maranville et al.

(1980) and Moll et al. (1982). Root: shoot ratios, harvest as well as response indices were also estimated.

2.5.7 Crop analyses

Dried crop samples were ground using Brabender laboratory mill with 1 mm grid. The analyses of most of the parameters briefly mentioned below have been considered in the cited sections. Total nitrogen and carbon were both analyzed using automated FP-2000 LECO analyser (LECO Corporation, St. Joseph, MI, USA) (Ref. section 2.1.7.3), while nitrate was analyzed using colorimetric SKALAR method (Skalar-Analytik GmbH, Erkelenz, Germany) as described by Schittenhelm and Menge (2006) (Ref. section 2.1.7.4). Water soluble carbohydrates (WSC) were analyzed by high performance liquid chromatography (HPLC) (Ref. section 2.1.7.2). Starch was analyzed by the method of Ewers (1973). Potassium was analyzed through the emission mode of atom absorption spectrophotometer (AASP), whereas phosphorus was also determined from the same extract using spectrophotometer, Zeiss-Braun-Analysenstrasse model, Germany at an extinction of 720 nm. Chloride anions were analyzed using ion chromatography (761 compact IC – Metrohm Ionanalytik, AG CH-9101 Herisaue, Sweden), following the method described by Small et al. (1975) (Ref. section 2.1.7.5).

2.5.8 Soil analyses

Soil was sampled from zones 1, 2, 3 and 4 situated a long the vertical section at depths of 2, 7, 15 and 27 cm. Replicate zones of the five injection-points were pooled together and mixed for analysis. DM was determined by drying 10 g soil at 105 ^{o}C in ceramic Porcelain crucible cups overnight and cooling in silica gel filled desiccators before reweighing upon cooling. About 25 g soil was extracted in 100 ml of 0.0125 M $CaCl_2$ in a rotary machine shaker for 1 h, paper-filtered and analyzed for NH_4^+ and NO_3^- concentrations using colorimetric SKALAR method (Skalar Analytik GmbH Erkelenz, Germany) (Ref. section 2.1.8.1). Phosphorus was measured using spectrophotometer while potassium was determined using emission mode of atom absorption spectrophotometer (Ref. section 2.1.8.2). Soil pH was determined on $CaCl_2$ extracted soil using pH meter following VDLUFA (1991) method (Ref. section 2.1.8.4).

2.5.9 Data analysis

Statistical analysis was performed using SAS program for windows version 9.1 employing general linear model (GLM). The presented data are averages of two seasons. Two way factorial analyses of variance were performed to compare mean differences among various parameters of pooled seasonal data sets of nitrogen treatments among the different temperature regimes besides individual seasonal comparisons. Similarly, one way ANOVA was performed to compare mean differences among the three nitrogen regimes within each temperature regime. Soil sampling zones were compared within and among LTR, MTR and HTR. Treatment means were considered significant when $P \leq 0.05$ and post ANOVA was performed using Tukey test to determine the significance level of means. Correlation analysis was performed wherever necessary. Shoot, grain and root chemical constituents were compared.

3 RESULTS

3.1 Stability of soil-injected liquid NH_4^+

3.1.1 Soil physical and chemical characteristics

To determine the texture of the soil mixture used for growing barley, the relative proportion of the different components were analysed. The soil was generally sandy-loam comprising 14.6% clay, 39.6% silt, 45.8% sand and 1.6% humus with a near neutral pH value of 7.4. Total nitrogen and carbon was 0.03% and 0.43%, respectively. Total nitrogen concentrations varied considerably during the season in the different zones of different treatments (Fig. 4a). Ammonium-based treatments recorded exceedingly high total nitrogen concentrations in zones 1 and 2a while zones 2b, 2c, 3 and 4 recorded low concentrations. Nitrate and non-fertilized control treatment equally reported low nitrogen recoveries. Zones 1 and 2b of ammonium treatments differed significantly from the rest at $P < 0.001$. While the interval between 11 and 25 DAS in nitrate treatment slightly increased, non-fertilized control recorded a decline during the same period (Fig. 4a). Though total nitrogen persisted within the soil, it reduced as the season progressed. Zones 1, 2a and 2b of ammonium treatments gradually decreased as the season progressed. Total carbon concentrations of ammonium treatments ranged between 0.84 and 0.95% in air-dried soil during the season. Nitrate and non-fertilized control recorded much lower initial carbon concentrations whose peaks at 25 and 32 DAS ranged between 0.89 and 0.95%. No peculiarity of this kind was observed among ammonium treatments at season onset (Fig. 4b). Large carbon: nitrogen (C/N) ratio imbalances were observed, but the exact fate of excess nitrogen was not clear.

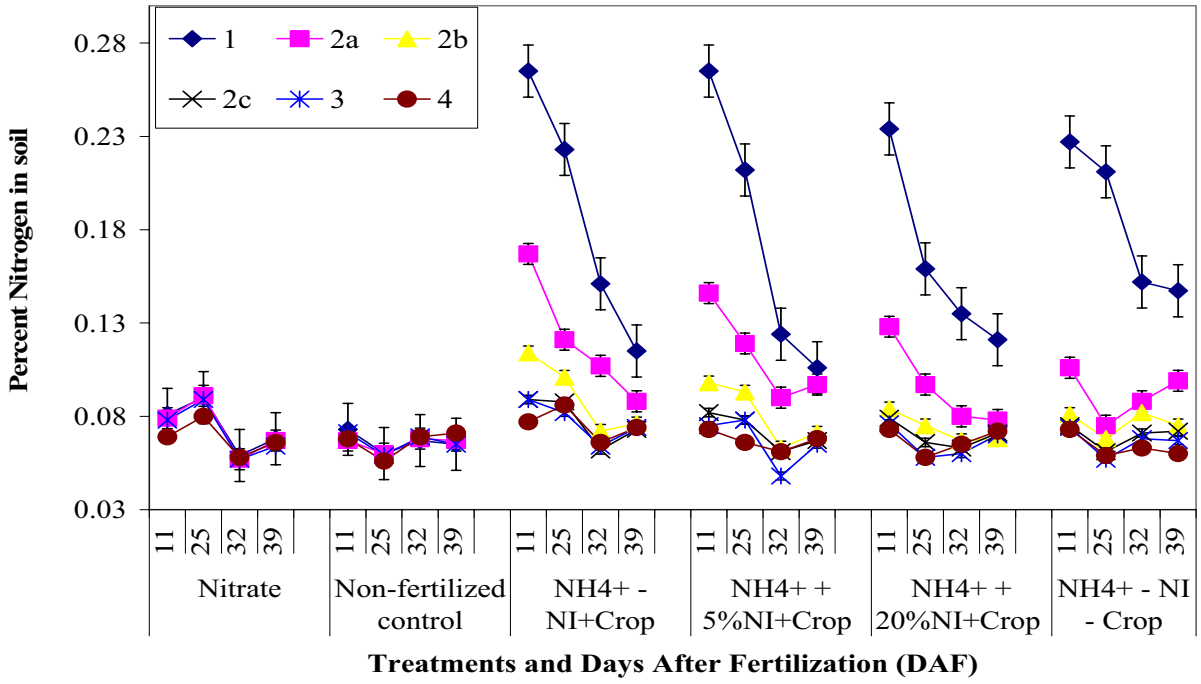

Fig. 4a: **Influence of various nitrogen forms and fertilizer application regimes on total soil nitrogen concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of season one.**

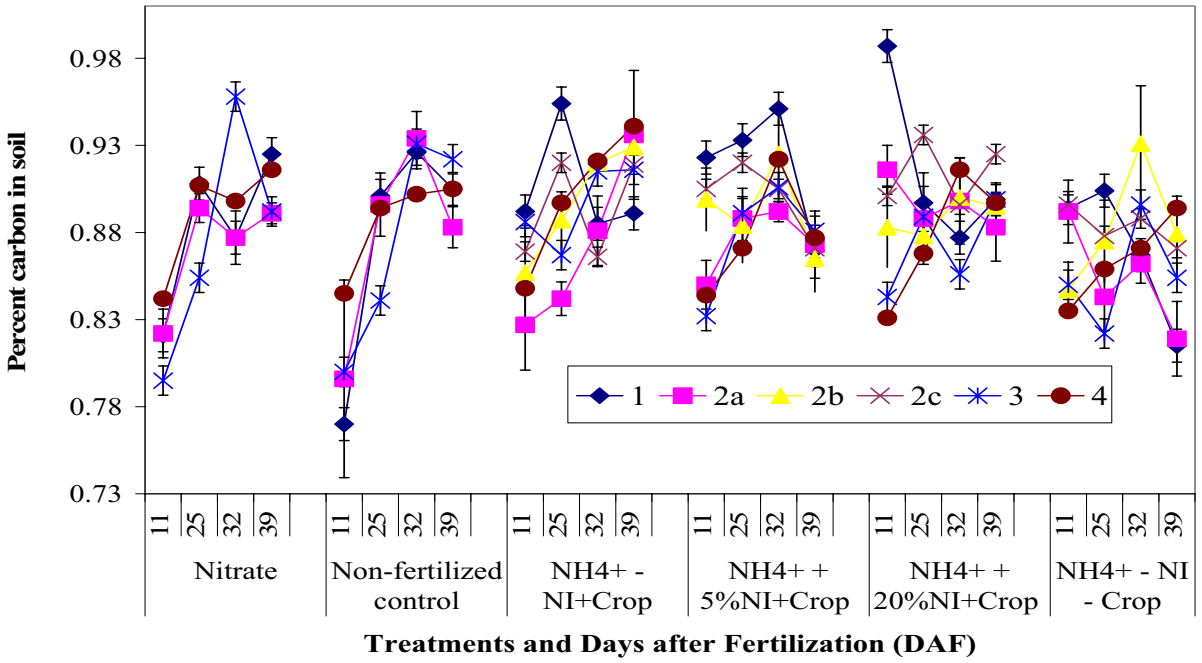

Fig. 4b: **Influence of various nitrogen forms and fertilizer application regimes on total soil carbon concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of season one.**

3.1.2 Concentration of soil-injected NH_4^+-N

To evaluate the stability of soil-injected liquid NH_4^+, its concentration was determined at different intervals during the cropping season. Ammonium was found to be dominant during the early part of the season (11 to 30 DAS). As the season progressed, its dominance rapidly decreased. As expected, no appreciable NH_4^+ concentrations were measured in non-fertilized and nitrate treatments since they were not fertilized by NH_4^+ (Fig. 5a). Among the NH_4^+ treatments, zone 1 had the highest NH_4^+-N concentrations of between 1300 and 1100 mg (Kg DM)$^{-1}$ in season one (Fig. 5a). The rapid decrease rate of NH_4^+-N was observed in season two as well (Fig. 5b). Nitrification inhibitor incorporation did not show any direct effect on the measured NH_4^+-N concentrations of reduction rate. However, its incorporation significantly suppressed the transformation of NH_4^{+-}-N into NO_3^--N. Zone 2a recorded wide variations that ranged from 500 and 100 mg (Kg DM)$^{-1}$ soil in NH_4^+-N concentration as the two seasons progressed, respectively, (Fig. 5a and b). Very low concentrations of NH_4^+-N were measured in zones 3 and 4.

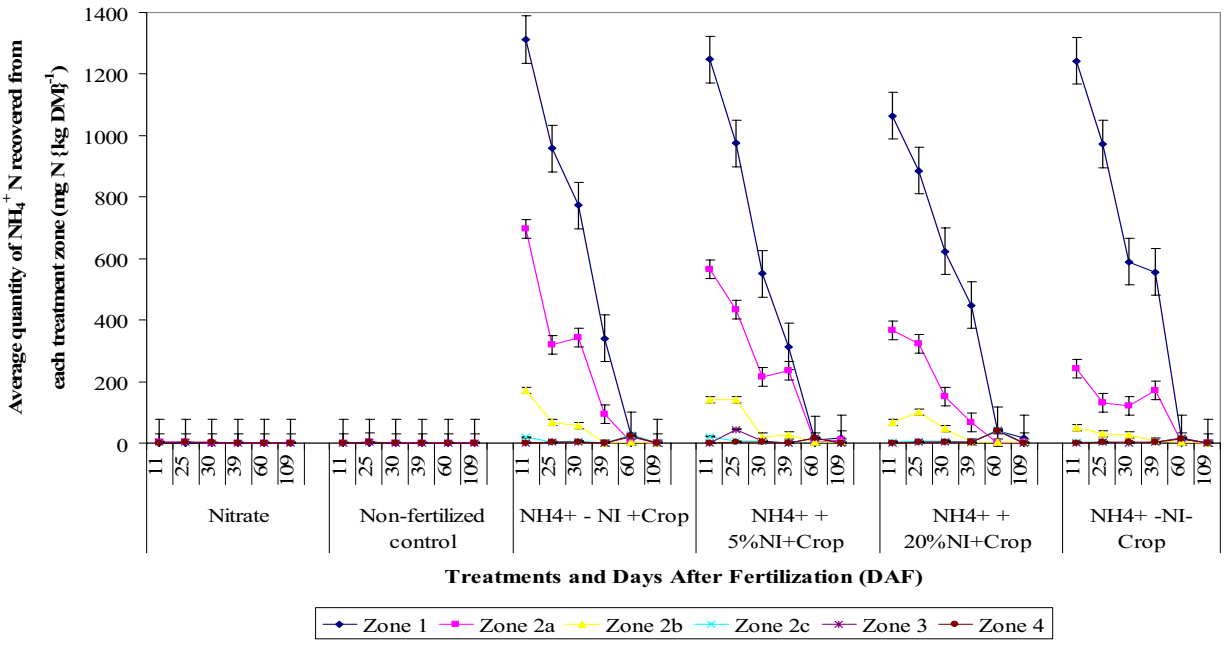

Fig. 5a: Influence of various nitrogen forms and fertilizer application regimes on NH_4^+-N concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of season one.

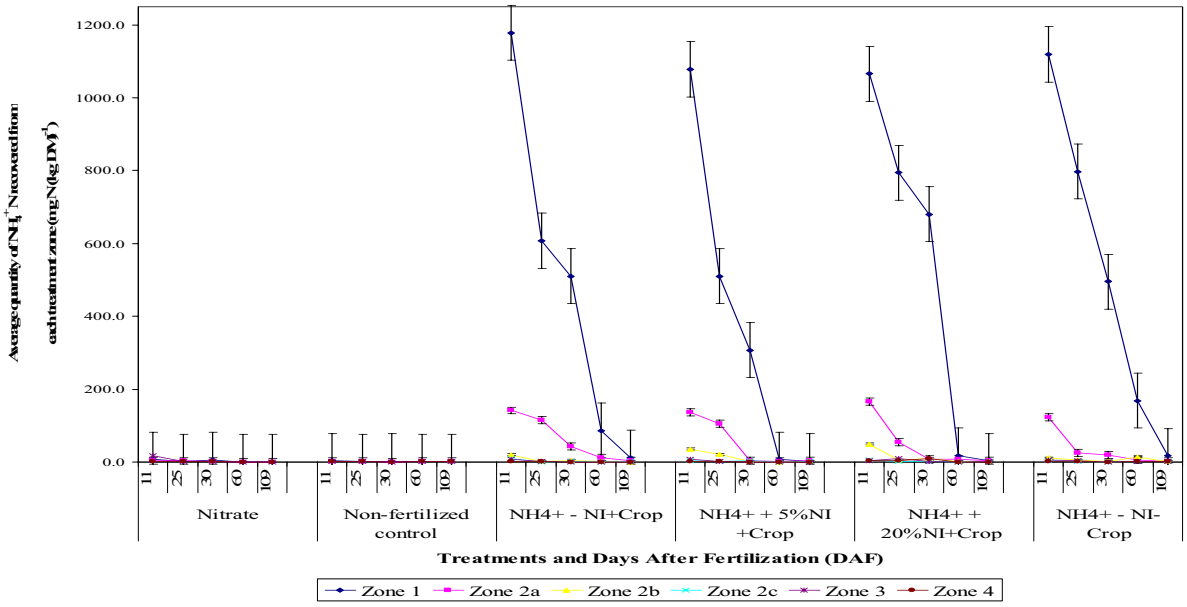

Fig. 5b: Influence of various nitrogen forms and fertilizer application regimes on NH_4^+-N concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of season two.

3.1.3 Concentration of NO_3^--N in the soil

To evaluate the availability of NO_3^--N for crop uptake, its concentrations were monitored in different treatments at varying intervals. Plant nitrogen concentrations and ammonia oxidation caused large variations in the NO_3^--N concentrations measured during the season (Fig. 6a and b). The two NH_4^+-based treatments without NI incorporation had higher concentrations of NO_3^--N, while those incorporated with NI had extremely low concentrations suggesting the occurrence of ammonia oxidation. The incorporation of 5 and 20% NI, Nitrapyrin® successfully suppressed NH_4^+-N transformation into NO_3^--N during the cropping season (Fig. 6a and b). The effectiveness of both low and high NI-incorporation concentrations of 5 and 20%, respectively, demonstrated the successful nitrification inhibition, hence improvement of its stability. Cropped NH_4^+ treatment without NI incorporation recorded lower NO_3^--N concentration peaks in comparison to uncropped NH_4^+ treatment without NI incorporation in zones 1 and 2a. Nitrate concentration peaks in the two NH_4^+ treatments incorporated with NI in season one did not even attain 50 mg N $(Kg\ DM)^{-1}$ (Fig. 6a), while the peaks in the second season

approached 60 mg N (Kg DM)$^{-1}$ (Fig. 6b). In both seasons, the measured NO_3^--N concentrations of NI incorporated treatments showed trends similar to those in non-fertilized control and NO_3^- treatments, albeit with low concentrations. In season one, the highest concentration attained in non-NI incorporated ammonium treatments was 120 and 230 mg N (kg DM)$^{-1}$ in those with and without crops, respectively, whereas the highest NO_3^--N concentration in NO_3^- treatment was 2 to 3 times less than those recorded in non-NI incorporated treatments with and without crops, respectively. NH_4^+ concentrations in zone 1 at 11 DAF of the 20% NI incorporated treatment experienced 17% NH_4^+-N reduction, whereas the 5% NI incorporated NH_4^+ treatment recorded a reduction of 22% at 25 DAF. The uncropped NH_4^+ treatment without NI incorporation recorded more than 50% NH_4^+ concentration reduction between 11 and 30 DAS.

3.1.4 Potential of nitrogen loss in CULTAN-fertilized soils

Recovery of high NO_3^--N concentrations in non NI-incorporated NH_4^+ treatments indicate that CULTAN-fertilized soils experience microbial metabolic transformation too. The presence of high concentrations of NO_3^-, the final product in the nitrification process suggest that CULTAN fertilized soils may as well experience NO_3^--N associated losses. The high NO_3^--N concentrations in CULTAN were mainly in zones 1 and 2a, whereas very low concentrations were found in zones 3 and 4 situated at 15 and 27 cm below the soil surface within the deeper parts of the container. The possible occurrence of NO_3^--N losses through leaching were not directly evaluated, but the finding that only lower NO_3^--N concentrations were measured in the deeper zones discounts the contribution of leaching as a major N loss contributor. Recoveries of NO_3^--N in non-fertilized control treatment even in the absence of any N fertilizer application suggested the occurrence of mineralization (Fig. 6a and b). Non NI-incorporation led to unabated nitrification which culminated into large NO_3^--N accumulation. However, presence of crops dramatically reduced the concentrations. Estimation based on comparison of peaks of NO_3^--N concentration in zones 1 and 2a of cropped and uncropped NH_4^+ treatments without NI-incorporation indicated that about 48 and 60% of NO_3^--N could have been taken by crops. Comparison of the highest peaks attained by cropped and uncropped non NI-incorporated NH_4^+ treatments were 9 and 36-fold higher than concentrations in NI-

incorporated treatments in season one, respectively. The highest concentration obtained in NI-incorporated NH_4^+ treatment was quite low and hardly exceeded 0.3 mg g^{-1} soil. This was lower than 1.23 and 2.16 mg g^{-1} soil recorded in non-NI incorporated treatments with and without crops, respectively, (Fig. 6a). Similar trends, but not of equal concentrations in crop N utilization were also obtained in the second season (Fig. 6b). These results provide first experimental evidence for the occurrence of mixed nitrogen nutrition in CULTAN fertilized soils, which were previously believed to predominantly supply NH_4^+-N to crops.

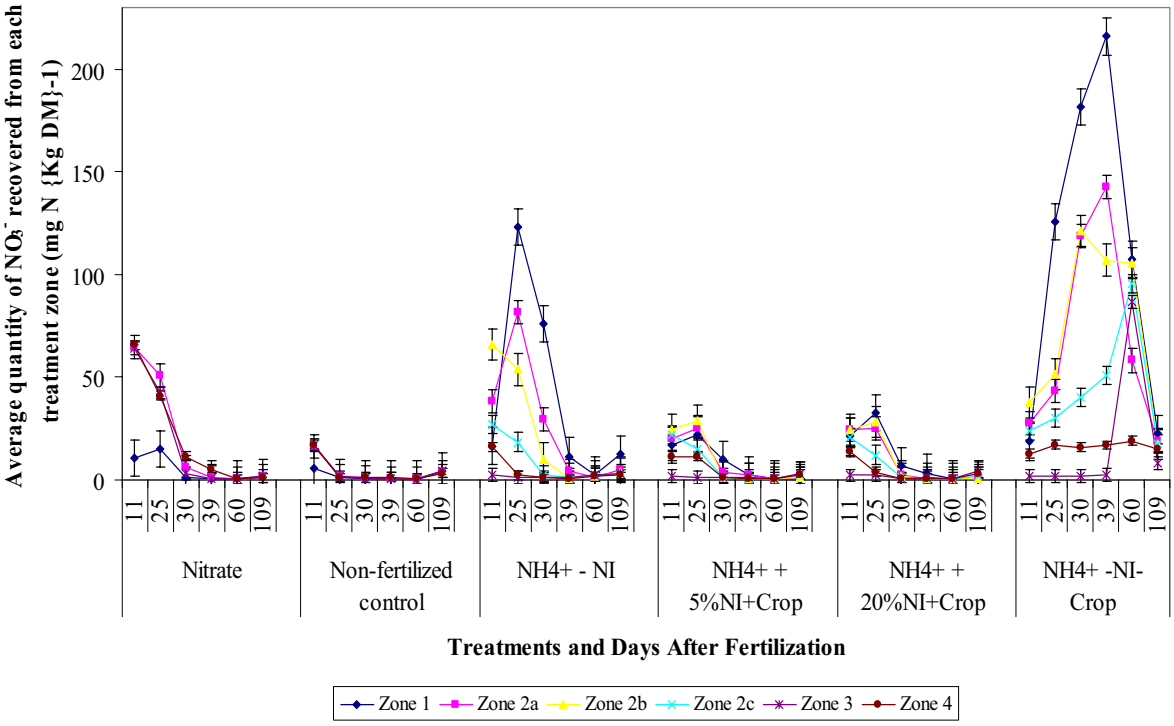

Fig. 6a: Influence of various nitrogen forms and application fertilizer regimes on NO_3^--N concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of season one.

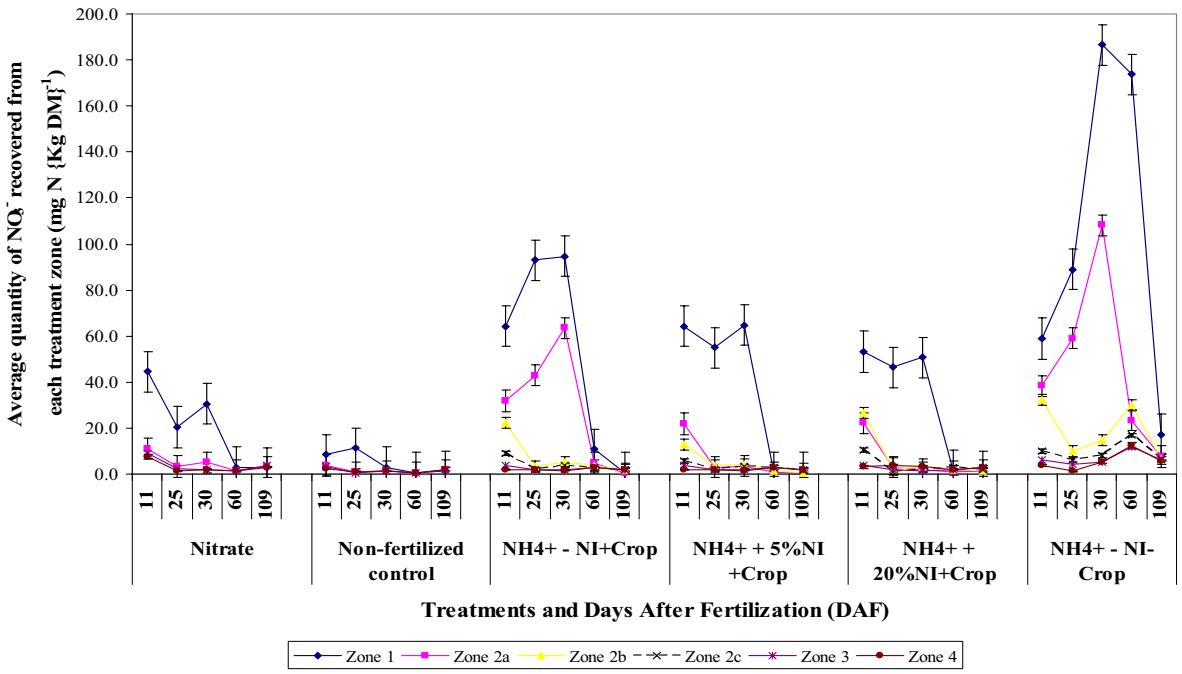

Fig 6b: Influence of various nitrogen forms and application fertilizer regimes on NO_3^--N concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of season two.

3.1.5 Influence of CULTAN fertilization on soil pH

To determine the influence of CULTAN-fertilization on soil pH, soil was sampled at different intervals for analysis. The analysis revealed that liquid NH_4^+-N injection into soil caused pH shifts from the initial near neutral of 7.4 at the start of the season to acidic values when NH_4^+-N dominated which later changed to alkaline values as NO_3^--N concentrations accumulated Fig. 7a and b). This demonstrates that soil pH values fluctuate over a large range during the season. Influence of CULTAN on the pH values was clearly shown by the pH shifts that corresponded closely to NH_4^+-N concentrations (Fig. 5a and b). Generally, soil pH shifted by 1 unit towards acidic values in zones 1 and 2a, which also had high NH_4^+-N concentrations. As NO_3^--N concentrations increased with decreasing NH_4^+-N concentration, the soil pH also shifted towards alkalinity. Nitrate and non-fertilized treatments had relatively constant pH value of about 7.4 in all zones throughout the season. Zones 1 and 2a had a larger pH shift which dominated in both seasons in NH_4^+-N treatments. Zones 1 and 2a of NH_4^+ treatments differed significantly from non-fertilized and nitrate treatments at $P < 0.001$.

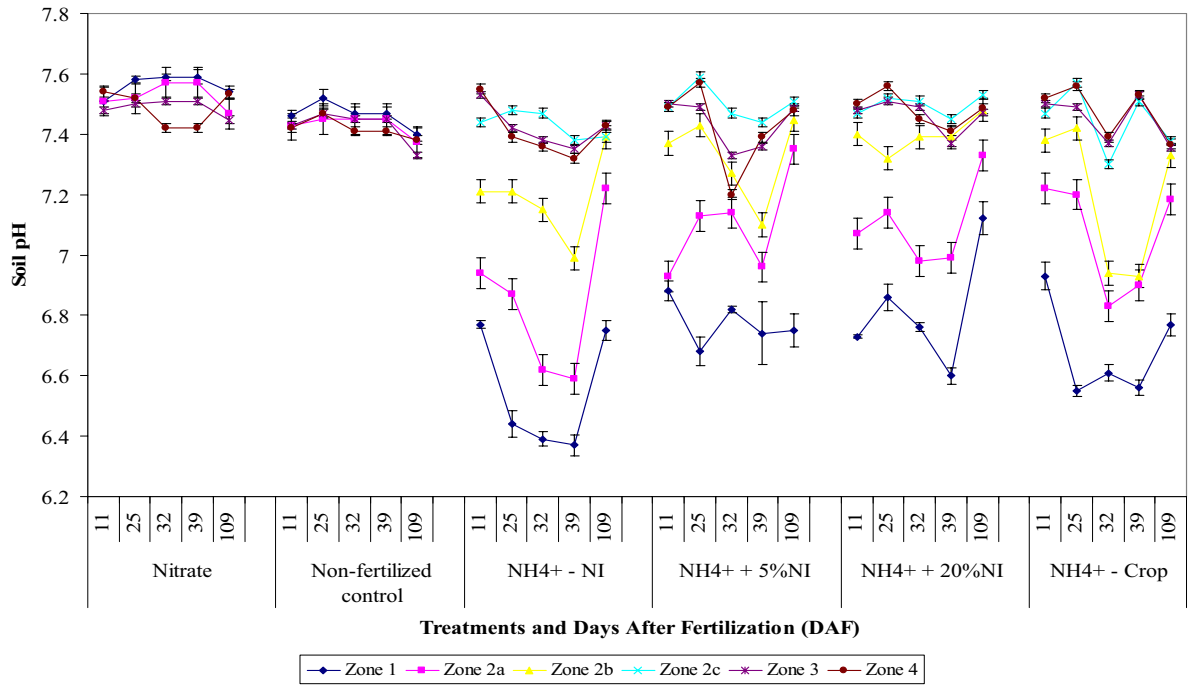

Fig. 7a: Influence of various nitrogen forms and fertilizer application regimes on soil pH values as a function of days after fertilization (DAF). Presented are means ±S.E. of season one.

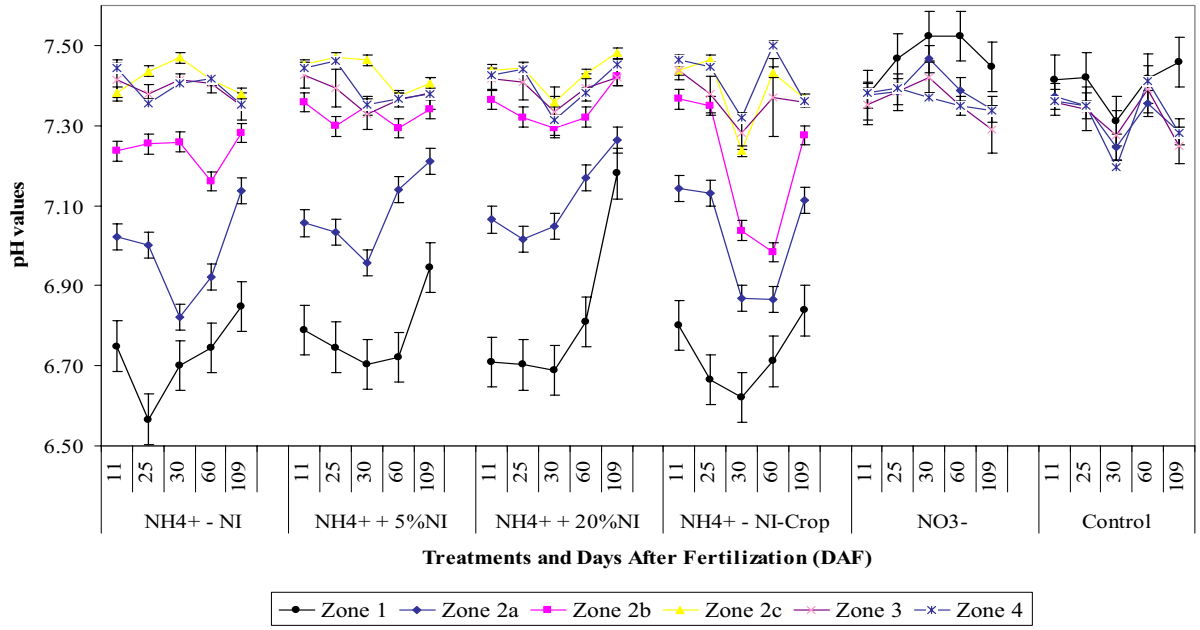

Fig. 7b: Influence of various nitrogen forms and fertilizer application regimes on soil pH values as a function of days after fertilization (DAF). Presented are means ±S.E. of season two.

