Environmental and Experimental Botany 147 (2018) 167–178

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Water-extractable humic substances speed up transcriptional response of maize roots to nitrate

Laura Zanin[a,*], Nicola Tomasi[a], Anita Zamboni[b], Davide Sega[b], Zeno Varanini[b], Roberto Pinton[a]

[a] Dipartimento di Scienze Agroalimentari, Ambientali e Animali, University of Udine, via delle Scienze 206, I-33100 Udine, Italy
[b] Dipartimento di Biotecnologie, University of Verona, Ca' Vignal 1- Strada Le Grazie 15, I-37134 Verona, Italy

ARTICLE INFO

Keywords:
Zea mays
Nitrate induction
NO_3-
Gene expression
Microarray
Transcriptomic response
Nrt
Nitrogen assimilation

ABSTRACT

Humic substances are known to positively influence plant growth and nutrition. In particular, the water-extractable fraction of humic substances (WEHS) has been shown to enhance nitrate acquisition, increasing the activity of high affinity nitrate uptake system. However, molecular bases of this physiological response are not clarified so far.

Thus, in the present work, the physiological effect of WEHS on nitrate acquisition in maize roots was correlated with changes in the root transcriptomic profile.

Results confirmed that WEHS caused a faster induction of a higher capacity to take up nitrate in maize roots. Comparing the root transcriptomic profile of *Nitrate*- and *Nitrate* + *WEHS*-treated plants with *Control* (-N) ones, more than 2000 transcripts appeared to be modulated only in the presence of WEHS. Among these, genes involved in nitrate transport and assimilation (*NRT1s, NRT2s, NAR2.1, NR, GS, GOGAT, CNX, UPM*) were strongly modulated by WEHS. Furthermore, also some genes known to be linked to the nitrogen limitation responses were affected by WEHS, as transcripts coding for transcription factors (as *LBD37, NIN-like protein, NFY-A, GRF5*) and enzymes of hormones' metabolism. The modulation of these transcripts might play a crucial role in coordinating the induction to nitrate, favouring its uptake and assimilation in WEHS-treated plants. The over-expression of nitrogen assimilatory genes by WEHS might led to an early feedback regulation of the high affinity nitrate transport system, as being operated by N-metabolites.

Results of the present work shed further light on the contribution of the organic soil component to the nitrogen use efficiency in crops.

1. Introduction

Humic substances (HS), comprising humic acids, fulvic acids and humin are the main components of soil organic carbon (Stevenson, 1994). Over the last decades a great deal of literature has shown that humic substances (HS) can stimulate plant growth and nutrition (Calvo et al., 2014; Rose et al., 2014). It has been estimated that the concentration of HS in the soil solution may reach 250 mg L^{-1} (Gerke, 1993) and some works provided evidence that, under hydroponics conditions even lower concentration of HS (5 mg L^{-1}) could determine beneficial effects on nutrition and growth of plants (Cacco et al., 2000; Canellas et al., 2002; Vaccaro et al., 2015; Pinton et al., 1999). It has been hypothesized that the biological activity of HS might be related to a direct interaction of HS with the roots, especially the low molecular size of fulvic acids might render plausible their movement towards the root surface and in the apoplastic compartment (Varanini and Pinton, 1995). Using ^{14}C-labelled HS (HA and FA), Vaughan and Ord (1981) registered the incorporation of radioactivity in pea roots as operated by

Abbreviations: ACO, aconitase; ADA2, adenosine deaminase 2; AGO1, Argonaute1; ANR1, Arabidopsis nitrate regulated 1; AP2, apetala 2; ARR9, type A response regulator 9; ASN, asparagine synthetase; BST1, bristled1; C2H2, Cysteine 2 Histidine 2; C4 and C8, transcriptomic profiles of *Control*-plants at 4 and 8 h respectively; CDS, coding sequence; cHATS, constitutive high-affinity transport system; CIPK23, CBL-interacting protein kinase 23; CK, cytokinin; CNX, cofactor for nitrate reductase and xanthine dehydrogenase; DER4, deformed root hairs 4; FA, fulvic acid; FC, fold change; GARP, Golgi associated retrograde protein; GOGAT, glutamine oxoglutarate aminotransferase; GRF5, growth regulating factor 5; GS, glutamine synthetase; HA, humic acid; HS, humic substances; iHATS, inducible high-affinity transport system; LBD37, LOB domain-containing protein 37; MATE, multidrug and toxin extrusion protein transporters; N, nitrogen; N4 and N8, transcriptomic profiles of *Nitrate*-plants at 4 and 8 h respectively; NAR, nitrate assimilation related; NFY-A, Nuclear transcription factor Y subunit alpha; NIN-like, nodule inception-like; NiR, nitrite reductase; NLP7, NIN like protein7; NR, nitrate reductase; NRT, nitrate transporter; NW4 and NW8, transcriptomic profiles of *Nitrate* + *WEHS*-plants at 4 and 8 h respectively; SAUR, small auxin-upregulated RNA; SD2-5, transcription factor S-domain-2 5; TDT, tonoplast dicarboxylate transporter; UPM1, urophorphyrin methylase 1; VPT, vacuolar phosphate transporter; WEHS, water-extractable humic substances

