Journal of Horticultural Science & Ornamental Plants 6 (3): 126-132, 2014
ISSN 2079-2158

DOI: 10.5829/idosi.jhsop.2014.6.3.1146

Influence of Nitrogen Forms on Nitrate and Nitrite Accumulation in the Edible Parts of Spinach (*Spinacia oleracea*, L.) Plant with Maintenance for Yield Production

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Abstract: Two field experiments were established during the two successive seasons of 2012 and 2013 to study the effect of different forms of nitrogen fertilizers (urea, ammonium nitrate and ammonium sulphate) and biofertilizer (rizobacterine) on growth, yield and nitrate and nitrite accumulation in the edible parts of spinach plant. Data indicated that fertilization with different forms of nitrogen alone led to significant differences in plant height (cm), number of leaves/plant, fresh and dry weights (g/plant) and total yield (ton/fed.) and the highest increment was achieved in case of ammonium nitrate in both seasons. It is clear that, there was no significant difference in plant height and number of leaves/plant when application of the biofertilizer with a half rate of nitrogen fertilizers by the two methods of application. On the other hand, addition a half rate of nitrogen fertilizers then biofertilizer led to significant increase in fresh and dry weights and yield as compared to application of rizobacterine as a seed treatment in both seasons. It is obvious that accumulation of nitrate and nitrite was increased by application the different forms of nitrogen fertilizers singly, however, it is considered hazardous to human health. Meanwhile, application of the biofertilizer with a half rate of nitrogen fertilizers at recommended dose reduced nitrate and nitrite accumulation in the edible parts of spinach plant and more reduction was detected by application of the biofertilizer as a seed treatment. Data also indicated that, addition half rate of ammonium sulphate and then the biofertilizer (T8) was the most efficient one in decreasing nitrate and nitrite fractions concomitant with an increase in dry matter and no significant difference in total yield against the corresponding control (T7) in both seasons and save about 50% of the cost of nitrogen fertilizer.

Key words: Nitrogen fertilizer · Biofertilizer · Nitrate and nitrite accumulation · Spinach

INTRODUCTION

The nitrogen form supplied affects the growth of both plant shoots and roots. Nitrogen supplied in the form of nitrate is frequently much more effective on the growth than the ammonium nitrogen. Plant nitrogen content is dependent on the form of nitrogen nutrition [1].

Most of the absorbed ammonium has to be incorporated into organic compounds in the roots, whereas the absorbed nitrate is mobile in the xylem and can also be stored in the vacuoles of roots, shoots and storage organs [2]. Finally, after NH_3^+ is produced, it is assimilated rapidly and only traces of NH_3 are normally found in most plants, in fact, greater than trace amounts in plant tissues are often toxic.

Neyra and Dobereiner [3] and Ishac [4] mentioned that the artificial inoculation of agriculture crops by dinitrogen-fixing bacteria has gained much popularity in increasing fresh and dry weights of crops and save about 50% of the cost of nitrogen fertilizer.

Some of the leaf crops grown for human consumption have been found to accumulate excessive NO_3^- and are possibly responsible for methemoglobinemia, particularly in babies [5]. Crops known to accumulate excessive NO_3^- in the forage with overlay liberal fertilization include spinach, lettuce and dill [6]. The adverse effect of high NO_3^- is actually due to NO_2^- as NO_3^- can be reduced to NO_2^- in the intestine of some animals and human infant during the first few months of life. Nitrite is rapidly absorbed into the blood where it oxidizes the Fe of

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hemoglobin to the ferric state forming methemoglobin which cannot function in oxygen transport. Moreover, some circumstantial evidence relating exposure to NO_3^- or NO_2^- to the incidence of cancer is available. Therefore, the present investigation was carried out to study the effect of different forms of nitrogen fertilizers and biofertilizer on nitrate and nitrite accumulation in the edible parts of spinach plant with maintenance for yield production.