3.1.6 Phosphorus and potassium availability under CULTAN

The concentration of extractable P and K were determined to assess how CULTAN-fertilization technique influences their availability. It was found that the injection of diammonium phosphate resulted in the occurrence of high concentrations of phosphates within zones 1 and 2a of NH_4^+-based treatments (Fig. 8a). The presence of large P concentrations in zones 1 and 2a closely corresponded to the pH shifts, but contrasted greatly to the gradual reducing trend, which was characteristic of NH_4^+-N (Fig. 5a and b). Though P concentrations reduced, the rate was not as rapid as in NH_4^+-N nutrition. Nitrate and non-fertilized treatments recorded less than 50 mg of P in all zones during the entire season despite the supply of additional P to balance out DAP supplied phosphorus. P remained localized within the injection zones due to its poor mobility within the soil (Fig. 8a). Zones 1 and 2a were significantly different to zones 3 and 4 along the vertical section as well as 2b at $P < 0.0012$. Ammonium treatments were statistically different from nitrate and non-fertilized control at $P < 0.001$. At end of the season, P concentrations were not yet exhausted.

The effect of CULTAN-fertilization on K was also determined. There were no large variations between the treatment zones (Fig. 8b). However, zone 1 K concentrations in all the NH_4^+ treatments except the uncropped without NI-incorporation that differed from the other three by retaining large concentrations. Fluctuations experienced by the three had trends closely resembling soil pH value shifts. Nitrate and non-fertilized control treatments witnessed reductions too, but the zone differences were not very large as compared to the three NH_4^+ treatments mentioned above (Fig. 8b). There were no wide disparities among the zones of different treatments as the season progressed. Its distribution was well among all zones with zone 1 recording slightly higher concentrations as compared to zones 3 and 4 located much deeper in the sowing container. This is in contrast to the trend observed among other macro-nutrients. However, all zones witnessed a reduction over the season. Nitrate fertilized treatment recorded more or less equal K concentrations in the different zones despite experiencing a rapid reduction. Cropped NH_4^+ treatments recorded large spatial and temporal differences, and the concentrations were higher in NH_4^+ predominated zones (Fig. 8b).

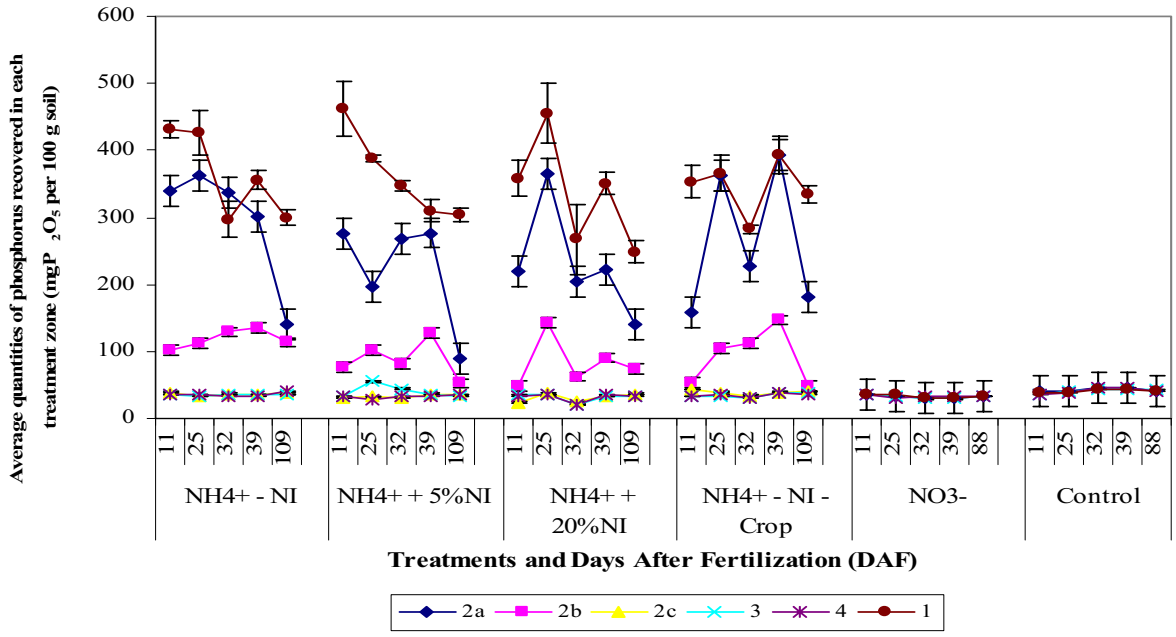

Fig. 8a: Influence of various nitrogen forms and fertilizer application regimes on phosphorus concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of season two.

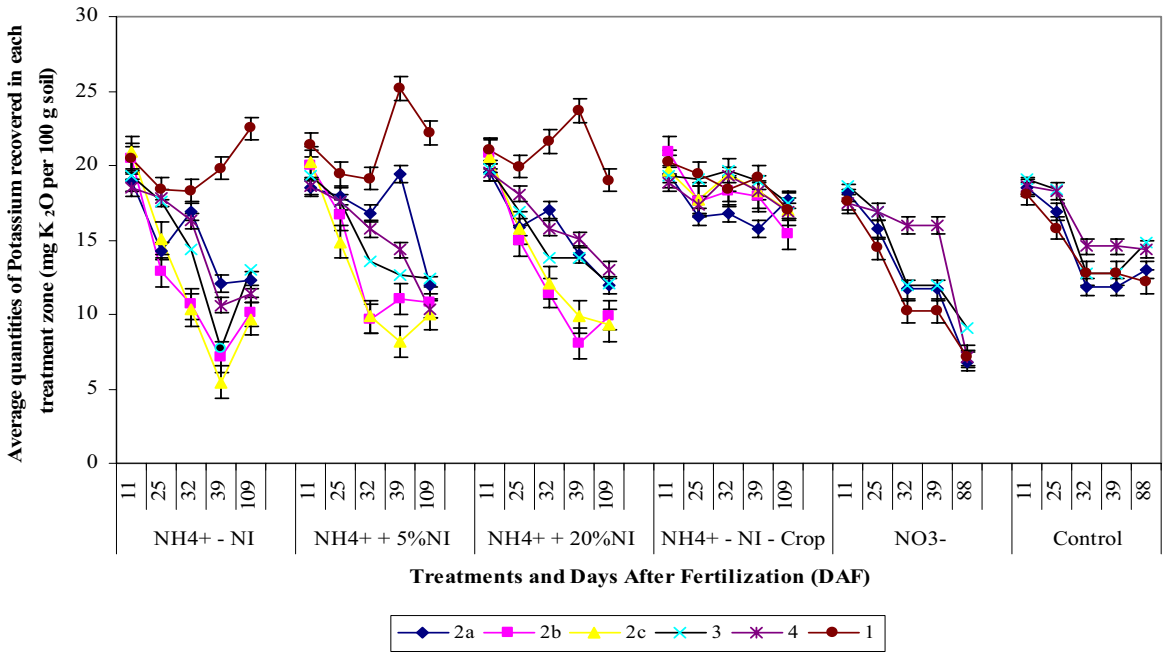

Fig. 8b: Influence of various nitrogen forms and fertilizer application regimes on potassium concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of season one.

3.1.7. Crop growth and yield responses to CULTAN-fertilization

To evaluate growth and yield responses of cereals to CULTAN fertilization, barley growth was monitored as the crop grew from seedling stage until physiological maturity. Barley seedlings successfully germinated near NH_4^+-N injection-points. However, they exhibited mild toxicity symptoms such as yellowish and deformed leaves, but they quickly regenerated and formed new leaves and resumed normal growth. No observable NH_4^+ toxicity symptoms were discernible on the aerial parts of CULTAN-fertilized crops at later stages (Fig. 9a). Besides aerial morphological responses, NH_4^+-fertilized crops developed intensively branched root architectural network. However, the roots proliferated around the injection-point, but did not penetrate through the injection-point (Fig. 9b). In contrast, nitrate and non-fertilized crops did not develop any intensive root network as witnessed in NH_4^+-injected treatments. Nitrate and non-fertilized control instead supported long, sparsely distributed roots (Fig. 9c). NH_4^+ injection-points were dominated by high NH_4^+-N concentrations, which in turn inhibited root growth due to NH_4^+ toxicity. Intensive root proliferation was clearly observable from 30 DAS.

Fig. 9a: Aerial morphological growth responses to CULTAN fertilized barley

Fig. 9a: Photograph showing late morphological growth responses of barley fertilized with various nitrogen treatments indicated by T1 – Nitrate, T2 – non NI-incorporated NH_4^+, T3 - NH_4^+ with 5%NI, T4 - NH_4^+ with 20%NI, T5 - non-cropped NH_4^+ without NI and Non-fertilized control.

Fig. 9b: Superior root growth under soil injected NH_4^+

Fig. 9c: Inferior root development under NO_3^-

Fig. 9 b-c: Photographs showing root growth architecture of barley fertilized through CULTAN which supported an intensively branched root architecture around NH_4^+ injection-points (b), and nitrate fertilized crops which had sparse root distribution (c).

3.1.7.1 Biomass accumulation and relative growth rates (RGRs)

To monitor the effect of CULTAN-fertilization on barley crop biomass accumulation, the actual biomass accumulations were determined. Besides this, the relative growth rates were calculated. The results show that both fertilized and non-fertilized crops steadily gained aerial biomasses as the season progressed (Fig. 10a). The fertilized crop aerial biomasses ranged between 150 to 200 and 170 to 230 g in seasons one and two, respectively. Non-fertilized control treatment had the least aerial biomass while NO_3^--fertilized crops recorded the highest, however, the biomass attained by NO_3^--fertilized crops was not significantly different from NH_4^+-fertilized crops. In both seasons, nitrate supported superior aerial biomass yields. Non-fertilized control treatment yielded 80 and 90 g in the first and second seasons, respectively (Fig. 10a). The percent shoot biomass yield gains at maturity (109 DAS) among fertilized in comparison to non-fertilized crops in the first season were 69, 50, 44 and 50% for nitrate, NH_4^+ treatment without-NI, NH_4^+ incorporated with 5 and 20% NI, respectively. Second season was not any different from the first season. No statistical differences were observed among the fertilized nitrogen treatments (Fig. 10a).

The crops in the two seasons had growth durations of 109 days. The relative growth rates (RGRs) showed two distinct exponential phases (Fig. 10b). Initially, the RGRs increased and attained optimal peak of 3 g d^{-1} at 30 DAS in season one and 4 g d^{-1} at 39 DAS in season two (Fig. 10b). The second exponential growth phase occurred at 60 and 66 DAS to attain 9 - 15 and 13 - 16 g d^{-1} in seasons one and two, respectively (Fig. 10b). The initial exponential phase coincided with tillering and booting between 25 and 30 DAS, whereas second phase was at grain formation from 60 DAS. Non-fertilized crop tiller numbers initially increased and remained constant from 30 and 39 DAS in seasons one and two, respectively (Fig. 10c). Wide variations in tiller numbers were observed between the two seasons. Season one had 10 tillers at 30 DAS while the second season recorded 40 tillers at 25 DAS. Non-fertilized crops supported equal number of surviving tillers at 80 DAS in both seasons (Fig. 10c).

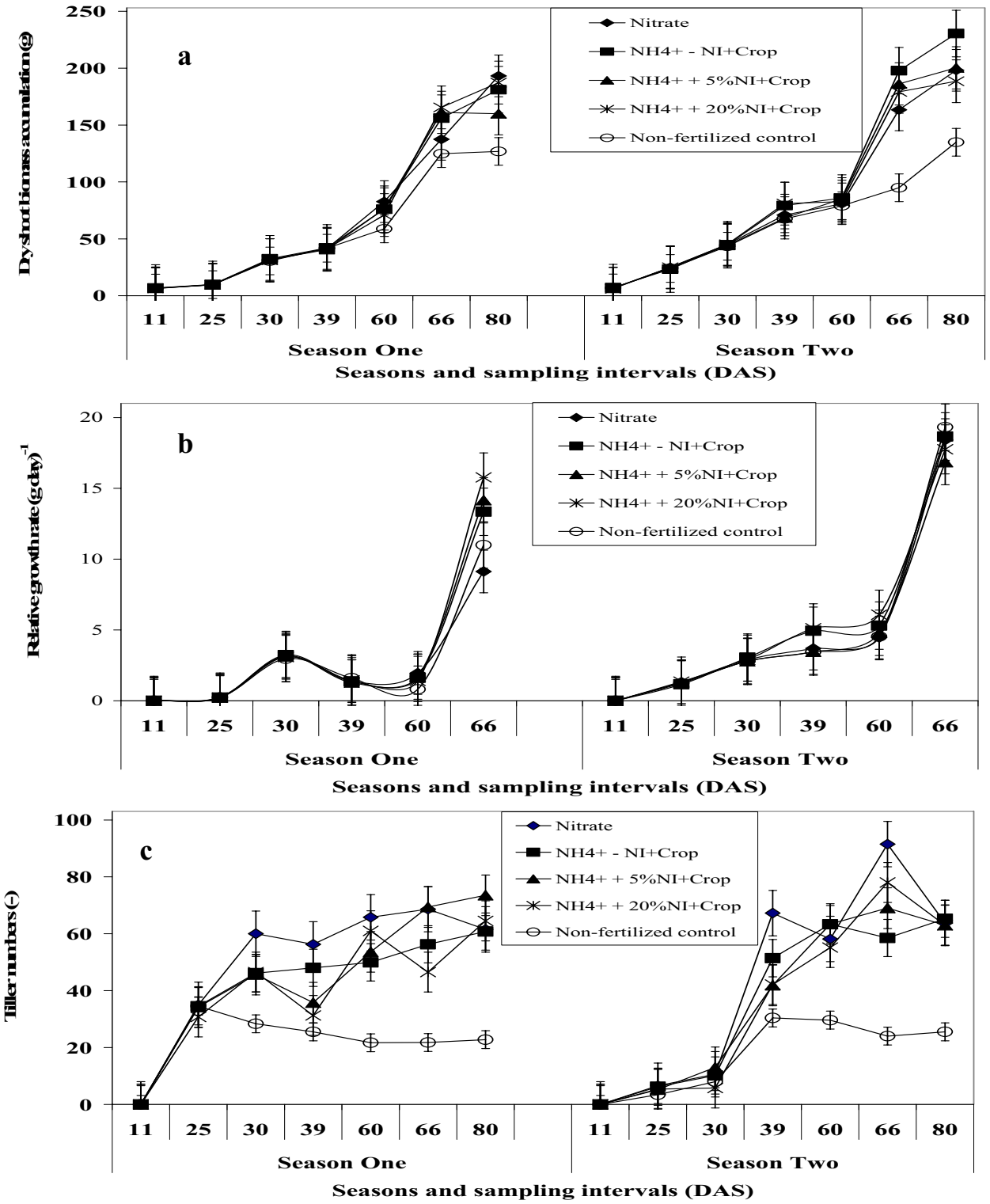

Fig. 10a –c: Influence of various nitrogen forms and fertilizer application regimes on shoot biomass accumulation (a), relative growth rates (RGRs) (b), and tiller numbers (c) as a function of days after fertilization (DAF). Presented are means ± S.E. of seasons one and two.

3.1.7.2 Barley grain yield and yield-forming factors

Grain yields of CULTAN-fertilized crops were compared with those fertilized through the conventional NO_3^--fertilization. The results indicate that CULTAN fertilized crops had comparable yields to those fertilized by NO_3^-, but yielded much higher grains than non-fertilized control. The three NH_4^+-fertilized treatments supported more or less similar grain yields ranging between 43 and 45 g in season one between 46 and 63 g in season two (Fig. 11a). The grain yields of fertilized crop attained in season one were not statistically different from each other. In season two, NO_3^- and NH_4^+ treatments without NI-incorporation yielded slightly higher grain weights of 62 and 63 g, respectively (Fig. 11a). However, the yields of fertilized treatments were not statistically different from each other. However, they were statistically different from the non-fertilized crops at $P < 0.001$ (Fig. 11a). The percent yield gains due to nitrate fertilizer application was 32 and 104% in comparison to non-fertilized control in seasons one and two, respectively. Ammonium treatment without NI-incorporation gained 34 and 108% in seasons one and two, respectively, while NH_4^+ treatments incorporated with 5 and 20% NI gained 25% in both treatments in season one and 53 and 59% in the second season (Fig. 11a).

The number of grains of fertilized treatments though was numerically different, although they were not statistically different (Fig. 11b). The grain numbers were between 1102 and 1298 in season one and 1094 and 1327 grains in the second season (Fig. 11b). Fertilized crops were significantly different from non-fertilized at $P < 0.003$. Yield-forming factors such as tillers also showed a trend similar to that observed for grain yields. Tiller numbers of fertilized crops ranged between 58 and 65 in season one. The same trend was reflected on the number of fertile ears that also ranged between 53 and 55 per treatment. Though the tiller numbers in fertilized crops were not statistically different from each other, they however, differed significantly from those of non-fertilized control at $P < 0.001$ (Fig. 11c). Fertile ears of NO_3^- and NH_4^+-fertilized crops were also statistically different from those of non-fertilized crops (Fig. 11d). The average number of tillers per plant at maturity was 3 among non-fertilized crops and 4 among the fertilized crops (Fig. 11c). NO_3^- and NH_4^+ treatment without NI-incorporation had 24 grains per ear in season two. Both 5 and 20% NI-incorporated treatments supported 23

while non-fertilized crops had 20. Thousand grain weights ranged from 39 to 44 g and 41 to 46 in seasons one and two, respectively.

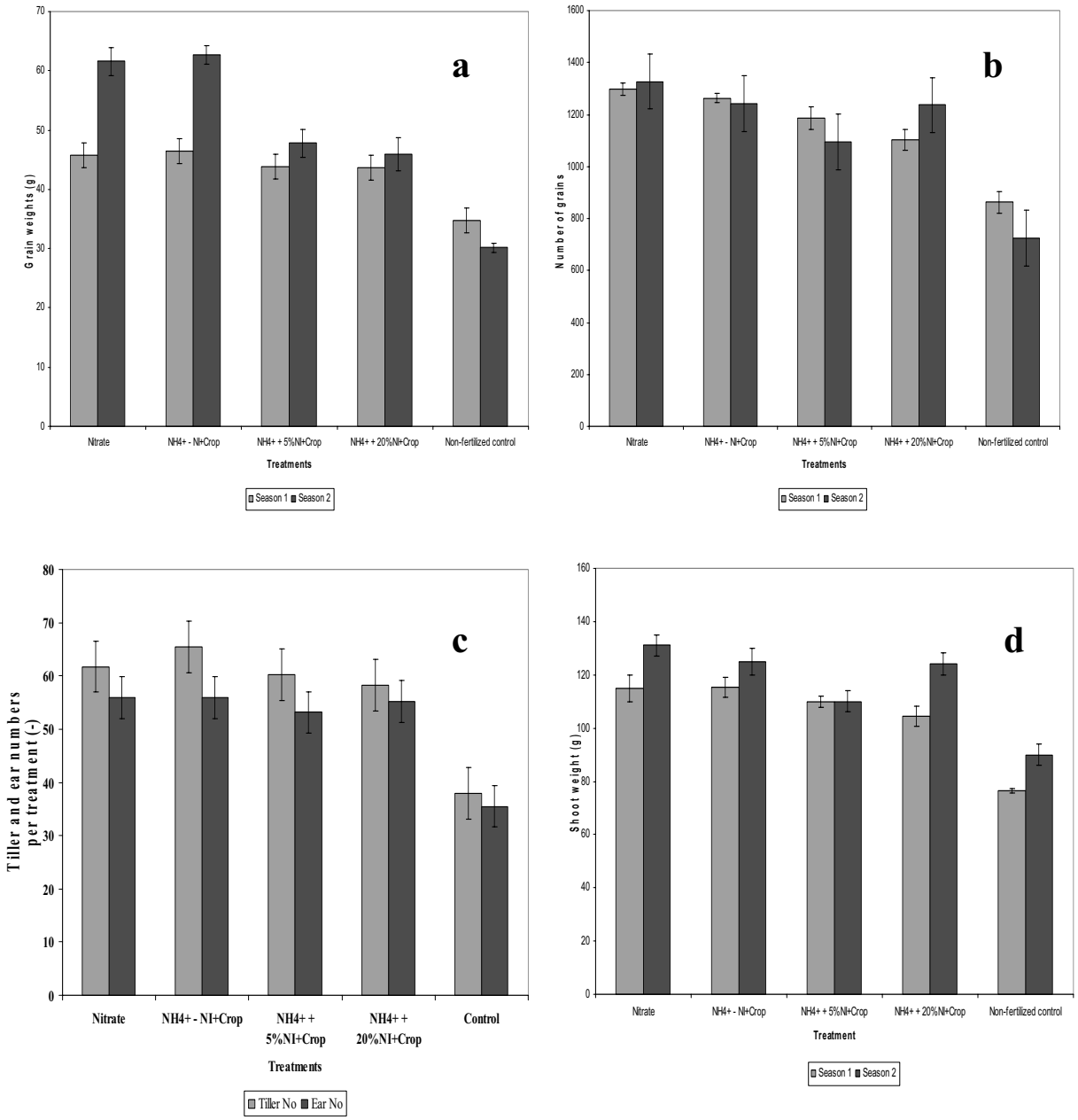

Fig. 11a – d: Influence of various nitrogen forms and fertilizer application regimes on grain yield weight (a), grain numbers (b) for seasons one and two. Tiller and ear numbers at crop maturity in season one (c) and aerial shoot biomass at crop maturity in seasons one and two (d) respectively, as a function of days after fertilization (DAF). Presented are means ± S.E. of seasons one and two.

3.1.8 Chemical composition of crops fertilized with different N forms

3.1.8.1 Crop nitrogen uptake and partitioning

Crop samples were analysed to assess nutrient concentrations within the tissues at different growth stages. Early growth stages accumulated high concentrations of different nutrients, which gradually reduced as the crop grew to attain maturity (Fig. 12a). Total nitrogen concentration of non-fertilized crop was lower compared to the fertilized crops in both seasons. Even though NO_3^--fertilized crop had slightly higher total nitrogen concentrations than NH_4^+-treated crops during the early growth stages, the concentrations at the late growth stages were not statistically different. While aerial biomasses increased with crop age, nitrogen concentration expressed a reverse trend. Total nitrogen concentration of fertilized crops decreased from 4.6 to 1.1% in season one between 30 and 60 DAS, which coincided with the end of tillering and the start of booting stage.

Similarly, season two had reductions from 5.9 to 3% (Fig. 12a). Both NO_3^- and NH_4^+ without NI-incorporation had equal NO_3^- concentrations of 11 mg NO_3^- g^{-1} DM, while 5 and 20% NI-incorporated treatments had 10 and 11 mg NO_3^- g^{-1} DM (Fig. 12b). Among the five treatments, NO_3^- concentration rapidly decreased to less than 1 at 66 DAS. The measurement of comparable NO_3^- concentration among CULTAN-fertilized crops in both seasons suggested that they could have taken up mixed nitrogen nutrition (Fig. 12b). Total N concentration in grains was higher in NH_4^+ without NI-incorporation which had (1.5% in DM) along side the two NH_4^+ treatments incorporated with 5 and 20% NI with 1.7% per DM than non-fertilized control which attained 1.2% in season one (Table 2). Nitrogen use efficiencies in season one grains ranged between 18 and 36% where $NH4^+$ without NI had the highest use efficiency. In season two, both 5 and 20% NI incorporated treatments had the highest NUE of 42% while nitrate recorded 16% (Table 2).

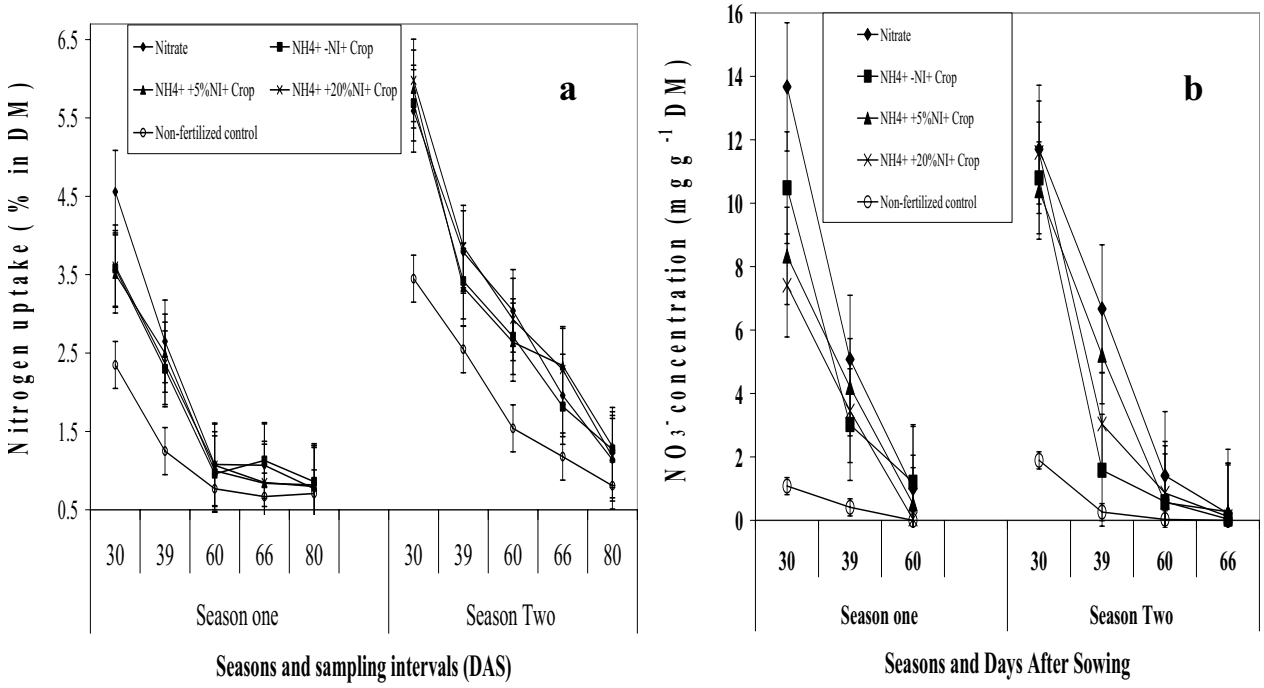

Fig. 12a-b: Influence of various nitrogen forms and fertilizer application regimes on total nitrogen concentrations (a) and nitrate concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of seasons one and two.

Table 2: Influence of various nitrogen forms and fertilizer application regimes on grain total N concentration at maturity. Presented are means ±S.E. of seasons one and two.

Treatments	Season one (% in DM)	Season two (% in DM)
Nitrate	1.399 ± 0.0952 a	1.608 ± 0.0188 ab
NH_4^+ - NI + Crop	1.483 ± 0.0390 a	1.540 ± 0.0146 b
NH_4^+ +5% NI + Crop	1.315 ± 0.0213 ab	1.740 ± 0.0468 a
NH_4^+ +20% NI + Crop	1.275 ± 0.0366 ab	1.651 ± 0.0351 a
Non-fertilized control	1.078 ± 0.0098 b	1.187 ± 0.0233 c
Pr > F	0.0021	0.0001

Means followed by same letters within similar columns are not significantly different from each other at ($p < 0.05$) Tukey test.

3.1.8.2 Shoot mineral concentrations

Different N forms influenced tissue ion concentrations. In general, cations dominated tissues of NO_3^--fertilized crops (Fig. 13f), whereas anion concentrations were higher in crops fertilized by NH_4^+ incorporated with nitrification inhibitor (Fig. 13a). The uptake of K^+, Ca^{2+} and Mg^{2+} in both seasons was higher in younger crops, but reduced as the crop biomass increased. Though no statistically different K^+ concentrations occurred among the fertilized crops, the non-fertilized crops recorded significantly lower concentrations in both seasons (Fig. 13a). Nitrate fertilized crops recorded higher Ca^{2+} and Mg^{2+} concentrations in comparison to other treatments during the entire growth duration despite decreases associated with biomass increases. Non-fertilized crops showed lower concentrations in comparison to NH_4^+-fertilized crops (Fig. 13b and c). On the contrary, non-fertilized crops had the highest P concentration while NO_3^--fertilized crops recorded the lowest concentrations (Fig. 13d). Sulphate did not show any clear trend, though NH_4^+ treatments had slightly higher concentrations between 30 and 60 DAS, and were both significantly different from the concentrations of non-fertilized control at $P < 0.0001$ (Fig. 13e).

Ammonium treatment incorporated with 20% NI recorded the highest chloride concentrations, while both NO_3^- and non-fertilized crops had the least in season one (Fig. 13f). Generally, second season crops took up lower quantities of chloride in comparison to the first, nevertheless, the proportions recorded in nitrate fertilized crops remained distinctively low (Fig. 13f). Total ash analysis indicated the presence of high ion concentrations in the early crop growth stages than the later stages (Fig. 14). Nitrate fertilized crops had higher concentrations of total ash while non-fertilized crop had the lowest total ash concentrations during the growth period between 30 and 60 DAS. However, the two treatments switched positions between 60 and 80 DAS (Fig. 14). NH_4^+-based treatments occupied intermediate positions between NO_3^- and non-fertilized crops. Though seasons one and two varied in total ash concentrations, both exhibited similar trends. Both seasons attained ranges between 16 to 19% of total ash concentration at 30 DAS (Fig. 14).

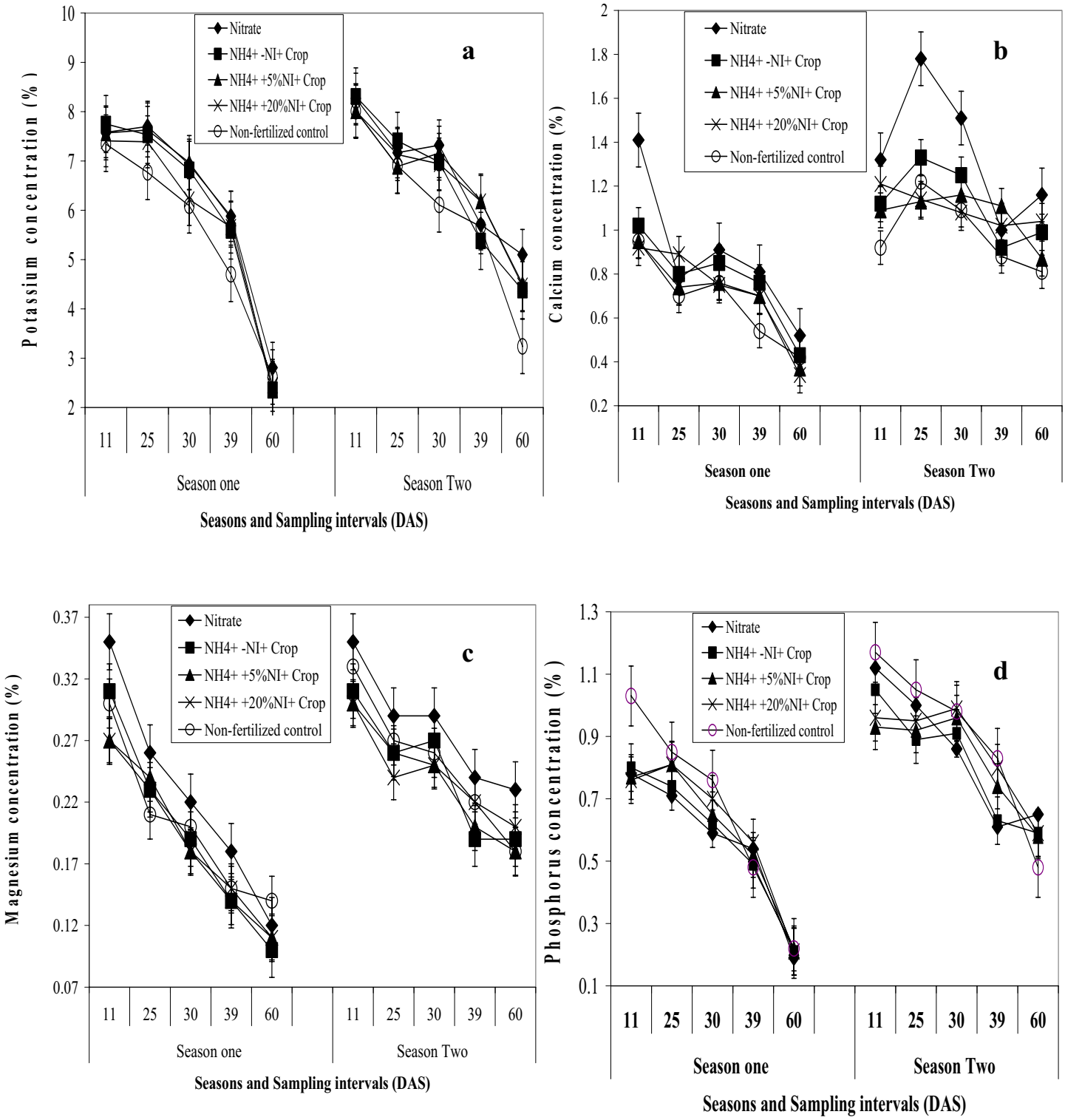

Fig. 13a-d: Influence of various nitrogen forms and fertilizer application regimes on Potassium (a), Calcium (b) Magnesium (c) and phosphorus concentrations of whole plants as a function of days after fertilization (DAF). Presented are means ±S.E. of seasons one and two.