* Corresponding author.
E-mail addresses: laura.zanin@uniud.it (L. Zanin), nicola.tomasi@uniud.it (N. Tomasi), anita.zamboni@univr.it (A. Zamboni), davide.sega@univr.it (D. Sega), zeno.varanini@univr.it (Z. Varanini), roberto.pinton@uniud.it (R. Pinton).

https://doi.org/10.1016/j.envexpbot.2017.12.014
Received 12 October 2017; Received in revised form 13 December 2017; Accepted 14 December 2017
Available online 16 December 2017
0098-8472/

two uptake components: one initial and rapid passive process, and a second slower, but continuous, active uptake dependent on metabolism. More recently Kulikova et al. (2014) demonstrated that tritium-labelled humic acids preferentially accumulated in the apices of root of wheat seedlings. However, the mechanisms by which HS are able to enhance the nutrient acquisition by plants are still under debate. Some authors have distinguished between indirect effect (as reducing soil compaction) and direct effects of HS (Varanini and Pinton, 2001). Among these last the improvement of the overall plant biomass, the root ion uptake, the rhizosphere acidification by roots, the nutrient allocation in leaves have been envisaged (Pinton et al., 1997; Pinton et al., 1999; Canellas et al., 2002; Nardi et al., 2002; Zanin et al., 2015a; Zamboni et al., 2016). For a sustainable agriculture, improving nutrient use efficiency in crops has become a research topic of great interest. In particular, new soil and crop management practices should be applied in modern agriculture to optimize the acquisition of nitrogen, the main nutrient required by plants and supplied as fertilizer to the crops. 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).

Previous evidence showed that humic molecules are able to stimulate the nitrate absorption and the activity of PM H^+-ATPase in roots (Albuzio et al., 1986; Keeling et al., 2003; Jannin et al., 2012). It has been suggested that the modulation of nitrate uptake by HS might involve their direct interaction with the activity PM H^+-ATPase (Varanini et al., 1993; Pinton et al., 1999) and in turn trigger concomitant changes in the root-to-shoot distribution of nitrate and cytokinin concentrations (Mora et al., 2010). However, some aspects of this beneficial interaction between HS and nitrate acquisition remains unknown. Up today, only few studies have been performed to understand the molecular mechanism undertaken to a short term exposure of plants to humic acids. Trevisan et al. (2011) analysed the early response of arabidopsis plants to earthworm-originating humic acids and identified 133 genes differentially modulated in treated plants in comparison to untreated control plants. In *Brassica napus* the effect of black peat originating humic acids influenced significantly the transcriptomic profile of treated plants after 3 and 30 days of treatment with the humic fraction (Jannin et al., 2012). In both studies the authors reported an enhancement of gene expression related to primary metabolism; although only in leaves of *Brassica,* an enhancement of N related metabolic changes by humic acid addition was observed (Jannin et al., 2012). Based on differences among nature of humic factions and the timing of treatments, the molecular mechanisms by which the humic acids are able in the short term to enhance nitrate acquisition are not described so far.

In the present study, we applied a fraction of humic acid extracted from peat (water-extractable humic substances, WEHS) to maize seedling hydroponically grown using a concentration conceivably present in agricultural soils (5 mg L^{-1} organic C). Time course experiments of nitrate uptake in roots of intact maize plants were performed and the root transcriptomic profiles of treated plants was analysed. Transcriptional changes of metabolic pathways for primary and secondary metabolism shed light on modifications occurring in maize plants when treated with the WEHS fraction.

