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TESI DI LAUREA

Molecular regulation of nitrate and ammonium response in maize roots

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ABSTRACT

Nitrogen (N) is fundamental for plant growth and development, since it represents an essential component of DNA, RNA, proteins and other metabolic components. Thus, nitrogen can be considered the most requested mineral nutrient in plants. Soil does not have sufficient N in available forms to support production levels, for this reason the application of nitrogen fertilization plays a key role in crop productivity. Only about 50% of the total N is harvested in the grain, and the remainder is lost from the plant-soil system, causing adverse impacts on environment and human health. Therefore, in order to increase the sustainability of agriculture and to reduce the environmental damage, improving nitrogen use efficiency (NUE) of crops represents a crucial point for reducing the N fertilizer application by ensuring high yields. However, the NUE molecular pathway components that act in plant response to nitrogen availability are still only partially characterized.

The nitrogen uptake and assimilation by plants can essentially occur in two forms: nitrate (NO_3^-) and ammonium (NH_4^+). Concerning nitrate, besides its role as a nutrient, it acts also as a signalling molecule since it is involved in the modulation of expression of several genes linked to plant development. The understanding of the molecular mechanisms at the base of root architecture adjustment in response to N fluctuations in soil seems to be necessary to enhance the ability of plants to capture the applied N fertilizer.

Recent studies displayed that the transition zone (TZ), located between the meristem and the elongation zone, represent a specific sensory zone able to integrate and re-elaborate external and internal signals in developmental response. Particularly, this zone seems to be the responsive zone to nitrate in maize root. Furthermore, it was shown that several early nitrate-responsive mechanisms at the base of root development, including primary root length, are mediated by the synthesis of nitric oxide, which is a free radical produced by the nitrate reductase enzyme (NR) and detoxicated by the involvement of haemoglobin (HB).

Moreover, it was reported that NO-mediated root apex response to nitrate is accomplished in cells of transition zone via integrative actions of hormones like auxin and strigolactones, but also through the involvement of reactive oxygen species (ROS) production.

In the present study, in order to discriminate between the specific effects of nitrate from those more generally attributed to nitrogen, root morphology and gene expression analyses

were conducted, particularly on 2 days-old maize seedlings growth for 24 hours in a N deprived solution and then transferred for additional 24 hours to nitrate or ammonium supplied medium.

Growth root measurements showed a decrease of primary root (PR) elongation in response to both ammonium and nitrate, after 24 h of treatment. Concerning lateral root (LR) development, a similar behaviour was displayed by seedlings fed on ammonium and nitrate supplied medium, which evidenced both an increase of LR primordia density. Root and shoot weights showed a significant increase in the case of nitrate provision, while ammonium treatment seems to cause an increase only at the root level.

Moreover, in order to better characterize the nitrate effects dependent by NO, a NO-scavenger (cPTIO) was also provided to nitrate-supplied root. The increase of PR lengths, LR densities and root and shoot weights after nitrate provision seemed to be NO-dependent, since all of these features were affected by cPTIO treatment.

A quantitative RT-PCR was also conducted to better characterize the molecular regulation of maize root response to N. Particularly, the expression pattern of NO-, ROS- and hormones related genes was analysed. Results brought to light a complex and sophisticated molecular mechanism of regulation at the base of root architecture adjustment, that require to be further deepened.

A RNA sequencing analysis by means of Illumina technique was conducted on the RNA root apex samples deriving from the maize seedlings fed for 24h on N-free/+nitrate/+ammonium/+cPTIO supplied media. It allowed us to obtain a huge number of differentially expressed genes putatively involved in the molecular pathway governing root growth development. Future work will be performed, starting from this dataset, to identify and better decipher novel components underlying the response of maize root to nitrate and ammonium.

RIASSUNTO

L'azoto (N) può essere considerato come il nutriente minerale più richiesto dalle piante, rappresenta infatti un elemento fondamentale per la loro crescita e il loro sviluppo in quanto è un costituente essenziale di DNA, RNA, proteine ed altri importanti componenti metabolici. La produttività delle colture agrarie dipende fortemente dall'utilizzo di fertilizzanti azotati, poiché la disponibilità di azoto nel terreno non è sufficiente per garantire elevate rese. Purtroppo, solo il 50% dell'azoto somministrato al terreno viene assorbito e utilizzato dalla pianta, e la rimanete parte viene persa dal sistema agricolo, causando problemi ambientali ma anche sulla salute umana. Nell'ottica del perseguimento di un'agricoltura sostenibile risulta quindi fondamentale puntare sul miglioramento della NUE (Nitrogen Use Efficiency), ovvero l'efficienza d'uso dell'azoto, permettendo così di diminuire l'applicazione dei fertilizzanti azotati garantendo comunque elevate rese produttive. Le componenti molecolari che governano la NUE e che agiscono modulando la risposta delle piante in funzione della diversa disponibilità di azoto risultano tutt'oggi comunque solo parzialmente caratterizzate. Le piante possono assorbire ed assimilare l'azoto prevalentemente sotto forma delle forme ioniche di nitrato (NO_3^-) ed ammonio (NH_4^+). Per quanto riguarda il nitrato, oltre al ruolo di nutriente, esso funge anche da importante molecola segnale nella modulazione dell'espressione di geni associati allo sviluppo della pianta. Approfondire il meccanismo molecolare alla base dell'adattamento dell'architettura radicale alla diversa disponibilità di azoto nel terreno risulta fondamentale per migliorare la capacità di assorbimento del fertilizzante azotato da parte della pianta.

In questo contesto, studi condotti recentemente hanno dimostrato che la zona di transizione, situata tra il meristema apicale radicale e la zona di allungamento, potrebbe rappresentare un centro sensoriale in grado di integrare stimoli esogeni ed endogeni, rielaborandoli in adeguate risposte di sviluppo. In particolare, la TZ potrebbe rappresentare la zona di percezione del nitrato nelle radici di mais. Inoltre, è stato dimostrato che molti meccanismi molecolari attivati in risposta della percezione del nitrato e che portano alla regolazione dello sviluppo della radice, compresa la crescita della radice primaria (PR), sono specificatamente mediati dalla produzione dell'ossido nitrico (NO), un radicale libero prodotto dall'enzima nitrato reduttasi (NR) e detossificato dall'emoglobina (HB).

