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Nitrate reductase in wheat plants grown under water stress and inoculated with *Azospirillum* spp.

H.M. EL-KOMY, M.A. HAMDIA and G.K. ABD EL-BAKI

Department of Botany, Faculty of Science, Minia University, Minia, 61519, Egypt

Abstract

The present investigation has been performed to evaluate nitrate reductase (NR) and nitrogenase activities as well as growth and mineral nutrition of wheat plants grown under drought stress and inoculated with different *Azospirillum* strains (NR^- and NR^+). Fresh, dry mass and water content decreased with decreasing soil moisture content, which was accompanied with low soluble sugars and soluble protein content and increase in the total amino acids content. *Azospirillum* inoculation with either bacterial strain (NR^- and NR^+) significantly increased the above characteristics even at 40 % moisture content. NR activity decreased in both the shoots and roots by decreasing soil moisture content. NR^+ strain exhibited increased root NR activity compared with uninoculated plants or inoculated with NR^- strain. However, plants inoculated with NR^-strain increased NR activity in the shoot more than in the root of the same plant and in the shoot of control plants. Inoculation with either NR^- and NR^+ *Azospirillum* strains gave higher nitrogenase activity than uninoculated control plants. The low N supply (0.5 mM) did not affect nitrogenase activity. NR^-strain was less effective than NR^+strain in promoting total N-yield, spike numbers and their mass per pot. *Azospirillum* inoculation exhibited no significant changes in wheat Mg^{2+} content. However, K^+ and Ca^{2+} have shown significantly increased values. *Azospirillum* beneficial effect on plant N balance and growth are most probably composed of multiple mechanisms and beneficial NR is one of them. The importance of *Azospirillum* NR^+strains for increasing wheat resistance to water stress is also supported by the obtained data.

Additional key words: amino acids, calcium, magnesium, nitrogen fixation, potassium, proteins, soil moisture content, sugars.

Introduction

In most soils, saline or non-saline, nitrogen is usually the most growth-limiting plant nutrient. Consequently, the addition of N usually improves plant growth and the yield of plants grown in arid climate (Van Hoorn *et al.* 2000). However, nitrogen uptake and consequently plant growth decreased with increasing drought-stress (Al-Rawahy *et al.* 1992, Hamdia and El-Komy 1998). Moreover, the danger of increasing soil salinity is likely to further limit the application of N to rangelands (Mohammed *et al.* 1989). Hence, the importance of biological fixation of nitrogen has increased (Zahran 1991, Katerji 2000, Ribaudo *et al.* 2001)

In attempts to gain information on the latter aspect, we have recently studied the possible role played by associative nitrogen fixing *Azospirillum* and *Bacillus* spp. on growth, mineral nutrition, nitrogen fixation and nitrogen uptake (using ^{15}N techniques) in maize and wheat grown under salinity stress (Hamdia and El-Komy 1998, Hamdia *et al.* 2000). These investigations were focused mainly on the effects of these rhizobacteria in nitrogen fixation and phytohormonal balance. The beneficial effects were correlated with the exogenous application of GA_3 (Hamdia and El-Komy 1998).

Azospirillum has been reported to improve N_2 supply in association with cereals even under field conditions, although the mode of interaction between plants and bacteria is not fully understood (Bashan and Holguin 1997). It is not clear wheather bacteria provide an excess of fixed nitrogen that is supplied to the plant. An alternative to N_2 fixation as an explanation for N accumulation following *Azospirillum* inoculation is bacterial nitrate reductase (NR) theory. *Azospirillum* is

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Abbreviations: ARA - acetylene reduction assay; CFU - colony forming units; N_2-ase - nitrogenase; NR - nitrate reductase.

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Fax: (+20) 86 342601, e-mail: heshamelkomy@hotmail.com

able to perform denitrification either *in vitro* or in association with the host plant (Danneberg *et al.* 1986, Boddey and Döbereiner 1988, Ribaudo *et al.* 1998).