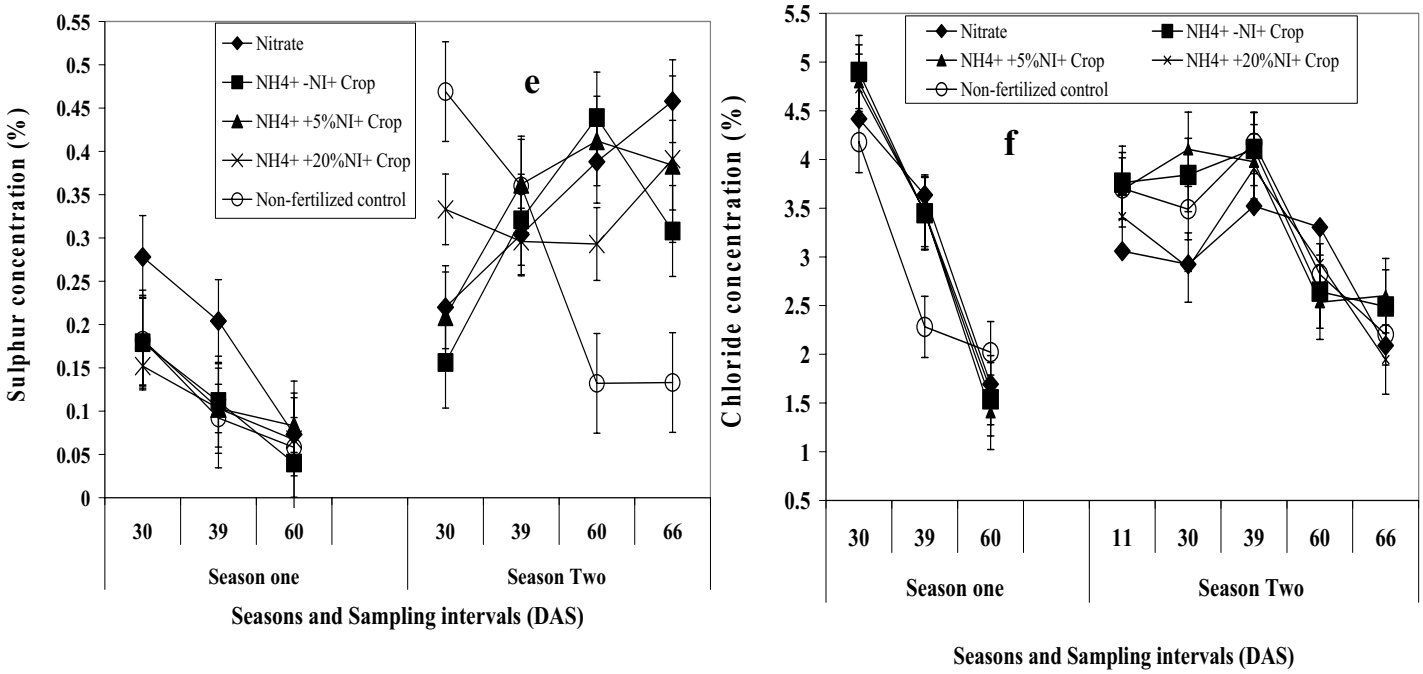

Fig. 13e-f: Influence of various nitrogen forms and fertilizer application regimes on sulphur (e) and chloride (f) concentrations of whole plants as a function of days after fertilization (DAF). Presented are means ±S.E. of seasons one and two.

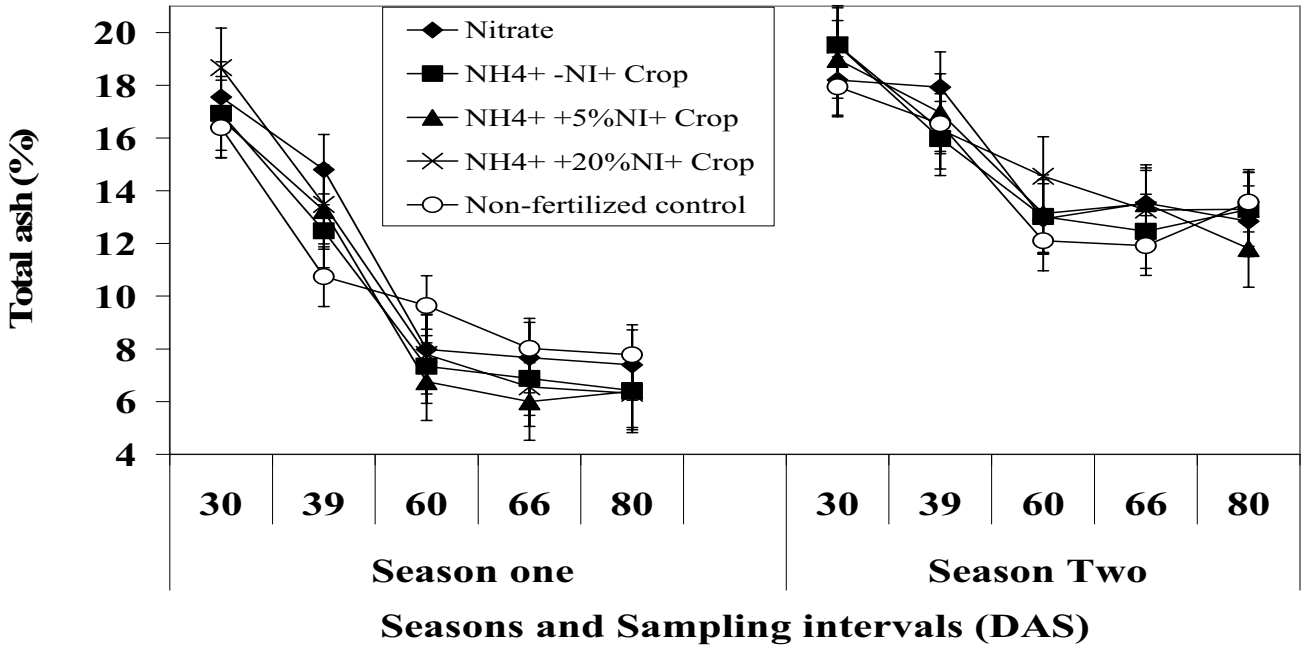

Fig. 14: Influence of various nitrogen forms and fertilizer application regimes on total ash of whole plant tissues as a function of days after fertilization (DAF). Presented are means ±S.E. of seasons one and two.

3.1.8.3 Leaf chlorophyll and carotenoid concentrations

The three pigment concentrations differed considerably between the two seasons. While season one crops had peak concentrations at 25 DAS after which, the concentrations drastically reduced (Fig. 15a-c). Season two crops had low concentrations at the initial sampling interval (25 DAS), which slightly increased at 30 DAS to attain the maximum peak (Fig. 15a-c). After 30 DAF, concentrations of the three pigments rapidly reduced as the season progressed. The sum of chlorophyll a and b concentrations expressed a trend similar to chlorophyll a alone (Fig. 15 a and b). Both nitrate and NH_4^+ treatment without NI-incorporation attained higher peaks of total chlorophylls a and b as well as in each individual pigment of season two grown crops at 30 DAF (Fig. 15a-d). Non-fertilized control hand the lowest concentration of each of the pigments. The concentration of chlorophyll a was highest followed by b while carotenoid concentration remained low. Nitrate and NH_4^+ treatment without NI-incorporation higher initial concentration of carotenoids than the rest of the treatments. The two NH_4^+ treatments incorporated with NI had low carotenoid concentrations, however, their concentrations were slightly higher than those measured in non-fertilize control (Fig. 15d). Concentrations of chlorophylls a and b differed significantly from each other among leaves of the same treatment at $P < 0.002$ in season one and $P < 0.001$ in season two (Fig. 15b and c). Rapid decreases of chlorophyll a and b concentrations resulted into lower chlorophyll a:b ratios which ranged between 2 and 3. The higher ratio of 3 occurred early in the season and it reduced to 2 towards the end of the season as the crops matured and the leaves senescenced.

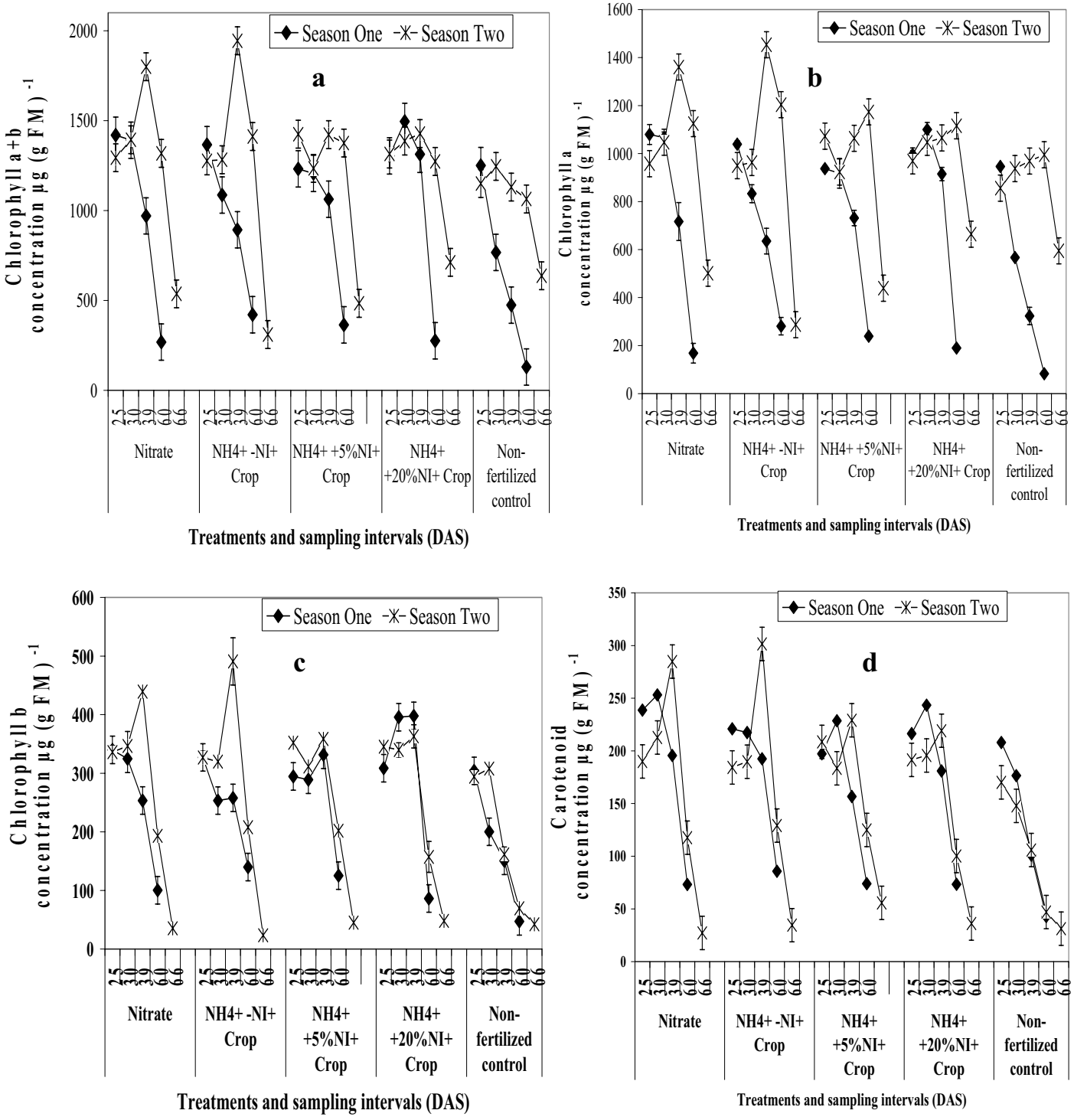

Fig. 15a-d: Influence of various nitrogen forms and fertilizer application regimes on chlorophyll a+b (a), a (b) b (c) and carotenoid (d) concentrations of leaves as a function of days after fertilization (DAF). Presented are means ±S.E. of seasons one and two.

3.1.8.4 Sugar concentrations

Irrespective of the nitrogen treatment, the crops accumulated appreciable concentrations of glucose, sucrose and fructose. Of the three sugars, glucose concentration was slightly higher in NO_3^--fertilized crop followed by fructose while sucrose had the lowest concentration among season one grown crops. In NH_4^+-fertilized crops, glucose was more abundant, while fructose and sucrose switched positions (Fig. 16a). Non-fertilized crops had low sugar concentrations in spite of their similar trends to those in NH_4^+ fertilized crops. Among all treatments, glucose decreased by about two- to five-fold between 39 and 60 DAS in NO_3^- and NH_4^+ fertilized crops, respectively. The highest glucose concentration was 1.4 while 0.2 mg (g DM)$^{-1}$ was the lowest among season one grown-crops. The lowest attainable glucose concentration was much lower than those of sucrose and fructose at corresponding crop growth stages of 60 and 66 DAS. The early crop growth stages (30 DAS) recorded highly variable sugar concentrations whereas in during growth period between 39 and 60 DAS, the concentrations of the three sugars were equal.

The totals of the three different sugars closely resembled individual trend of glucose (Fig. 16a). Contrary to observations made in the first season, the second season crops underwent initial sugar concentration increase from 0.3 mg (g DM)$^{-1}$ at 25 DAS to attain a peak of 1 mg (g DM)$^{-1}$ at 30 DAS regardless of N form fertilizer applied. Upon attaining the peaks, the sugar concentrations drastically dropped to negligible levels, except in NO_3^--fertilized crop whose concentrations were low though detectable (Fig. 16b). The ascending order of the crop sugar concentrations of season two crops was sucrose, fructose and glucose. Similarly, totals of the three sugar concentration trends are similar to that observed for glucose (Fig. 16b). Although significant differences occurred in terms of sugar concentrations of different sugar types within each treatment during the early growth stages at $P < 0.001$, no similar observations were made when comparisons among the different treatments were made.

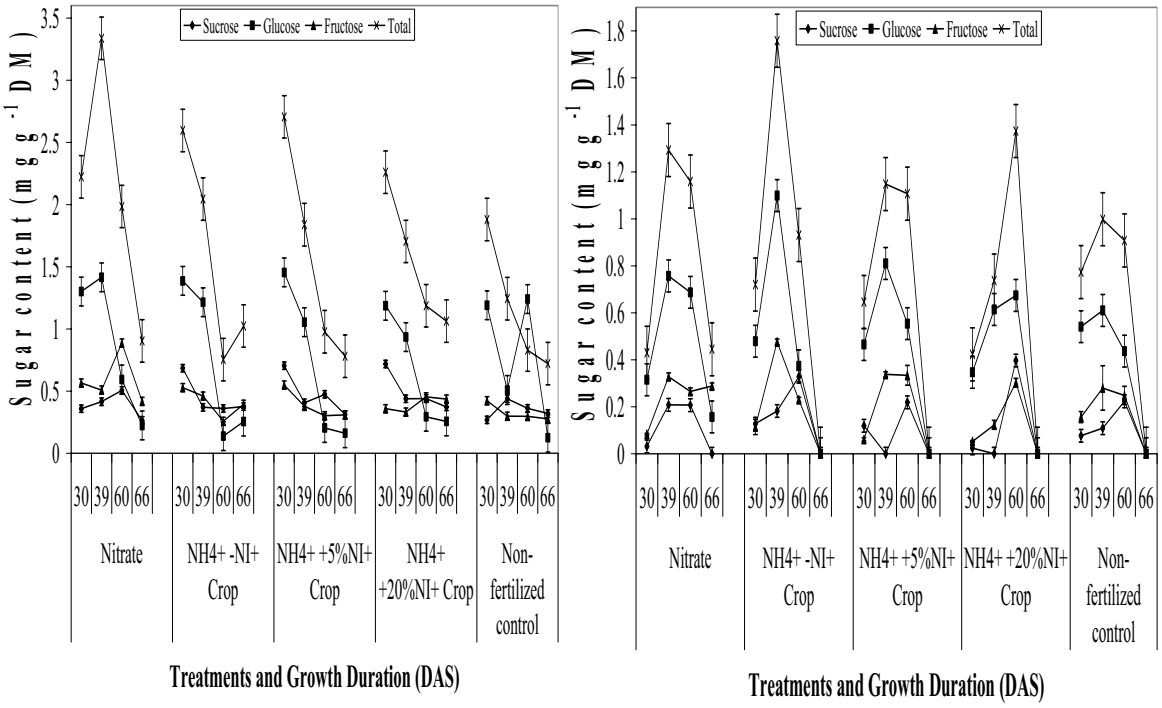

Fig. 16a-b: Influence of various nitrogen forms and fertilizer application regimes on barley sugar concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of seasons one and two.

3.1.8.5 Organic acid concentrations

Malic acid was the most abundant organic acid followed by citric while succinic acid had lowest concentration in both seasons (Fig. 17a). Between 25 and 60 DAS growth period, malic acid concentrations rose rapidly in all the treatments regardless of the N form applied. Citric acid concentration slightly decreased between 30 and 39 DAS, then increased once again as the crop approached 60 DAS in season one (Fig. 17a). On the other hand, season two crops expressed a rapid and steady increase in organic acid concentrations between 30 and 60 DAS (Fig. 17b). Malic was the most abundant organic acid, while very low succinic quantities accumulated in both seasons. Citric acid was intermediate and its concentration fluctuated was between 1.5 and 4 ml (g FM)$^{-1}$ in both seasons in all treatments. Nitrate fertilized crops along side NH_4^+ treatment without NI-incorporation had high concentrations of organic acid pools in both seasons, while non-fertilized control and NH_4^+ treatment incorporated with 20% NI had the lowest concentrations in both seasons (Fig. 17a and b). Moreover, crops fertilized with nitrate

together with those fertilized by NH_4^+, but without NI-incorporation had the lowest ratios of total sugars to organic acids, while the non-fertilized control and NH_4^+ incorporated with 20% NI had slightly higher ratios.

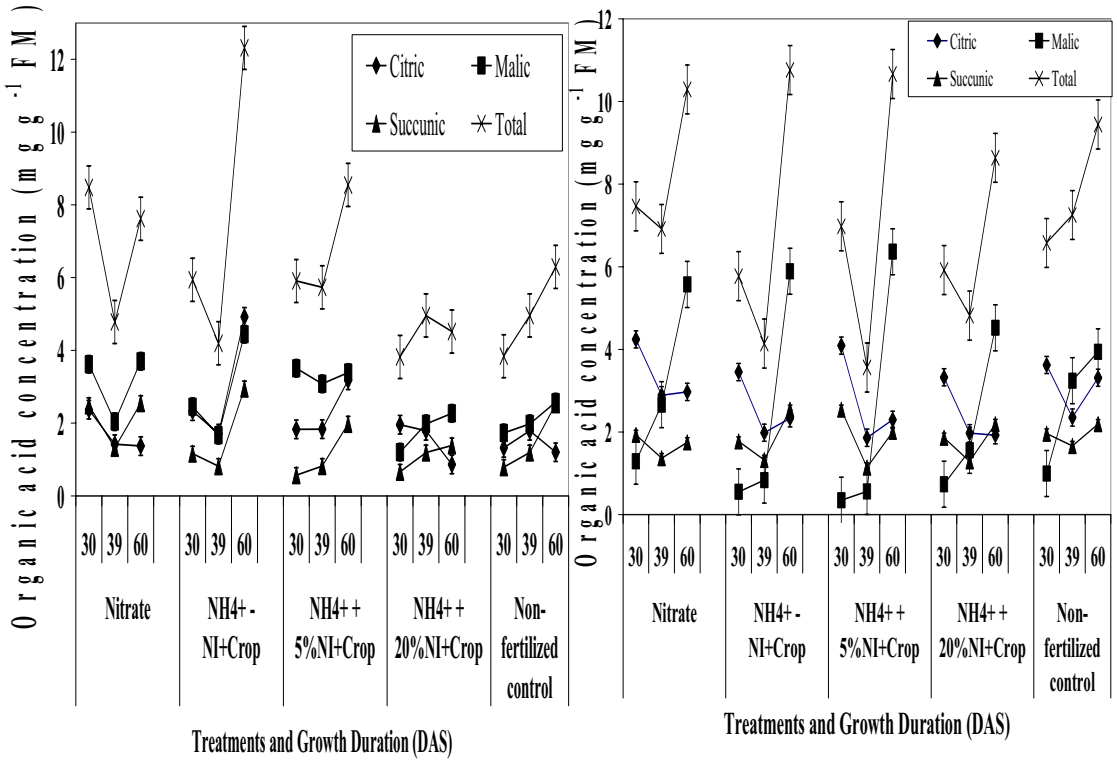

Fig. 17a-b: Influence of various nitrogen forms and fertilizer application regimes on the crop organic acid concentrations as a function of days after fertilization (DAF). Presented are means ±S.E. of seasons one and two.

3.2 Bacterial community diversity and responses to CULTAN fertilization

3.2.1 Bacterial community detection

To assess the response of bacterial communities to CULTAN fertilization, total bacterial DNA was extracted from soils fertilized by different nitrogen forms as well as non-fertilized control included as a comparison check. Quantification of genomic DNA using Pico green did not show any statistical differences among different sampling zones of various treatments. Amplification of undiluted genomic DNA by universal bacterial primers generated expected PCR products whose sizes were 391 bp length. Upon separation, most of the expressed bands were weak while others were completely invisible (Fig. 18a). Dilution of genomic DNA by ten-fold resulted into amplification of visible DNA products (Fig. 18b). Three separately amplified PCR products were pooled and analysed using single strand conformation polymorphism (SSCP) technique.

3.2.2 Spatial dynamics of detected bacterial communities

To separate the amplified PCR products into individual amplicons, SSCP technique was deployed. The generated SSCP profiles of small subunit (SSU) rRNA showed the occurrence of spatial variability among the bacterial communities. Ammonium based treatments had 15 clearly distinguishable bands while nitrate and non-fertilized control revealed only five bands at 30 DAF (Fig. 19a and b). Clearly observable bands in zone 2c were 10 while zone 3 had 11 at 30 DAF in ammonium treatments. The banding pattern variability reveals that not only band numbers reduced with distance from the injection point, their staining intensities (dominance) also decreased. Majority of the strongly staining bands detected in zone 2a among ammonium based treatments were absent in both nitrate and non-fertilized control and were unique to ammonium based treatments. Apparently, the five bands observed in both nitrate and non-fertilized control was common to all zones of both ammonium and non-ammonium based treatments. No unique banding patterns were observed among various zones of nitrate and non-fertilized control. The three ammonium treatments showed similar banding patterns regardless of the presence or absence of crops as well as incorporation or non-incorporation of nitrification inhibitor at 30 DAF (Fig. 19a and b). The banding pattern of uncropped NH_4^+ treatment without NI-incorporation along side nitrate and non-fertilized control had an exceptionally large cloud of non-separated band mass at 30 DAF (Fig. 19b). The intensity of band staining dominance among ammonium based treatments

reduced with distance from the injection point, where zone 2a located within the injection zone expressed a strong staining intensity in comparison to zones 2c and 3 with lesser staining intensity. Zone 2a situated at a closer proximity to injection point had a higher number of bands compared to zones 2c and 3 located spatially away from the injection point. Banding pattern among the three replications of each treatment zone were highly reproducible (Fig. 19a and b). Coincidentally, the presence of numerous bands along side their dominance occurred in ammonium treatments, in zones dominated with high ammonium concentrations coupled up with acidic pH values (Fig. 5a and 7a).

To evaluate the banding patterns of different zones and treatments, digital band cluster analyses of SSCP gels were compared using Gelcompar software. The bands from cropped NH_4^+ with 5% NI and without NI-incorporation sampled at 30 DAF were grouped into two distinct categories (Fig. 19c). Treatment and zone similarities estimated by UPGMA similarity matrices based upon estimated Pearson correlation coefficients relative to percent band coverage area showed zone 2a to be dominated by bands associated with high NH_4^+–N concentrations. Replications of zone 2a in ammonium with 5% NI-incorporation and without NI were separated from each other at 65.9 and 69.1% in treatment with 5% NI-incorporated and non-NI incorporated NH_4^+ at 30 DAF, respectively (Fig. 19c). Distinctions between treatments and zones confirm the reproducibility of banding patterns in the replicate zones. Zone 2a cluster of the two treatments showed 53.6% similarity at 30 DAF. The three replicates of zone 2c of each ammonium treatment were equally distinctly separated, but these two groups shared 60.7% similarity index. Zone 3 did not form any separate and distinct group. Clustering by treatments rather than zones was observable among nitrate, non-fertilized control and non-NI incorporated NH_4^+ treatment without crop (Fig. 19d). Zone 2a of nitrate and ammonium without NI-incorporation clustered together at 67 and 45%, respectively (Fig. 19d). Digital band clusters of non-NI incorporated NH_4^+ treatment without crop grouped NH_4^+ treatment zones together (Fig. 19d). They formed a cluster at 45% similarity index. Both nitrate and non-fertilized control were grouped together at 66% similarity index but, later separated into its own grouping based on treatment irrespective of zones at 86.5% and 87.3%, respectively.

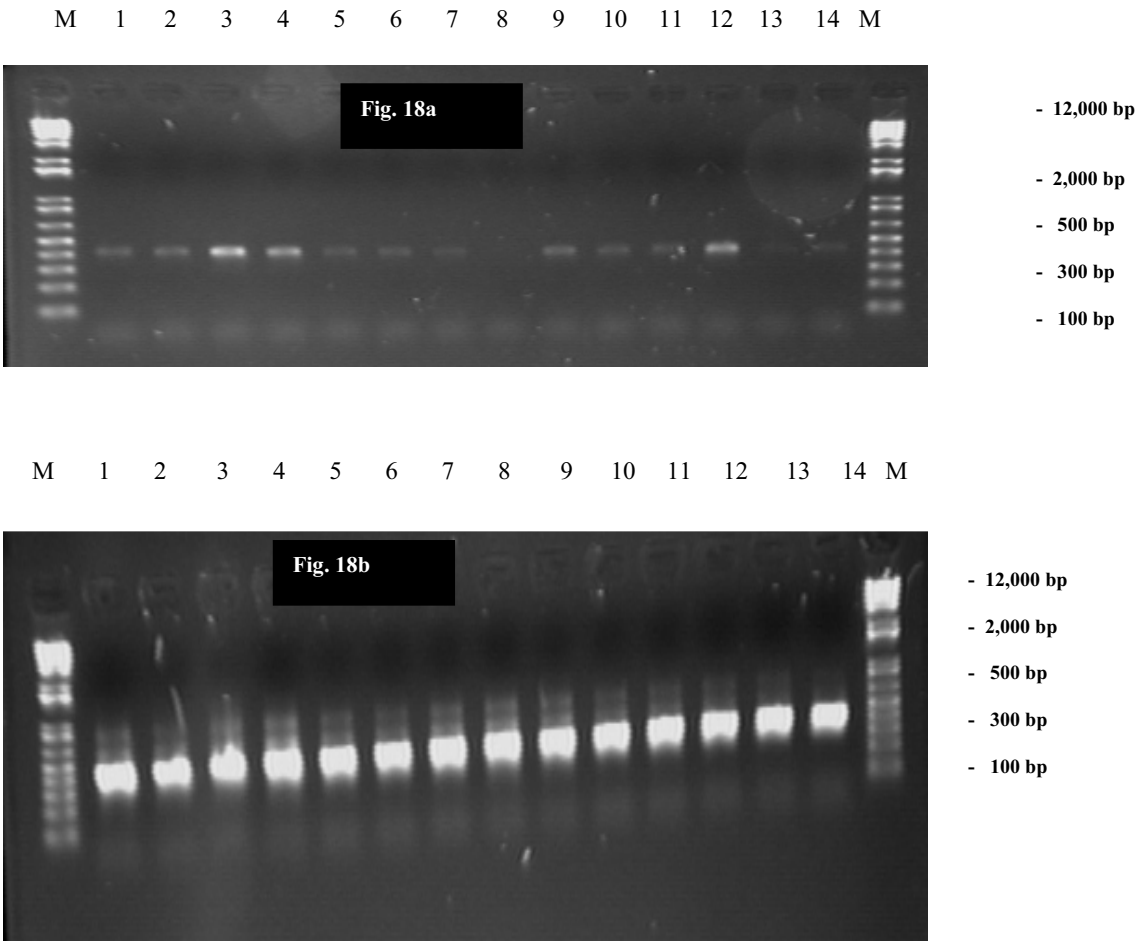

Fig. 18a - b: PCR products amplified from undiluted bacterial genomic DNA (a) and PCR products generated from ten-fold DNA dilution (b) as revealed by electrophoresis on 1% agarose gel. Lanes 1-3, 4-6, 7-9, 10-12 and 13-14 are three replicates of bacterial DNA extracted from nitrate, NH_4^+ -NI+ Crop, NH_4^+ +5%NI+ Crop, NH_4^+ -NI- Crop and non-fertilized control respectively at 30 DAF.