Le risposte che si osservano a livello delle cellule della TZ a seguito della percezione del nitrato e che risultano essere mediate dalla produzione di ossido nitrico vedono l'entrata in

gioco dell'azione coordinata di fitormoni, quali auxina e strigolattoni, e delle specie reattive dell'ossigeno (ROS).

In questo studio sono state eseguite analisi sulla morfologia della radice e di espressione genica allo scopo discriminare gli effetti specifici indotti dal nitrato da quelli attribuibili più generalmente all'azoto. Le analisi sono state condotte su piantine di mais coltivate per 24 ore in una soluzione privata di azoto, per essere poi trasferite per altre 24 ore in mezzi di crescita addizionati di nitrato o ammonio.

I risultati relativi alla crescita della radice hanno mostrato un allungamento della radice primaria sia a seguito del trattamento con il nitrato che con l'ammonio. Anche nel caso delle radici laterali (LR) è stato osservato un aumento della loro densità conseguente ad entrambi i trattamenti. Il peso di radici e germogli ha mostrato un aumento significativo in seguito al trattamento con il nitrato, mentre il trattamento con l'ammonio ha determinato un aumento di peso solo a livello radicale.

Inoltre, per meglio caratterizzare gli effetti del nitrato mediati dalla produzione di ossido nitrico, la soluzione di crescita caratterizzata dalla presenza del nitrato è stata integrata con il cPTIO, uno 'spazzino' dell'ossido nitrico. L'allungamento della radice primaria, così come l'aumento della densità delle radici laterali e del peso di radici e germogli è stato influenzato dalla presenza del cPTIO, suggerendo quindi che l'effetto osservato sia mediato specificatamente dalla produzione dell'ossido nitrico.

Attraverso una RT-PCR quantitativa è stato valutato il pattern di espressione di geni coinvolti nella produzione e detossificazione dell'ossido nitrico (NO) e delle specie reattive dell'ossigeno (ROS) e di ormoni correlati, allo scopo di caratterizzare la regolazione molecolare della radice di mais in seguito alla percezione dell'azoto. È stato così evidenziato un complesso e sofisticato meccanismo molecolare alla base della regolazione dell'architettura radicale che necessita di essere più approfonditamente investigato.

Attraverso la tecnica Illumina è stata inoltre condotta un'analisi di RNAseq sui campioni di RNA derivanti dall'apice radicale delle piantine di mais coltivate per 24 ore in quattro mezzi di crescita diversi (-N, $+NO_3^-$, NH_4^+, ($+NO_3^-$) +cPTIO). I risultati hanno rivelato un elevato numero di geni differenzialmente espressi che possono quindi essere putativamente coinvolti nel meccanismo molecolare che governa la crescita e lo sviluppo della radice. Studi successivi saranno condotti sui dati così ottenuti per identificare e meglio

caratterizzare i componenti molecolari coinvolti nella risposta della radice di mais specificatamente a nitrato ed ammonio.

1. INTRODUCTION

1.1 Nitrogen in Environment and Agriculture

Nitrogen (N) is the mineral nutrient most requested by plants, since it represents an essential component of DNA, RNA, proteins, but also ATP and other metabolic components, thus it is essential to plant growth (Andrews *et al.* 2013).

N forms about 78% of Earth's atmosphere, but in the form of molecular dinitrogen (N_2) which is inert and cannot be used by plants. For this reason, dinitrogen must be fixed into forms like ammonium (NH_4^+) and nitrate (NO_3^-) (Fowler *et al.* 2013)

Naturally, N_2 is commonly fixed by two processes (Puri *et al.* 2018):

- one process is the atmospheric fixation in which the energy contained in lightning breaks dinitrogen molecules; in this way, the atoms are released, and they are able to combine with oxygen resulting in the formation of oxides of N (NO_x); then, these oxides of N are carried to the Earth through the rain and give rise to nitrates;
- the second process is the biological fixation (BNF), in which microorganisms known as diazotrophs break down the triple bond of N_2 using a complex of enzyme called nitrogenase, giving rise to ammonia (Bottomley and Myrold 2007).

Particularly, soil nitrogen is present in three major forms: organic matter, soil organisms and microorganisms, and mineral-N forms in soil solution, like ammonium (NH_4^+) and nitrate (NO_3^-) (Cameron *et al.* 2013).

Nitrogen fertilization is a common practice in agricultural production and it plays a key role in the obtainment of the desired crop yields because soils do not have sufficient N in available forms to support production levels (McAllister *et al.* 2012). The limited content of N and its heterogeneous and dynamic concentration in the soil depends on several factors like pH, temperature, presence of microrgansims and chemical features (López-Arredondo *et al.* 2013). For this reason, synthetic N is widely applied to enhance worldwide crops production (Andrews *et al.* 2013).

The artificial fixation process that allows to convert N_2 into nitrogenous fertilizers like urea and ammonium is known as the Haber-Bosch process. This process, that has been developed in 1913, consume a large amount of fossil fuel and it works thanks to a catalyst (iron with a small amount of aluminium added), at high pressure (as much as 5.06 × 107 Pa) and at high temperature (600–800 K). It gives rise to ammonia, which is combined with other elements in order to produce nitrogenous fertilizers (Puri *et al.* 2018).

Unfortunately, the residual nitrate in soil profile resulting from the application of synthetic N is susceptible to lose with consequent significant negative impacts on the environment (Cameron *et al.* 2013). Indeed, only about 30-50% of the total N applied is actually harvested in the grain (Raun and Johnson 1999) and the remainder is lost from the plant-soil system (Good and Beatty 2011).