The input of reduced nitrogen to plant is determined by activity of nitrate reductase, which catalyses the first step and determines rate of this assimilating process. Several environmental factors like temperature, water stress as well as the nitrogen source influence synthesis and activity of this enzyme (Ourry *et al.* 1992, Munjal *et al.* 1997, Sagi *et al.* 1997).

Therefore, this investigation has been performed to evaluate nitrate reductase and nitrogenase activity as well as growth and mineral nutrition of wheat plants inoculated with different *Azospirillum* strains (NR^- and NR^+) grown under drought stress.

Materials and methods

Azospirillum lipoferum 137, a nitrate reductase positive strain (NR^+), kindly supplied by Prof. Vasyuk, Russian Academy of Sciences, St. Petersburg, Russia, and *Azospirillum brasilense* W/S, a nitrate reductase negative strain (NR^-) isolated from wheat histosphere (El-Komy 1992) were used in this study. Nitrite formation and denitrification ability of these bacteria were tested according to Neyra *et al.* (1977). Bacterial strains were grown in malate medium supplied with 0.2 g dm^{-3} yeast extract for 20 h at 30 °C on a shaker at 200 rpm. Cells were harvested at the logarithmic phase by centrifugation, washed twice in sterile demineralized water and then used as inoculum of amount of 1 cm^3 = 10^7 CFU per seed.

Seeds of wheat (*Triticum aestivum* L. hybrid VI) were surface sterilized by immersion in mixture of ethanol 96 % and 95 % H_2O_2 (1:1, v/v) for 3 min, followed by several washings with sterile distilled water and germinated on wet sterile filter papers in Petri dishes for 3 d in the dark. Six germinated seeds were transplanted into pot containing 5-kg of a mixture of sand and garden clay soil in a ratio of 1:2 (m/m). Seedlings of the first group were inoculated with NR^+ bacterial strain, whereas the second group of pots was inoculated with NR^- strain and pots of the third group were left without inoculation as control. Pots were equally nitrogen fertilized in the form of NH_4NO_3 at 0.5 mM concentration. At the beginning of the second week after sowing, plants were thinned down to three per pot and the soil moisture content was adjusted to 100, 80, 60, and 40 % of the field capacity. Pots were arranged in a complete randomized design with six replicates for each treatment. After 120 d of sowing the plants were harvested. Shoot, root and spikes were detached separately. Shoots and roots were then dried in an oven at 70 °C to constant mass, then weighed and ground for further analysis.

Densities of *Azospirillum* populations were determined in root histosphere (crushed roots after had been washed and shaken for 2 min in ethanol) at harvesting using the dilution plate method on DN-medium supplemented with Congo Red (0.25 % aqueous solution, Rodrigues-Caceres 1982).

Nitrogenase activity (*in situ*) was assayed in a defined washed root fresh mass of inoculated and uninoculated plants. The acetylene reduction assay (ARA) was applied as described by Turner and Gibson (1980), using a gas chromatograph *ATIUNICAM 610-GLC* (UK) equipped with a glass column filled with activated alumina. The ARA was performed after 24-h incubation with 10 % acetylene. Results were expressed as nmol(C_2H_4) g^{-1}(f.m.). Nitrate reductase activity (*in vivo*) was determined by the method of Jaworski (1971) as follows: one-gram sample of fresh plant tissue was incubated for 30 min at 30 °C in assay medium (pH 7), then boiled at 100 °C for 5 min. The nitrite was then determined colorimetrically using sulphanilic acid and α-naphthylamine solution at 520 nm with a *55B Perking Elmer* spectrophotometer (UK).

Total N content of dry shoot mass was determined after Kjeldahl digestion, and the total N yield was calculated according to Rennie (1980).

The contents of chlorophyll *a* and *b* and carotenoids were determined using the spectrophotometric method of Metzner *et al.* (1965). Saccharides were determined by the anthrone-sulfuric acid method (Fales 1951), amino acids according to Moore and Stein (1948), and soluble protein according to Lowry *et al.* (1951). Proline was determined according to Bates *et al.* (1973). Sodium and potassium were determined by the flame photometric method (Williams and Twine 1960) using *Corning 410* flame photometer (Essex, England), calcium and magnesium by the versene titration method (Schwarzenbach and Biedermann 1948). Experimental data were subjected to one way analysis of variance and the means were separated by the least significant difference (LSD).