Fig. 19a

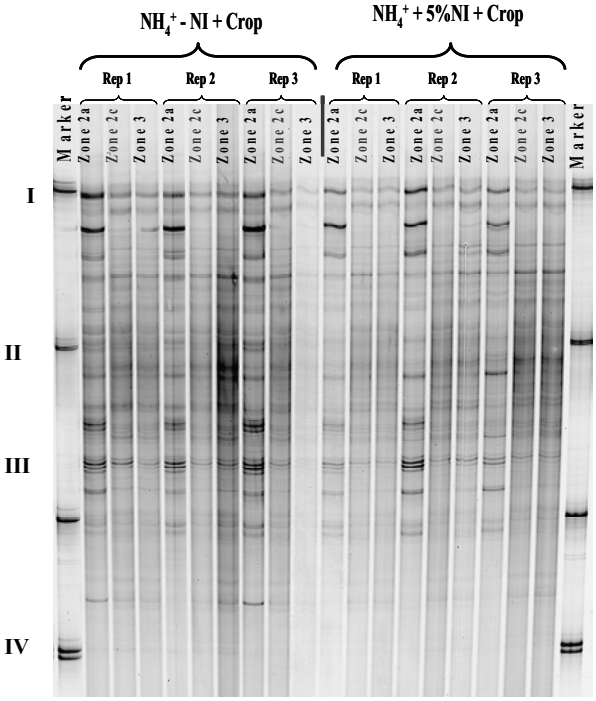

Fig. 19b

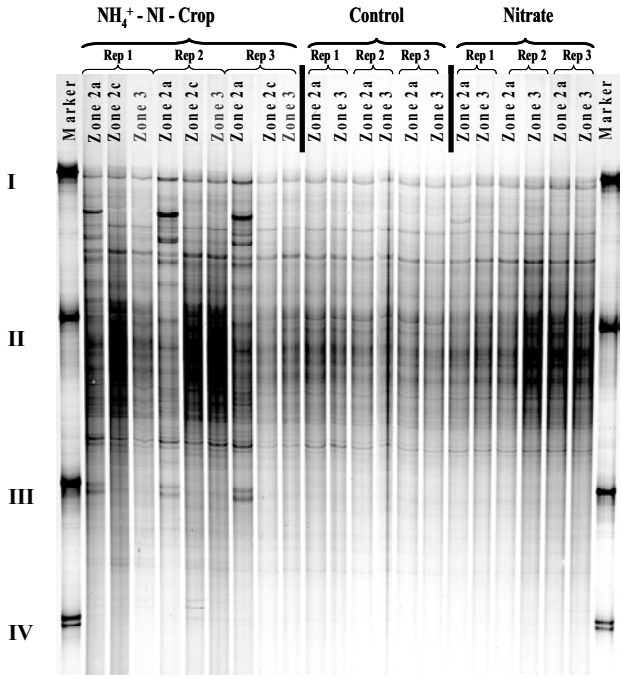

Fig. 19a - b: SSCP gels showing band profiles of bacterial DNA extracted from nitrogen treatments, NH_4^+-NI+Crop and NH_4^++5%NI+Crop (a), as well as NH_4^+-NI-Crop, Nitrate and Non-fertilized control (b) from each of the three different zones, 2a, 2c and 3 at 30 days after fertilization (DAF)

Fig. 19c

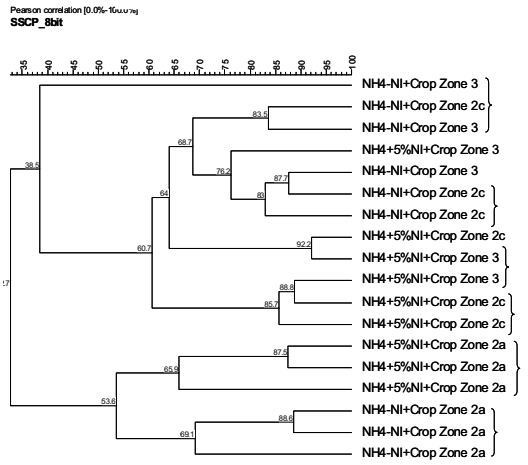

Fig. 19d

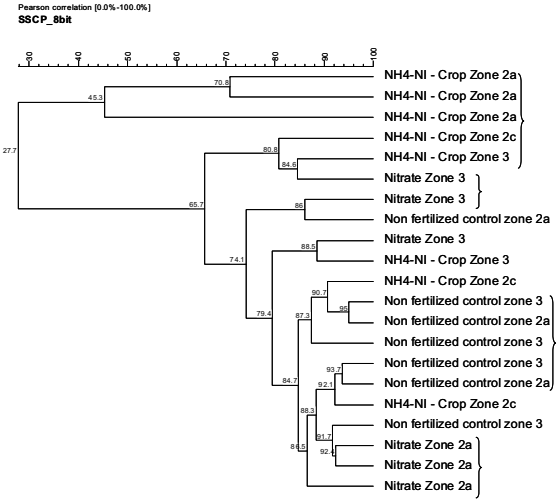

Fig. 19c - d: Digital band clusters of SSCP gels showing band profiles of bacterial DNA extracted from nitrogen treatments, NH_4^+-NI+Crop and NH_4^++5%NI+Crop (c), as well as NH_4^+-NI-Crop, Nitrate and Non-fertilized control (d) from each of the three different zones, 2a, 2c and 3 at 30 days after fertilization (DAF)

3.2.3 Temporal dynamics of detected bacterial communities

There were no banding pattern differences observed with regard to nitrogen treatments applied, zone sampled, nitrification inhibitor incorporation and presence or absence of crops at 109 DAF (19 e and f). All treatments expressed similar banding patterns among different replicate zones. Non-fertilized control treatment had banding profiles similar to those of fertilized treatments indicating that fertilizer application did not have an effect on the detected bacterial communities at the end of the season. Each treatment zone revealed occurrence of five bands with equal staining intensities in zones 2a, 2c and 3 (19 e and f). Each of the five bands appeared as duplets besides expressing uniform staining intensities. Migration positions occupied by bands detected at 109 DAF corresponded to those of common bands across all zones irrespective of nitrogen treatment at 30 DAF, which were also uniformly common to ammonium, nitrate and non-fertilized control treatments. Another striking difference in the profiles of ammonium treatments which consistently had reproducible unique bands in zone 2a at 30 DAF was the total disappearance of these bands at 109 DAF (19a, b, e and f). The disappearance of most of bands observed in ammonium treatments at 30 DAF had a corollary effect of reduced number of bands from 15 to five at 109 DAF. Only eight clearly observable bands were detected at 60 DAF (data not shown). The absence of banding profile differences among treatments at 109 DAF irrespective of previous N fertilization status at the end of season corroborated results of ammonium concentration which equally decreased to very low levels (Fig. 5a). This is in sharp contrast to the concentrations recorded in the early season (30 DAF) when ammonium concentration predominated. This resulted into a complex banding profile consisting of more and dominant bands. Digital band clusters of NH_4^+ without NI-incorporation and with 5% NI incorporation sampled at 109 DAF did not distinguish zones. However, the two treatments had band profiles that closely resembled with a similarity index of 82.2%. These further clustered into sub-groups at 92.6, 93.2, 93.0 and 94.5% similarity indices (Fig. 19g). The digital band clusters of non-NI incorporated NH_4^+ treatment without crop, nitrate and non-fertilized control clustered together both by treatment and zones (Fig. 19h). The non-NI incorporated NH_4^+ treatment without crop formed a large cluster at 83.7%, which further subdivided at 94.5 and 92.1% to regroup zones 2a and 2c, respectively (Fig. 19h). Nitrate and non-fertilized control formed separate clusters at 78.1 and 89.1% similarity indices. Nitrate further subdivided into zones 2a and 3 at 90.4 and 89.7% (Fig. 19h). Non-fertilized control separated into zones 2a and 3 at 94.9 and 96.5% similarity indices, respectively (Fig. 19h).

Fig. 19e **Fig. 19f**

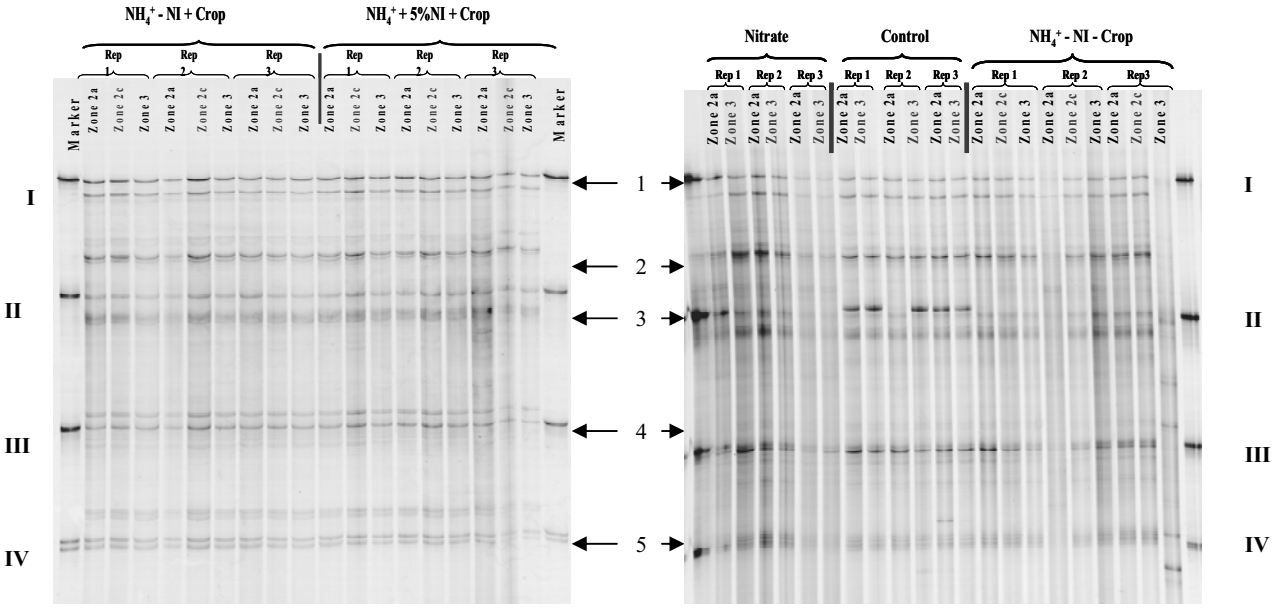

Fig. 19e - f: SSCP gels showing band profiles of bacterial DNA extracted from nitrogen treatments, NH_4^+-NI+Crop and NH_4^++5%NI+Crop (e), as well as NH_4^+-NI-Crop, Nitrate and Non-fertilized control (f) from each of the three different zones, 2a, 2c and 3 at 109 days after fertilization (DAF)

Fig. 19g **Fig. 19h**

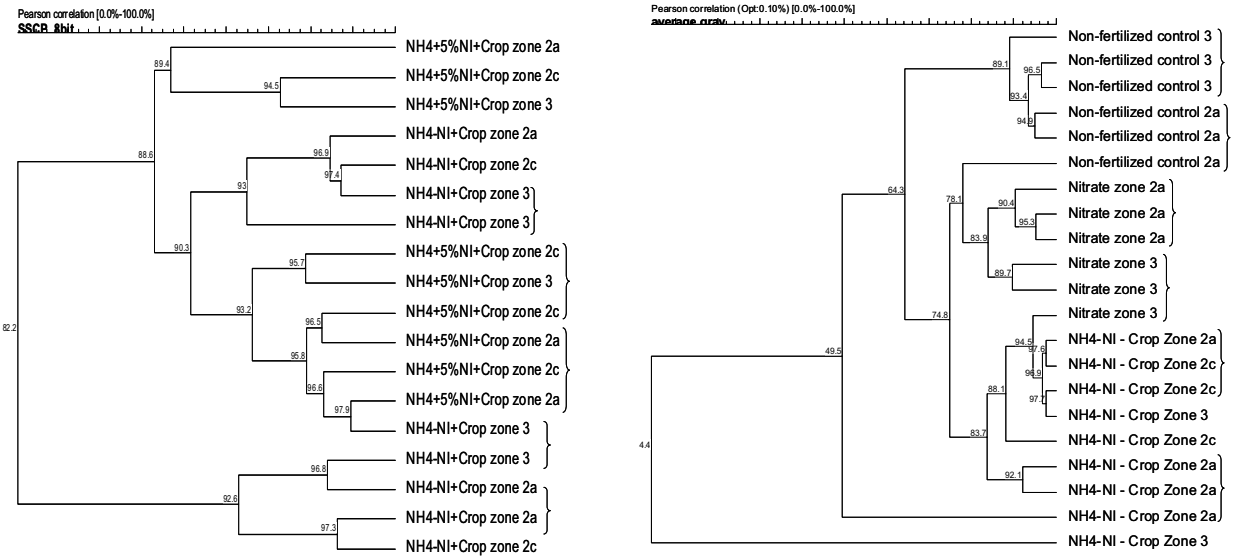

Fig. 19g - h: Digital band clusters of SSCP gels showing band profiles of bacterial DNA extracted from nitrogen treatments, NH_4^+-NI+Crop and NH_4^++5%NI+Crop (g), as well as NH_4^+-NI-Crop, Nitrate and Non-fertilized control (h) from each of the three different zones, 2a, 2c and 3 at 30 days after fertilization (DAF)

3.2.4 Characterization of CULTAN-associated bacterial communities

To characterize CULTAN associated bacterial groups, some representative bands were selected and sequenced so as to provide a clue of the occurring bacterial community. Most of the clone bands migrated to same positions of the excised parent genomic DNA band (data not shown). Duplicate clones of bands that migrated equal distances yielded more or less identical nucleotide sequences indicating close genetic relatedness. This was true for bands on the same gels irrespective of the nitrogen treatment. Comparisons of bacterial sequences with their published closest relatives in Genebank database through BLAST revealed the presence of a diverse bacterial community. Majority of the retrieved sequences expressed close relationships to known cultured bacterial groups while others related to unknown and uncultured groups. The strongly staining double bands in NH_4^+ treatments at 30 DAF yielded sequences that were 100% similar to *Arthrobacter* sp. accession number was DQ173010. Additional *Protebacteria* phylum representatives comprised *beta, alpha, gamma* and *delta* clusters. The recovered clusters represented uncultured *Pseudomonas* belonging to *gamma-Proteobacteria* whose similarity index to DQ887931 was 99%. Other members of the phylum were *Xanthomonas* and *Lysobacter* spp. both of which expressed equal similarity indices of 99% to DQ177466 and AB161359, respectively. *Alpha-Proteobacteria* was represented by uncultured *Agrobacterium* sp. with a similarity index of 99% to accession number AJ295444 while the uncultured *delta-Proteobacteria* exhibited a similarity index of 99% to AY921695. *Alpha-Proteobacteria* sub-phyla represented by uncultured bacterium whose similarity index was 97% to AJ318184. Other bacterial groups such as *Streptomyces* sp., *Flavobacterium* sp., *Bacillus* sp., *Sporoasrcina* sp. were also recovered.

3.2.5 Composition of CULTAN-associated bacterial communities

Essentially, *Proteobacteria* phylum was the single most commonly retrievable among the 40 analyzed sequences. This phylum accounted for 42.9% of the analyzed sequences and it was twice the number of *Firmicutes*-like or *Actinobacteria*-like sequences. The two were equal in proportionality to each other and their total populations were equivalent to that of *Proteobacteria*. *Bacteroidetes* accounted for 14% of the identified bacterial groups and had the lowest number of representatives. The distribution within different classes showed that *gamma-Proteobacteria* accounted for 23.8% and was the most common bacterial group followed by *Actinobacteria* with 21.4% while *Clostridia* and *Bacteroidetes* accounted for 16.7

and 11.9%, respectively. *Alpha-* and *beta-Proteobacteria* were 7.1% each, whereas *delta-* and *alpha-Proteobacteria* each accounted for 2.4%. *Firmicutes* was represented by members of two classes, *Clostridia* and *Bacilli. Clostridia* were the most commonly retrievable group. It accounted for 80%, out which orders *Thermoanaerobacteriales* and *Clostridiales* accounted for 50 and 40%, respectively. Class *Bacilli* accounted for 20% of the sequenced *Furmicutes*; however, it represented a paltry 4.8% of all the retrieved bacterial sequences. However, this was equivalent to the sum of both *delta-* and *alpha-Proteobacteria*. The above bacterial composition suggests the presence of a diverse bacterial community supported within a narrow spatial zone and within a highly heterogeneous ecological niche possibly made available by CULTAN fertilization.

3.2.6 Phylogenetic relationships of characterized CULTAN bacterial groups

Selected PCR-product phylogenetic analyses of 16S rRNA gene sequences revealed the presence of *beta-Proteobacteria* among other dominant soil bacterial groups (Fig. 20). The sequences were grouped into four main clusters. The first cluster comprised *Xanthomonas*-like sequences while the second cluster was composed of *Bacilli*-like sequences. The third cluster comprised *Arthrobacter*-like sequences while the fourth group was made up of uncultured bacterial grouping. The phylogenetic relationship is quite similar to the BLAST derived identities. It is worth noting that the majority of *Xanthomonas*-like sequences were recovered from zone 2a within the highly concentrated NH_4^+ zone. Sequences which showed close relationship to species like *Pseudomonas* and *Arthrobacter* grouped in clusters 1 and 3, respectively. They were commonly retrievable in the early season sampled soils. It is probable that these species were tolerant to concentrated NH_4^+ and could have participated in its detoxification. In the later part of the season, which corresponded to 109 DAF, the dominant bacterial groups were *Thermoanaerobacteriales, Flavobacteriales* and a few more members of *Proteobacteria.*

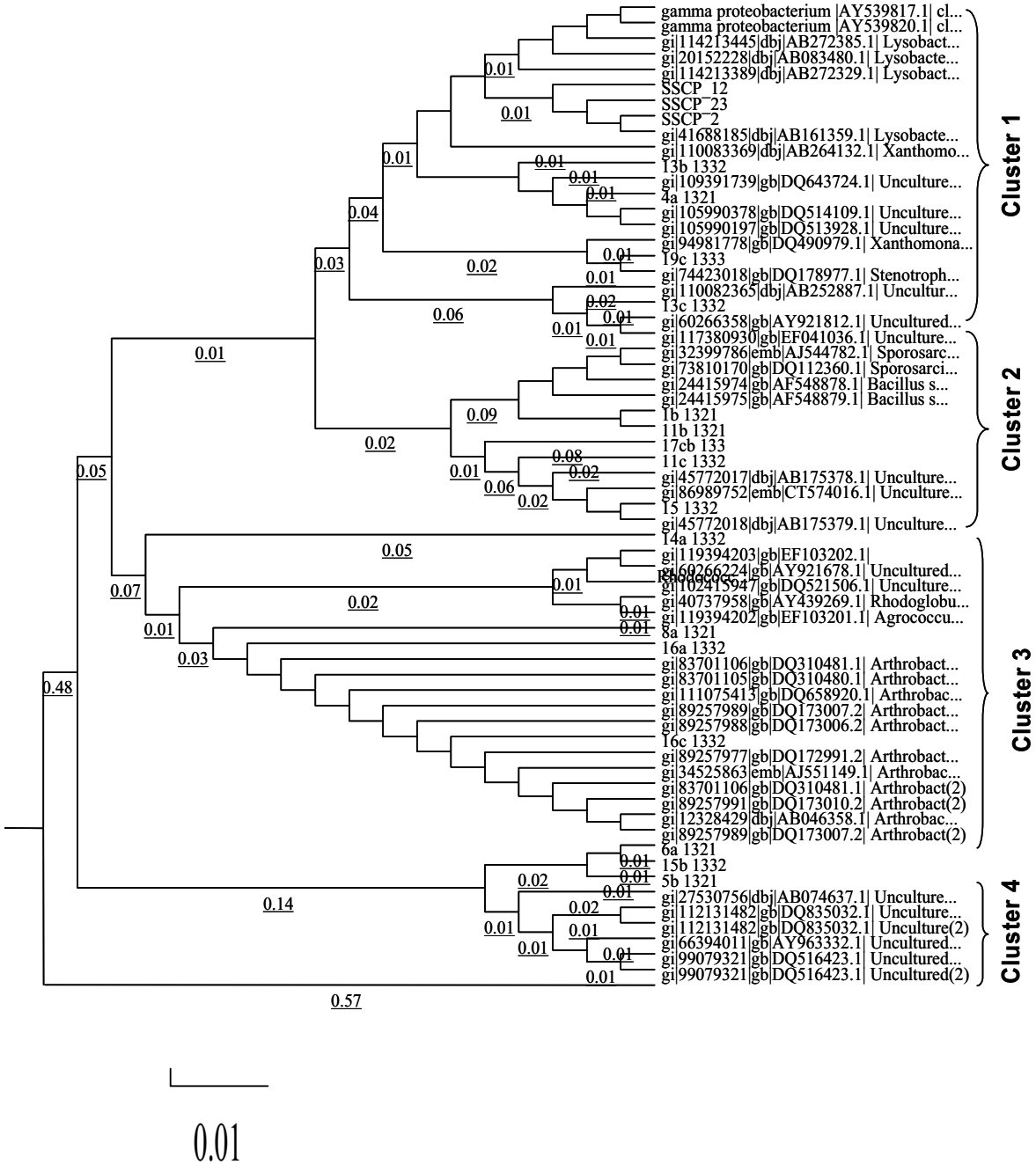

Fig. 20: Phylogenetic cluster analysis of bacterial community based on the 16S rRNA gene sequences retrieved from CULTAN-fertilized treatment and comparison checks

3.3 Biodiversity of AOB associated with CULTAN-fertilized soils

Attempts were made to evaluate the occurrence of ammonia oxidizing bacteria (AOB) by targeting ammonia monooxygenase subunit A (*amoA*) gene encoding the active site of the unique enzyme found in nitrifying bacterial groups to complement 16S rRNA gene analysis since it does not give any bacterial functional roles. The utilized primer pair yielded DNA products of about 491 base pair length on 1 kb ladder. The amplified environmental products corresponded well with those of *Nitrosospira multiformis* selected for use in this study as a model ammonia oxidizing bacterium. Some of the obtained bands in SSCP profiles generated from DNA extracted from zones 2a and 3 corresponded to bands of known AOB, hence the confirmation that this bacterial group occurred in CULTAN-fertilized soils (Fig. 21a and b). The changes corresponded quite closely to NH_4^+ -N concentrations as indicated by the SSCP banding gels along side soils analysis results (Fig. 5a and 7a).

3.3.1 Spatial changes of ammonia oxidizing bacteria under CULTAN fertilization

To evaluate the effect of CULTAN on AOB on a spatial scale, zones 2a and 3 were analyzed. The banding pattern of AOB was generally uniform within similar zones and slightly different in different zones (Fig. 21a and b). The spatial distribution of AOB within zone 3 at 109 DAF had a complex banding pattern among the five treatments, with one strong staining band commonly found among the five treatments (Fig. 21b). To enable comparison of zones 2a and 3 sampled at 30 DAF, a single gel was developed. The gel showed a very complex banding pattern comprising seven clearly observable bands. Only two bands were strongly staining, though a total of nine were observable, except they were quite faint (Fig. 21a). Both nitrate and non-fertilized control too expressed distinct bands in both zones 2a and 3. There were two strongly staining bands however, there was no band corresponding to positive control, *N. multiformis* (Fig. 21a). Besides the single strongly staining band found in NH_4^+ treatment incorporated with 5% NI, two other weak bands were seen. Similar band numbers were observed in zone 3 of the nitrate and non-fertilized control at 30 DAF (Fig. 21b). Digital band cluster analysis showed that positive control, *N. multiformis* grouped together with some of the environmental genomic samples (Fig. 21c). Most of the treatments and zones did not exhibit any specific grouping. However, zone 3 of both nitrate and 5% NI incorporated NH_4^+ treatment clustered together at 98.7 and 95.4% similarity indices, respectively (Fig. 21c).

Fig. 21a

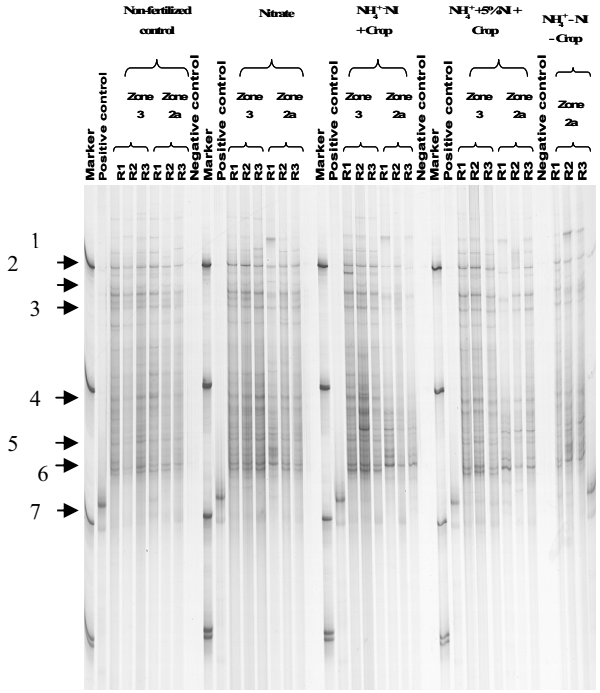

Fig. 21b

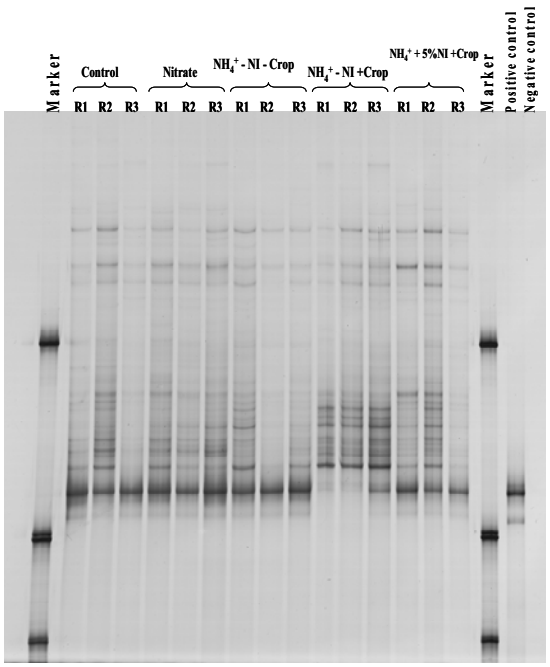

Fig. 21a-b: SSCP gels showing band profiles of bacterial DNA extracted from zones 2a and 3 sampled at 30 DAF in non-fertilized control, nitrate, NH_4^+-NI+crop, NH_4^++5%NI+crop and NH_4^+-NI-crop treatments (a) and zone 2a of the five treatments sampled from zone 2a at 109 days after fertilization (DAF) (b)

Fig. 21c

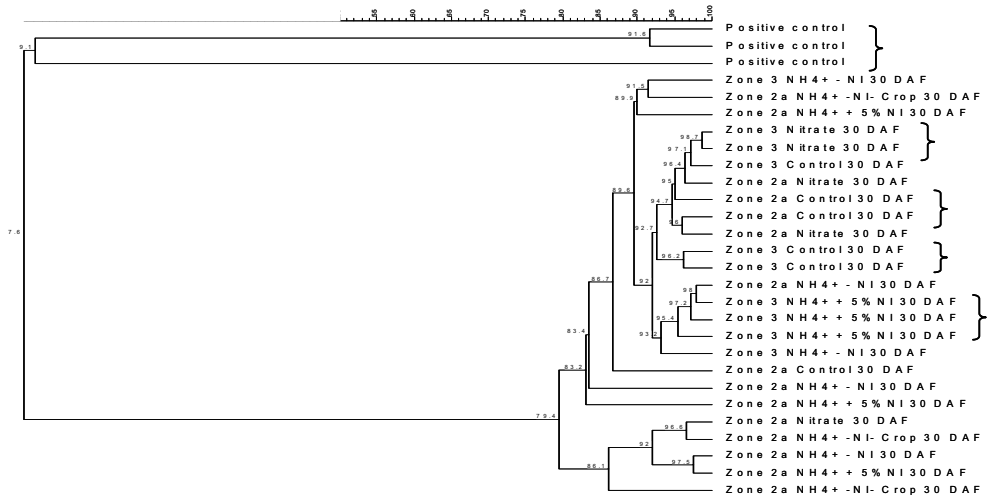

Fig. 21c: Digital band clusters of SSCP gel showing band profile comparison among *amoA* bacterial genes extracted from zones 2a and 3 sampled at 30 DAF in non-fertilized control, nitrate, NH_4^+-NI+crop, NH_4^++5%NI+crop and NH_4^+-NI-crop treatments. Gel 21b had a similar clustering, though not shown.

3.3.2 Temporal dynamics of ammonia oxidizing bacteria under CULTAN fertilization

The *amoA* genes were detected in both zones 2a and 3 of the CULTAN-fertilized soils. Nitrate and non-fertilized control treatments too showed *amoA* gene bands in both zones (Fig. 22a and b). Zones 2a showed the presence of 13 clearly visible bands, and some were unique to particular treatments (Fig. 22a). On the other hand, zone 3 had 12 visible bands, but a few could not be detected at different temporal scales (Fig. 22b). Zone 3 bands whenever detected were strongly staining than zone 2a. Non-fertilized control did not show any clear band presence at 109 DAS. NH_4^+ treatment incorporated with 5% NI, non-fertilized control and nitrate revealed the presence of 12 visible bands (Fig. 22a and b). Bands corresponding to *N. multiformis* were, however, absent in both zones 2a and 3 of NH_4^+ incorporated with 5% NI, non-fertilized control and nitrate treatments at 30 DAF (Fig. 22a and b). The bands corresponding to *N. multiformis* were nonetheless detected in both zones among the three treatments at 60 DAF. Nitrate and non-fertilized control did not show the presence of these bands at 109 DAF. While nitrate and NH_4^+ treatment incorporated with 5% NI supported larger AOB diversities in zone 2a, non-fertilized treatment hand only a single band occurring at 109 DAF. The migration position of the band corresponded to that of *N. multiformis* (Fig. 22a). In zone 3, only samples collected at 30 DAF showed a large diversity, a time when NH_4^+ treatment incorporated with 5% NI had most of its bands detected (Fig. 22b). A single band was detected in zone 3 at 60 and 109 DAF samples of nitrate and 5% NI incorporated treatment. In non-fertilized control, it corresponded to *N. multiformis* at 60 DAF and non at 109 DAF (Fig 22b). Whenever present, *N. multiformis* was the most strongly staining band. It was also the furthest migrating band among the detectable bands in the treatment and zones. No band was detected in zone 3 of the non-fertilized control at 109 DAF (Fig. 22b). *N. multiformis* was present in zone 3 only at 60 DAF and was dominant in all the three treatments.

Fig. 22a

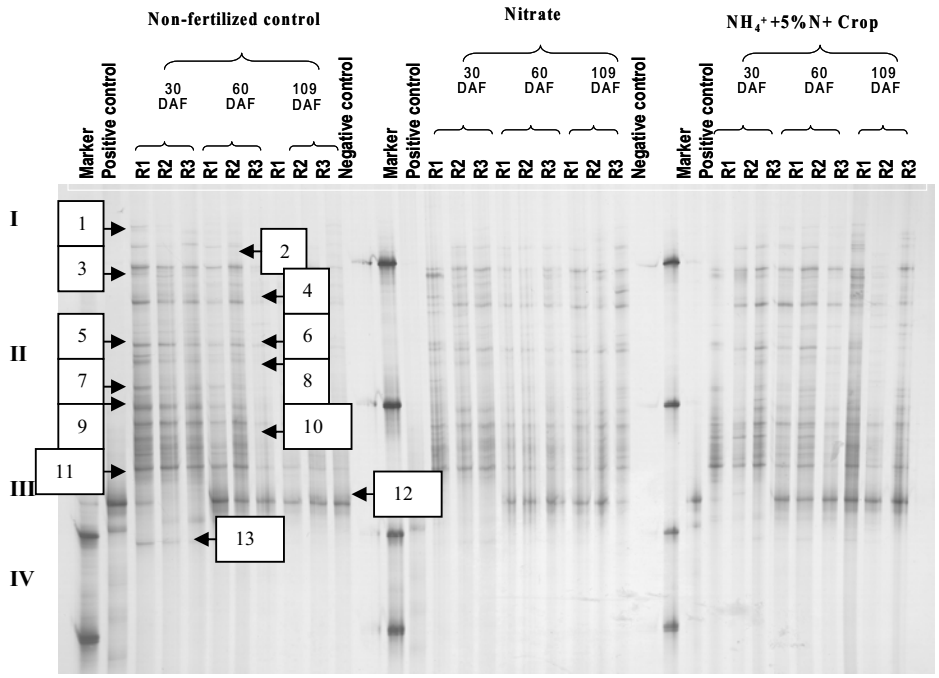

Fig. 22b

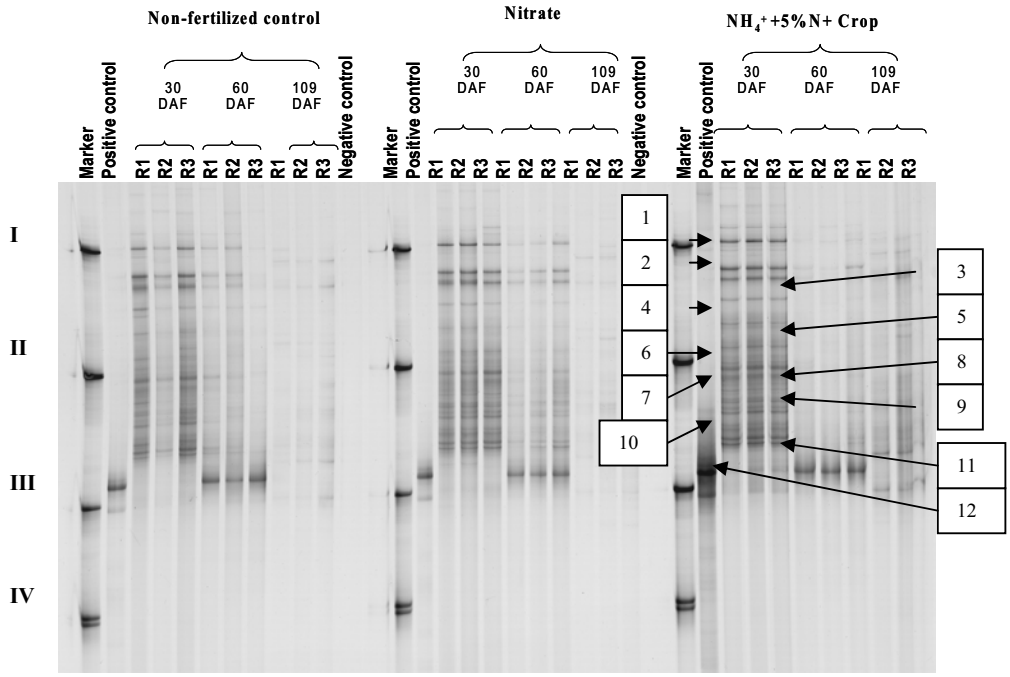

Fig. 22a-b: SSCP gels developed from *amoA* genes amplified from DNA extracted from zone 2a (d) and zone 3 (e) of non-fertilized control, nitrate and NH_4^++5%NI+ crop treatments at sampling intervals of 30, 60 and 109 DAF.