Particularly, the low N assimilation by plants is associated with N loss due to leach (i.e. removal in drainage water), denitrification (i.e. transformations into gaseous forms), and ammonia volatilisation (Nacry *et al.* 2013). Most of the ammonia that is volatilised is returned to the Earth's surface through wet deposition (i.e. dissolved in rainwater) or dry deposition (i.e. attached to particulate matter) causing acidification and eutrophication of natural ecosystems, thus representing an indirect source of nitrous oxide greenhouse gas emissions (Cameron *et al.* 2013). Nitrate leaching losses from soil into water represent a loss of soil fertility, but also a threat to the environment and to human health (Howarth 1988; Di and Cameron 2002; Andrews *et al.* 2007; Goulding *et al.* 2008) (Fig.1). In fact, nitrate entering the drink water is associated to the risk of methaemoglobinaemia in babies and to cancer and heart disease (Grizzetti *et al.* 2011). Nitrate entering rivers and lakes can also contribute to eutrophication, leading to algae blooms and loss of fish (Smith and Schindler 2009). Moreover, nitrous oxide emissions into the atmosphere contribute to the depletion of the ozone layer and contribute to climate change (Cameron *et al.* 2013).

Overall, losses of nitrogen from the soil/plant system cause adverse impacts on the environment as well as reduce soil fertility and plant yield (Cameron *et al.* 2013).

Therefore, in order to increase the sustainability of agriculture and to reduce its impact on the environment, the development of new technologies and best management agricultural practices are clearly desirable.

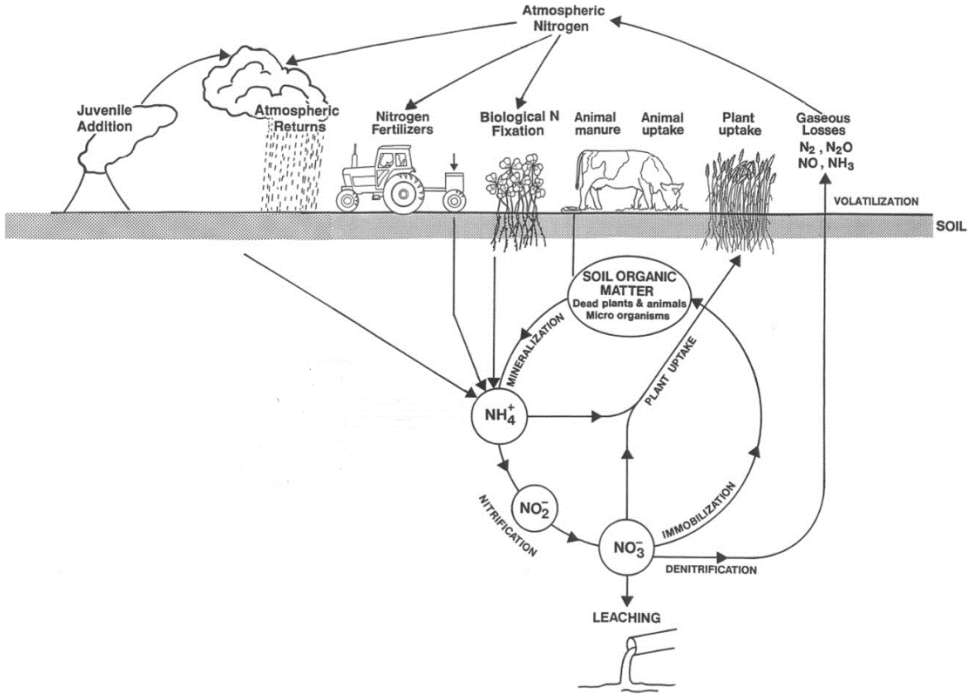


Fig 1. *The nitrogen cycle (Cameron 1992).*

1.2 Nitrogen Use Efficiency

As mentioned above, the application of synthetic nitrogen fertilizer to farmland resulted in an increase in crop yields but with considerable negative impacts on the environment (Good and Beatty 2011). For this reason, it seems necessary to improve the nitrogen use efficiency (NUE) of crops, in order to increase yields by decreasing the applied N fertilizer (Lynch and Brown 2012). NUE is a complex term to define, because endogenous but also exogenous environmental factors can modulate N metabolism, like for example soil properties and management practices, nature of N source, climate (Moll *et al.* 1982) and the steps of N acquisition (Hirel *et al.* 2007). However, NUE in composed by the N uptake efficiency (NUpE), which is the efficiency of absorption/uptake of supplied N, and N utilization efficiency (NUtE), which is the efficiency of assimilation and remobilization of plant N to ultimately produce grain (Good *et al.* 2004).

The common definition of plant NUE is the grain yield per unit of supplied N. It is also represented by the product of NUpE and NUtE:

$$NUE = NUpE \times NUtE = \text{grain yield (Gw)/supplied N (Ns)}$$

This means that through the improvement of NUpE and/or NUtE it could be possible improve the NUE (Han *et al.* 2015).

Several approaches have been developed in improving NUE, for example by breeding cultivars with a high nutrient use efficiency and using efficient crop managing practices. In case of maize, it was oserved that in low N conditions this crop shows a better NUE (Machado and Fernandes 2001), but under low N supply a significant reduction of yield is inevitable (Presterl *et al.* 2003).
Another approach in improving NUE is represented by the overexpression of the genes involved in N uptake and assimilation, but this approach has not always had positive effects on yields (Good *et al.* 2004).

In conclusion, it seems clear that the cultivation of crops with higher NUE would be the best way to increase crop yield by guaranteeing the preservation of the environment.
Neverthless, little is known about the genetic mechanisms regulating NUE, therefore further studies will be necessary to identify the NUE molecular pathway components that act in plant response to nitrogen availability**.**

1.3 N acquisition: the key role of the root architecture

As sessile organisms, plants need to rapidly adapt to the variable environmental conditions, therefore plant root system shows an elevated plasticity in response to environment (Kong *et al.* 2014). The root system and its architecture are crucial for plant growth, indeed it represents a key role in essential functions like anchorage to the soil but also water and nutrients acquisition (Hodge *et al.* 2009).
Via different gene network plants can control the root architecture, as the orientation by regulating the root angle, but also the rate of growth and the degree of root branching (Kochian 2016). Therefore, RSA and other characteristic that are involved in the ability of roots to capture available N from the soil may affect NUE.
The effects of nitrate on root growth are complex and appear to be strongly variable depending on the nitrate concentration in the soil, but also on the plant species and on the exposure times (Linkohr *et al.* 2002; Zhao *et al.* 2007; Gifford *et al.* 2008; Andrews *et al.* 2013). A model was proposed in *Arabidospis* by Krouk *et al*. (2010b) to explain the role of nitrate in regulation of lateral root growth, in which the stimulation of growth involves the phytormone auxin.
To enhance the ability of plants to capture the applied N fertilizer is important to understand the molecular mechanisms by which plants adjust their root system architecture in response

to the variable concentrations of N in the soil, in order to develop crop varieties with improved and more efficient nutrient acquisition under limiting conditions (Kochian 2016). In view of the environmental problems mentioned before, we could use these knowledges to increase crop plant yields meanwhile using less N fertilizers, in order to implement a sustainable agriculture that allows to safeguard the environment.