Results

Decreasing soil moisture content decreased plant dry mass and water content (Table 1). Inoculation of plants with any of the tested *Azospirillum* spp. (NR^+ or NR^-) resulted in significant increases in plant dry mass. This stimulatory effect was more pronounced in root mass up to 2-fold in plants inoculated with (NR^{+}) than in shoot mass. Application of the (NR^{+}) also significantly enhanced the plant water content in both shoots and roots. The photosynthetic pigments were decreased with decreasing soil moisture content (Table 1), while plant bacterization resulted in pronounced increases in photosynthetic pigments compared with non inoculated plants in most treatments.

Nitrogen content in shoots and roots was decreased by decreasing soil moisture content, however, *Azospirillum* inoculation significantly elevated the total shoot and root N-yield. It is interesting to indicate that NR^{+} strain gave the highest N-yield, especially in the root system.

Soluble saccharides and soluble protein contents were markedly decreased by decreasing soil moisture content when compared with plants grown under 100 % saturation of field capacity. *Azospirillum* inoculation significantly increased soluble saccharides and soluble proteins in both the shoots and roots of wheat. Such increases were markedly prominent at lower soil moisture contents (60 and 40 %). Total amino acids and proline contents ran parallel to each other and markedly increased with increasing soil moisture content in roots but remained almost unchanged in shoots. It appears that NR^{+} strain significantly increased the plant tissue proline content, whereas, NR^{-} strain lowered the proline content (Table 2).

Magnesium content also decreased with decreasing soil moisture content in both shoot and root systems (Table 3). *Azospirillum* inoculation had insignificant effects on Mg^{2+} content. However, potassium and

Table 1. Effect of *Azospirillum* (NR^{+} and NR^{-} strains) inoculation at different soil moisture (100, 80, 60, and 40 % of field capacity) on shoot and root dry mass [g plant^{-1}], total N-yield [mg(N) plant^{-1}], water content [% f.m.], and pigment contents [μg g^{-1}(d.m.)] of 120-d-old wheat plants.

Moisture		Shoot		Root		Water content		Pigments		
[%]		d.m.	N-yield	d.m.	N-yield	shoot	root	Chl *a*	Chl *b*	Car
100	control	9.3	83.7	8.9	101.2	3.7	1.6	3.9	0.9	0.47
80		7.5	67.5	8.2	98.4	3.8	1.9	3.2	1.4	0.38
60		6.3	69.3	6.7	60.3	1.9	1.3	2.9	1.2	0.83
40		4.1	45.1	2.5	28.0	1.9	0.9	2.8	1.4	0.82
100	NR^{+}	9.7	136.4	15.6	160.8	4.7	9.1	3.3	1.6	0.97
80		9.4	126.2	18.9	176.7	6.7	13.5	3.7	1.6	0.96
60		8.9	120.2	11.8	129.8	5.2	10.2	3.6	1.7	0.86
40		5.6	71.6	3.8	57.0	1.9	1.7	3.6	1.1	0.68
100	NR^{-}	11.6	139.2	11.0	154.0	3.3	1.2	3.1	1.2	1.02
80		9.2	128.8	9.6	105.6	3.0	1.9	2.6	1.2	0.64
60		7.1	78.1	6.2	62.0	2.5	2.0	3.2	1.3	0.79
40		4.9	46.5	2.1	27.3	1.3	0.7	2.9	1.2	0.65
LSD$_{0.05}$		1.4	11.2	1.2	13.7	0.2	0.1	0.1	0.3	0.09

Table 2. Effect of *Azospirillum* inoculation at different soil moisture contents on contents of soluble saccharides, soluble proteins, amino acids, and proline [mg g^{-1}(d.m.)] content of shoots and roots of 120-d-old wheat plants.