3.3.3. AOB clustering and phylogenetic relationships

Digital band clustering of *amoA* genes amplified from DNA samples of zone 2a harvested at intervals of 30, 60 and 109 DAF were distinctively grouped based on sampling time. Sampling intervals at 30, 60 and 109 DAF clustered together at 61.8%, 71.7% and 86.8%, respectively, indicating that soil samples harvested at the same interval shared quite similar banding patterns (Fig. 23a). Each treatment formed a clade within the sampling interval cluster. The positive control AOB too grouped together and shared a similarity index of 97.7% (Fig. 23a).

Zone 3 samples equally categorized into 30, 60 and 109 DAF at similarity indices of 82.6%, 72.3% and 35%, respectively (Fig. 23b). However, 30 and 109 DAF samples were more related to each other at 31.2% than 60 DAF samples which branched much earlier at 14.5%. All the three replications of each treatment formed a sub-group within the sampling interval (Fig. 23b).

A few *amoA* gene PCR-products were selected, cloned and sequenced to characterize the ammonia oxidizing bacteria associated with the CULTAN-fertilized soils (Fig. 24). Out of the 16 selected bands, only four cloned into the vector. The retrieved sequences were *Nitrosospira*-like (Fig. 24). One soil sample yielded sequences that clustered together with *Nitrosospira multiformis*, which was used as positive control (Fig. 24). Even though the retrieved sequences were *Nitrosospira*-like, they grouped separately, which suggests that there could be sub-clusters.

Fig. 23a

Fig. 23b

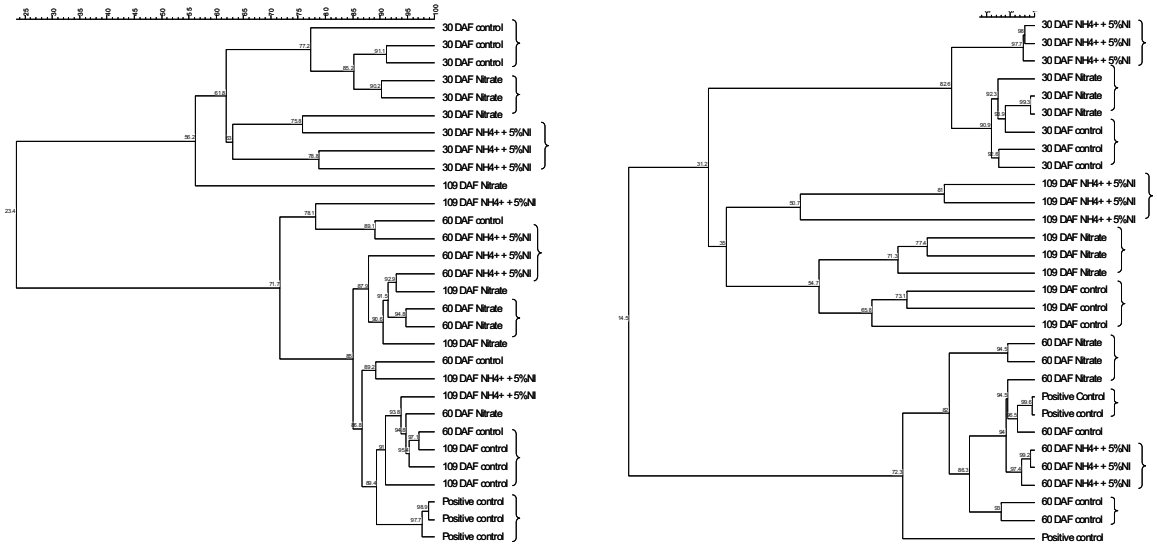

Fig. 23a-b: Digital band clusters of *amoA* SSCP gels amplified from DNA extracted from zone 2a (d) and zone 3 (e) of non-fertilized control, nitrate and NH_4^++5%NI+ crop treatments at sampling intervals of 30, 60 and 109 DAF.

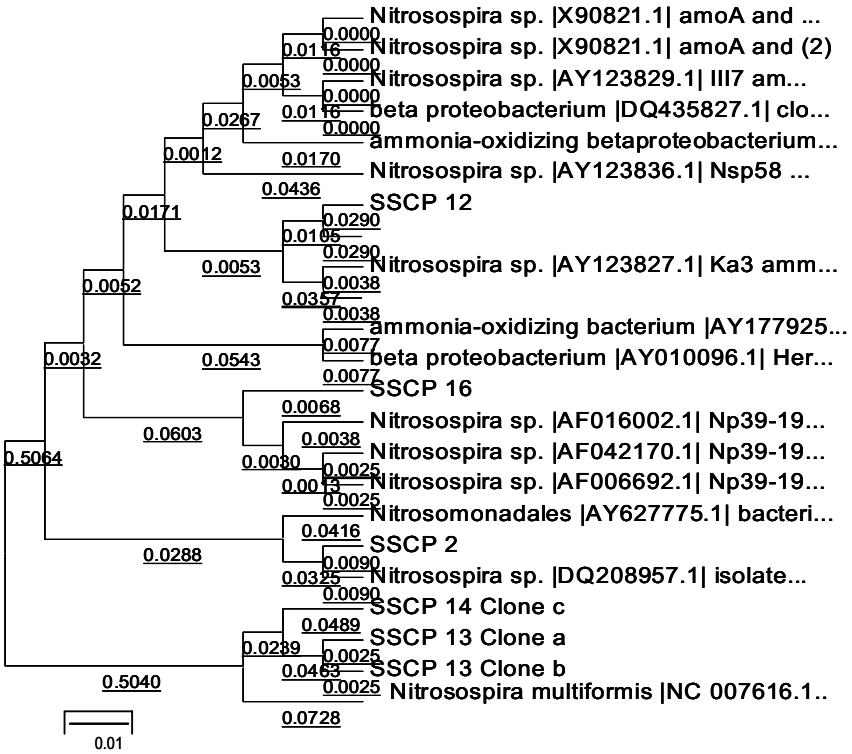

Fig. 24: Phylogenetic relationships of ammonia oxidizing bacteria based on *amoA* gene sequences retrieved from CULTAN fertilized treatments along side comparison checks of nitrate and non-fertilized control

3.4 Population abundance of AOB in CULTAN-fertilized soils

AOB specific primers targeting *amoA* gene of 491 bp length were used to optimize the threshold cycle ($C_{t.}$). The amplification efficiency of AOB positive control was ensured by performing four serial dilutions of *N. multiformis amoA* genes used as standard in Real-time (quantitative) polymerase chain reaction (qPCR). The threshold cycle (C_t) from the standard curves derived from *N. multiformis amoA* genes were used to estimate *amoA* genes in CULTAN-fertilized soil samples (Fig. 25a). The regression coefficients (R^2 values) of the standard curves were greater than 0.99 in all qPCR assays with *N. multiformis* (Table 3). Though performed separately, each qPCR assay expressed more or less similar slopes. C_t is the point where accumulated amplicons are significantly above background fluorescence level. It is proportional to calculated gene copy numbers of the positive control (5.08×10^6 *amoA* gene copy numbers μL^{-1}). The estimated and calculated gene copy numbers closely corresponded and hence its adoption in AOB population estimation. The standard curves ranged over four orders of magnitude (5.08×10^3 and 5.08×10^6 μl^{-1}) and C_t occurred within the exponential phase of PCR cycle (Fig. 25a). A linear regression of C_t values of *amoA* genes was used to develop a standard curve. Calibration curve was developed by plotting logarithm of gene copy numbers against detected DNA concentration during amplification.

The non-template control (NTC) occurred between 26 and 31 cycles. However, in a few cases, qPCR assays gave NTC curves with lower C_t values than environmental samples, suggesting that NTC had more *amoA* genes than soil samples. In such cases, the assays were not used for AOB population estimate. When undiluted genomic DNA was used for qPCR amplification, no products were obtained due to inhibition (Fig. 26a). On dilution, well amplified bands were obtained (Fig. 26b). In some cases, non-target DNA was amplified (Fig. 26a). A high level of reproducibility among the standard curves was obtained (Table 4). Attempts to assess amplification biases were made by performing concurrent qPCR amplification assays of two separately serially diluted samples. The developed curves generated expressed high levels of congruency with duplicate dilution rates appearing as mirror images of the other (Fig. 25a). Analyses of covariance between the standard curve slopes showed no statistical differences and they were thus adopted in the estimation of AOB abundance in CULTAN-fertilized soils along side non-fertilized control and sole nitrate which were included as comparison-check treatments.

Attempts were made to estimate AOB population abundance at the start and end of season, coinciding with 30 and 109 DAF, respectively. Gene copy numbers of NH_4^+ fertilized soils were more abundant as compared to nitrate and non-fertilized control both at the beginning and end of the season (Table 3). Apparently, uncropped NH_4^+-treatment without NI-incorporation had the lowest AOB population abundance of 1.09 x 10^6 *amoA* gene copy number (g soil)$^{-1}$ at 30 DAF among NH_4^+-based treatments. Cropped NH_4^+-treatment without NI-incorporation had the highest gene copy number of 3.29 x 10^6 *amoA* gene copy number (g soil)$^{-1}$ at the beginning of the season (Table 3). Nitrate and non-fertilized control had the lowest *amoA* gene copy numbers. The gene quantities were 3.78 x 10^5 and 3.31 x 10^5 *amoA* gene copy numbers (g soil)$^{-1}$, for nitrate and non-fertilized control, respectively.

A trend similar to that observed among the 30 DAF samples was repeated at 109 DAF (Table 3). The cropped NH_4^+ treatment without NI predominated in AOB population abundance. It had 1.08 x 10^6 *amoA* gene copy numbers (g soil)$^{-1}$ at 109 DAF. NH_4^+ treatment incorporated with 5% NI had the second largest estimated AOB population of 1.80 x 10^5 *amoA* gene copy numbers (g soil)$^{-1}$. Nitrate and uncropped NH_4^+ treatment each supported two-fold less AOB population in comparison to NH_4^+ treatment incorporated with 5% NI at 109 DAF (Table 3). The highest AOB gene copy numbers at 109 DAF supported by cropped NH_4^+ treatment was equal to the estimated gene copy numbers of uncropped NH_4^+ treatment without NI of 1.09 x 10^6 *amoA* gene copy numbers (g soil)$^{-1}$ attained at 30 DAF (Table 3). Uncropped NH_4^+ treatment had the greatest reduction of about 12-fold whereas the cropped NH_4^+ treatment had 3-fold reduction between 30 and 109 DAF (Table 3).

Analysis of DNA melting temperatures of environmental samples and *N. multiformis* showed the occurrence of more than one melting point peak. This suggested a possible presence of more than one *amoA* gene of unknown AOB or presence of co-extracted contaminants. The MP peaks of *N. multiformis* corresponded to those of soil samples. The MP peaks were between 75.5 to 79.7 and 87.0 to 88.8 ^oC for the first and second peaks, respectively (Fig. 25b). Analysis of qPCR assay products on agarose showed the presence of a diffuse band of a different size from the expected *amoA* gene of 491 bp length (Fig. 25a), suggesting a possible amplification of non-target *amoA* gene.

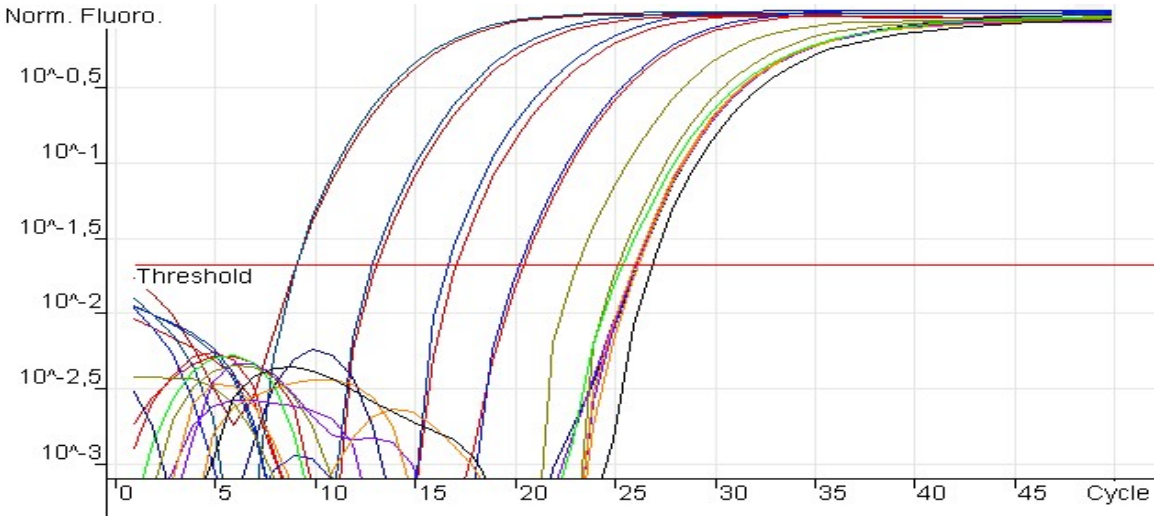

Fig. 25a: Amplification curves generated during the measurements of serially diluted bacterial DNA using Real time PCR. The dilution rates were 10, 100, 1000, 1000, 10000 and the last blue curve is NTC.

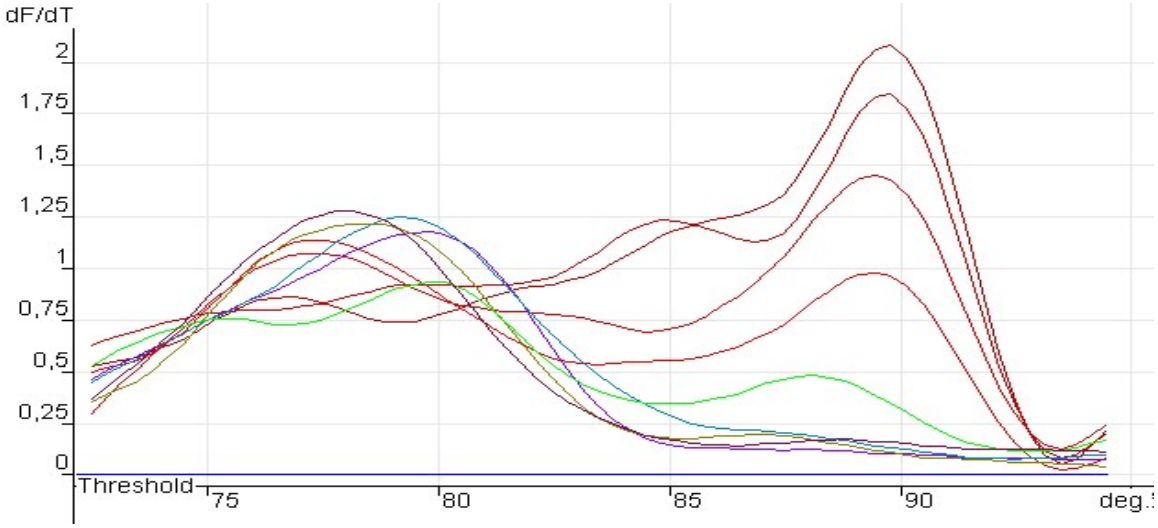

Fig. 25b: Melting curves obtained from amplified serially diluted bacterial DNA using Real time PCR.

Fig. 26a

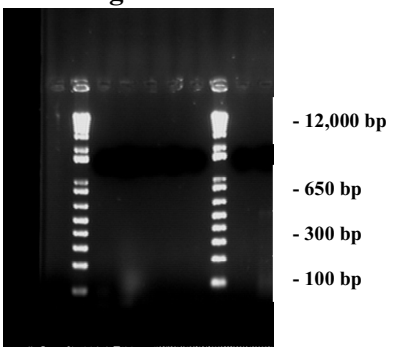

Fig. 26b

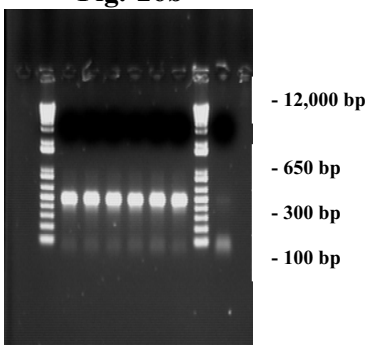

Fig. 26c

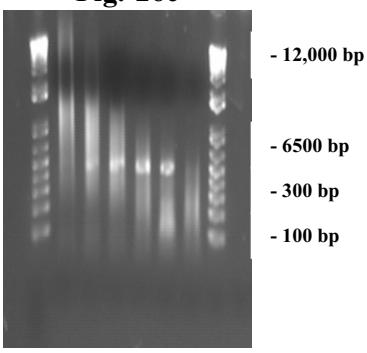

Fig. 26a - c: Inhibition of amplification during Real-time PCR (a) amplified ammonia oxidizing bacterial *amoA* genes (b), and amplification of non-target DNA

Table 3: Abundance of ammonia-oxidizing bacteria *amoA* gene copy numbers (μL^{-1}) in different nitrogen fertilized treatments together with a non-fertilized control comparison-check treatment

Nitrogen treatments	Threshold cycles (C*t*)		*amoA* gene copy numbers (g soil)$^{-1}$	
Zone 2a	30 DAF	109 DAF	30 DAF	109 DAF
Non-fertilized control	30.82	26.77	3.78×10^5	6.36×10^4
Nitrate	30.92	26.11	3.31×10^5	7.92×10^4
NH_4^+-NI-Crop	29.72	26.05	1.09×10^6	8.76×10^4
NH_4^+-NI+ Crop	28.23	23.21	3.29×10^6	1.08×10^6
NH_4^++5%NI+ Crop	29.44	25.46	1.35×10^6	1.80×10^5

Table 4: Regression coefficient (R^2), amplification efficiency (E), y-intercept value (b) and slope (m) of four standard curves adopted for qPCR amplification of zones 2a and 3 of 16S rRNA sampled at 30 and 109 days after fertilization (DAF)

Parameters	Assay one	Assay two	Assay three	Assay four
	30 DAF	109 DAF	30 DAF	109 DAF
R^2	0.998	0.999	0.999	0.999
E	0.86	0.84	0.82	0.68
b	41.09	34.54	40.57	46.83
m	-3.70	-3.78	-3.84	-4.42

3.5 Growth temperature effect on grain yield of CULTAN fertilized barley

3.5.1 Barley growth duration and shoot height

In an attempt to determine the impact of temperature regimes on growth duration and stem elongation, the growth durations and stem lengths were monitored. It was found that different growth temperatures caused varied growth durations with crops under LTR requiring 6 months to attain maturity while those in HTR needed 4 months (Fig. 27a). MTR crops took about 5 months (160 days) to mature (Fig. 27a). Besides these, clearly discernible growth responses existed between fertilized and non-fertilized crops. Fertilized crops expressed increases in stem lengths as growth temperatures reduced (Fig. 27b). When stem lengths of fertilized crops under LTR and HTR were compared, ammonium and nitrate fertilized crops experienced 24.5 and 20% increases, respectively (Fig. 27b). The stem lengths of the fertilized crops were not significantly different from each other within the same temperature regime. Non-fertilized crops did not express any stem length variations in response to temperature regime changes, and had equal lengths of 60 cm. This suggested that nitrogen fertilization irrespective of the form applied improved shoot length across the three regimes with reducing temperature levels. Under LTR, ammonium and nitrate experienced 40 and 36% shoot length increases, respectively, in comparison to 12 and 13% recorded by HTR grown crops (Fig. 27b). MTR grown crops were intermediate. Fertilized crop shoot lengths were significantly different from non-fertilized crops at $P < 0.001$ in each growth regime.

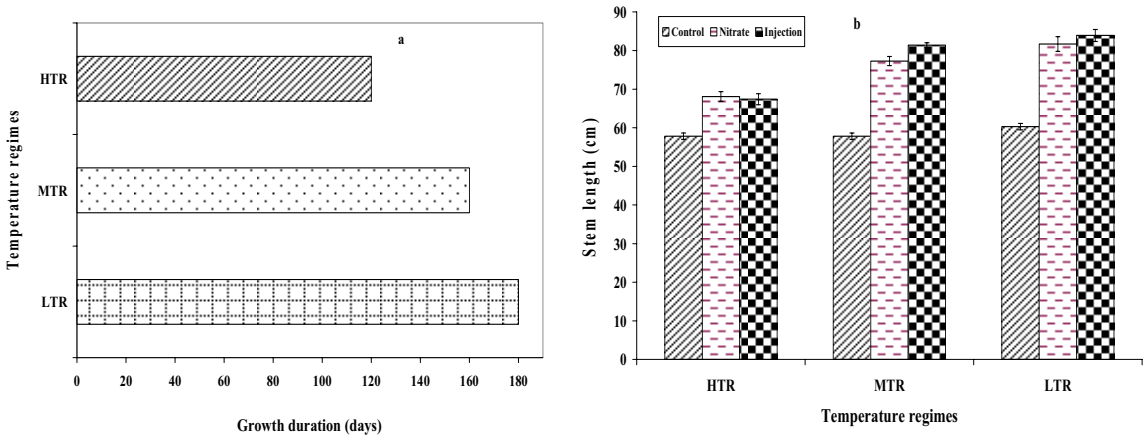

Fig. 27a-b: Barley growth durations under (a) and stem lengths (b) in response to the interaction between nitrogen treatments and growth temperatures.

3.5.2 Biomass accumulation and partitioning

To investigate the response of barley root and shoot growth responses to CULTAN fertilization, root growth architecture and accompanying root and shoot biomasses were assessed at the end of the season. It was observed that ammonium fertilized crops developed intensive architectural root network around the injection-points. In contrast, nitrate fertilized crops did not develop any similar intense root network. Besides the intensity of root growth around the toxic injection point, no roots penetrated through the injection zone. Instead the roots proliferated around the peripheral regions with lower ammonium concentrations. Shoot biomasses of the three N treatments exhibited increasing trends with decreases in growth temperatures (Fig. 28a). Similarly, root biomass production also showed an increasing trend with decreases in growth temperature (Fig. 28b). Shoot biomasses were significantly different from each other at $P < 0.001$. Both shoot and root biomasses of non-fertilized crops too increased with temperature decrease (Fig. 28a and b). Dry root biomass improvement due to N fertilizer application in LTR was 191.4 and 154.7% for ammonium and nitrate fertilized crops, respectively (Fig. 28b). Moreover, ammonium treated crops supported 14.4% higher root biomass over nitrate in LTR. Ammonium and nitrate fertilized crops under MTR recorded more or less equal shoot biomass yields of 857 and 870 g per container, respectively. The effect of nitrogen fertilization on shoot as experienced by nitrate and ammonium in terms of biomass gains was 127.5 and 86%, respectively, when compared to non-fertilized crops under HTR (Fig. 28a). Root biomasses too responded similarly. Shoot biomass gains in LTR in comparison to HTR were 194, 94, and 32% for ammonium, nitrate and non-fertilized control (Fig. 28a). Similar trends were observed in root biomass increases caused by decreases in growth temperatures. The gains were 61, 11 and 104% for ammonium, nitrate and non-fertilized control (Fig. 28b). Even though both shoot and root portions responded to changes in temperature regimes, the effects were more pronounced in shoot than root. Non-fertilized crops in LTR recorded the highest root: shoot ratio of 0.18 while nitrate was lowest with 0.08 whereas ammonium recorded 0.14. Changes in biomass partitioning caused shifts in root: shoot ratios under HTR in comparison to those in LTR while MTR was intermediate. These ratios illustrate that non-fertilized and ammonium fertilized crops in LTR partitioned higher biomasses to roots with less apportioned to shoot.

3.5.3 Grain yield and yield forming factors

To evaluate the impact of growth temperature and nitrogen forms on barley grain yield, both total grain weights and numbers were compared within and among the temperature regimes. The results clearly demonstrated that the total grain weights and numbers were influenced by growth temperatures as well as nitrogen fertilization (Fig. 29a and b). Both grain weights and numbers increased with decreasing growth temperatures (Fig. 29a and b). Grain yields of non-fertilized crops were more or less equal among the three temperature regimes with about 2000 grains that weighed about 100 g per treatment. Fertilized crop grain yields within the same growth regimes were, not significantly different from each other, but were significantly different from non-fertilized crops at $P < 0.001$. The agronomic NUE attained by ammonium and nitrate fertilized crops in HTR was 105 and 133%, respectively. NO_3^- fertilized crop attained 14% higher grain weights over NH_4^+ fertilized crops grown in HTR, while nitrate and ammonium fertilized crops in MTR attained equal gains of 122 and 126%, respectively. The agronomic NUE was even higher under LTR where nitrate and ammonium grain weights gained 214 and 227%, respectively (Fig. 29a). The percent gain in grain numbers by nitrate and ammonium fertilized crops under HTR was 151 and 145%, whereas LTR had 171 and 179%, respectively (Fig. 29b). Similarly, the percent gain in grain yield weights attributed to temperature effect was 40, 13 and 7% under LTR for ammonium, nitrate and non-fertilized control in comparison to HTR. Generally, LTR fertilized crops yielded more grains with slightly lower average weights of 0.043 g compared to HTR grains whose average weights were 0.045 g. Non-fertilized crops compensated for reduced grain numbers by sustaining high average grain weights of 0.045 g. Thousand grain weights ranged between 40 and 45 g, where non-fertilized crops expressed heavier grains. The three nitrogen treatments attained equal harvest index of 0.2 in HTR. Both ammonium and nitrate fertilized crops under LTR had 0.1 while the non-fertilized crops attained 0.2.

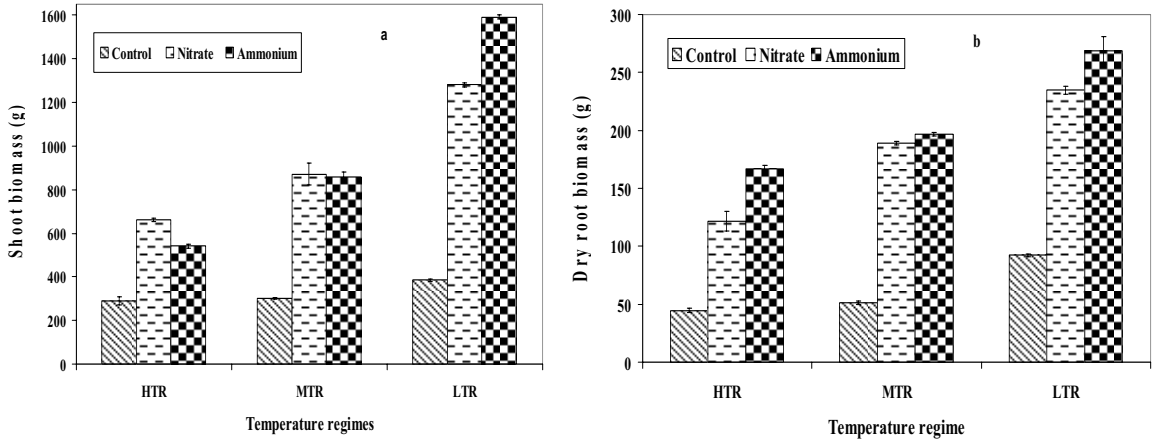

Fig. 28a-b: Shoot (a) and root biomasses (b) of barley crop fertilized by different nitrogen forms and subjected to three different growth temperatures.

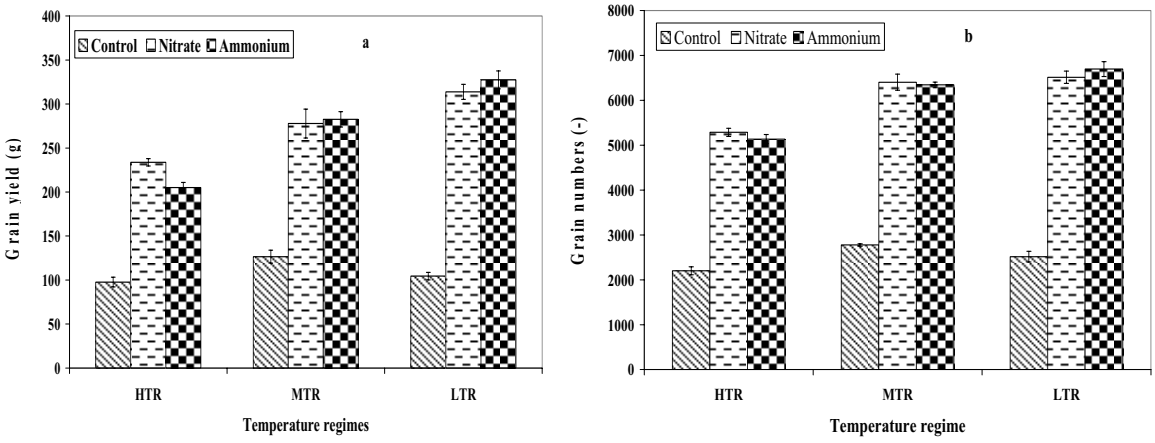

Fig. 29a-b: Total barley grain weights (a) and numbers (b) attained by the three different nitrogen fertilization regimes in the three different growth temperatures.

3.5.4 Response of yield-forming factors to temperature and CULTAN fertilization

To determine the effect of growth temperatures and nitrogen forms on yield forming factors, tiller numbers as well as ear numbers (fertilized tillers) per treatment were counted and compared. The results confirmed that tiller numbers directly influenced grain yield (Fig. 30a). Fertilized crops had more numbers of surviving tillers at physiological maturity than non-fertilized crops. Tiller numbers of non-fertilized crops were not influenced by growth temperatures. The average number of tillers per plant in non-fertilized treatment was three across all the temperature regimes (data not shown). However, tiller numbers increased among fertilized crops as growth temperatures reduced (Fig. 30a). Ammonium injection supported more tiller numbers under both LTR and MTR in comparison to NO_3^-. Interestingly, NO_3^- was superior to NH_4^+ under HTR (Fig. 30a). Tiller numbers per plant supported by nitrate were five and six in HTR and LTR while ammonium had six and seven in HTR and LTR, respectively (data not shown). Despite the infertility of some tillers, most treatments attained appreciable fertility levels. Tiller bearing ears directly influenced the attained grain yields, whereas both fertile and infertile tillers contributed to the recorded shoot biomasses (Fig. 30b). The results illustrated that ear bearing tillers directly impacted upon grain yields. Nitrate fertilized crops in HTR recorded 27% infertile tillers, whereas ammonium and non-fertilized control had 10 and 18%, respectively. The percentage of infertile tillers among ammonium and non-fertilized crops in LTR were 11 and 18%, respectively, whereas nitrate fertilized crops recorded 7%.

