Introduction

Nitrate is the prevalent form of available nitrogen in well-aerated soil, however the concentration of this anion is very variable due to its high soil mobility and the microorganism activities (Forde and Clarkson, 1999). The ability of plants to adapt their uptake systems to the changing environmental conditions could be an important factor for their nitrogen use efficiency (NUE) and hence for their growth and fitness. Humic substances are known to positively influence plant growth and nutrition; particularly, low-molecular fractions have been shown to increase NO_3^- uptake and PM H^+-ATPase activity (Pinton et al., 1999) and alter expression of related genes (Quaggiotti et al., 2006). In this work, a water-extractable low-molecular humic fraction (WEHS) has been tested for its ability to affect molecular physiology of nitrate uptake in two maize inbred lines with different NUE (Locci et al. 2001).

Materials and methods

Maize *(Zea mays* L., inbred line Lo5 or T250) seeds were germinated over aerated 0.5 mM $CaSO_4$ solution in a dark growth chamber at 27°C. After 3 days, the seedlings were transferred into an aerated solution containing 0.5 mM $CaSO_4$ in a controlled climatic conditions (day/night photoperiod, 16/8; light intensity, 220 µE $m^{-2}s^{-1}$; temperature (day/night) 25/20 °C; RH 70 to 80 %). After 2 days, seedlings were treated with KH_2PO_4 0.175 mM, $MgSO_4$ 0.1 mM, KCl 0.005 mM, $FeSO_4$ 0.002 mM, H_3BO_3 0.0025 mM, $MnSO_4$ 0.0002 mM, $ZnSO_4$ 0.0002 mM, $CuSO_4$ 0.00005 mM, H_2MoO_4 0.00005 mM, $CaSO_4$ 0.4 mM, 0.2 mM NO_3^- and 0.025 mM NH_4PO_4 with or without 5 mg C_{org} L^{-1} WEHS (isolated as described by Pinton et al., 1999) for 0, 2, 4, 6, 8, 12 or 24 hours.

Time courses of net NO_3^- uptake by root were determined as NO_3^- depletion from the solution containing 0.5 mM $CaSO_4$ and 0.2 mM NO_3^-, as described by Cataldo et al. (1975). In parallel, expression levels of genes involved in nitrate acquisition were evaluated by means of Real-time RT-PCR experiments as described in Tomasi et al. (2009). Gene studied (accession number in brackets) and primers (PCR efficiency in brackets) used were the following: *high affinity nitrate transporter 2.1* (*NRT2.1*; unpublished) 5'-GATCGACGATCACCTATACCTC-3' and 5'-GTGCTCCGTTGACATGAG-3' (74.7 %); *PM H^+-ATPase 2* (*MHA2*; U09989), 5'-TCCGACTGTTGTTTGTCGAG-3' and 5'-CACCGACTCCATCCTCATCT-3' (87.05 %); *nitrate reductase 2 (NR2*; U20450) 5'-GGTGAAGGTCAACGTGTGC-3' and 5'-CGGTCTCGAGGTGCTTCT-3' (90.15 %); and as housekeeping gene, an ribosomal protein gene (*RPL17*; AF034948) 5'-AAAGTCTCGCCACTCCAATG-3' and 5'-ACGTCCAAGCCTTTCACATC-3' (80.02 %).

Results

Net NO_3^- uptake rates were measured on the whole root system of each of the two inbred lines in a 0-24 hours time span of contact with nitrate in the presence or absence WEHS. Figure 1 shows that the presence of WEHS altered the well-known pattern of net uptake rates following exposure of roots to the anion. In particular, the humic fraction caused an acceleration