Moisture		Soluble sugars		Soluble proteins		Amino acids		Proline	
[%]		shoot	root	shoot	root	shoot	root	shoot	root
100	control	26.1	24.4	27.1	23.3	1.8	1.6	0.172	0.178
80		25.7	19.8	21.2	20.0	1.6	1.9	0.176	0.182
60		18.6	18.1	17.5	16.4	2.3	4.3	0.232	0.217
40		15.7	15.9	14.6	13.3	2.4	4.9	0.333	0.437
100	NR^{+}	27.6	28.9	26.9	38.3	1.4	2.3	0.246	0.250
80		28.7	25.9	27.9	34.9	1.6	2.9	0.135	0.234
60		21.4	23.7	29.4	28.6	1.6	4.7	1.020	0.219
40		22.1	24.5	18.7	20.2	2.4	4.8	0.894	0.155
100	NR^{-}	25.5	29.5	22.2	22.3	1.7	2.6	0.226	0.128
80		23.9	26.2	26.3	22.7	1.7	3.0	0.253	0.148
60		21.8	22.3	20.5	21.1	3.3	1.7	0.128	0.057
40		20.8	21.2	15.4	24.1	2.9	1.8	0.109	0.068
LSD$_{0.05}$		2.8	3.1	4.4	6.5	1.3	0.01	0.001	0.020

calcium ions were more or less unchanged in control uninoculated plants with the drop in humidity neverthless, plant bacterization significantly increased their contents in both shoots and roots.

Nitrate reductase activity in control uninoculated plants was decreased by decreasing soil moisture content (Table 4). Inoculation of the wheat plants with NR$^+$ strain led to the increase in NR activity in roots as compared with control uninoculated plants or plants inoculated with NR$^-$strain. However, inoculation of the plant with *A. brasilense* NR$^-$ strain increased NR activity in the shoot system compared with controls and roots of plants inoculated with NR$^-$strain.

Nitrogenase activity and bacterial counts were decreased at low soil moisture content. Plants inoculated with the NR$^+$ strain showed higher nitrogenase activity, but less bacteria colonized the plant histosphere compared with NR$^-$ strain. Inoculation with NR$^-$ strain resulted in the highest bacterial numbers colonizing the histosphere, but it fixed less nitrogen compared with NR$^+$strain.

Table 3. Effect of *Azospirillum* inoculation at different soil moisture contents on mineral content [mg g^{-1}(d.m.)] in shoots and roots shoots and roots of 120-d-old wheat plants.

Moisture [%]		Shoot K^+	Ca^{2+}	Mg^{2+}	Root K^+	Ca^{2+}	Mg^{2+}
100	control	5.2	3.8	2.4	2.6	4.5	2.1
80		6.1	4.0	2.4	2.6	4.5	2.1
60		5.5	4.5	2.7	5.4	4.0	2.7
40		4.8	5.5	2.7	6.3	5.0	2.4
100	NR$^+$	6.9	5.0	2.1	5.2	5.5	2.4.
80		9.3	7.0	2.9	4.3	9.0	2.6
60		11.7	6.5	2.1	6.6	8.0	2.6
40		7.6	7.0	3.3	6.6	6.5	3.6
100	NR$^-$	7.4	6.0	2.4	4.7	4.5	2.1
80		8.9	6.0	2.1	6.4	7.0	3.0
60		6.6	5.0	2.4	6.0	6.0	2.4
40		5.5	5.5	1.8	7.8	5.5	1.8
$LSD_{0.05}$		1.3	1.8	2.0	1.3	2.1	1.1

Table 4. Effect of *Azospirillum* inoculation at different soil moisture contents on nitrate reductase (NR) activity [μmol (NO_2) g^{-1}(f.m.) h^{-1}]; nitrogenase (N_2-ase) activity [nmol g^{-1}(f.m.)], total bacteria counts in the histosphere (log number), spike number [plant^{-1}] and spike mass [g plant^{-1}] of 120-d-old wheat plants.