3.5.5 Carbon exchange rates and SPAD

To assess the influence of growth temperature and nitrogen forms on crop physiological process performance such as photosynthesis, the carbon exchange rates (CERs) were estimated at flowering stage and compared among the nitrogen and temperature regimes. Nitrogen fertilization significantly impacted upon the photosynthetic rate, but no statistical differences were observed among the temperature regimes of the fertilized crops (Fig. 31a). CER among nitrate fertilized crops consistently decreased as growth temperature decreased, though the margin was quite low. Similarly, SPAD values were equal among the fertilized crops at 40 SPAD values across the fertilized crops while non-fertilized crops oscillated between 20 and 30 SPAD values across the three temperature regimes (Fig. 31b).

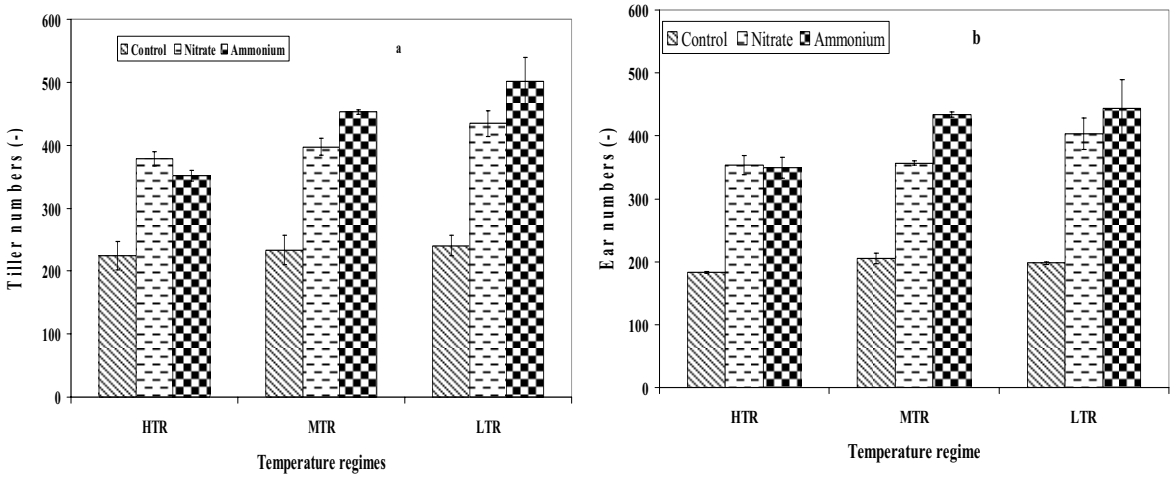

Fig. 30a-b: Total number of barley tillers (a) and ear bearing tillers (b) at crop maturity in the three nitrogen treatments across the three temperature regimes.

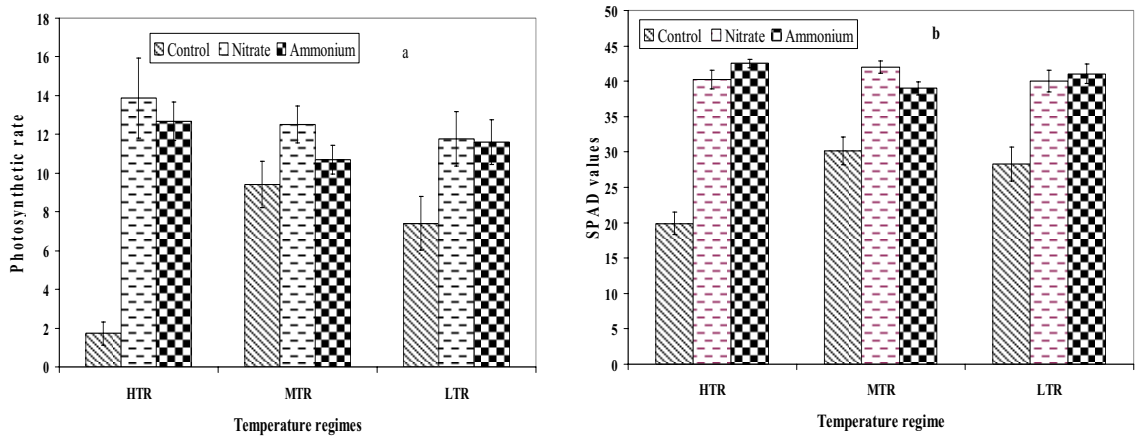

Fig. 31a-b: Average photosynthetic rates (a) and SPAD values of barley at flowering stage in the three nitrogen treatments across the three temperature regimes

3.5.6 Soil and crop nutrient concentrations

Soil sample analysis revealed the occurrence of both NO_3^- and NH_4^+ N nutrition forms among the three nitrogen treatments in each temperature regime, albeit at low concentrations (Table 5). Zones 1 and 2 supported high phosphorus concentrations than 3 and 4 among the three temperature regimes (Table 5). Soil pH level analysis indicated that most samples from different zones were more or less within the neutral range irrespective of the temperature regime. The only exceptions were ammonium treatments under HTR and MTR in zones 1 and 2 that experienced acidic pH values (Table 5). K^+ concentrations were quite stable across the temperature regimes. However, K^+ in non-fertilized soils increased with increasing depths.

Attempts were also made to assess the effect of temperature on mineral nutrient availability within soil and concentrations with the crops at the end of the season. Crop chemical constituent analysis showed that shoot portions had higher K^+ concentrations than grains and roots irrespective of nitrogen treatment (Table 6). Nitrate and non-fertilized crop K^+ concentrations significantly differed among the three growth temperature regimes at $p < 0.002$, whereas ammonium fertilized crops were not significantly different. Chloride concentrations were higher in the shoot of ammonium than nitrate and non-fertilized crops (Table 6). Higher chloride concentrations were taken up by HTR grown crops than those under the other two temperature regimes. Crop NO_3^- concentration was consistently low and insignificant irrespective of plant tissue (Table 6). Plant tissue total N was highest in grain with very low amounts recovered in the shoot portion. Growth temperatures influenced plant N partitioning with roots of HTR grown crops accumulating higher concentrations than those under both MTR and LTR. Both grain and shoot N concentrations were not influenced by either nitrogen or temperature treatment (Table 6). Neither glucose nor fructose was detected in both root and grain samples, although stems recorded appreciable concentrations (Table 7). Low fructose concentrations were available in stems, roots and grains, and their proportions substantially increased with decreases in temperature regimes among the nitrogen treatments. Starch concentrations in grains were rather constant at about 66 mg g^{-1} DM in the three growth temperatures irrespective of the nitrogen form supplied (Table 7). None was detected in stems while only traces were available in root samples across the temperatures (Table 7). Starch concentrations in grains were statistically different from those in roots at $p < 0.0002$.

Table 5: Concentrations of ammonium, nitrate, phosphorus, potassium nutrients and pH values of soil samples from three different temperature regimes at the end of the season upon fertilization with two different nitrogen forms

Temp	Nitrogen	Zone	NH_4^+	NO_3^-	PO_4^-	K^+	pH
HTR	**Control**	1	1.27±0.09 aA	1.96±0.82 aA	33.45±2.37 bB	4.55±0.09 aA	7.4±0.10 aA
		2	1.16±0.02 aA	2.33±0.55 aA	45.07±3.06 bA	6.75±0.59 aA	7.3±0.09 aA
		3	1.24±0.03 aA	3.71±1.40 aA	40.07±4.26 aA	8.38±1.13 aA	7.3±0.06 bA
		4	1.18±0.04 aA	3.21±1.19 aA	36.30±1.24 abA	8.45±0.80 aA	7.3±0.05 aAB
	Nitrate	1	1.16±0.89 aA	2.50±0.67 aA	31.05±2.11 bA	3.35±0.50 aA	7.7±0.10 aA
		2	1.17±0.09 aA	2.08±0.72 aA	42.68±5.59 bA	3.73±0.19 aA	7.7±0.09 aB
		3	1.02±0.02 bA	3.42±1.19 aA	40.45±1.58 aA	5.08±0.25 bA	7.5±0.06 aA
		4	0.97±0.02 bA	3.53±2.34 aA	44.50±5.13 aA	5.30±0.51 bA	7.4±0.07 aB
	Ammonium	1	1.40±0.18 aA	2.74±1.11 aA	236.7±32.2 aA	5.05±0.71 aA	6.5±0.28 bA
		2	1.18±0.05 aA	6.96±2.67 aA	170.1±38.9 aA	6.25±1.25 aA	6.5±0.16 bB
		3	1.06±0.04 bA	2.58±0.70 aA	30.83±0.99 aA	5.05±0.59 bA	7.4±0.06 abA
		4	1.05±0.08 bA	2.09±0.92 aA	26.25±0.76 bA	5.83±0.19 bA	7.4±0.11 aA
MTR	**Control**	1	1.18±0.06 aA	2.41±1.27 aA	43.4±2.14 bA	4.70±0.90 aA	7.5±0.90 aA
		2	1.21±0.09 aA	1.83±1.37 aA	43.1±3.24 bA	5.70±1.07 aA	7.5±0.09 aA
		3	1.18±0.07 aA	0.57±0.31 aA	38.9±2.77 aA	6.80±1.05 aA	7.5±0.07 aA
		4	1.18±0.06 aA	0.62±0.40 aA	39.5±4.63 aA	8.85±1.49 aA	7.5±0.05 aA
	Nitrate	1	1.17±0.07 aA	1.70±0.76 aAB	39.8±4.49 bA	4.33±0.54 aA	7.4±0.04 aB
		2	1.14±0.05 aA	2.85±0.96 aAB	40.0±4.65 bA	5.03±1.18 aA	7.4±0.03 aB
		3	1.18±0.04 aA	2.05±0.83 aA	48.3±4.60 aA	6.22±1.27 aA	7.5±0.07 aA
		4	1.24±0.08 aA	1.34±0.62 aA	39.4±2.40 aA	6.30±1.17 aA	7.4±0.03 aB
	Ammonium	1	1.23±0.04 aA	3.08±1.97 aA	200.3±14.8 aA	5.28±1.29 aA	6.8±0.14 bA
		2	1.24±0.04 aA	4.31±2.59 aA	206.9±14.6 aA	6.88±0.77 aA	6.6±0.08 bB
		3	1.26±0.07 aA	2.43±1.43 aA	35.43±3.49 aA	6.00±0.67 aA	7.4±0.07 aA
		4	1.14±0.01 aA	0.32±0.17 aA	30.93±1.84 aA	6.53±0.83 aA	7.5±0.05 aA
LTR	**Control**	1	1.08±0.02 aA	0.56±0.42 aA	36.6±2.56 bAB	5.35±0.55 aA	7.4±0.08 aA
		2	1.17±0.02 aA	0.88±0.51 aA	40.5±1.93 aA	8.50±0.57 aA	7.3±0.07 bA
		3	1.21±0.06 aA	1.08±0.63 aA	40.6±3.77 aA	10.3±0.68 aA	7.3±0.09 bA
		4	1.30±0.14 aA	1.52±0.74 aA	41.2±6.10 aA	11.5±0.86 aA	7.3±0.06 bB
	Nitrate	1	1.17±0.70 aA	1.70±0.76 aB	39.8±4.89 bA	4.33±0.54 aA	7.4±0.40 aAB
		2	0.76±0.25 aA	0.07±0.05 aB	34.8±1.92 aA	4.65±0.69 bA	7.6±0.04 aA
		3	1.07±0.36 aA	0.05±0.03 aB	43.3±2.38 aA	6.38±0.41 bA	7.6±0.04 aA
		4	1.29±0.70 aA	0.36±0.31 aA	40.2±2.33 aA	6.63±1.51 bA	7.7±0.07 aA
	Ammonium	1	1.15±0.18 aA	0.44±0.30 aA	183.3±20.4 aA	6.40±1.18 aA	7.0±0.09 bA
		2	0.83±0.30 aA	0.62±0.37 aA	147.0±55.1 aA	6.45±0.57 abA	7.2±0.09 bA
		3	0.88±0.23 aA	0.61±0.36 aA	56.5±26.88 aA	5.90±0.52 bA	7.4±0.04 abA
		4	0.97±0.22 aA	0.87±0.42 aA	30.70±0.67 aA	7.85±0.49 abA	7.5±0.03 abA

Means followed by same small and capital letters within same columns are not significantly different from each other within the same and among different temperature regimes, respectively ($p < 0.05$) Tukey test.

Table 6: Chemical composition of grain, shoot roots harvested from three nitrogen treatments at the end of the season in the three temperature regimes

		HTR			MTR			LTR		
		NH4	**NO3**	**Control**	**NH4**	**NO3**	**Control**	**NH4**	**NO3**	**Control**
Grains	**P**	0.46 ± 0.01 aA	0.47 ± 0.01 aA	0.46 ± 0.01 aA	0.40 ± 0.02 aA	0.40 ± 0.01 aA	0.39 ± 0.01 aA	0.36 ± 0.02 aA	0.35 ± 0.02 aA	0.36 ± 0.01 aA
	K	0.70 ± 0.04 aA	0.72 ± 0.04 aA	0.65 ± 0.05 aA	0.79 ± 0.06 aA	0.82 ± 0.09 aA	0.56 ± 0.04 bB	0.60 ± 0.01 aB	0.60 ± 0.01 aB	0.61 ± 0.03aAB
	Cl-	8.42 ± 3.46 aA	8.11 ± 3.25 aA	6.07 ± 2.34 bB	7.03 ± 2.43 aB	6.57 ± 2.41abA	5.52 ± 1.91 bB	7.23 ± 2.61 aA	7.83 ± 2.80 aA	6.31 ± 2.10 aB
	Total N	1.68 ± 0.03 aA	1.63 ± 0.02 aA	1.47 ± 0.05 aA	1.43 ± 0.05 aA	1.43 ± 0.06aA	1.44 ± 0.05 aA	1.46 ± 0.05 aA	1.41 ± 0.05 aA	1.29 ± 0.05 bB
	NO_3^-	0.01 ± 0.001aA	0.01 ± 0.001aA	0.01 ± 0.001 aA	0.01 ± 0.001aB	0.05 ± 0.002 A	0.04 ± 0.003 aA	0.00 ± 0.001aB	0.03 ± 0.002 A	0.00 ± 0.001 aA
Shoot	**P**	0.152 ± 0.02 aA	0.107 ± 0.01 bA	0.107 ± 0.01 bA	0.105 ± 0.01 aA	0.106 ± 0.01 aA	0.075 ± 0.01 aC	0.051 ± 0.00 aC	0.065 ± 0.01 aC	0.046 ± 0.00 aC
	K	2.567 ± 0.11abA	2.636 ± 0.14 bA	3.07 ± 0.20 aA	2.414 ± 0.22 aA	2.387 ± 0.03 aB	2.262 ± 0.18 aB	1.6945 ± 0.08 aC	1.674 ± 0.08 aC	1.636 ± 0.10 aC
	Cl-	108.78 ± 47.1 bA	94.505 ± 39.4abB	116.31 ± 7.66 aA	78.124 ± 28.7 aB	80.265 ± 32.3 aB	74.606 ± 26.9 aB	70.743 ± 30.0 bB	59.805 ± 23.6 aC	64.997 ± 26.5 abC
	Total N	0.748 ± 0.01 aA	0.768 ± 0.03 aA	0.725 ± 0.03 aA	0.605 ± 0.03 bA	0.685 ± 0.05 aA	0.623 ± 0.04 bA	0.650 ± 0.04 aA	0.668 ± 0.05 aA	0.633 ± 0.04 aA
	NO_3^-	0.053 ± 0.02 aA	0.055 ± 0.02 aA	0.015 ± 0.02 aA	0.045 ± 0.05 aA	0.103 ± 0.08 aA	0.00 ± 0.00 aB	0.00 ± 0.00 aB	0.00 ± 0.00 aB	0.009 ± 0.01 aB
Root	**P**	0.181 ± 0.01 bA	0.155 ± 0.01abA	0.213 ± 0.03 aA	0.161 ± 0.03 aA	0.140 ± 0.02 aB	0.166 ± 0.04 aA	0.168 ± 0.02 aA	0.134 ± 0.01 aB	0.122 ± 0.01 aA
	K	0.567 ± 0.29 aA	0.240 ± 0.04 aA	0.283 ± 0.06 aA	0.299 ± 0.07 aA	0.318 ± 0.06 aA	0.247 ± 0.04 aA	0.261 ± 0.03 aA	0.227 ± 0.02 aA	0.257 ± 0.01 aA
	Cl-	1.428 ± 0.41 aC	1.555 ± 0.59 aC	1.405 ± 0.54 aC	4.014 ± 2.03 aB	3.863 ± 1.99 aB	3.458 ± 1.75 aB	7.524 ± 4.14 aA	7.395 ± 3.63 aA	6.064 ± 3.32 aA
	Total N	1.170 ± 0.04 aA	1.083 ± 0.03 aA	1.073 ± 0.05 aA	0.955 ± 0.05 aA	0.968 ± 0.03 aA	0.880 ± 0.05 aA	0.910 ± 0.05 aA	0.910 ± 0.07 aA	0.838 ± 0.05 aA
	NO_3^-	0.054 ± 0.02 aA	0.036 ± 0.01 aA	0.056 ± 0.02 aA	0.083 ± 0.03 aA	0.079 ± 0.04 aA	0.097 ± 0.05 aA	0.047 ± 0.02 aA	0.059 ± 0.02abA	0.020 ± 0.01 bA

Means followed by same small and capital letters across rows are not significantly different from each other within the same and among different temperature regimes, respectively ($p < 0.05$) Tukey test.

Table7: Sugar concentrations recorded from grain, shoot and roots of nitrogen treated barley harvested from three temperature regimes

		HTR			MTR			LTR		
		NH4	**NO_3^-**	**Control**	**NH4**	**NO_3^-**	**Control**	**NH4**	**NO_3^-**	**Control**
Grains	**Sucrose**	1.39 ± 0.39 aA	0.95 ± 0.04 aA	1.10 ± 0.25 aA	1.72 ± 0.36aA	1.76 ± 0.35 aA	0.98 ± 0.15 aA	1.027 ± 0.75aA	1.39 ± 0.95 aA	1.98 ± 0.67 aA
	Glucose	0.00 ± 0.00 aA	0.00 ± 0.0 aA	0.00 ± 0.00 aA	0.00 ± 0.00aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA
	Fructose	0.00 ± 0.00 aA	0.06 ± 0.01 aA	0.00 ± 0.00 aA	0.06 ± 0.02aA	0.064 ± 0.01 aA	0.053 ± 0.01 aA	0.05 ± 0.01aA	0.09 ± 0.01 aA	0.02 ± 0.01 aA
	Stacrch	67.4 ± 1.39 aA	66.7 ± 1.19 aA	66.9 ± 1.90 aA	66.5 ± 0.99aA	67.7 ± 1.17 aA	67.2 ± 1.80 aA	67.3 ± 1.42aA	66.0 ± 0.74 aA	66.5 ± 2.04 aA
Shoot	**Sucrose**	0.00 ± 0.00 bB	0.13 ± 0.08 bB	0.46 ± 0.11 bB	0.65 ± 0.26aB	0.94 ± 0.27 aA	1.36 ± 0.19 aA	1.58 ± 0.22aA	1.47 ± 0.22 aA	1.84 ± 0.12 aA
	Glucose	1.20 ± 0.56 aA	0.49 ± 0.29 aA	2.19 ± 0.23 aA	2.13 ± 0.56aA	2.68 ± 0.12 aA	2.39 ± 0.47 aA	1.63 ± 0.27aA	1.73 ± 0.32 aA	1.67 ± 0.22 aA
	Fructose	0.19 ± 0.02 aC	0.17 ± 0.06 aC	0.99 ± 0.14 aC	2.18 ± 0.56aB	3.30 ± 0.76 aB	4.19 ± 0.73 aB	5.14 ± 0.20aA	5.75 ± 0.87 aA	5.22 ± 0.16 aA
	Stacrch	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.00 ± 0.00aA	0.00 ± 0.00 aA	0.00 ± 0.00 aA
Root	**Sucrose**	0.02 ± 0.00 aA	0.11 ± 0.09 aA	0.13 ± 0.08 aA	0.04 ± 0.00aA	0.11 ± 0.07 aA	0.20 ± 0.12 aA	0.13 ± 0.08aA	0.21 ± 0.11 aA	0.22 ± 0.06 aA
	Glucose	0.00 ± 0.00 aA	0.00 ± 0.00 aA	0.01 ± 0.01 aA	0.07 ± 0.04aA	0.07 ± 0.01 aA	0.08 ± 0.05 aA	0.08 ± 0.01aA	0.21 ± 0.15 aA	0.10 ± 0.06 aA
	Fructose	0.00 ± 0.00 aC	0.02 ± 0.02 aC	0.08 ± 0.05 aC	0.18 ± 0.17aB	0.16 ± 0.09 aB	0.21 ± 0.12 aA	0.25 ± 0.13aA	0.324 ± 0.11 aA	0.17 ± 0.10 aB
	Stacrch	1.99 ± 0.14 aA	1.41 ± 0.47 aA	1.66 ± 0.19 aA	0.89 ± 0.37aA	1.21 ± 0.31 aA	1.19 ± 0.56 aA	0.94 ± 0.38aA	0.96 ± 0.36 aA	0.85 ± 0.44 aA

Means followed by same small and capital letters across rows are not significantly different from each other within the same and among different temperature regimes, respectively ($p < 0.05$) Tukey test.

4 DISCUSSION

4.1 Stability of soil-injected liquid NH_4^+

Results generated in this study have demonstrated the capability of a single injection of liquid ammonium fertilizer applied through the CULTAN technique to supply sufficient N for barley production from sowing through seedling emergence to physiological maturity. In addition, the results revealed the presence of concentrated NH_4^+–N depots within the injection points. The successful implementation of this fertilization technique has illustrated the potential of CULTAN as an alternative fertilizer application in comparison to conventional methods such as broadcasting or top dressing where nitrate fertilizers are applied on the soil surface thus necessitating split application to match crop N demand at different growth stages by ensuring adequacy through staggered applications with a view to minimizing losses (Sowers et al., 1994). Split application however, increases the number of farm operations, which eventually reduce the profit margins. CULTAN is thus understood to be ecologically benign and economically sound as it reduces the number of operations and N losses within the soil through the formation of concentrated NH_4^+–N depots.

4.1.1 Establishment of NH_4^+–N deposition zones

The formation of concentrated NH_4^+–N deposition zones within the injection point (Fig. 5a-b) was in agreement with the finding on Sommer (2000) who also observed the formation of a 'sorption-complex' zone. This could have resulted from the unspecific binding of NH_4^+ by the 2:1 clay interlayer. This in effect impacted upon the mobility of the ions within the soil in this study in a similar manner as noted by Sommer (1999). The high concentrations of NH_4^+ – N could have perhaps helped in the reduction of N losses commonly caused by leaching and denitrification of NO_3^-–N. NH_4^+–N adherence onto clay matrix and humus within the injection zone enabled the development of concentrated deposition zones whose diffusion to the outer zones was low. These findings are in agreement with those reported on wheat and sugar beet by Sommer (2000), maize and beans by Feng et al. (1997) as well as barley by Schittenhelm and Menge (2006). While coining the term, controlled uptake long term ammonium nutrition (CULTAN), Sommer

(2000) relied upon the retention of high NH_4^+–N concentration ensured by its sorption onto clay matrix. However, the unbound free NH_3 within the injection-point causes toxicity among the non-tolerant crops, which further facilitates the regulation of its uptake. Presently, there are different models of modern farm implements used for CULTAN technique fertilizer application (Baker et al., 1989). Photographs showing fingered spoked-wheels and injection shares have been provided (Fig. 2). Additional types include tines or knives which are equipped with nozzles for mechanized NH_4^+-N application into soil. They are adapted for large scale mechanized farm operations.

The stability of NH_4^+–N indicated by its predomination during the early season could have been facilitated by the formation of NH_4^+ sorption-complex zone within the injection-point. These highly concentrated NH_4^+ -N zones resulted into the formation and presence of locally enriched NH_4^+–N patches within the injection points coupled up with acidic pH levels (Fig. 5a-b and 7a-b). Since plants rely mainly upon root interception of nutrients to meet their requirements (Miller and Cramer, 2004), the young barley crops growing at distances far away from the injection zones could have been constrained in their acquisition of the injected NH_4^+–N. On the other hand, seedlings emerging at distances very close to the injection-point came into contact with the highly toxic zone, which probably caused the exhibited mild visual NH_4^+–N toxicity symptoms. NH_4^+–N diffuses slowly in soil and thus leads to low mobility (Owen and Jones, 2001). The low diffusion rate is a constraint to the distribution of NH_4^+ (Barber, 1984). The low diffusion offers an insight into potential constraint of NH_4^+-N distribution and subsequent hindrance to its accessibility by roots of young plants growing at distances away from the injection point. This same phenomenon of low NH_4^+–N mobility along side its toxicity possibly resulted into a corollary effect of slow uptake and/or release, a pillar upon which long-term N supply to crops relies (Sommer, 2000). The short-term constraint of NH_4^+-N inaccessibility could have been overridden by the long-term N availability when roots accessed the NH_4^+-N within the injection depots. The observed growth responses in the early period of NH_4^+-N fertilized crops used in this study could have been caused by the inaccessibility of NH_4^+–N by barley roots away from the injection-points or growth suppression by the high concentrations. Despite the few limitations associated with NH_4^+-N, it has been reported by Colmer and Bloom (1998) that whenever accessed, NH_4^+–N is readily taken up in large quantities as compared to NO_3^-–N. It could be suggested that the

rapid NH_4^+–N concentration reduction resulted from use by bacteria or crops among other fates (Fig. 5a-b). This could be an indication of its preference for uptake by barley roots or rapid immobilization by soil microbes as noted by (Kuzyakov et al., 2000).

The possible inaccessibility of NH_4^+–N by crops away from the injection zones therefore emphasizes the need to establish correct NH_4^+-N injection spacing for proper deposition of fertilizer within intervals that would allow easy access due to the overlap of the developing depots within NH_4^+–N diffusion zones. In addition to this, the injection depth and the concentrations to be used are both crucial considerations to be made. An injection depth of between 7 and 15 cm below the soil surface has been proposed as optimal for cereal crops (Sommer, 2000), though this may vary greatly with crop species and soil type among other factors. Proper inter and intra-row sowing spaces and well adjusted injection distances can ensure a rather uniform distribution of the injected N. Though the low NH_4^+–N diffusion rate limits its expansion into the peripheral zones, the same factor ensures its stability within the sorption complex which plays a great role in its transformation suppression.

The incorporation of nitrification inhibitor successfully suppressed the nitrification process. Considerable evidence has revealed the involvement of specific metal cofactors in causing nitrification inhibition (Campbell and Aleem, 1965). Copper is thought to be the main cause of the inhibitory effects exhibited by Nitrapyrin® [2-chloro-6-(trichloromethyl) pyridine] (Powell and Prosser, 1986). Effectiveness of nitrification inhibitors relies upon their abilities to act as alternative substrates (Rasche et al., 1990). Nitrapyrin under aerobic oxidation can produce 6-chloropicolinic acid while on the other hand it can undergo a reductive dehalogenation under anaerobic condition to produce 2-Chloro-6-dichloromethyl-pyridine (Vannelli and Hooper, 1992). The incorporation of 5 and 20% NI effectively suppressed the nitrification process. Mahli and Nyborg (1986; 1992) witnessed yield improvements with NI incorporation. However, (Maschner, 1995) has reported that the oxidation product of Nitrapyrin when taken up by crops may cause toxicity effects. It is not clear whether such interactive effects could have occurred on the barley crops as a result of the NI incorporation. Despite this potential negative effect, nitrification inhibitors have practical importance in increasing crop yield through the improvement of crop nitrogen use efficiency.

4.1.2 Nitrogen forms available in CULTAN-fertilized soils

The N form taken up by a plant has far reaching consequences not only in its metabolism, but also on the short and long-distance transport processes (Schubert and Yan, 1996). In this study, non-ammonium based treatments (nitrate and non-fertilized control) retained NO_3^-–N as the dominant N form, even though the quantities witnessed in non-fertilized treatments were quite low. The occurrence of NO_3^-–N in non-fertilized control suggested the occurrence of N mineralization. It is though not clear if the same could have occurred in the NO_3^-–N and NH_4^+–N fertilized treatments. Nevertheless, the ability of barley crop to survive in the non-fertilized control treatment until physiological maturity confirmed the occurrence of sufficient N concentrations necessary to support basic plant functions and eventual grain yield formation. In comparison to these treatments, application of diammonium phosphate fertilizer through CULTAN made available large concentrations of phosphorus in addition to NH_4^+. Though NH_4^+–N dominated during the early season, the presence of high phosphorus concentrations within the rooting zone could have also contributed to the observed root proliferation. However, the recovery of high NO_3^-–N concentrations in CULTAN fertilized treatments without NI-inhibitor incorporation suggested the occurrence of more than one nitrogen form commonly referred to as mixed N nutrition (Fig. 6a-b).

On the other hand, NH_4^+–N treatments incorporated with 5 and 20% NI were effectively suppressed from experiencing reasonable nitrification and thus retained NH_4^+-N as the predominant N form. The recovery of high NH_4^+–N concentrations within zones of 5 and 20% NI-incorporated treatments indicated the presence of localized NH_4^+–N regions particularly with regard to the injection point. Given that the injected NH_4^+– N greatly influenced the total soil nitrogen while total carbon remained relatively constant, it created imbalances in the carbon : nitrogen ratio. It was not clear where and how the excess N availed through CULTAN fertilization was disposed. However, evaluation of N dynamics regulating factors like microbial activity in CULTAN fertilized soils, especially the role played by ammonia oxidizing bacteria in transforming NH_4^+ into NO_3^- indicated their influence on the extractable N. Essentially, this is the first experimental evidence

reporting the occurrence of mixed N nutrition under CULTAN which has mostly been presumed to provide sole NH_4^+.

In this study, NH_4^+–injected treatments were dominated by high NH_4^+–N especially during the early season after which the occurrence of large NO_3^-–N concentrations were recorded from 30 DAF among the treatments without NI-incorporation (Fig. 6a-b). Apparently, this period which recorded the highest NO_3^-–N concentration coincided with the occurrence of the largest bacterial community diversity in general and more specifically the highest number of ammonia oxidizers. However, there were no microbial diversity differences between 5% NI-incorporated and non-NI incorporated treatments whereas they were inhabited by a large diversity of bacteria at 30 DAS (Matoka et al., 2007a). The study demonstrated the accumulation of large NO_3^-N, however, it was not examined to what extent N losses could have been contributed to by leaching (Rao and Popham, 1999) and denitrification (Zumpft, 1997). Nitrification process occurrence in non NI-incorporated treatments notwithstanding the recovery of low NO_3^-–N levels within zones 3 and 4 below the injection-point discounts major contribution of NO_3^-–N losses through leaching. Therefore, if any NO_3^-–N losses could have occurred, it could have majorly resulted from denitrification whose estimates are not known since it was not attempted.

The mode of irrigation adopted in this study where water was supplied from bottom of the sowing containers through openings to the upper layers of the soil where NH_4^+ injection was performed at a depth of 7 cm could have had an influence. It would be important to assess what the situation is likely to be under field condition where lysimeters are installed to determine how much N loss occurs potentially throughout the entire season. The presence of high NO_3^- concentrations in uncropped NH_4^+ treatment without NI incorporation could be explained by the absence of crops to utilize the nutrients. Differences between uncropped and cropped NH_4^+ treatments gave a rough estimate of NO_3^-–N taken up by crops on the assumption that denitrification was negligible especially under well aerated soil conditions. The occurrence of N form nutrition shift in contrast to the earlier presumptions that CULTAN fertilization with NH_4^+–N provided mainly NH_4^+ for crop uptake seem not to be always the case. It was however, not clear which of the two N forms occurring in the mixed N nutrition under

CULTAN fertilization was preferred for up take. Our findings closely resemble those of Kirk and Kronzucker (2005) who also reported the occurrence of a mixed N nutrition within the rhizosphere of flooded rice fields.