1.3.1 N uptake and transport

Plants can take up and assimilate nitrogen essentially in two forms: nitrate and ammonium. The first step of the nitrogen acquisition is represented by the uptake of nitrate/ammonium from the soil; they are taken up actively into root cells by different sets of plasma membrane-localized transporters (Krapp *et al.* 2011).

Particularly, nitrate and ammonium uptake into roots involves two physiological mechanisms (Glass *et al.* 2002) (Fig. 3):

- The low-affinity high-capacity transport system (LATS)
- The high-affinity low-capacity transport system (HATS).

LATS is active at elevated concentration of nitrate and ammonium (> 0,5 mM). On the other hand, when nitrate and ammonium concentrations is low (< 0,5 mM) the other system is activated (Wang *et al.* 2012).

Four families of transporters and channels are implicated in the uptake and transport of nitrate in plant organisms (Léran *et al.* 2014; Noguero and Lacombe 2016; O'Brien *et al.* 2016):

- Nitrate Transporter1/Peptide Transporter (NPF) family
- Nitrate Transporter 2 (NRT2) family
- Chloride Channel (CLC) family
- Slow Anion Associated Channel Homolog (SLC/SLAH) family.

Several transporters of NPF family, which encodes the LATS proteins, and NRT2, which encodes the HATS proteins, are involved in nitrate uptake from soil/external media (Kant 2018) (Fig.2).

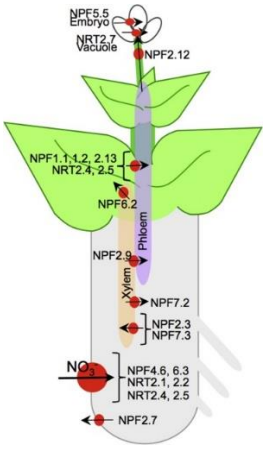


Fig 2. *Nitrate transporters of NPF and NRT2 families and their localisation in plant (Kant 2018).*

The CLC family, which encodes two tonoplast H^+/NO_3^- antiporters and the SLAC1/SLAH family, which encodes two guard cell anion channels, are responsible, for the efflux, transport and allocation of nitrate within the plant (Wang *et al.* 2012).

In order to be activated, NRT2 requires the presence of companion proteins, NRT3 (NAR2) (Okamoto *et al.* 2006; Dechorgnat *et al.* 2010; Pii *et al.* 2016).

The uptake of ammonium at low external concentrations (HATS) requires members of the AMT (AMmonium Transporter) gene family (Yuan *et al.* 2007).

Currently, no genes encoding the LATS proteins for ammonium transport have been described, however recently a protein encoding a putative candidate for this type of transport has been identified in soybean (Chiasson *et al.* 2014).

In *Arabidopsis* the NPF family is characterized by 53 known genes and up to 139 genes in higher plants, divided in to eight subfamilies (Chiasson *et al.* 2014; Noguero and Lacombe 2016).

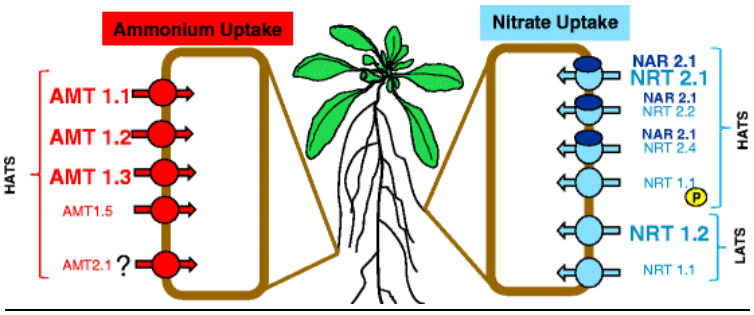


Fig. 3. *Representation of the membrane transporters involved in the root uptake of nitrate and ammonium in Arabidopsis thaliana plants. Transporters highlighted with big letters are play the major role in nutrient uptake. Question marks represent unconfirmed results (Nacry el al., 2013).*

1.3.2 N assimilation

Once nitrate is taken up into the plant, it is assimilated into amino acids through a series of reactions facilitated by a well-known suite of enzymes, although when nitrate is sufficiently available it can be temporarily stored in vacuoles before assimilation (Kant 2018) (Fig. 4). As mentioned above, the nitrate assimilation can occur either in the root or in the shoots. However, this depends on the plant species; in most plants, such as *Arabidopsis*, at high soil nitrate availability, nitrate assimilation occurs mainly in the shoots because of the best energy efficiency (Krapp *et al.* 2011).