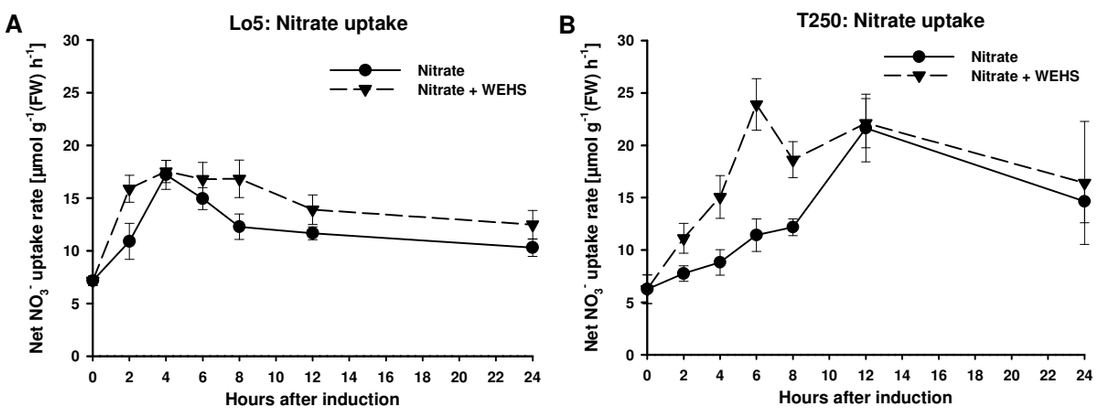

Figure 1. Time course of net nitrate uptake rate in roots of two maize inbred lines exposed to the anion in presence or absence of 5 mg C_{org} L^{-1} WEHS. Seedlings of Lo5 (panel A; high NUE) and T250 (B; low NUE) lines were transferred into an uptake solution containing 0.2 mM NO_3^- at the indicated time intervals. Data are means ± SD (n = 5).

of the increase in net nitrate uptake rate in both lines, almost halving the time needed to reach the maximal uptake capacity; as a tendency, values of uptake remained higher in WEHS-treated