4.1.3 Barley root growth responses to CULTAN fertilization

Barley root growth under CULTAN fertilization showed morphological responses expressed by root architectural change. The NH_4^+ fertilized crops developed a root architecture dominated by intensively networked system with short and heavily branched structure. Along side these, several short and thin root structures which could have been involved in NH_4^+–N uptake from the toxic regions were also observed (Fig. 9b). This resulted into an enhanced barley root proliferation around NH_4^+–N injection-point. Moreover, the root mass formed a ring around the injection zones, but they did not penetrate through the toxic NH_4^+–N injection zone. During the early season when NH_4^+–N dominated as the major N form, the injection core (zone 2a) remained devoid of any root growth. The root growth inhibition perhaps resulted from the high NH_4^+–N toxicity within the injection-point caused by free unbound NH_3. However, this zone was surrounded by several highly branched roots with dense network which could have possibly aided NH_4^+-N uptake from the toxic zone. On the contrary, non-fertilized control and NO_3^-– fertilized crops did not express similar root growth morphological features as those observed under CULTAN. Nitrate and non-fertilized treatments were dominated by sparsely distributed and less branched roots (Fig. 9c).

The presence of high NH_4^+-N concentrations was presumably the cause of root growth inhibition within the injection point/core (zone 2a) (Fig. 5a-b; 9b). The absence of root penetration through the toxic zone showed the effectiveness of high NH_4^+-N inhibition of root growth in concert with soil pH (Fig. 7a-b). However, the mechanism of how this occurs is not well understood. Furthermore, the morphological, physiological and molecular effects of NH_4^+, NO_3^- and inorganic phosphate concurrence to crops fertilized with CULTAN method have not been fully evaluated. Elsewhere, fertilized local soil patches forming pockets of rich NH_4^+ and NO_3^- have been individually shown to enhance root proliferation (Zhang and Rengel, 1999; Zhang et al., 1999). More recently, Wach-Liu (2006) reported the response of roots to NH_4^+, NO_3^- and inorganic

phosphates. This array of information has sparked off new questions with regard to CULTAN fertilization technique. It is not clear, which of the nutrients actually causes the observed crop growth responses. It is also not yet known if there could be occurring synergism or antagonism with regard to nutrient uptake. Generally, barley root plasticity response to rich ammonium dominated regions could have improved its exploration of the soil patches enriched with both nitrogen and phosphorus. In addition, this feature could have enabled the crop to effectively compete for the limited soil resources especially with the soil occurring microbes. The preferential shoot growths within habitats rich in essential growth resources such as light have been reported (Lepik et al., 2005). Zhang and Forde (2000) noted that exposure of plant to localized NH_4^+, NO_3^- and inorganic P sources cause root proliferation within the nutrient rich zones.

The spatial variations in N distribution within the CULTAN fertilized treatments, especially the presence of low N in zones far away from the injection point potentially aid N occurrence in patches when diffusion zones fail to overlap. The gradual transformation of NH_4^+–N to NO_3^-–N highlights the occurrence a shift in N-form and quantity available for crop uptake over space (zones) and time (different periods of the season). Drew and Saker (1975) reported that the response of barley to localized NO_3^-–N resulted from a combination of increased lateral root numbers and their elongation, whereas Zhang and Forde (1998) observed that localized NO_3^- increased lateral root numbers of Arabidopsis. Zhang and colleagues noted that these responses could be caused by a signaling effect of NO_3^- ion itself rather than down-stream metabolites resulting from its assimilation (Zhang et al., 1999). Wach-Liu et al. (2006) emphasized that shifts caused on root growth due to their modifications are general expressions of both intrinsic and extrinsic factors. Probably, inhibition of barley crop root elongation under CULTAN fertilization due to the highly concentrated NH_4^+–N, which trigger heavy lateral root branching as a compensatory strategy to access NH_4^+–N whose uptake is reported to be rapid at root apex (Bloom et al., 2003).

Nitrate has been reported to regulate plant growth by signalling through two distinct pathways namely, an external positive and internal negative mechanism (Zhang and Forde, 2000). The observation of root growth proliferation under CULTAN fertilization due to the direct contact between the roots and enriched NO_3^- patches, a

phenomenon referred to by Zhang and Forde (2000) as localized stimulatory effect could have occurred around the injection zones that underwent the nitrification process. In addition to the localized stimulatory effect, both NH_4^+ and NO_3^-–N availability could have also influenced crop growth through nutrition mediated effects. An alternative explanation for the intense rooting system under CULTAN could be increased metabolic activity within the roots which probably led to growth stimulation thus causing influx of carbohydrates and auxin production. Since the crops recorded very high chlorophyll concentrations during the early crop growth stages when NH_4^+ uptake could have predominated (Fig. 15a-b), the pigments could have equivalently supported high photosynthetic rates. This might have possibly supplied adequate amounts of carbohydrates required for NH_4^+ assimilation. However, the chlorophyll concentrations decreased rapidly an indication that senescence was experienced by the crops among all the treatments, though at different periods of onset. The presence of low carotenoid concentrations indicated the absence of light stress (Fig. 15d).

Since CULTAN technique provides mainly NH_4^+ in the early season and thereafter shifts to a mixed N nutrition with the accumulation of NO_3^- (Matoka et al., 2007b), it was not clear which of the N form could have contributed majorly to the observed suite of root growth responses. Converse to this, root growth inhibition in the highly concentrated NH_4^+–N depot zones could have been caused by a local inhibitory effect working in concert with the acidic pH values. The possibility of long-distance signal occurrence as suggested by Zhang et al. (1999) cannot be ignored given the fact high tissue NO_3^- concentrations were also observed in some of the treatments especially in ammonium without NI-incorporation (Fig. 12b). It is not known whether any of these mechanisms was involved in causing the observed responses. Further research is warranted to elucidate the cause of the observed root growth responses and possible effects of hormones under CULTAN fertilized crops.

4.1.4 Barley aerial growth responses to CULTAN fertilization

CULTAN fertilization technique relies upon NH_4^+ sorption onto 2:1 clay interlayer. The unbound free ammonia cause toxicity, which suppress root growth into the injection-zones and thus guarantee N uptake hence optimizes N utilization by coupling carbohydrate during the assimilation into nitrogenous compounds, which are

eventually translocated into the shoot (Weimer and Sommer, 1990). When taken up in sufficient concentrations, NH_4^+–N supports normal plant growth, whereas high concentrations cause toxicity symptoms (Gerendas et al., 1997; Britto et al., 2001). Generally, ammonium toxicity results from pH imbalances besides anion/cation imbalance (Chaillou et al., 1991) and/or energy drain resulting from the efflux of the ion (Britto and Kronzucker, 2002). Even though ammonium toxicity may be widespread among crops (Britto et al., 2001), their threshold levels to express symptoms are manifested differently among species (Schortemeyer et al., 1997). Theoretically, it has been deduced that the use of only one form of N fertilizer can derive soil pH away from the optimum. However, in some cases, the relief obtained by buffering pH could only be partial (Breteler, 1973), and in many other instances it may be absent (Cox and Reisenauer, 1973; Van Beusichem et al., 1988). It is thus plausible to suggest that barley cv. Maresi used in the study could have benefited from pH-buffering capacity offered by the soil within the regions with acidic pH levels where they grew.

In addition, uptake of mixed N nutrition could have ameliorated NH_4^+ toxicity symptoms. Since ammonium rich soils are typically low in pH (Vitousek et al., 1982), it has thus been proposed that acid tolerance could be a prerequisite for NH_4^+ uptake (Schubert and Yan, 1997), and hence plants which are able to tolerate some level of acidity have the potential of benefiting from NH_4^+-N. Two examples of cereal crops exhibiting varying adaptation thresholds to ammonium such as rice, tolerant (Wang et al., 1993) and barley, susceptible (Britto et al., 2001) express large variations among cultivars. This highlights the fact that tolerance or sensitivity of crops to NH_4^+-N (Schortemeyer et al., 1997) and different growth stages (Vollbrecht et al., 1989) also occur. In spite of rice being NH_4^+-N tolerant crop, high concentrations of NH_4^+-N has been reported to cause toxicity symptoms expressed as yellow leaves and suppressed growth (Liao et al., 1994). In this regard, it is highly probable that N form taken was a mixture of the two (Fig. 6a-b).

Though the barley seedlings regenerated to recover from the observed mild visual symptoms, they however did not seem to restore their chemical constitutions. For instance, NH_4^+ treatments incorporated with 5 and 20% NI took up high concentrations of chloride (Cl^-) (Fig. 13f) and P (Fig. 13d) than the sole NO_3^- and NH_4^+ treatment without NI incorporation fertilized crops. Similar observations were made with respect to the total

ash concentrations (Fig. 14). A peculiar NO_3^--N characteristic observed was the accumulation of large organic acid quantities particularly in nitrate and NH_4^+ treatment without NI-incorporation (Fig. 17a-b). On the other hand, it is thought that the low accumulation of organic acids in NH_4^+ based treatments could have resulted from their use in NH_4^+ assimilation as carbon sources. The corresponding high accumulations of NO_3^- in the tissues of crops fed by NO_3^- -N dominated treatments with nitrate and NH_4^+ treatment without NI-incorporation though non-significant, had substantial accumulations of organic acids (malate and citric) might have acted as osmotic regulators. While the 5 and 20% NI-incorporated treatments demonstrated mild ammonium toxicity symptoms, NH_4^+ treatment without NI incorporation behaved almost like a NO_3^- fertilized crop. This could have been due to high NO_3^--N concentrations. On the reverse, when compared to nitrate fertilized crops, the NI incorporated treatments had lower concentrations of inorganic cations like K^+, Ca^{2+} and Mg^{2+} (Fig. 13a-c).

The cation/anion imbalances found especially in the early crop growth phases in the crops fertilized by NI-incorporated NH_4^+ treatments was further supported by the high total ash concentrations in the corresponding treatments (Fig. 14). A similar observation was made by Scherer et al. (1982). The switching of N sources from NO_3^- to NH_4^+ has been proposed to be the major cause of 'ammoniacal syndrome' (Chaillou et al., 1986), through concurrent NH_4^+ and NO_3^- uptake to alleviate NH_4^+ toxicity symptoms (Houdusse et al., 2005). In deed, extensive evidence is available that NH_4^+ and not NH_3 is the major form of ammoniacal N form taken up, although, passive efflux of NH_3 could also occur (Britto and Kronzucker, 2002). These findings especially those from NH_4^+ treatments incorporated with 5 and 20% NI which expressed significant alterations in the crop chemical constitution pointed to barley crop responses where NH_4^+-N dominated treatments exhibited physiological changes associated with ammonium toxicity even though the visual symptoms became invisible with NO_3^- availability.

The application of NH_4^+ as a major N source has important implications in rhizosphere pH regulation, since the up take of this cation results into a strong acidification. In contrast, the up take of NO_3^- results into net alkalinisation of soil, albeit, at much slower rate than that of acidification associated with NH_4^+ uptake (Maschner et al., 1991). Furthermore, bacterial activity can rapidly convert NH_4^+ to NO_3^- through the nitrification process, thus affecting acidification and the consequent supply of NH_4^+. This

can cause acidification regardless of whether NH_4^+ is taken up by plant roots or not. The net acidification occurrence when NH_4^+ is fertilized and the net alkalinization associated with NO_3^- uptake can also cause differences in solubility, concentration, ionic N form availability and mobility in the soil (Maschner, 1991). Since crop uptake of NH_4^+ increases with increasing pH, high soil pH could exacerbate NH_4^+ toxicity effects (Findenegg, 1987). The availability of NH_4^+ may have also been severely limited by unspecifically binding onto the 2:1 clay interlayer upon replacing K^+. Further, it could have been fixed within the micaceous clay soil minerals besides exploitation by micro-organisms (Lewis, 1986). The problem of limited NH_4^+ availability may be partially overcome in agriculture through additional application of K^+ which increases its availability by competing it for binding sites within the soil thus increasing the exchangeable quantities (Haynes and Goh, 1978) thus prompting its effective utilization, though this could compromise NH_4^+-N sorption complex formation.

4.1.5 Possible mechanisms involved in mixed N nutrition

Growth and yield of most crop species have been reported to be superior under mixed NH_4^+ to NO_3^--N (Wiesler, 1997; Kirk and Kronzucker, 2005). Co-provision of NH_4^+ and NO_3^--N are known to alleviate ammonium toxicity effects (Deignan and Lewis, 1988; Gill and Reisenauer, 1993; Britto *et al.,* 2001). Mixed N forms have the ability to induce synergistic growth responses surpassing maximal growth rates of either N source at a range of between 40 to 70% under hydroponic conditions (Weissman, 1964), with slight increases in soil grown crops (Gill and Reisenauer, 1993). However, in some cases, synergistic responses are absent, while in some cases growth inhibitions occur (Dijk and Eck, 1995). In the current study, the attainable barley grain yields were not superior though comparable to those obtained in nitrate fertilized crop. Moreover, this was obtained despite recoveries of large mixed N nutrition availability especially in NH_4^+ treatment without NI incorporation. This was intriguing and warrant further investigations to unravel possible mechanisms influencing N form uptake and assimilation under CULTAN.

It is known that NO_3^--N absorption is inducible (Tischner, 2000). Barley can effectively attain full NO_3^- uptake induction in 24 h (Kirk and Kronzucker, 2005). However, the presence of both NH_4^+ and NO_3^--N could repress NO_3^- absorption and

assimilation, while NH_4^+ could be stimulated. The occurrence of cation/anion imbalances in crops fertilized through CULTAN is a sign of ion antagonism (Fig. 13a-f). Two possible mechanisms of NH_4^+ involvement in NO_3^- inhibition could have resulted. The first could have been the direct effect of NH_4^+ on NO_3^- reduction and the uptake system associated with it and secondly, the involvement of K^+ as an intermediate regulator of NO_3^- accumulation in the shoot tissue and its translocation. It has been reported that sub-cellular distribution of N absorbed by plants fed by either NH_4^+ or NO_3^- -N could translocate more than 50% NO_3^- -N to the shoot while the loss from the root through efflux was less than 50% (Britto and Krozucker, 2004).

Until recently, it was unclear whether NH_4^+-N absorbed was entirely assimilated within the roots or some were transported into the shoot (Tobin and Yamaya, 2001). Schjoerring et al. (2002) reported that up to 11% of shoot translocated N could be NH_4^+-N. The relatively low free NH_4^+-N translocation within shoots of NH_4^+ -sensitive crops such as barley possibly suggest that NO_3^--N might have facilitated NH_4^+-N assimilation in some way. Though NO_3^- specific induction of additional pathways for NH_4^+-N assimilation exist (Kronzucker et al., 1999; Britto and Krozucker, 2004), it is however, not clear if this was experienced by barley used in this study. The recovery of high NO_3^- concentrations in NH_4^+ treatment NI inhibitor could have resulted into NO_3^--N uptake suppression as suggested by Kronzucker et al. (1999). NH_4^+-N uptake could have also been stimulated by NO_3^--N as was observed by both Rideout et al. (1994) and Saravitz et al. (1994). Nevertheless, the energy demand for uptake and assimilation of NH_4^+ is less intensive as compared to NO_3^- (Bloom et al., 1992).

The absence of strong NH_4^+ toxicity symptoms suggest uptake of mixed N nutrition resulting from ammonium oxidation. Even though mixed N uptake could have occurred given the presence of higher NO_3^- concentrations in CULTAN than nitrate treatments, the absence of superior grain yield under CULTAN which offered partial NO_3^- in addition to NH_4^+ was not clear. This is not in line with the findings of Kirk and Kronzucker (2005) who reported increased rice yields when partial NO_3^- became available within the rhizosphere of paddy rice initially predominated by NH_4^+. On one side, because NH_4^+ is normally assimilated within roots after acquisition of carbon-skeletons to prevent toxicity, the formed amine compounds are thereafter translocated to

the aerial parts of the plant. Nitrate on the other side, could have been taken up and directly assimilated or stored in the shoot (Maschner, 1995). High demand for the carbon skeletons by NH_4^+ fertilized crops could have contributed to the cause of inferior grain yields against the expectation of superior grain yield under mixed N forms. This could have been caused by equal survival of tiller numbers along side the number of fertile ears (Fig. 11c). Similarly affected was the shoot biomass (Fig. 11d) which also remained relatively similar among the fertilized treatments regardless of the N form applied. The low yields cannot be attributed to deficiency in the carbon skeletons since the photosynthetic pigments were quite high with the potential of supporting high carbon exchange rates (Fig. 15a-b). The soluble sugar concentrations decreased with decrease in chlorophyll concentration as the crops grew. On the contrary, organic acid concentrations increased. Nonetheless, organic acid concentrations were relatively low in NH_4^+ treatments with 5 and 20% NI-incorporation indicating the possibility that the largest N form taken up by the crops in this treatment was NH_4^+. It is also evident that some NO_3^- could have been taken up given the presence of high organic acid concentrations. Nevertheless, the affinity of nitrate system for NO_3^--N uptake has been reported to be much less than that of ammonium system for NH_4^+-N uptake (Glass et al., 1997; 2001). Moreover, the induction of NO_3^- absorption in the presence of either NH_4^+ or NO_3^- has been reported (Aslam et al., 1992; 1993). The results reported herein strongly suggest a paradoxical mixed N nutrition under CULTAN fertilization.

Further research work is still needed to elucidate the mechanisms involved in the uptake and assimilation of CULTAN fertilized nutrition so as to realize its full potential. Additional amendments can be made to improve its effectiveness or stability among other considerations. Incorporation of agricultural inputs such as herbicides, pesticides, fungicides, both macro and micro-nutrients that are limiting in particular soil types for certain crops could be attempted under CULTAN fertilization technique. Currently, the thrust of realizing the full potential of this fertilization method is to understand its basic concepts so as to guide future field applications and implications on crop production, economics and ecological components. The best approach would be participatory on-farm trials with farmers who have adopted the technique on their fields for commercial crop production in different ecological zones among different crops.

4.2 Bacterial community responses to CULTAN fertilization

4.2.1 Occurrence of bacterial communities within CULTAN fertilized soils

The study demonstrated the occurrence of bacterial communities in CULTAN fertilized soils. Ammonium-based treatments experienced bacterial community shifts as the season progressed. These findings contrast with previous reports emphasizing that CULTAN fertilized ammonium inhibit microbial habitation and activity, especially within the toxic zones (Sommer, 2000). These results have demonstrated the occurrence of a large diversity of bacterial communities as revealed by cultivation-independent DNA extraction for molecular finger-printing while targeting 16S rRNA genes. The study also recorded the highest bacterial community diversity and strongly staining (dominant) bands within the same injection zones, which coincided with high NH_4^+-N concentrations coupled up with acidic pH values that were until recently presumed to be toxic to bacteria. The bacterial diversity and dominance closely corresponded to NH_4^+-N concentrations on both temporal and spatial scale. Throughout the cropping season, nitrate and non-fertilized control treatments supported similar bacterial community diversities. Visible bands in these treatments were commonly present in ammonium treatments too indicating that NH_4^+ treatments had additional specific unique bands associated with them in addition to the commonly present bands.

The observed bacterial community within the concentrated NH_4^+-N rich zones of CULTAN fertilized treatments did not persist throughout the season. The banding complexity and prominence during the early season (at 30 DAF) reduced with diminishing NH_4^+ -N concentrations as the season progressed to 109 DAF. These results are supported by the findings of Pommerening-Röser (2001) who also found a strong linkage between microbial phylogenetic groups to the environmental conditions. Even though these results revealed occurrence of bacterial communities, no information could be derived from it to show the functional diversity of the occurring bacteria. It is for this reason that *amoA* gene was analyzed to establish the role played by the CULTAN associated bacteria in NH_4^+ transformation.

4.2.2 Functional responses of AOB in CULTAN fertilized soils

The predicted existence of ammonia oxidizing bacteria was ultimately confirmed by the amplification and characterization of bands generated, which gave *Nitrosospira*-like sequences. The study not only revealed that AOB were present in CULTAN fertilized soils, they were abundant too. The diversity of AOB also indicated shift on a time scale. All treatments expressed presence of AOB bands throughout the entire season except the non-fertilized control. Some *amoA* bands could not be detected early, but were present at later periods of the season. The detection of *amoA* genes which gave *Nitrosospira*-like sequences indicated the presence of ammonia oxidizer bacteria. There were four distinct AOB clusters generated from *amoA* phylogenetic tree. One of the four clusters comprised clone sequences retrieved from environmental samples and standard AOB, *N. multiformis* (Fig. 24). Since previous studies have categorized *N. multiformis* as a member of *Nitrosospira* cluster 3a that are sensitive to high ammonia concentrations, either their presence or active participation in ammonium oxidation is not clear. However, the presence of high NO_3^- concentrations indicated the occurrence of bacterial activity. These findings are in agreement with those of Webster et al. (2005) who also reported nitrification in sheep grazed grassland soil samples.

The adoption of *amoA* gene has in the recent past become a useful molecular marker for studying ammonium oxidizers (Francis et al., 2003; Calvo et al., 2005). To date, autotrophic AOB of the beta-and gamma-*Proteobacteria* are the known most important contributors to aerobic ammonia oxidation (Purkhold et al., 2000). These organisms comprise only a small fraction of microbiota (Phillips et al., 2000). Ammonia monooxygenase (AMO) comprises three subunits, AMO-C, AMO-B and AMO-A. The three subunits have different structures and arrangements within cell membrane/periplasmic space along side varying sizes with AMOC being 31.4 kDa while AMOB is 27 kDa and AMOA with 38 kDa (Hooper et al., 1997). The three AMO subunits from autotrophic ammonia oxidizers are encoded by *amoC*, *amoB* and *amoA* genes of the *amo* operon (Sayavedra-Soto et al., 1996). The *ß-Proteobacteria* possesses multiple copies of *amoA* (Norton et al., 2002) in addition to non-operon copies of *amoC* gene (Sayavedra-Soto et al., 1996). Though multiple gene copies have been

observed among *ß-proteobacteria*, they are nearly identical. While *Nitrosomonas* strains usually carry two gene copies, most *Nitrosospira* strains possess three (Klotz et al., 1997). Since *amoA* gene encodes the active site of ammonia monooxygenase (McTavish et al., 1993), it has been extensively used in the detection and analysis of ammonia oxidizers, from natural environments (Aakra et al., 2001) as well as enrichment cultures (Juretschko et al., 2002). The wide adoption of *amoA* has been facilitated by the development of specific PCR primers for its amplification (Sinigalliano et al., 1995; Rotthauwe et al., 1997; Webster et al., 2002).

Essentially, *amoA* has some merits over 16S rRNA in the comparison of genetic diversity. Given the fact that it encodes a protein that is involved directly in ammonia oxidation, the genetic differences noticed are more likely to be of functional importance to nitrification process. The rate of molecular divergence in *amoA* exceeds that of 16S rRNA, hence allowing for greater resolution of genetic differences in natural populations (O'Mullan and Ward, 2005). Additionally, *amoA* is a unique AOB molecular marker because, unlike many other biogeochemically important functional genes such as *nir*S that are involved in the denitrification process, which undergo significant horizontal gene transfer (Zumft, 1997), *amoA* has not been reported to undergo any appreciable horizontal transfer. Conversely, *amoA* phylogeny appears to be coherent with 16S rRNA sequence based phylogenies (Purkhold, 2000), thus suggesting either very minimal or absence of horizontal gene transfer among the group members. In addition, it has been reported that *amoA* gene compared to 16S rRNA gene topologies simplify inferences on the evolutionary history of the organisms based upon the functional relationships (O'Mullan and Ward, 2005). This gene has the advantage of linking up microbial phylogeny and their physiological activities on interface with environmental and nutritional factors. In this regard, the use of *amoA* gene to analyze the CULTAN fertilized soils supplemented the information attained from 16S rRNA gene analysis of the soil samples.

4.2.3 Effect of bacterial communities on N form in CULTAN fertilized soils

The rapid decrease in NH_4^+-N concentrations suggest its utilization by crops or microbes (Fig. 5a-b). The formation of high NO_3^--N concentrations in the injected NH_4^+-N further confirmed the occurrence of nitrification given that the process is carried out by the nitrifying bacteria (Prosser, 1989). Nitrate detection in non-NI incorporated

treatments which resulted in the availability of mixed N nutrition within NH_4^+-N treatment contrasts with the perceived notion that no nitrification would occur in these zones. Nitrification normally occurs as a two-step process carried out by two distinct groups of bacteria; ammonia oxidizing bacteria (AOB) that convert ammonia to nitrite, nitrosyfiers, and nitrite oxidizing bacteria (NOB), the true nitrifiers that convert nitrite to nitrate (Prosser, 1989). Since ammonia oxidation is the first step in nitrification, it is quite important in N cycle. It results in the formation of NO_3^--N through microbial activity (Kowalchuk and Stephen, 2001; Prosser and Embley, 2002).

Nitrification process is an essential component of N cycle that links the most reduced to the most oxidized inorganic N forms (Francis et al., 2003). Its rate is normally influenced by factors such as substrate concentration, pH, temperature and oxygen availability (Jones and Hood, 1980). Therefore, the recovery of NO_3^--N in the tested soil samples suggested a possible interaction of the aforementioned factors to enable the transformation of ammonium to nitrate. Though the increase in NO_3^--N availability within the soil is important for plant nutrition (Maschner, 1995), it also has a considerable impact on groundwater pollution owing to leaching (McCaig et al., 1999) besides denitrification associated with N-gas losses (Zumpft, 1997). Therefore, CULTAN fertilization technique having been developed on the precept that it would predominantly supply crops with NH_4^+-N while it suppresses its transformation into NO_3^--N by adsorbing onto clay particles thus forming toxic sorption-complex depots (Sommer, 2000). This creates a contradiction to the initial goal of CULTAN fertilization which was supposed to remain as ammonium. The presence of high NO_3^- concentrations necessitates adoption of strategies that could improve NH_4^+ stability in the sail. One of the possible nitrification mitigation methods is the incorporation of NI to improve NH_4^+-N stability through the suppression of the nitrification (Bremner and McCarty, 1993).

4.2.4 Bacterial community shift occurrence in CULTAN fertilized soils

Another significant observation made from this study was the bacterial community shift within the season. The change from somewhat 'ammonium tolerant/adapted' bacterial groups that were able to survive or regain vitality within the highly concentrated NH_4^+-N zones dominated the early part of the season. Bands

corresponding to the tolerant strains possibly induced by high NH_4^+ concentrations were not detected as NH_4^+ concentration decreased. They were replaced by bands deemed to be from representatives of bacterial groups 'sensitive to high ammonium concentration' (Fig. 19a-b). The occurrence of biodegraders represented by *Pseudomonas, Arthrobacter* and *Clostridia* sp. portrayed a classical example of 'substrate affinity response' or 'adaptation/ tolerance to high NH_4^+ concentrations' as reported by Webster et al. (2005). The rather systematic shift of bacterial communities from one ecologically distinct grouping (domination by biodegraders) to other physiologically different groups could have presumably occurred due to the dynamics of the various niche conditions. Another potential contributor to these changes could have also been soil pH. The occurrence of members belonging to *Clostridiales* and *Thermoanaerobacteriales* suggested involvement of microbes with the ability to metabolize /biodegrade the highly toxic NH_4^+ so as to detoxify it into lesser toxic forms or concentrations (Matoka et al., 2007b). The absence of *Flavobacteriales* during early part of the season indicated a possible inhibition by high NH_4^+ concentrations as reported by Koops et al. (2003).

Similarly, bands corresponding to *N. multiformis* were also conspicuously absent from zone 2a at 30 DAF while they existed and even predominated over the other bands in zone 3 at the same harvest period. Interestingly, this period coincided with the high NH_4^+ in zone 2a, whereas zone 3 corresponded to low NH_4^+ concentrations. While the occurrence of the dominant 'tolerant' bacterial groups in zone 2a is thought to have resulted from 'substrate affinity' induced by NH_4^+ availability as found by Webster et al. (2005). The absence of *N. multiformis* during the early period (30 DAF) could have been caused by 'ammonia inhibition' or 'low growth rate' that is characteristic of *Nitrosospira* sp. (Kowalchuk et al., 1999). Though bacterial community shifts were observed, no corresponding significant nitrification rate influences could be attributed to it. It seems like the early appearing bacterial groups were mainly involved in NH_4^+ detoxification because of their physiological metabolic versality enables them to use less preferred nutrient sources under scarcity. Avrahami et al. (2003) have showed the occurrence of AOB community structural shifts which they attributed to effects of NH_4^+ fertilization. Elsewhere, nitrifying bacteria have been reported to exhibit different substrate concentration sensitivities (Suwa et al., 1994), hence providing support for the possible microbial selection which probably caused the shifts observed in our studies. Moreover,

the bacterial community composition and functional diversity shifts have been reported in sheep grazed fields that experienced large urine depositions which upon hydrolysis resulted into the formation of highly enriched regions with NH_4^+ which subsequently caused large bacterial community changes (Webster et al., 2005). It is also known that nitrifying bacteria can survive long periods of dormancy during times of substrate limitation (Stein and Arp, 1998). This offers a possible explanation to the retrieval of *amoA* sequences from the non-fertilized soil samples (Fig. 21a-b, 23).

4.2.5 Possible influences of soil pH on microbial community

Ammonia oxidation can be inhibited at high substrate concentrations in natural environments (Prosser, 1989). However, similar substrate inhibitions of ammonia oxidation have been reported in wastewater systems too (Sharma and Ahlert, 1997). The main cause of ammonia oxidation inhibition is usually free ammonia (NH_3) rather than the total ammonium (NH_4^+) concentration (Anthonisen et al., 1976). Furthermore, the ratio between the ionized (NH_4^+) and non-ionized form (NH_3) depends on the pH. Toxicity of NH_4^+ too depends on environmental pH (Princie et al., 1998). The observed pH changes in CULTAN fertilized treatments from near neutral, 7.4 to acidic (6.3) before finally rising to 7.6 is thought to have contributed to the existing bacterial community responses (Fig. 7a). Ammonia oxidation is an acidogenic reaction (Stein and Arp, 1998). In the presence of excess ammonium, pH of the zone decreases during ammonia oxidation until it is sufficiently low enough that further ammonia oxidation is prevented. If the starting pH is lower, the amount of ammonia which can be oxidized to reach a limiting pH is decreased. Thus at lower pH values, not so much ammonia can be oxidized as observed by Stein and Arp (1998) in an incubation study. Elsewhere, nitrification has been reported to be inhibited by pH levels below 4 under natural conditions (De Boer, 1989). Since we never, attained pH ranges lower than 6, it is not clear what direct impact it could have had on the bacterial community and activity, but its effects could have possibly been exaggerated by the interaction effects with other factors like temperature, oxygen concentration to influence NH_4^+ ionization state and toxicity level (Princie et al., 1998). Earlier studies found closely related *Nitrosospira* sequences in both neutral and acid soils, although some sequences were more common in one soil type than the other (Stephen et al., 1996).

Under laboratory conditions, the optimal growth of AOB occurs under neutral to moderately alkaline conditions with pH ranges of 7.5 to 8.0 and their activities have been shown to cease below pH 5.5. The inability of AOB to cope with strong acidic condition is thought to be primarily based upon the 'unavailability of substrate' (ammonia) with decreasing pH values since decreasing pH levels increasingly facilitate protonation of ammonia (Suzuki et al., 1974). The absence of bands corresponding to *Nitrosospira multiformis* among the replicates of zone 2a which recorded high NH_4^+-N concentrations allude to this fact. Furthermore, experimental observations have shown that ammonia (NH_3) and not ammonium (NH_4^+) is the substrate catalyzed by ammonia monooxygenase enzyme during the first step of ammonia oxidation (Frijlink et al., 1992; Vannelli and Hooper, 1992). It therefore suggests that the soil injected NH_4^+ was not directly available to AOB in the same form and hence needed to be deionized or reduced to NH_3 which is mainly a pH regulated activity (Stein and Arp, 1998). The deionized form is the substrate used by AOB in the first oxidation step (Purkhold et al., 2000; Aakra et al., 2001). Unavailability of the oxidation substrate (ammonia) under strong acidic pH levels causes AOB community 'starvation' within a plentiful NH_4^+ resource. Nitrite, the substrate NOB oxidize to nitrate could have also undergone protonation to produce nitric acid which disproportionate to nitrate and gaseous nitric oxide with decreasing pH levels. Furthermore, when present at elevated concentrations under low pH values, free nitric acid negatively affects the growth and activity of nitrifying bacteria (Anthonisen et al., 1974). A more recent and detailed study by Webster et al. (2005) who delved into physiological activities of *Nitrosospira* cluster 3 classified this group into two categories, namely *Nitrosospira* cluster 3a and b. The category represented by *Nitrosospira* 3a were sensitive to high ammonia concentration, whereas *Nitrosospira* cluster 3b representatives and *Nitrosomonas eurpaea* were tolerant to high ammonia concentrations. It should be noted that the high NH_4^+concentrations cause pH reduction while NH_3 raises pH towards alkalinity. The dominance of *Nitrosospira* in our samples demonstrates their ubiquitous distribution and wide physiological versatility.