The assimilation of nitrate (NO_3^-) involves at first a reduction to nitrite (NO_2^-) by the cytosolic enzyme nitrate reductase (NR) (Fischer *et al.* 2005) as showed below:

$$NO_3^- + NAD(P)H + H^+ + 2e^- \rightarrow \quad NO_2^- + NAD(P)^+ + H_2O.$$

Nitrite (NO_2^-) is toxic to plant cells and it is immediately reduced into ammonium (NH_4^+) by the plastidal nitrite reductase (NiR) (Miflin 1974). By using reduced ferrodoxin (Fd) as electron donor, this enzyme catalyses the transfer of six electrons to NO_2^- (Sakakibara *et al.* 2012)*:*

$$NO_2^- + 6Fdred + 8H^+ + 6e^- \rightarrow \quad NH_4^+ + 6Fdox + 2H_2O$$

Ammonium produced by nitrite reduction but also ammonium deriving by direct root uptake or from the secondary metabolism (photorespiration) are mainly assimilated in the plastid by the sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Cren and Hirel 1999; Miflin and Habash 2002). Particularly, ammonium is at first combined with glutamate to form glutamine, through an ATP-dependent reaction catalysed by GS:

$$Glu + NH_4^+ + ATP \rightarrow \quad Gln + ADP + Pi$$

Through the activity of GOGAT, glutamine then reacts with the 2-oxoglutarate giving rise to two molecules of glutamate (Suzuki and Knaff 2005):

$$Gln + 2\text{-oxoglutarate} + Fdred\ (NADH + H^+) \rightarrow \quad 2Glu + Fdox\ (NAD^+)$$

Subsequently, assimilated nitrogen can enter a range of amination or deamination reactions leading to the production of amino acids (Trucillo Silva *et al.* 2017).

Once N has been assimilated in organic forms, it is transported throughout the plant predominantly as glutamine and glutamate, but also as asparagine and aspartate. Two aminotransferase enzymes are requested for the conversion of glutamine to asparagine and glutamate to aspartate: asparagine synthetase (AS) and aspartate aminotransferase (AspAT), respectively.

Amino acids are further utilized in the synthesis of proteins and enzymes required for the molecular pathways that govern the plant growth and development.

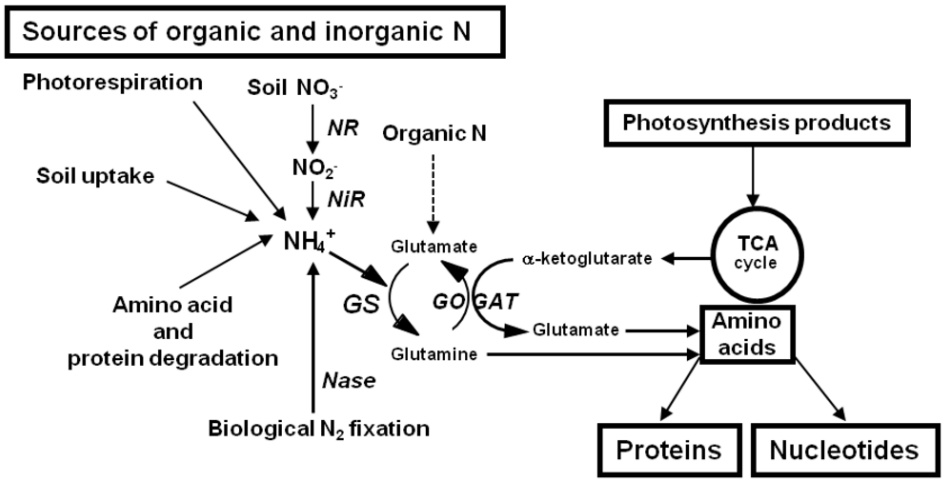


Fig. 4. *The assimilation of nitrogen (Hirel et al. 2011).*

1.3.3 Nitrate sensing and signalling

Nitrate early sensing in plant seems to be mediated directly by nitrate transporters, namely the dual-affinity NO_3^- transporter NRT1.1/CHL1 (NPF6.3) and the high-affinity NO_3^- transporter NRT2.1 (Gojon *et al.* 2011). NPF6.3 is involved in the modulation of the expression of the other transporter NRT2.1: in case of short term supply of nitrate NPF6.3 can up-regulate NRT2.1, on the contrary, under long term high nitrate conditions, it acts in the feed-back repression of NRT2.1 (Muños *et al.* 2004; Bouguyon *et al.* 2015).

Besides its role as a nutrient element, nitrate acts as an important signalling molecule in several physiological and molecular processes in plants, through the activation of a wide range of genes involved in several processes, such as:

- genes responsible for its own transport and assimilation, such as NR, NiR and many nitrate transporters (Wang *et al.* 2000; Bouguyon *et al.* 2012; Krapp *et al.* 2014),
- genes governing germination by relieving seed dormancy (Alboresi *et al.* 2005),
- genes promoting leaf expansion (Walch-Liu *et al.* 2000),
- genes inducing flowering
- genes regulating root development, growth and architecture (Zhang *et al.* 1999; Zhang and Forde 2000).

Particularly, transcriptome analysis in *Arabidopsis thaliana* have been revealed that the expression of 10% of the genome is under the control of nitrate (Wang *et al.* 2000; Bouguyon *et al.* 2012; Krapp *et al.* 2014).

The overall network and its regulation of signal cascades in response to nitrate is not yet completely understood. However, several transcription factors and kinases involved in regulating nitrate transporters and assimilatory genes have been recently identified, such as NLP7, SLP9, TGA1, TGA4, TCP20, LBD37, LBD38, LBD39, CIPK8, and CIPK23(Kant 2018).

NPL7 (Nodule Inception-like protein 7), a member of RWK-RK transcription family, was shown to be involved in nitrate signalling, transport and assimilation (Marchive *et al.* 2013). SPL9 (Squamosa Promoter-binding-like Protein 9) is involved in the modulation of the expression of several nitrate transporters and assimilatory genes, such as NPF6.3, NRT 2.1, NRT 2.2 and NIR (Krouk *et al.* 2010c). It was shown that TGA1 and TGA4 transcription factors accumulate in roots in response to nitrate treatment, suggesting a putative role in root development (Alvarez *et al.* 2014). As regards to TCP20, it has been demonstrated to have a role in the regulating of lateral root development, directing root growth to nitrate rich areas (Guan *et al.* 2014).

The three homologous transcription factors LBD37, LBD38 and LBD3 are zinc-finger DNA binding protein belonging to the LATERAL ORGAN BOUNDARY DOMAIN family; they are suggested to contribute in nitrate signalling because their expression is strongly and rapidly induced by NO_3^- and they repress many N-regulated genes in response to high NO_3^- supply (Rubin *et al.* 2009).