Moisture [%]		NR shoot	root	N_2-ase	Total count	Spike number	Spike mass
100	control	1.81	1.42	41.6	1.6	8.5	7.4
80		2.12	1.31	46.4	1.4	7.7	4.2
60		1.23	1.20	37.8	1.1	5.7	3.6
40		1.01	0.91	20.7	0.8	6.5	2.8
100	NR$^+$	1.91	1.61	95.1	5.3	9.7	13.4
80		2.81	1.52	70.2	5.4	8.0	10.7
60		2.12	1.41	55.0	3.1	6.9	6.7
40		1.61	1.31	32.0	3.0	7.0	5.8
100	NR$^-$	2.21	1.41	130.0	4.8	13.4	11.8
80		2.62	1.21	90.0	4.9	10.7	7.2
60		1.13	1.01	75.0	3.2	7.1	7.9
40		1.63	0.72	40.0	2.2	6.8	3.7
$LSD_{0.05}$		0.12	0.08	9.3	0.16	1.3	1.6

Discussion

Fresh mass, dry mass and water content of wheat plants decreased with decreasing soil moisture content, these changes were accompanied with a drop in the contents of soluble sugars and soluble proteins. *Azospirillum* inoculation with either bacterial strain (NR$^+$ or NR$^-$) significantly increased the above mentioned growth characteristics even at the lowest moisture content tested (40 %). Since at this moisture content nitrogen fixation and bacterial counts were at their minimum values (Table 4), it can be assumed that the stimulatory effect on growth parameters might have occurred due to other factors than nitrogen fixation. Several research groups attributed the increase in plant growth following *Azospirillum* inoculation to the production of growth promoting substances (IAA, IBA, and several gibberellins) by *Azospirillum* spp. (Omay *et al.* 1993, Bashan and Holguin 1997). Supporting this view, when the variously salinized maize plants were sprayed with 100 μg g^{-1} GA_3 or inoculated with *Azospirillum,* a pronounced stimulatory effect on growth parameters was obtained at all NaCl levels used (Hamdia and El-Komy 1998). These results also showed that *Azospirillum* inoculation significantly increased the chlorophyll content. Similar findings were reported earlier by Hegazi (1983) who attributed the increase in growth parameters following *Azospirillum* inoculation to general improvement of physiological status of the inoculated plants including the chlorophyll content.

The observed drop in saccharide content as well as in protein content in shoots and roots of water-stressed wheat plants was accompanied by a marked increase in the total amino acids content. These results are in accordance with previously reported findings of Devitt *et al.* (1987). It is worthy to point out that in our experiment when wheat plants were inoculated with various *Azospirillum* strains the opposite effect occurred. Saccharides as well as soluble proteins progressively increased, especially at lower soil moisture. This was accompanied by a increased contents of amino acids, especially in the root system inoculated by NR^+ strain and in the shoot system of plants inoculated by NR^- strain. Thus *Azospirillum* inoculation might play an important role in the protein biosynthesis, either by direct nitrogen supply (through fixation of nitrogen) or indirectly by the accumulation of nitrite (as a result of nitrate reductase activity) especially at low soil moisture contents (60 and 40 %).

Nitrate reductase (NR), the first enzyme in the nitrate assimilation pathway, is a limiting factor of plant growth and development (Solomonson and Barber 1990) and it is influenced by a variety of environmental factors (Crawford 1995). Reports on NR activity in plants are contradictory. Cramer and Lips (1995) reported that NR was slightly inhibited by salinity in tomato roots, while leaf NR decreased sharply. However, Martinez and Cerda (1989) showed that NR activity increased in tomato and cucumber leaves with exogenous NO_3 concentration. Results presented in this investigation (Table 4) indicate that low soil moisture content decreased both shoot and root nitrate reductase activity. Our results are in accordance with the findings of Peuke *et al.* (1996) and Abd El-Baki *et al.* (2000). These authors showed that NR activity decreased by increasing salt stress, moreover, NR itself is a major signal affecting NR expression and activity under salinity.