4.2.6 Bacterial community structure restoration from CULTAN effects

The bacterial community analyzed while targeting 16S rRNA showed a tendency to regain original population structure reminiscent to that retained in the nitrate and non-

fertilized treatments throughout the season (109 DAF). The revelation of a similar bacterial community structure among all the treatments and zones seemed to have been a gradual process between 30 from 109 DAF (Fig. 19a-b). Somewhat, the soil conditions also returned to the original state before the establishment of the CULTAN experiment after 109 days. Princie and colleagues who on observing bacterial community shifts to pH and NH_4^+ experiments re-established original conditions and witnessed bacterial community restoration. They thus concluded that the effects were due to pH and NH_4^+ concentration changes (Princie et al., 1998). However, they observed that NH_4^+ induced shift was reversible once the concentrations returned to the original level, whereas community structural changes due to pH extremes were irreversible (Princie et al., 1998). Even though efforts of the bacterial communities to restore their structures in our studies were generally attempted along side diminishing NH_4^+ concentrations, it is not clear if possible internal cellular alterations were also restored. However, it can be suggested that the restoration of the bacterial community structure suggests the occurrence of endogenous stability over the season. The complex interactions of environmental factors which were expressed as community structural changes and restoration attempts warrant further evaluations. These findings suggest that CULTAN effects are neither permanent in soil nor are the bacterial community changes. However, it would be interesting to monitor such shifts and restoration of both soil and bacterial community structures in long term CULTAN fertilizer application regimes. This may provide guidance on NH_4^+ injection intervals of specific concentrations or incorporation of nitrification inhibitors.

4.2.8 Potential use of nitrification inhibitors to suppress AOB activity in CULTAN

In the current study, no substantial nitrification rates were recorded in NH_4^+ treatments incorporated with NI since there was an enhanced inhibitory effect on the oxidation of ammonia conferred by NI-incorporation (Fig. 6a-b). However, no significant differences occurred between 5 and 20% NI-incorporations. Application of NI suppress NH_4^+ transformation to NO_3^- and has thus become a common agricultural practice aimed at reducing N losses caused by leaching and/or dentrification (Bremner and McCarty, 1993). NI should be highly specific to ammonia oxidizers, non-toxic to plants and non-target organisms and must have ability to move along with the fertilizer within the soil besides being affordable (Wolt, 2004). They have been reported to improve nitrogen use efficiency (Ferguson et al., 2003), an effort attributed to the enhanced knowledge on its

mode of action in inhibiting oxidation (McCarty, 1999). Since ammonia oxidation is catalyzed by ammonia monooxygenase (AMO), a key enzyme among all aerobic ammonia oxidizers (Rotthauwe et al., 1997), (Nitrapyrin®) NI-incorporation into injected ammonium effectively suppressed nitrification thus providing further evidence that microbes actually inhabited the ammonium dominated injection zones contrary to the report by Sommer (2000). Nitrification inhibition mechanism mainly relies on the broad substrate range of AMO during the catalytic oxidation and the inhibitory effects by many compounds that occur due to competition for the active sites (McCarty, 1999).

Considerable evidence has revealed the presence of specific metal cofactors in active sites. Copper has been demonstrated to play a key role in this respect (Gunsalus et al., 1975). Lees (1946) showed inhibition of ammonia oxidation in soil using copper-chelating agents such as thiourea and allylthiourea, whereas Campbell and Aleem (1965) found reversal of Nitrapyrin®/ N-Serve [2-chloro-6-(trichloromethyl) pyridine] inhibitory effects by adding copper. Powell and Prosser (1986) as well as Rasche et al., (1990) recently illustrated the influence of copper on the inhibitory properties of Nitrapyrin® with regard to ammonia oxidation and concluded that NI effectiveness rely on their abilities to act as alternative substrates. Such alternative substrates influence AMO enzyme activity by directly binding and/or interacting with AMO hence interfering with the supply of reductant needed for ammonia monooxygenase activity or by oxidation of substrates to give products that are highly reactive that can inactivate AMO enzymes (Keener and Arp, 1994). Nitrapyrin has a weak mechanism-based enzyme inhibition on the substrate with a large indiscriminate binding site of its aerobic oxidation product (6-chloropicolinic acid) to membrane proteins (Vannelli and Hooper, 1992). Under anaerobic conditions, Nitrapyrin undergoes reductive dehalogenation to produce 2-Chloro-6-dichloromethyl-pyridine (Vannelli and Hooper, 1992). Nitrate accumulation was high in the cropped ammonium treatment without NI, but was even much higher in the uncropped ammonium treatment in comparison to the nitrate treatments. The information highlights the relevance of NI incorporation into NH_4^+ fertilizers.

4.3 Ammonia oxidizing bacteria abundance in CULTAN fertilized soils

The adoption of qPCR method enabled a reliable detection and quantification of *amoA* gene copy numbers from CULTAN fertilized soils (Table 3). Most of the selected bands confirmed the occurrence of ammonia oxidizing bacteria within the injection point (zone 2a) at the beginning and end of the season. However, there seem to have occurred co-amplification of non-targeted genes along side *amoA* genes. This became apparent with the presence of double/triple melting point peaks on the DNA melting temperature curves (Fig. 25b). Analysis of qPCR assay products on agarose also indicated the presence of non-targeted gene amplification (Fig. 26c). Furthermore, failure of some of the selected bands chosen for cloning and sequencing to reamplify with specific *amoA* primers supports this view. Though the primers used have been reported to be specific and sensitive to *amoA* gene amplification (Rotthauwe et al., 1997), co-amplification of non-targeted genes even at high annealing temperatures of 62 ^{o}C has also been reported (Dionisi et al., 2002). These results herein should therefore be treated with caution. The occurring false positives in NTC qPCR assays were however, adjusted from the gene copy numbers in the environmental samples as reported by Dionisi et al. (2002) who too reported the occurrence of false positives. In contrast to our results, a high level of specificity with the same primer set has been reported (Avrahami et al., 2003; O'Mullan and Ward, 2005; Leninger et al., 2006). It thus remains unclear what could have caused the co-amplification of the non-targeted genes. However, the failure of DNA amplification from undiluted samples could have occurred due to inhibition by co-extracted contaminants such as humic acids. This has been demonstrated to commonly occur in DNA extracted from environmental samples (Dohrmann and Tebbe, 2004).

4.3.1 Relative AOB population abundance in CULTAN fertilized soils

The abundance of ammonia oxidizing bacteria was high in all the treatments, ranging from 3.31 x 10^5 to 3.29 x 10^6 and 6.36 x 10^4 to 1.08 x 10^6 *amoA* gene copy numbers g $^{-1}$ soil at the beginning and end of the season, respectively (Table 3). These results are within reported AOB population ranges found in agricultural soils (Hermansson and Lindgren, 2001; Mendum et al. 1999). All the three ammonium fertilized soils were predominant over nitrate and non-fertilized treatments with regard to AOB population abundance both at 30 and 109 DAF. However, all the treatments

witnessed *amoA* gene copy number decline at the end of the season in comparison to those at the beginning. The high AOB abundance levels and declining trend over time are closely associated with the high NH_4^+ presence presumed to have improved its bioavailability in the CULTAN treatments. It is noteworthy mentioning that NI-incorporation at 5% did not have any significant effect on the obtained *amoA* gene copy numbers (Table 3, Fig. 5A). Our results are supported by those of (Leninger et al., 2006 and Okano et al., 2004) both of whom too reported close associations between N nutrition presence and bioavailability with AOB population abundances.

Generally, fertilized soils have been variously reported to support high ammonia oxidizing bacterial population sizes (Mendum et al., 1999). Additionally, the presence of NH_4^+ and its concentrations might have supported high AOB population abundance (Bruns et al., 1999). The finding that cropped NH_4^+ treatment without NI-incorporation supported much higher *amoA* gene copy numbers both at the beginning and end of the season suggested potential role played by crop root in the excretion of exudates that are rich in nutrients besides possibly altering the rhizospheric zones which could have resulted into suitable niches that enabled AOB population to flourish. Microbial access to root exudate supplies carbon and nitrogen which are important nutrients for their growth and activity (Kowalchuk et al., 2000). Duineveld et al. (2001) also suggested that roots create highly heterogeneous ecological niches that allow bacteria to colonize small soil patches without necessarily competing for resources. The findings of Lu et al. (2001) too support the view that crop presence enhances microbial activity and growth. Our findings together with those cited herein strongly suggest that root exudates can positively impact upon AOB population abundance even where no diverse groups have been observed.

4.3.2 Potential of ammonia oxidation by non-*Proteobacteria*

Previously, only aerobic bacteria were known to have been involved in the nitrification steps of ammonia oxidation (Purkhold et al., 2000; 2003) and nitrite oxidation (Bartosch et al., 1999). These bacterial groups are reported to be related along the evolutionary tree (Teske et al., 1996) and coexist in natural environments (Francis et al., 2003). Comparative 16S rRNA gene sequencing analysis has revealed that ammonia oxidizers constitute two monophyletic assemblages (Head et al., 1993). The first

assemblage is characterized by *Nitrosococus oceanus*, and *N. halophilus* in *gamma Protebacteria* while the second group comprising majority of known AOB belong to *beta Protebacteria* (Purkhold et al., 2000; Bano and Hollibaugh, 2000). The later group is taxonomically grouped into two genera, *Nitrosomonas* and *Nitrosospira* (Head et al., 1993). Apart from the two monophyletic assemblages within *Proteobacteria* division, recent findings have shown ammonia oxidation under anaerobic conditions to be carried out by a group of bacteria belonging to *Planctomycetales* (Jetten et al., 1998; Sliekers et al., 2004). Freitag and Prosser (2003) too recently reported the occurrence of anaerobic oxidation of ammonia by *Planctomycetales* within an anoxic marine sediment where *beta-Proteobacteria* of *Nitrosomonas*-like sequences were capable of utilizing dinitrogen oxide to perform anoxic ammonia oxidation. The anaerobic ammonia oxidation (anammox) process has currently gained a lot of popularity in industrial application (Chouari et al., 2003; Strous et al., 2006).

The most startling finding was the recent demonstration which showed that autotrophic ammonia oxidation is not restricted only to the domain bacteria (Park et al., 2006), since ammonia oxidizing archaea (AOA) belonging to phylum *Crenarchaeota* too can also carry out the same process (Francis et al., 2005; Wuchter et al., 2006). Much more surprisingly, the AOA have even been found to occur more abundantly in some environmental soil samples than their AOB counterparts (Leninger *et al.,* 2006). Despite the divergent evolutionary pathways taken by AOB and AOA which has led to their classification into different domains, bacteria and archaea, respectively, it is now evident that they are more functionally related (Leninger et al., 2006). In comparison to AOB and NOB which phylogenetically belong to the same, *Nitrobacteriacea,* but are distinctly functionally divergent is hence a clear demonstration of how interrelated phylogenetic evolution and physiological activities are. With regard to CULTAN fertilized soils, it is not known if such non-*Proteobacterial* groups were present and if so, what their contribution to the recorded nitrification rates could have been. Nonetheless, the possible involvement of these microbes under CULTAN cannot be ruled out and may warrant future evaluation.

4.4. Effect of growth temperature on CULTAN-fertilized barley crop grain yield formation

4.4.1 Effects of temperature on CULTAN-fertilized barley growth responses

Barley biomass accumulation commenced slowly after emergence and increased to attain a peak at physiological maturity. This period of growth was characterized by an orderly sequence of ontogenic events that precipitated into coordinated initiation, appearance and establishment of structures that eventually formed the mature crop. The timing of these events, together with their initiation rates determined the manner in which the accumulating biomass was partitioned into different structures and the proportions subsequently stored in the grain. The growth duration of six months experienced by crops under LTR to attain maturity in comparison to four months under HTR led to significant biomass and grain yield variations. The accumulation of photosynthetic products by fertilized crops over varying growth periods under LTR and HTR also contributed to the attained yield differences despite their rather similar N status as revealed by SPAD measurements. Grain, shoot and root biomass yields supported by injected ammonium were superior to nitrate treatment under both LTR and MTR. Though grain and biomass yields of ammonium treatment were inferior to nitrate fertilized crops under HTR, they were not significantly different from each other. These results indicate that ammonium fertilization compared favourably to nitrate fertilized crops across the three temperature regimes. These findings are in agreement with those of Trnka et al. (2004) who observed that growth under high temperatures produced lower yields in comparison to those grown under low temperatures. The low yields under high temperature regimes resulted from the rapid crop growth through the different phenological phases culminating into shorter growth duration, while low growth temperatures provided adequate growth duration which facilitated grain formation and biomass accumulation.

4.4.2 Temperature effects on grain yield of CULTAN-fertilized barley

Tillering was highly responsive to temperature and nitrogen forms applied. Its influence on grain yield components emphasize its contribution to the attained grain yield (Yan et al., 1998; Li et al., 2003). Moreover, barley grain yield is mainly influenced by grain

numbers rather than grain sizes which remain relatively constant and quite stable components (Frank et al., 1992). Since grain yield is genetically controlled by a number of quantitative trait loci (QTL) whose sensitivity to environmental factors such as temperature is quite varied (Bezant et al., 1997; Kato et al. 2000), the interaction effect of nitrogen forms and temperature further influenced barley growth responses to directly or indirectly impact upon the attainable yields. Tiller dynamics was caused by both temperature and nitrogen fertilizer applied. Garcia et al. (1994) too observed that cultivar and nitrogen fertilizer applied influenced barley tillering ability. Though the total tiller numbers contributed to the recorded shoot biomasses, only fertile surviving tillers directly contributed to the attained grain yields. These results are supported by those of Garcia et al. (1996) who reported a strong correlation between longer growth durations and tiller survival when provided with sufficient nitrogen nutrition. Higher grain numbers per ear was compensated for by less heavy grains, thus expressing a negative correlation between grain numbers per ear and the average grain weights. This observation is supported by the findings of Frank et al. (1992) who reported a trade-off between grain numbers and weights.

4.4.3 Fate of CULTAN-fertilizer upon injection into soil

Along side its contribution in grain yield improvement, partial NO_3^- occurrence is thought to play a key role in ammonia toxicity alleviation (Houdusse et al., 2005), though the mechanism remains unknown (Britto and Kronzucker, 2002; Cruz et al., 2003). Although partial NO_3^- is associated with enhancement of plant tissue NH_4^+ assimilation, the pathway involved is yet to be unravelled (Redinbaugh and Campbel, 1993). The induction of enzymes by NO_3^- involved in NH_4^+ assimilation has been proposed (Kronzucker et al., 1999). In fact, these findings form the basis of rice bioengineering aimed at improving its N uptake and assimilation (Britto and Kronzucker, 2004). Wang and Below (1996) found a correlation between cytokinines and tillering under mixed N nutrition and concluded that plant hormones could be involved in tiller initiation and development. This line of argument is backed up by the suggestion that ammonia toxicity remediation could be due to changes in plant hormone balance (Gerandas et al., 1997). Tillering among CULTAN fertilized crops particularly under LTR indicated the occurrence of an interaction effect between temperature and nitrogen nutrition. Higher

tiller numbers among fertilized LTR grown crops experienced mutual shading from the neighbouring tillers. This triggered competition for light resources that led to the development of longer stem growth. The findings are supported by those of Ballare et al. (1994) who reported competition for light among monocultures upon detection of their neighbours. Such long stems could immensely contribute to grain yield losses due to lodging in fields.

4.4.4 Potential of CULTAN-fertilization for crop production

The study has demonstrated the potential of CULTAN technique as an alternative crop fertilization method. The ability of a single CULTAN injection operation to provide adequate N nutrition necessary for the sustenance of crop growth throughout the entire season emphasizes its comparative advantage in an attempt to minimize the number of farm operations from the usual three or four undertaken to administer split nitrate fertilizers as reported by Sowers et al. (1994) to one as demonstrated in this study. The corollary effect of fewer farm operations can lead to reduced operation costs with improved profit margins. Since NH_4^+ usually adsorbs onto clay soil and thus has the potential of suppressing nitrogen losses through leaching and denitrification processes besides reducing environmental pollution hence improving nitrogen use efficiency (Raun and Johnson, 1999). CULTAN injected NH_4^+ has been reported to cause root growth inhibition within the injection core, but enhances root growth proliferation along the periphery of the injection point (Sommer, 2000). Similar observations were made in the current study. Root architectural modifications occurred in CULTAN injected treatments. Aerial ammonia toxicity symptoms appeared during the early growth stage, but the crops recovered as the season progressed. Elsewhere, the occurrence of ammonia toxicity symptoms when NH_4^+ dominates has been reported (Sommer, 2000; Schittenhelm and Menge-Hartmann, 2006). Zhang and Rengel (2002) too made similar observations in wheat fertilized through NH_4^+ banding. The provision of NH_4^+ dominated N nutrition through CULTAN technique was for a long time presumed to supply only NH_4^+-N (Sommer, 2000). It was however, until recently when it was demonstrated that upon injection, NH_4^+ rapidly decreased while NO_3^- accumulated in appreciable proportions leading to the presence of a large concentration of mixed N nutrition in the absence of nitrification inhibitors (Matoka et al., 2007b). The presence of mixed N nutrition under

CULTAN fertilization caused significant improvement in the root architecture. Elsewhere, altered root architectural morphology has been associated with improved crop nutrition which led to enhanced grain yields (Britto and Kronzucker, 2004).

4.4.5 Possible effects of temperature on CULTAN-fertilization

Another possible effect of temperature was its influence on ammonia oxidation rate since bacterial activity is highly dependent on environmental temperatures. The rate of nitrification under low or high temperature regimes seem to have varied. It could be presumed that HTR probably experienced early commencement of nitrification leading to the occurrence of mixed N nutrition much earlier in the season when the crop demand for N was still low, whereas low temperatures in LTR could have delayed the commencement of nitrification due to temperature effect on microbial activity. This might have facilitated synchronization of mixed N availability to crop demand. Avrahami et al. (2003) has reported a close correspondence between ammonia oxidation rate and environmental temperatures. They corroborated the extent of N gas emissions to temperature and nitrogen forms (Avrahami et al., 2002). In essence, high or low temperatures in this study could have impacted upon the commencement and/or rates of nitrification and N form availability. The stepwise increment of growth temperatures possibly facilitated both crop growth and ammonia oxidation. Though the mechanism underlying growth rate alteration as a function of temperature is so far not well understood, reduced growth rates in environments with temperatures below optima are thought to result from low nutrient uptake capacity (White et al., 1987). On the other hand, reduced growth rate under low temperatures is viewed not to be caused by low rates of nutrient uptake *per se*, but rather limited increase in plant volume due to temperature-sensitive mechanisms that impede cell expansion (Thomas et al., 1989). The limited crop volume thus led to diminished nutrient uptake demand occasioned by slow growth rate (Thomas et al., 1989). Macduff and Johnson, (1991) emphasized that depressed growth rates are a cause rather than consequence of lower nutrient supply rates. The interplay between N nutrition and temperature seem to impact upon yield and yield forming structures.

Both N forms were recovered in the analyzed soil samples. The crops exhausted the two N forms since very low levels were available in the three temperature regimes at the end of the season. Soil pH, phosphate and nitrate concentrations in nitrate treatment were influenced by temperature regimes. Though not significantly different, NO_3^- persisted in NH_4^+ treatments at slightly higher concentrations than NH_4^+ itself, thus suggesting NH_4^+ could have been either transformed into NO_3^- and only traces were recovered or much of it was taken up by either microbes or crops. NH_4^+ is usually preferred for uptake by crops since it requires less assimilative energy in comparison to NO_3^- (Bloom et al., 1992) and the occurrence of an exponential respiratory increase with increasing temperature (Murata and Iyama, 1963) which consume a large proportion of recently manufactured photosynthates could have caused the variations observed among the temperature and N form treatments. Since rise in day temperatures raise both photosynthesis and respiration at temperature ranges below optimum, whereas increasing night temperatures or rising day temperatures when above growth optima cause drastic increases in respiration rate alone without corresponding increases in the photosynthetic rates (White, 1973), which could have led to mobilization of non structural carbohydrates comprising water soluble sugars and starch to support respiration (Fulkerson and Donaghy, 2001). This could be a possible explanation for the low quantities of glucose and fructose reserves recovered among crops grown under HTR in comparison to LTR. Less soluble sugar recovery among HTR grown crops in comparison to LTR suggest the role different respiratory rates play with regard to growth temperatures experienced.

It can be generally concluded from this study that growth temperatures interact with CULTAN injected liquid NH_4^+ to cause a suite of growth responses such as growth duration alterations, tillering and ear formation which have a great impact on grain and biomass yields. Crop growth rate among different phenological stages influenced grain yield and yield forming structures which significantly affected yield output. Microbial transformation of NH_4^+ into NO_3^- and the possible occurrence of mixed N nutrition could have immensely contributed to grain and biomass yield superiority among CULTAN fertilized crops over sole NO_3^- fertilized crops.

5. SUMMARY

Injection of concentrated liquid ammonium nutrition into soil is an agricultural practice aimed at mitigating nitrogen losses. In Germany, it is referred to as controlled uptake long term ammonium nutrition (CULTAN) technique. In many soils, nitrate is mobile because as an anion, it is not bound to negatively charged surfaces of clay minerals or organic soil compounds. In contrast to phosphate, it is not specifically adsorbed. Nitrate fertilization may thus result in leaching and presents a potential environmental hazard. Ammonium ions, on the other hand, may be unspecifically bound to negatively charged surfaces or even fixed in inter-layers of 2:1 clay minerals. Following the injection of concentrated liquid ammonium into soil, ammonium is the predominant N nutrition form suggesting its stability in soil. After 30 days, though ammonium persisted in the soil, its concentration had drastically decreased. Unspecifically bound ammonium may be exchanged and readily oxidized to nitrate by nitrifying bacteria. Bacterial community occurrence within the perceived toxic ammonium injection-zones facilitated transformation of ammonium to nitrate. This resulted into a rapid reduction of ammonium due to microbial as well as crop uptake. Along side the reductions in ammonium concentrations, nitrate concentrations increased rapidly as the season progressed.

The incorporation of nitrification inhibitor (Nitrapyrin®) improved the stability of injected liquid ammonium by suppressing the nitrification process. However, the inhibitor did not express any direct effect on ammonium concentration reductions. It suppressed the transformation of ammonium into nitrate. Non-incorporation of the inhibitor caused the formation of high concentrations of nitrate. The transformation of ammonium to nitrate under CULTAN fertilization suggests that the technique offers mixed nitrogen nutrition contrary to earlier assumptions that it supplies predominantly ammonium. The nitrogen switch from predominant ammonium to mixed nitrogen through the availability of partial nitrate is deemed to have played a role in the alleviation of ammonium toxicity symptoms commonly associated with crops fertilized with ammonium. Though mixed nitrogen nutrition is usually reported to produce superior grain yields, none was recorded in this study.

This study has provided the first molecular evidence demonstrating the occurrence of bacterial communities in CULTAN-fertilized soils contrary to the presumption that ammonium injected zones remain highly toxic and never allow any microbial inhabitation and/or any substantial activity. In addition to this, the presumed toxic zones with high ammonium concentrations and acidic pH levels supported the highest diversity of bacterial communities. This diversity shifted spatio-temporally with regard to distance from injection zones and time after the injection was performed. Analysis of ammonia monooxygenase sub-unit A (*amoA*) gene revealed the occurrence of ammonia oxidizing bacteria (AOB) within these same toxic zones where their functional role is thought to have mainly been nitrogen transformation, especially in non-inhibitor incorporated ammonium treatments. Despite the presence of a large bacterial diversity in the early part of the season, it decreased as the season progressed. Though temporal bacterial community shifts also occurred (30 and 60 days after fertilization) with decreasing ammonium concentration and changing pH levels. The banding pattern seemed to have been restored to the original patterns of nitrate and non-fertilized control. This suggests the occurrence of an endogenous stability of the bacterial community structure over the season. This is an indication that CULTAN fertilization effects are not permanent, but only transient. The population of ammonia oxidizing bacteria in the treatment experiencing 'mixed nitrogen nutrition' had the highest estimates of population abundance. This illustrates that high nitrification rates *per se* did not support the high population occurrence, but rather supported root exudation, which could have influenced the rhizosphere by improving microbial nutritional resources and suitable niches.

It can be generally concluded from this study that growth temperatures interact with CULTAN-injected liquid ammonium to cause a suite of growth responses such as growth duration alterations, tillering and ear formation which have a great impact on grain and biomass yields. Crop growth rate among different phenological stages influenced grain yield and yield forming-factors which significantly affected yield output. The role of temperature on microbial activity, especially the nitrification of injected liquid ammonium can not be ruled out. The possible occurrence of mixed nitrogen and crop growth durations could have also contributed to superior grain and biomass yields under low temperatures. These findings could be useful in the temperate and tropical regions.

6. ZUSAMMENFASSUNG

Auswirkung von Ammonium-Infektionsdüngung auf Bodenbakterien-Gemeinschaften und Einfluss der Temperatur auf die Kornertragsbildung von Gerste (*Hordeum vulgare* L.)

Die Injektion hochkonzentrierter Ammonium-Lösungen in den Boden ist eine landwirtschaftliche Praxis, die eine Verminderung von Stickstoffverlusten zum Ziel hat. In Deutschland ist sie unter der Bezeichnung Controlled Uptake Long Term Ammonium Nutrition (CULTAN)-Düngetechnik bekannt. Nach Injektion in den Boden adsorbiert NH_4^+ an Tonminerale der Bodenmatrix unter Bildung eines Komplexes hoher NH_4^+-N Konzentration mit toxischer Wirkung auf Pflanzenwurzeln. Unmittelbar zur Injektion und einige Tage danach war NH_4^+-N die vorherrschende N-Form, was auf seine Stabilität im Boden hindeutet. Nach 30 Tagen war NH_4^+-N weiterhin im Boden nachweisbar, seine Konzentration hatte jedoch schnell abgenommen. Das Auftreten von Bakteriengemeinschaften innerhalb der vermeintlich toxischen Zonen der Injektionsdepots ermöglichte die Transformation des NH_4^+-N. Dieses führte zu einer schnellen Abnahme des NH_4^+-N sowohl aufgrund bakterieller Aktivitäten als auch der Aufnahme durch die Pflanzen. Mit der Abnahme der NH_4^+-N-Konzentration war eine schnelle Akkumulation von NO_3^--N verbunden. Die Inkorporation eines Nitrifikationsinhibitors (Nirapyrin,) verbesserte zwar die Stabilität des injizierten NH_4^+-N durch Unterdrücken des Nitrifikationsprozesses, war jedoch ohne Einfluss auf die Abnahmerate des NH_4^+-N. Ohne die Inkorporation von NI erfolgte die Nitrifikation unvermindert und hatte die Bildung großer NO_3^--N – Akkumulationen für die Pflanzenaufnahme zur Folge. Die Transformation von NH_4^+- in NO_3^--N legt nahe, dass die CULTAN-Düngetechnik eher eine gemischte N-Ernährung darstellt, im Gegensatz zu früheren Annahmen, die auf einer vorherrschenden NH_4^+-N-Ernährung basierten. Der Wechsel von einer vorherrschenden NH_4^+-N zu einer gemischten N-Ernährung könnte eine Rolle gespielt haben bei der Abschwächung von NH_4^+-N-Toxizitätssymptomen, die im allgemeinen bei Pflanzen auftreten, die unter alleiniger NH_4^+-N-Ernährung kultiviert wurden. Während bei gemischter N-Ernährung generell von höheren Kornerträgen berichtet wird, konnte in der vorliegenden Untersuchung keine Erhöhung beobachtet werden.

Die vorliegende Untersuchung hat den ersten molekularbiologischen Nachweis geliefert, der das Auftreten von Bakteriengemeinschaften innerhalb von mit CULTAN gedüngten Böden demonstriert. Die Ergebnisse stehen damit im Gegensatz zu der bisherigen Annahme, dass die Depot-Zonen hoch toxisch bleiben und keine mikrobielle

Besiedlung und /oder substantielle Aktivität erlauben. Darüber hinaus besaßen die vermeintlich toxischen Zonen mit hoher NH_4^+-N-Konzentration und sauren pH-Werten die höchste Diversität in den Bakteriengemeinschaften. Diese Diversität veränderte sich räumlich und zeitlich in Hinblick auf die Entfernung vom Injektionszentrum beziehungsweise auf die Zeit nach Injektion. Die Analyse des *amo*A-Gens belegte das Auftreten Ammonium-oxidierender Bakterien (AOB) innerhalb derselben toxischen Zonen. Es ist anzunehmen, dass ihre funktionale Rolle im wesentlichen die N-Transformation war, insbesondere bei Injektionen ohne NI-Inkorporation. Im Gegensatz zur großen Bakterien-Diversität zu Beginn der Kultur, reduzierte sich die Diversität im weiteren Kulturverlauf. Obwohl zeitliche Verschiebungen in den Bakteriengemeinschaften erfolgten (30 und 60 Tage nach Düngung) bei abnehmender NH_4^+-N-Konzentration und sich verändernden pH-Werten, erreichten sie einen stabilen Status, bei dem die Gemeinschaften in allen Zonen und Behandlungen einheitlich wurden, unabhängig von der gedüngten N-Form, was eine endogene Stabilität der Struktur der Bakteriengemeinschaft während der Kulturphase nahe legt. Dieses ist ein Hinweis darauf, dass die Auswirkungen der CULTAN-Düngung nicht dauerhaft, sondern nur vorübergehend existent sind. Die Population der AOB war in derjenigen Behandlung am vielfältigsten, bei der sich eine „gemischte N-Ernährung“ entwickelte, insbesondere in der Behandlung mit Pflanzenbesatz. Das deutet darauf hin, dass hohe Nitrifikationsraten *per se* nicht das Auftreten vielfältiger Populationen unterstützen. Eher hätten die Wurzeln der Pflanzen Exudate erzeugt und damit die Nahrungsversorgung verbessert und zusätzlich durch die Schaffung von Nischen die Konkurrenz verringert haben können. Es gibt Hinweise für das Auftreten einiger nicht-identifizierter Ammonium-Oxidierer in den untersuchten Zonen. Die Beobachtung multipler Peaks der DNA-Schmelzpunkte scheint diese Annahme zu unterstützen.

Die Interaktion von Wachstumstemperaturen und injiziertem Ammonium-Flüssigdünger (CULTAN) hatte eine Abfolge physiologischer und morphologischer Reaktionen auf das Wachstum zur Folge, wie z.B. auf die Wachstumsdauer. Die Rate des Pflanzenwachstums während einzelner phänologischer Phasen hatte einen großen Einfluss auf den Kornertrag und auf ertragsbildende Strukturen wie Triebe, Ähren und Kornzahlen wie auch Körnergewichte, woraus sich signifikante Veränderungen des Ertrags ergaben. Eine Rolle der Temperatur auf die mikrobiologische Aktivität,

insbesondere die Nitrifikation des injizierten NH_4^+-N, kann nicht ausgeschlossen werden. Das mögliche Auftreten gemischter N-Ernährung und die Dauer des Pflanzenwachstums könnten die Ursache für hohe Erträge unter niedrigen Temperaturen gewesen sein. Diese Ergebnisse könnten für gemäßigte und tropische Regionen von Nutzen sein.

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DEDICATION

To my sons

Jerry Matoka Mboya

and

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