Furthermore, two calcineurin B-like interacting protein kinases (CIPK8 and CIPK23) have been identified as nitrate regulators, suggesting the potential role of calcium in nitrate signalling. Particularly, they are induced by nitrate, and it was shown that their induction

is lower in npf6.3 mutants, thus suggesting that they might enter in the signalling pathway in a NPF6.3 dependent-manner (Ho *et al.* 2009; Hu *et al.* 2009).

1.4 Maize

Maize (*Zea mays*) is a cereal grain first domesticated by indigenous peoples in southern Mexico about 7000 years ago (Ranum *et al.* 2014).

It is estimated that world population will increase in the following years, reaching 9.5 billion by the year 2050 (Kochian 2016). Maize is one of the major world crop production and therefore it is expected to give an important contribution to human nutrition in the next few decades (Hirel *et al.* 2007).

Maize can be processed into a variety of food and industrial products, including animal feed, starch, sweeteners, oil, beverages, glue, industrial alcohol, and fuel ethanol (Ranum *et al.* 2014).

Particularly, considering the last 50 years (since 1961 to 2016*)*, the total cereals world production has increased from about 876 Mt to 2848 Mt (FAOSTAT, 2018*).* Regarding maize, the productivity shows an increase from 205 Mt to 1060 Mt, with a total harvested area in 2016 equal to 187 Mha (FAOSTAT, 2018).

Furthermore, maize and especially the maize root system represents an excellent study model for its capability to capture water and nutrient from the soil, which are then allocated in the aerial part of the plant (Hochholdinger *et al.* 2017).

The root systems can be divided into two types: tap root system and fibrous root system (Hochholdinger *et al.* 2004). Dicots, such as *Arabidopsis thaliana* and the crop *Brassica rapa*, mainly form a tap root system which comprise a single embryonically initiated primary root (PR) and post-embryonically initiated lateral roots (LRs). On the other side, maize (Fig.5), as a monocot crops, has a fibrous root system which is characterized by embryonic roots formed during embryogenesis and postembryonic roots initiated after germination. The embryonic root system comprises a single primary root (PR) and several number of seminal roots (SRs), while postembryonic root system comprises shoot-borne roots which dominate the root system of adult plants, and later roots (LRs) (Pacheco-Villalobos and Hardtke 2012; Smith and De Smet 2012).

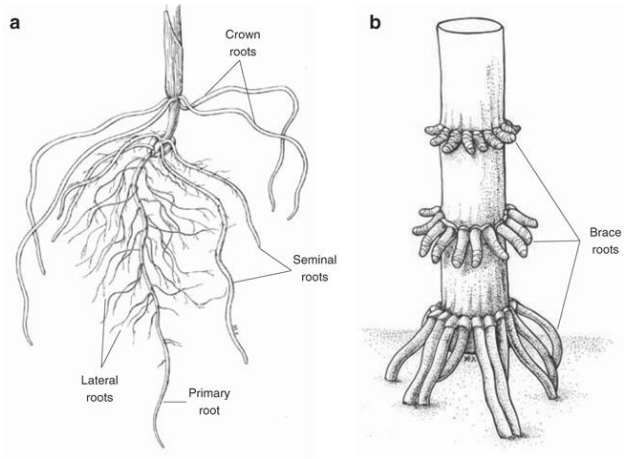


Fig. 5 *Maize root system at different developmental stages.* ***A:*** *Embryonic primary and seminal roots and postembryonic lateral and crown roots are already visible in 14-day-old wild type maize seedlings.* ***B:*** *Aboveground shoot-borne brace roots of a 6-week-old plant (Hochholdinger 2009.)*

Regarding nitrate, it seems to have a doubtful role in regulating the roots growth, especially on PR, depending on its concentration and time of exposure (Trevisan *et al.* 2014) (Table 1). For this reason, future studies are needed to more deeply understand the maize RSA adaptation to NO_3^- fluctuations.

Authors	Species	Treatments	Effect on PR length
Tian et al.	*Zea mays* L.	Plants were grown in nutrient solution containing several NO_3^- concentration (0.05–20mM). The lengths of the primary roots were recorded after 12d.	Inhibition (> 5mM)
Tian et al.	*Zea mays* L.	Seedlings were incubated in the solutions containing different concentrations of NO_3^- (0.05–20mM) and the root length was measured after 12d of incubation.	No effects (0- 0.5mM) Inhibition (> 5mM)
Zhao et al.	*Zea mays* L.	Seedlings were grown in varying concentrations of NO_3^- (0.1–10mM) for 7d and then exposed to 0.1 and 1mM NO_3^- for 48h. The root length was measured after the incubation.	Inhibition
Manoli et al.	*Zea mays* L.	Primary root growth of 8-d-old seedlings grown in 6 different solutions (1mM NO_3^-, - NO_3^- and NO-donors/scavengers) were monitored for 24–48h.	Stimulation

Tab.1 *Overview of the papers reporting results on primary root (PR) response to nitrate treatments in Zea mays (Trevisan et al., 2014)*

These data and observations underline the importance of maize not only from the production point of view, but also as a study model plant. For this reason, it seems important to improve our knowledge on maize, primarily at the root system level, from a physiological but also molecular point of view, to guarantee an increase of productivity that

goes hand in hand with an increase in the population, but also in order to maximize yield by reducing environmental damage and agricultural practices costs.

1.5 Nitric oxide in maize root nitrate response

In order to ensure a sustainable agriculture, the selection of cultivars better adapted to low nutritional inputs represents a key point. For achieving this purpose, understanding the physiological and molecular basis of the plants response to nitrogen is fundamental.