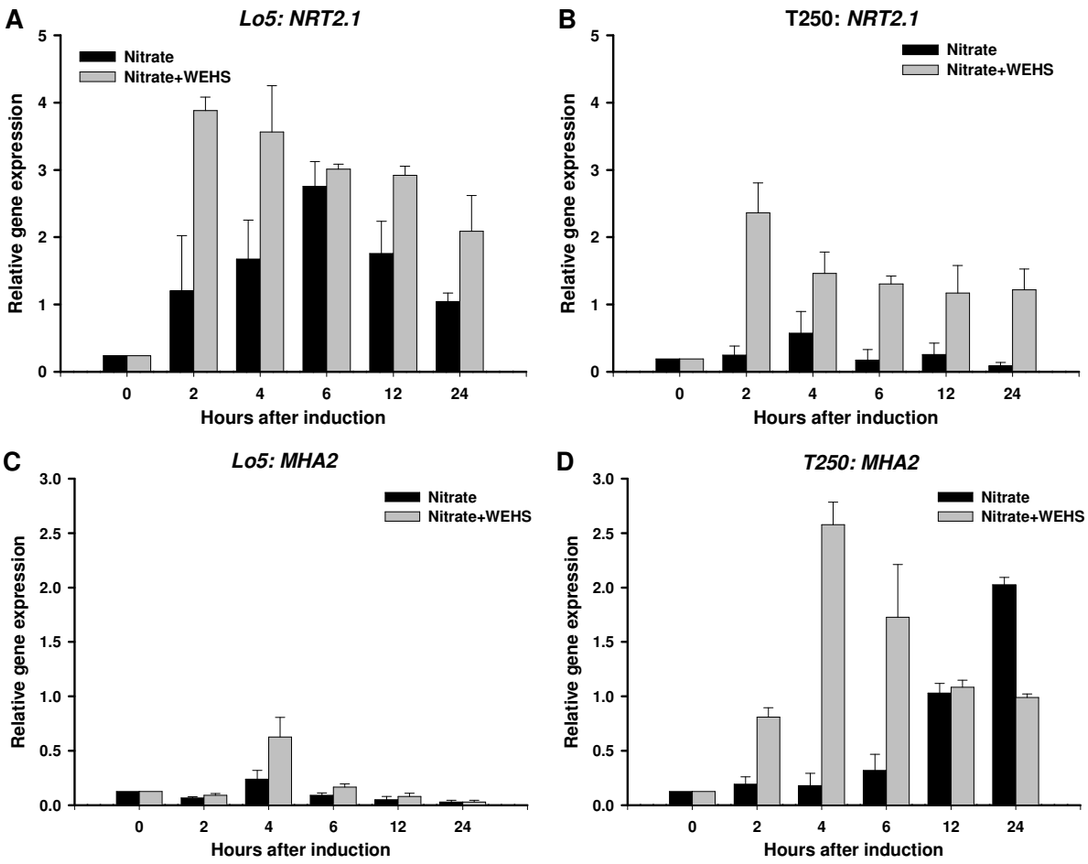

*Figure 2. Time course of expression levels of a high-affinity nitrate transporter (*NRT2.1*; A, B) and a plasma-membrane H^+-ATPase (*MHA2*; C, D) genes in roots of two maize inbred lines exposed to nitrate in presence or absence of 5 mg C_{org} L^{-1} WEHS. Gene expression levels were determined by Real-time RT-PCR at time intervals. Data are means ± SD (n = 3) and were calculated on the basis of expression levels of the housekeeping gene at time 0.*

roots as compared with control roots.

To investigate the transcriptional changes involved in the modulation of responsiveness to nitrate by WEHS, expression levels of some genes related to the acquisition and assimilation of the anion, were analyzed (Figure 2 and 3).

Changes in expression levels of the high-affinity nitrate transporter gene (*NRT2.1*) in roots of WEHS-treated plants followed the same pattern similar to that observed for the net uptake for both genotypes (Fig. 2A and B). Also a gene coding for a PM H^+-ATPase isoform (*MHA2*), that generates the proton-motive force for symporter-mediated nutrient uptake, was up-regulated by the presence of WEHS in the root bathing solution (Fig. 2C and D).

In parallel, the expression of the first enzyme in the nitrate assimilation pathway was monitored. Figure 3 shows that WEHS treatment caused a rapid increase in *nitrate reductase 2* transcript levels which followed a biphasic pattern for both lines. Expression levels were much higher and more quickly occurring in the high Lo5 line (high NUE) than in the other.

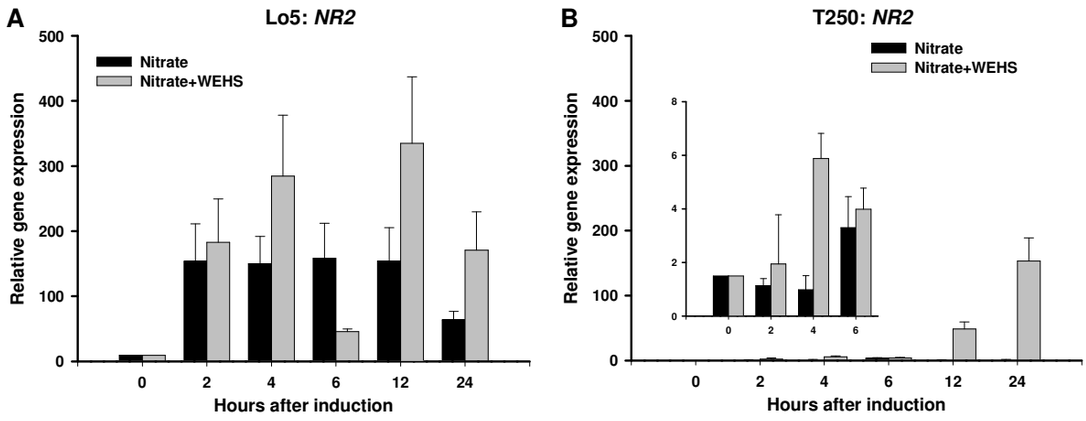

*Figure 3. Time course of expression levels of a nitrate reductase (*NR2*) gene in roots of two maize inbred lines exposed to nitrate in presence or absence of 5 mg C_{org} L^{-1} WEHS. Gene expression levels were determined by Real-time RT-PCR experiments at time intervals. Data are means ± SD (n = 3) and were calculated on the basis of expression levels of the housekeeping gene at time 0.*

Discussion

Nitrogen nutrition is one of the key limiting growth factors for cereals, mainly because of their low Nitrogen Use Efficiency (NUE) (Raun and Gohnson, 1999). The improvement of NUE is also becoming urgent to produce these crops in a more cost-effective and eco-compatible way. This could be done by breeding for high NUE at low fertilizer input and by optimizing plant-soil relationships in order to improve the acquisition of native and applied nitrogen. Soil humic substances have been shown to promote nutrient acquisition, including nitrate uptake; however, molecular basis of this behaviour have not been clarified so far.