MATERIALS AND METHODS

Field Experiment: This study was performed in the experimental farm of the Faculty of Agric., Ain Shams Univ., at Shoubra El-Keima, Cairo, Egypt, during the two growing seasons of 2012 and 2013 in sandy, loam soil. Mechanical and chemical analyses of the cultivation soil were determined before sowing for both seasons in Central lab., Faculty of Agric., Ain Shams Univ. as show in Table (1) [7]. Seeds of spinach (*Spinacia oleracea* cv. Saloneky) were kindly obtained from Agriculture Research Center, Ministry of Agric., Dokki, Cairo, Egypt. The experiment was established as completely randomized block design with three replicates. There were 27 experimental plots; the area of each one was 6 m^2 (3 × 2 m). Seedbed preparation practices; plowing (chisel plow twice), leveling and ridging were achieved before sowing. Seeds were sown on the 12^{th} of October in the two growing seasons. All other agricultural practices; irrigation, weed control and harvest were applied as recommended by Ministry of Agric., Egypt.

The fertilization with calcium superphosphate (15% P_2O_5) at 200 kg/fed and potassium sulphate (50% K_2O) at 75 kg/fed were added at two equal amounts after three and five weeks from sowing. Moreover, commercial micronutrient fertilizer as Stimofull (contained 0.17% Fe-0.085 % Mn-0.03% Zn-0.085% Cu-0.001% Mo-0.045% B produced by Zeneca Agro. S.A. Costa Brava) was sprayed periodically every 15 days commencing 15 days after sowing at the rate of 1g/L. Pest control program as well other culture procedures were made as normal practices. Regarding nitrogen fertilization treatments, ammonium sulphate (20.5% N) at 250 kg/fed (as recommended) and other nitrogen sources were added at two equal amounts after three and five weeks from sowing (T1, T4 and T7). The different forms of nitrogen fertilization and biofertilization were applied as follow:

T1. Urea at normal rate 104.08 kg/fed [51.3 kg N] (Add in two batches).
T2. Half rate of urea in the date of the first batch + Rizobacterine (commercial bio-fertilizer contain a complex of Azospirillum and Azotobacter bacteria) at 200 g/fed in the date of the second batch.
T3. Rizobacterine as seed treatment at 200 g/fed was mixed with Arabic gum to form a seed coating mixture + half rate of urea in the date of the second batch.
T4. Ammonium nitrate at normal rate 153.1 kg/fed [51.3 kg N] (Add in two batches).
T5. Half rate of ammonium nitrate in the first batch + Rizobacterine in the date of the second batch.
T6. Rizobacterine as seed treatment + half rate of ammonium nitrate in the second batch.
T7. Ammonium sulphate at normal rate 250 kg/fed [51.3 kg N] (Add in two batches).
T8. Half rate of ammonium sulphate in the date of the first batch + Rizobacterine in the date of the second batch.
T9. Rizobacterine as seed treatment + half rate of ammonium sulphate in the date of the second batch.

Growth Characters: Five plants were randomly taken from each treatment at harvesting age (60 days after sowing). The following growth characters were determined: plant height (cm), number of leaves/plant and

Table 1: Physical and chemical analysis of the sandy loam soil used in two seasons

-----	Mechanical analysis (Particle size distribution)							Soluble cations (ppm)			
Season	C. sand %	F. sand %	Silt %	Clay %	Texture	pH	EC mmhos/cm	Na^+	K^+	Ca^{++}	Mg^{++}
1st	28.7	37.9	16.5	16.8	Sandy loam	7.7	3.92	17.6	1.74	10	9.5
2nd	27.4	39.2	15.3	17.9		7.3	3.41	16.2	1.62	11	9.63

----		Available micronutrients (ppm)					Soluble anions (ppm)			
Season	Total nitrogen %	Copper	Zinc	Manganese	Iron	-----	Cl^-	CO_3^-	HCO^-	SO_4^-
1st	2.42	4.44	4.98	72.5	47.67	-----	9	-	1.3	28.9
2nd	2.11	3.21	5.12	69.6	45.33	-----	10	-	1.4	27.6

top fresh and dry weights (g/plant), in addition to estimation total yield (ton/fed.) of the edible parts of spinach plant.

The statistical analysis of data was performed using (SAS V.9.1) computer software program [8] and Duncan's new multiple range tests was used to differentiate between means as described by Duncan [9].

Chemical Analysis: Spinach sampled from the leaves was randomly taken from three replicates of each treatment after 60 days from sowing (suitable stage for green consumed). All samples were oven dried at 70°C for 48 h and were ground to determine nitrate and nitrite content.

Nitrate and nitrite levels in the plants were determined according to the method of Sen and Donalds [10].