Thanks to a cDNA-AFLP approach with the aim to characterize the transcriptional regulation of maize root in response to nitrate supply and starvation, Trevisan *et al.* (2011) revealed an interesting profile of expression of two genes encoding a maize nitrate reductase (NR) and a nonsymbiotic haemoglobin (nsHb), which displayed a coordinated spatio-temporal regulation of expression in root epidermal cells after nitrate provision. NR is a cytosolic enzyme which reduce nitrate to nitrite, but when high nitrite levels are produced, for instance as a result of the external rapid increase of nitrate, NR also acts on nitrite promoting the biosynthesis of NO (Gupta *et al.* 2011). Besides in O_2 transport, nsHb seem to play a key role in nitric oxide intracellular detoxification (Perazzolli *et al.* 2004; Crawford and Guo 2005). In fact, nitric oxide is a free radical which, as well as serving as a signal in regulating several physiological events, must also be kept at a steady level to avoid damage due to its toxicity (Dordas *et al.* 2003; Perazzolli *et al.* 2004). NO is considered to be a general plant signal since it has a key role in several physiological and developmental processes, such as root organogenesis (Pagnussat *et al.*, 2002), formation of adventitious roots (Pagnussat *et al.* 2003), lateral root development (Correa-Aragunde *et al.* 2004), and root hair formation (Lombardo *et al.* 2006). NO is also involved in biotic and abiotic stress response and phytohormones cross-talk (Durner and Klessig 1999; Wojtaszek 2000; Beligni and Lamattina 2001; Lamattina *et al.* 2003). Another interesting role of NO found out by Correa-Aragunde *et al.* (2004) is its implication with auxin hormone in the signalling pathway of lateral root formation in tomato.

Finally, Trevisan *et al.* (2011) found out a newly role of nitric oxide (NO) as a key player in nitrate signalling, particularly in the early response to nitrate in maize primary root. Moreover, nitrate reductase and nonsymbiotic haemoglobin seemd to be involved in the control of NO homeostasis in response to nitrate with their coordinated activity. Subsequently, Manoli *et al.* (2014) tried to better understand the role of NO in the maize root response to nitrate availability fluctuations. The results confirmed the key role of NO as an early signal of nitrate perception. Furthermore, the authors by analysing the spatial

regulation of expression of the genes involved in the NO steady-state level, brough to light that when nitrate was provided, a significant increase of transcripts of these genes was observed in the transition zone, located between the meristem and the elongation zone (Fig. 6). From this finding it would seem that NO synthesis preferably occurs in the cells of the transition zone, therefore this zone seems to be the most nitrate responsive zone of maize root. Moreover, the authors also suggested that nitrate provision could affect root lengthening by involving NO generated through NR enzyme.

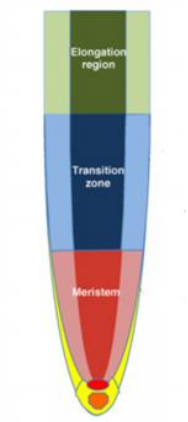


Fig.6 *Zonation of maize primary root (Baluska et al., 2010)*

To summarize, these findings suggest that NO is produced by NR as an early consequence of nitrate provision, and given its toxicity, its steady-state level is guaranteed by the coordinated induction of nsHbs. Furthermore, this cooperation between NR and nsHb in the control of NO homeostasis seems to take place specifically in the transition zone, participating in the signalling pathway involved in the root lengthening after nitrate provision.

1.6 The role of TZ in nitrate sensing and primary root elongation

In the past Charles Darwin (1880) proposed the idea of the existence of a "plant brain" localized at the level of the root apex, which acts as a command centres, able to interpret the external environmental stimuli (Baluška *et al.* 2004). The soil system represents a sort of protection against air environment and animals that feed on plants, thus the localization during the evolution of this sensory centre in the root apices find good reasons (Baluška *et al.* 2004). As mentioned above, the root apex structure is characterized by a distinct zonation, which is composed by a meristem zone and a rapid cell elongation zone (EZ)

separated by a transition zone (TZ) (Baluška *et al.* 2010) (Fig.7). The concept of transition zone was introduced by Baluska *et al*. (1990) and indicates a specific part of maize root apex in which cells, localized between the mitotically division and elongation regions, show an interesting cytoarchitecture, with centralised postmitotic nuclei and radial perinuclear microtubules (MTs) extending to the cell periphery (Baluška *et al.* 2001) (Fig. 7).

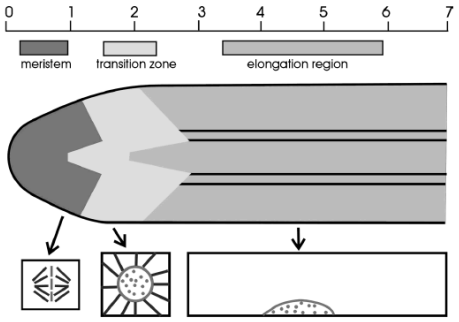


Fig. 7 *Each regions of root apex are characterized by a unique cytoarchitecture: meristematic root cells continually assembling and disassembling mitotic spindles; transition zone cells with central nuclei and radial perinuclear Mts; elongation zone cells, with elongated nuclei appressed against the cell wall (Baluška et al. 2001)*.

The particularly cytoarchitecture of transition zone cell represent an optimal configuration for the first perception and the following transmission of the signals, both to and from the nuclei. Moreover, these cells are not occupied in division or elongation, therefore can focus their resources to perceive and elaborate the environmental stimuli (Baluska *et al*., 2004). For these reasons, the transition zone could be considered the specific sensory zone already citated by Charles Darwin, since it is able to integrate and re-elaorate both environmental and endogenous signal (i.e. hormones) in order to trigger the appropriate growth root response (Mugnai *et al.* 2012). Particularly, in this zone the cells coming from the meristem zone, before entering the elongation zone, undergo into a series of cytoarchitecture and physiology changes that lead to a rearrangement of the actin cytoskeleton (Baluska *et al*., 2010). Specially, it was shown that the cells of the apical part (distal) of transition zone can re-enter the cell cycle, on the contrary the cells belonging to the basal (proximal) part can rapidly enter the fast elongation zone (Verbelen *et al.* 2006; Baluška *et al.* 2010; Baluška and Mancuso 2013).

It was demonstrated that cells of the TZ are very sensitive to several stimuli, such as gravity (Masi *et al.* 2015), osmotic stress (Baluška and Mancuso 2013), aluminium (Peres da Rocha Oliveiros Marciano *et al.* 2010; Sivaguru *et al.* 2013) and auxin (Mugnai *et al.* 2014).