In the present work the role of a water-extractable low-molecular weight humic fraction (WEHS) in modulating nitrate uptake and assimilation has been investigated. Using two inbred lines characterized by a different responsiveness to nitrate exposure, we could confirm that WEHS causes a faster increase in the net nitrate uptake rate, independently of the genotype considered. This effect appears to be due, at least in part, to a transcriptional regulation of a gene encoding for a high-affinity nitrate transporter. These higher uptake rates could be reasonably sustained by a higher activity of the plasma membrane proton pump that generates the electrochemical gradient for anion transport (Santi et al., 1995; Santi et al., 2003); indeed, this assumption could be somehow supported by the expression data of a PM H^+-ATPase isoform, the effect being particularly evident for the T250 inbred line.

NRT2.1 gene expression remained at high levels even when a feedback regulation should have occurred, thus indicating that WEHS treatment could poise the tissue for higher nitrate accumulation. This could be accomplished by a faster metabolic utilization; interestingly a dramatic increase in the expression level of a nitrate reductase gene was observed in WEHS treated plants. It might also possible that the following steps (e.g. GS-GOGAT) are also enhanced in order to remove feedback active products.

In conclusion, it appears that humic substances can favour nitrogen acquisition by improving uptake and assimilation of nitrate. Data presented indicate the importance of considering the interactions between roots and soil components in order to get a better understanding of nutrient use by plants and to improve agricultural practices aiming at reducing input of fertilizers.

Acknowledgments

Research was supported by grant from Italian M.I.U.R (P.R.I.N.) to R.P. and Z.V.

References

Cataldo DA, Haroon M, Schrader LE et al. Rapid colorimetric determination of nitrate in plant tissues by nitratation of salicylic acid. Commun. Soil Sci. Plant Anal. 1975; 6:71-80.

Forde BG, Clarkson DT. Nitrate and ammonium nutrition of plants: physiological and molecular perspectives. Adv. Bot. Res. 1999; 30: 1-90.

Locci G, Santi S, Monte R et al. Involvement of plasma membrane H^+-ATPase in nitrate uptake by maize genotypes. In: WJ Horst et al. eds. Plant nutrition - Food security and sustainability of agro-ecosystems. Dordrecht, Netherland: Kluwer Academic Publishers; 2001: 184-185.

Pinton R, Cesco S, Iacolettig G et al. Modulation of NO_3^- uptake by water-extractable humic substances: involvement of root plasma membrane H^+-ATPase. Plant Soil 1999; 215:155-161.

Quaggiotti S, Ruperti B, Pizzeghello D et al. Effect of low molecular size humic substances on nitrate uptake and expression of genes involved in nitrate transport in maize (*Zea mays* L.). J. Exp. Bot. 2004; 5: 803-813.

Raun W, Gohnson GV. Improving nitrogen use efficiency for cereal production. Agron. J. 1999; 91: 357-363.

Santi S, Locci G, Pinton R et al. Plasma membrane H^+-ATPase in maize roots induced for NO_3^- uptake. Plant Physiol. 1995; 109: 1277-1283.

Santi S, Locci G, Monte R et al. Induction of nitrate uptake in maize roots: expression of a putative high-affinity nitrate transporter and plasma membrane H^+-ATPase isoforms. J. Exp. Bot. 2003; 54:1851-1864.

Tomasi N, Kretzschmar T, Espen L et al. Plasma-Membrane H^+-ATPase-dependent citrate exudation from cluster roots of phosphate-deficient white lupin. Plant Cell Env. 2009; 32: 465-475.