Extraction of Sample:

- Weight 10 g of leaves well homogenized sample and blend for 5 minutes with 70 ml distilled water and 12 ml 2% NaOH solution.
- Adjusted the pH of the suspension to 8.
- Heat in an oven (50–60°C) with occasional stirring. Add 10 ml $ZnSO_4$ solution and maintain temperature at 50°C for additional 10 minutes.
- Add 2% NaOH solution until appearance of white precipitate.
- Cool and transfer dilute solution to 200 ml measuring flask and complete the volume with distilled water.
- Filter through filter paper No.1.
- Collect the filtrate and complete the analysis on the same day.

Determination of Nitrate:

- Mix 10 ml aliquot of the filtrate with 5 ml NH_4Cl buffer (pH 5) and then pass it through cadmium colum with 15 ml distilled water and collect combined effluent and wash in 50 ml volumetric flask.
- Add 5 ml 60% acetic acid and 10 ml colour reagent was prepared by mixing equal volumes of sulfuric acid solution and N-(1-naphthyl) ethylene-diamine reagent, just before use.
- Dilute to volume with distilled water, mix and let stand for 25 minutes in the dark.
- Read absorbance at 550 nm.

Determination of Nitrite:

- Transfer 10 ml aliquot of the filtrate to 50 ml volumetric flask.
- Add 9 ml NH_4Cl buffer (pH 5), 5ml 60% acetic acid and 10 ml colour reagent was prepared by mixing equal volumes of sulfuric acid solution and N-(1-naphthyl) ethylene-diamine reagent, just before use.
- Dilute to volume with distilled water, mix and let stand for 25 minutes in the dark.
- Read absorbance at 550 nm.

RESULTS AND DISCUSSION

Plant Height: Regarding to the first season, Table (2) shows that the application of fertilization with urea (T1) or ammonium nitrate (T4) led to significant increase in plant height in comparison with the plants that fertilized with ammonium sulphate (T7), while there is no significant difference in length between the plants that fertilized either with urea or ammonium nitrate.

On the other hand there is no significant difference in spinach plant height when using biofertilizer with a half rate of mineral or organic fertilizer even biofertilizer was applied as seed treatment or after mineral or organic fertilizer was added. The same trend was achieved in the second season with an increase in the plant height in all treatments. In this respect, Kirkby and Mengel [1] reported that the nitrogen form supplied affects the growth of both plant shoots and roots. Nitrogen supplied in the form of nitrate is frequently much more effective on the growth than in ammonium nitrogen. Plant nitrogen content is dependent on the form of nitrogen nutrition. The same author mentioned that urea can be classified according to its effect on plant metabolism and growth somewhere between nitrate and ammonium forms of nitrogen.

Number of Leaves: Table (2) shows that adding the mineral fertilization alone (T4 or T7) significantly increased number of leaves/plant as compared to organic fertilization with urea (T1) in both seasons. The organic fertilization treatments (T1, T2 and T3) did not show any significant differences, in the number of leaves per plant in both seasons. Also, adding bio fertilization as a seed treatment or after mineral fertilization did not present any significant differences in the number of leaves per plant,

Table 2: The effect of different forms of nitrogen fertilizers and biofertilizer on growth characters of spinach plants at harvesting age during the two growing seasons

	1st Season					2nd Season				
Treatments	Plant height cm	Number of leaves/Plant	Fresh weight g/Plant	Dry weight g/Plant	Yield ton/fed.	Plant height cm	Number of leaves/Plant	Fresh weight g/Plant	Dry weight g/Plant	Yield ton/fed.
T1	44 A	13 CDE	45.41 B	6.30 B	4.5B	48 A	15 CDE	50.50 B	5.46 B	5.2B
T2	34 D	13 CDE	32.44 F	3.92 DE	3E	38.3 C	15 CDE	37.66 F	3.80 E	3.5E
T3	35 CD	14 BCD	26.46 G	3.39 F	2.7F	39 C	16 CD	31.56 G	2.86 F	3F
T4	45 A	15.7 A	47.52 A	7.07 A	4.9A	49 A	17.6 A	53.03 A	5.93 A	5.5A
T5	35 CD	14.7 AB	42.97 C	5.78 C	4.4C	39.3 C	16.6 AB	48.16 C	4.86 C	5C
T6	34.7 CD	14.3 BC	35.20 E	5.64 C	3.4D	38.6 C	16.3 BC	40.23 E	4.26 D	4.3D
T7	39.7 B	15 AB	37.83 D	3.65 EF	3.6D	43.6 B	17 B	43.03 D	3.86 E	4.5D
T8	35.3 C	13 CDE	37.00 D	4.30 D	3.5D	39 C	15 CDE	42.10 D	4.96 C	4D
T9	34.7 CD	12.7 E	23.52 H	2.85 G	2.4G	38.6 C	14.6 E	29.00 H	2.90 E	3F