An alternative to N_2 fixation as an explanation for N accumulation and growth enhancement following *Azospirillum* inoculation in cereals is bacterial nitrate reductase (NR) theory (Boddey and Döbereiner 1988, Bothe *et al.* 1992, Ribaudo *et al.* 1998). Results of the present study (Table 4) indicated that wheat plants inoculated with NR^+ strain, exhibited increased root NR activity compared with those of uninoculated plants or inoculated with NR^- strain. However, plants inoculated with NR^-strain showed more NR activity in shoots than roots of the same plant and the leaves of control plants. These results are in agreement with findings of Ferrira *et al.* (1987) and Boddey and Döbereiner (1988). The last authors showed that when the wheat plants were inoculated with *Azospirillum brasilense* strain 245 and its NR^- mutant, NR activity of wheat leaves was increased by inoculation with NR^- mutants compared with parental NR^+strain. This phenomenon indicates that the effect of some *Azospirillum* strains on plants is not solely *via* N_2 fixation hence NR^+ and NR^- strains have this ability (see Table 4), but rather to an increase in these nitrate assimilation. The high NR activity in the shoots of plants inoculated with NR^- strain indicated that the nitrate was translocated to the top in the unreduced form, whereas inoculation with NR^+ strain aided nitrate reduction in the roots causing a decrease in NR activity in the shoots (Boddey and Döbereiner 1988). Our results (Table 4) have also showed that NR activity in the shoots of plants inoculated with NR^+ strain was higher than that in control uninoculated plants.

Our data have also showed that wheat plants inoculated with either NR^+or NR^- *Azospirillum* strains gave higher nitrogenase activity and histosphere colonization than uninoculated control plants (Table 4). Nitrogenase activity obtained in control plants could be attributed to bacterial cells, which were not entirely eliminated by seed surface sterilization (El-Komy 1992). The low N application (0.05 mM) did not affect nitrogen fixation in the washed roots. Inoculation with NR^+ strain gave higher nitrogen fixation and lower histosphere colonization compared with NR^- strain. These findings agree with results of Ferrira *et al.* (1987), and could be explained on the basis that nitrogenase activity measured by the ARA comes mainly from bacterial cells colonizing the rhizosphere (washed roots). This activity can be affected by root sterilization. Moreover, Danneberg *et al.* (1986) concluded that both the nitrogen fixation and nitrate reductase in *Azospirillum*-wheat association are strain-dependent.

Data presented in this investigation indicate that plant yield was influenced by soil moisture content. Plant bacterization significantly enhanced yield criteria (Table 4). Results also showed that NR^- strain was less effective than NR^+ strain in promoting inoculation responses of total N-yield, spike numbers and their mass. These results are in accordance with the results obtained by many investigators (Boddey and Döbereiner 1988, Bothe *et al.* 1992, Ribaudo *et al.* 1998). Moreover, stimulation of nitrate reductase activity promoted NO_3 reduction and its subsequent assimilation into protein and thus increased the plant yield (Grattan and Grieve 1993).

Azospirillum inoculation did not affect wheat Mg^{2+} content. However, K^+ and Ca^{2+} contents significantly increased. Bashan *et al.* (1990), observed variable effects of *Azospirillum* inoculation on mineral accumulation. However, Stancheva *et al.* (1995) suggested that the enhancement in uptake of NO_3^- and K^+ by *Azospirillum* inoculation caused an increase in foliar dry matter and accumulation of minerals in stems and leaves. During the reproductive period, these minerals might have been transferred to the panicles and spikes and finally resulted in a higher yield. It has further been suggested that increased mineral uptake by *Azospirillum*-inoculated wheat roots is provided by enhancement in proton efflux activity (Bashan 1990) which is directly related to the balance of ions in plant roots (Sarwar 1998).

In conclusion, data presented here indicate that the mode of action of *Azospirillum* is most probably composed of multiple mechanisms and potential nitrate reductase is one of them as previously suggested by Bashan (1998). The importance of *Azospirillum* NR^+ strains for increasing wheat resistance to water stress is also supported by the obtained data.

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