2. Materials and methods

2.1. Plant material and growth conditions

Maize plants (*Zea mays* L., PR33T56, Pioneer Hybrid Italia S.p.A.) were hydroponically grown as previously described in Zanin et al. (2015b). Therefore, after germination over aerated 0.5 mM $CaSO_4$ solution, maize seedlings (3-day-old) were transferred into aerated hydroponic system and under controlled conditions (16/8 h light/dark cycle, 220 μmol m^{-2} s^{-1} light intensity, 25/20 °C temperature, 70–80% relative humidity). After 2 days, maize plants (5-day-old) were transferred to a N-free nutrient solution (μM: $CaSO_4$ 500; KH_2PO_4 175; $MgSO_4$ 100; NaFe-EDTA 20; KCl 5; H_3BO_3 2.5; $MnSO_4$ 0.2; $ZnSO_4$ 0.2; $CuSO_4$ 0.05; Na_2MoO_4 0.05); only in *Nitrate* and *Nitrate + WEHS* treatments, nitrogen was added to nutrient solution in form of calcium nitrate, 0.5 mM $Ca(NO_3)_2$, with or without 5 mg C_{org} L^{-1} WEHS (as described by Pinton et al., 1999). WEHS fraction was previously characterized as reported in Tomasi et al. (2013). Using potassium hydroxide (KOH), the pH of solution was adjusted to pH 6.0. Hence, four nutritional treatments were tested: nutrient solution without any source of N (*Control* treatment), nutrient solution without any source of N and containing 5 mg C_{org} L^{-1} WEHS (*Control + WEHS* treatment), nutrient solution containing 1 mM nitrate (*Nitrate* treatment) or nutrient solution containing 1 mM nitrate plus 5 mg C_{org} L^{-1} WEHS (*Nitrate + WEHS* treatment). After 1 h from the beginning of the light phase, calcium nitrate and/or WEHS were added to nutrient solution (T_0 = 0 h of treatment). The treatments lasted up to 24 h (for physiological and molecular analyses) or up to 2 days (for morphological observation); during these periods samples of plants were harvested and used for the analyses described below.

2.2. Morphological evaluation of maize roots

Morphological analyses were performed on maize plants after 2 days of treatment with different nitrogen sources (7-day-old). At the end of the experiment, for each treatment plants were weighted and pictures of shoots and roots were taken (one representative picture is shown in Supplemental Figs. S1-S2).

The acidification activity of maize roots was visualized on gel using the pH indicator bromocresol purple, as described by Zanin et al. (2017). The analyses of root systems were performed using WinRHIZO™ software, 2015a Pro version (Regent Instruments Inc.) using the root morphology mode on three independent biological replicates.

2.3. Measurement of net high-affinity nitrate uptake

After 0, 2, 4, 6, 8, 10 and 24 h of treatment, the roots of intact plants were sampled to evaluate the net influx of nitrate by depletion from a solution containing 200 μM KNO_3 for 10 min as described by Pinton et al. (1999). Changes in nitrate concentration were determined spectrophotometrically at 410 nm as described by Cataldo et al. (1975).

2.4. Microarray analyses

The extraction of RNA from maize roots was performed using Invisorb Spin Plant RNA kit (Stratec Molecular) following manufacturer's instructions. After 4 and 8 h of treatment, maize roots from three independent biological replicates were used for microarray analysis, the quality of RNA was checked using Bioanalyzer Chip RNA 6000 series II (Agilent). The cDNA synthesis, microarray hybridization and the following steps were performed according to the NimbleGen Arrays User's Guide (www.nimblegen.com) on a NimbleGen microarray chip (maize chip 12 × 135 K Arrays from Roche). The maize chip allowed the detection of 59756 predicted transcripts, based on reference genome B73 (ftp.maizesequence.org/current/filtered-set/ZmB73_5b_FGS_cdna.fasta.gz). The chip description is available on Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo) under the series entry (GPL17540). The chip was scanned at 532 nm using an Axon GenePix 4400 (Molecular Devices) and GenePix Pro7 software (Molecular Devices) according to the manufacturers' instructions. Images were analysed and raw signals were normalized as previously reported in Zanin et al. (2015b) through RMA analysis (Irizarry et al., 2003). The Bioconductor software (Gentleman et al., 2004) allowed the analysis of normalized data using the R programming language (Ihaka and Gentleman, 1996). The use of linear model analysis (Smyth, 2005) with LIMMA package and Bayesian correction identified the differentially expressed probes (adjusted P-value ≤ 0.05, n = 3, FC ≥ |2.00|). All microarray expression data are available on GEO