Means with the same letter are not significantly different at 5% level.

while significant increment in the number of leaves/plant was achieved by adding any of the mineral fertilization alone (T4 or T7) as compared to T6 or T9 respectively in both seasons. In this regard, Abd-Elmoniem *et al.* [11] showed that lettuce plant growth increased according to applied N form, in the following order: calcium and potassium nitrate > ammonium nitrate > urea > ammonium sulphate. Marschner [2] showed that ammonium generally inhibits cation uptake and can depress growth by inducing a deficiency of magnesium or calcium.

Fresh and Dry Weights and Total Yield: As shown in Table (2), there was a significant difference in fresh and dry weights and yield between the plants which fertilized with urea (T1) or ammonium nitrate (T4) or ammonium sulphate (T7) alone. It is clear that ammonium nitrate fertilization led to the highest increase in fresh and dry weights and yield while ammonium sulphate fertilization was the least in both seasons.

The data reveal that adding half rate of organic (T2) or mineral fertilization (T5 & T8) first and then adding bio fertilization led to significant increase in fresh weight and yield as compared to the application of the bio fertilization as a seed treatment in both seasons. Fertilization with half rate of urea (T2) or ammonium nitrate (T5) first, then bio fertilization led to significant increase in dry weight as compared to adding a bio fertilization as a seed coating (T3 and T6) respectively in the two seasons with the exception of ammonium nitrate fertilization in the first season where there is no significant difference by adding bio as a seed coating or after mineral fertilization (T6 & T5).

As for ammonium sulphate fertilization, adding half rate of it first and then the bio (T8) has led to a significant increase in the dry weight as compared to mineral fertilization alone or adding bio as a seed coating (T7 or T9) in both seasons. On the other hand, fresh weight and yield of the same treatment (T8) was not significantly different than the corresponding control (T7) in the two seasons. The results agree with Hammad *et al.* [12] and Mahdi [13] who reported that application of high N rates increased the fresh and dry weights of spinach aerial organs. In addition, Dashti *et al.* [14] indicated that application biofertilizer in plants compared to untreated plants increased 48 and 40% of plant yield in a greenhouse and field experiment, respectively.

Nitrate and Nitrite Accumulation: Fig. (1) reveals the effect of different forms of nitrogen fertilizers and biofertilizer on nitrate and nitrite concentrations of spinach leaves. It could be noticed that the highest nitrate and nitrite concentrations were achieved in case of urea treatments and ammonium nitrate fertilization singly (T1, T2, T3 and T4) and the reverse was true in case of ammonium sulphate treatments (T7, T8 and T9) in both seasons.

It is clear that application of biofertilizer as a seed treatment and then adding half rate of the organic or mineral nitrogen fertilizers (T3, T6 and T9) reduced nitrate and nitrite concentration of spinach leaves at harvesting age as compared to nitrogen forms application at recommended dose (T1, T4 and T7) respectively during the two growing seasons. Therefore, the role of biofertilizer in the reduction of nitrate and nitrite accumulation in the edible parts of spinach plants was clear as compared to different forms of nitrogen fertilizers application at recommended dose, however, it is considered hazardous to human health. It is clear from the data that application of biofertilizer as seed treatments (T3, T6 and T9) gave the best results for the reduction of nitrate and nitrite accumulation.

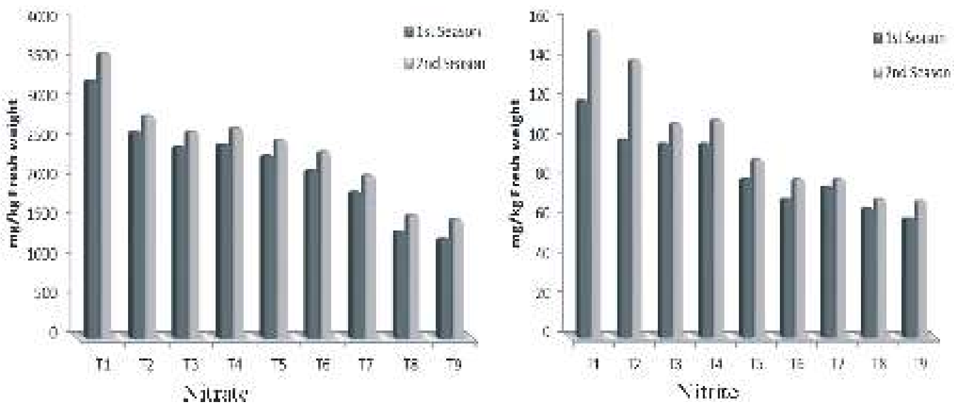

Fig. 1: Effect of different forms of nitrogen fertilizers and biofertilizer on nitrate and nitrite concentrations of spinach leaves at harvesting age during the two growing seasons.

Apart from urea treatment alone (T1), the limits detected for nitrate in the rest treatments are within the legal limits (>3000 mg/kg FW for spinach) recommended by European Union regulations (Commission Regulation [EU] No. 1258/2011) [15], thus, from the point of view of nitrates, top growth of spinach plant under these treatments are safe, especially, when separation the petiole which accumulate high nitrate concentration, prior to preparation of vegetable food, may appreciably reduce the extent of nitrate consumption by humans.

It is interesting to note that adding half rate of ammonium sulphate and then the biofertilizer (T8) was the effective treatment, since it produced 97.2% and 88.9% of the yield and reduced nitrate concentration 27.3% and 24.6% of the corresponding control (T7) in the two successive seasons respectively. Moreover, the same treatment achieved higher dry matter than the control (T7) in both seasons and save about 50% of the cost of nitrogen fertilizer.

In this concern, a number of factors influence nitrate and nitrite accumulation in vegetables such as nitrate supply from the soil, environment such as light intensity, photoperiod and temperature and depends on the time of application and the amount and composition of the fertilizers applied [16-18]. The nitrate content varies in various parts of a plant and with physiological age of the plant [19]. In addition, Alfredo *et al.* [20] showed that the nitrate content was dependent on the agricultural production system, while for nitrites, this dependency was less pronounced.

The present data are in agreement with previous results of Hemmat *et al.* [21] who reported that, on average the nitrate content of spinach leaves ranged from 601.4 mg/kg FW in control; treatment to 5353.3 mg/kg FW in the highest urea fertilizer rate (200 kg/ha). Urea was hydrolyzed rapidly following its application to soil. Ammonium accumulates in the application zone and pH increases due to the consumption of H^+. The resultant pH in most soil ranges from 7.0 to 9.0 [22]. As soil pH rises, the proportion of NH_3 over NH_4 increases and volatilization can occur when urea is surface applied [23]. The rate of nitrogenous fertilizer, resulting in accumulation of nitrate in plants, uptake exceeds the rate of its reduction to ammonium [24]. Jan and Kolota [25] found that the lowest accumulation of nitrates and vitamin C was associated with the application of ammonium sulphate and Entec-26. In addition, inhibition of nitrate by ambient ammonium was reported by Pan *et al.* [26] who suggested that mechanism of ammonium inhibition is implicated directly affects nitrate reduction in the tissues through inhibiting nitrate reductase (NR) activity and the uptake system associated with it. Zhou *et al.* [18] mentioned that, the order of nitrate accumulation in the Cole leaves, upon application of different nitrogenous fertilizers, is: urea > ammonium carbonate > ammonium nitrate > ammonium sulphate.

In addition, Sheng [27] showed that microbial fertilizer can control nitrate content of vegetables effectively. It's of utmost importance from the angle of environmental sanitation and the production of green food. Hanafy Ahmed *et al*. [28] reported significant decrease in nitrate accumulation by the plants treated with nitrobien, biogien and rizobactrein. In addition, Kawthar *et al.* [29] showed that the most promising treatment is biofertilizer combined with 50% recommended dose of nitrogen fertilizer, however, detected pronounced increase in potato tuber yield with the reduction of nitrate and nitrite accumulation which consider a health hazard to human and save about 50% of the cost of nitrogen fertilizer.

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