Another important finding was reported by Manoli *et al.* (2014), which demonstrated the central role of the transition zone in the control of NO homeostasis. As reported in the previously paragraph, this interesting part of root apex seems to be the most nitrate responsive zone, in which the coordinated activity of NR and nsHB regulate the NO steady-state level. Furthermore, the authors suggest that the NO_3^- supply influences the root elongation in a NO-dependent manner. Going deeper, nitrate seems to have an opposite effect on root elongation according to its concentration, stimulating or inhibiting the elongation at concentration up to 1 mM and higher than 1 mM, respectively (Trevisan *et al.*, 2014). Considering these findings, it would seem that nitrate provision activates its own sensing by stimulating NO production, which is synthesized at the level of the transition zone cells, starting a signalling pathway that leads to the adaptation of root growth basing on nitrate availability (Manoli *et al.*, 2014).

Several authors reported evidences of a possible role of NO as a key element in molecular pathway involved in a number of cytoskeleton-mediated processes, such as root delevopment, vescicle trafficking, root hair, tip growth, gravitropic bending and actin-dependent endocytosis (Lombardo *et al.* 2006; Kasprowicz *et al.* 2009; Kolbert *et al.* 2010). For this reason, it could be hypostasized that the regulation of root elongation deriving from the rearrangement of cytoskeletal because of NO production in response to nitrate (Trevisan *et al.*, 2014) (Fig. 8).

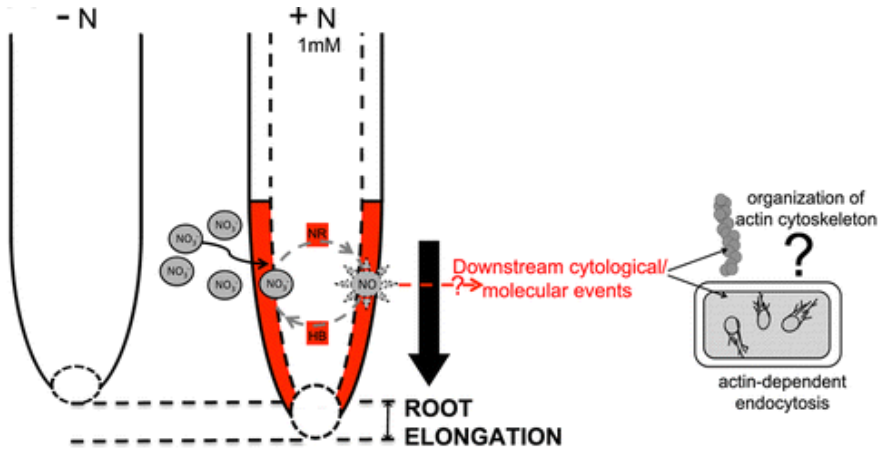


Fig.8 *NO-mediated nitrate regulation of primary root elongation (Trevisan et al., 2014).*

1.7 Molecular components of the TZ in early nitrate response

By means of a RNA seq analysis and following qRT-PCR validation, Trevisan *et al.* (Trevisan *et al.* 2015) tried to understand more deeply the role of the root transition zone in response to nitrate, particularly in the primary root growth of maize seedlings. The transition zone transcriptional profile was extrapolated in response to provision of NO_3^- 1 mM for 2 hours. The study led to identify several nitrate-responsive genes representing the starting point for better understand the molecular pathway which occurs in transition zone in response to nitrate. Several genes known to be responsive to nitrate in *Arabidopsis* (Canales *et al.* 2014) were found also in maize, indicating an overlap among maize and *Arabidopsis.*

Analysis showed a differential regulation in response to nitrate treatment of genes annotated as reactive oxygen species (ROS) related. This finding underlines the already known role of ROS in nutritional response (Shin *et al.* 2005; Krapp *et al.* 2011), suggesting to the authors the possible role of ROS as signal molecules to indicate the presence of nitrate at the level of TZ cells.

Genes belonging to cytochromes P450 gene superfamily and orthologues of the *Arabidopsis Max1*, *Max3* and *Max4* genes, which are involved in strigolactone biosynthesis (Gomez-Roldan *et al.* 2008; Umehara *et al.* 2008; Zhang *et al.* 2014), were clearly identified in this study as downregulated by nitrate supply. Moreover, it was observed a downregulation of a gene encoding a PDR protein, which acts as a cellular exporter of SL, facilizing the SL distribution to the site of action (Kretzschmar *et al.* 2012). Another important finding concerning the SL hormone was the revelation of the downregulation of a protein, AP-2, which is involved in endocytoses for the regulation of transport in plants of indole-3-acetic acid (IAA) (Yamaoka *et al.* 2013). These findings confirm the already known overlaps among auxin and SL (Cheng *et al.* 2013) and the involvement of SLs in the early nitrate signalling in maize TZ.

Besides AP-2, other components of auxin signalling were identified, supporting the role of auxin in nitrate root response (Vidal *et al.* 2010).

The study also uncovered the role of another class of hormones, the brassinosteroids (BR), in the nitrate response pathway. Particularly, it was observed the up-regulation in response to nitrate supply of two genes, *BR1* (Brassinosteroid insensitive 1), which encodes for a BR

receptor as demonstrated by Trevisan *et al*. (2011), and *BAK1* (BRI1-Associated Receptor Kinase 1).

Several transcripts putatively involved in cytoskeletal organization were identified, grouped as cytoskeletal protein binding, microtubule cytoskeleton organization, tubulin binding, vesicle-mediated transport, ARF GTPase activator activit. Among them, an unknown protein with an ArfGAP- like domain was chosen for further analysis because its function seemed to be new in the contest of nitrate response. ARF act as regulators of vesicular trafficking and actin remodelling (Du and Chong 2011), but also interfere with auxin transport (Manoli *et. al*., 2016). The transcription of ArfGAP-like in meristem but also in TZ is strongly induced by nitrate (Trevisan *et al*., 2015). These findings suggest a role of this protein in nitrate network response, through cytoskeleton modifications and auxin polar localization (Manoli *et al*., 2016). Beside genes involved in cytoskeletal organization, several genes related to cell wall structure and composition (i.e. genes encoding XGs, endotransglycosylases, polygalacturonases, and glycosyltransferases) were identified as involved in the regulation of early nitrate response in root TZ (Trevisan *et al*., 2015). Among them, further studies were focused on XGs. At first, it was demonstrated that nitrate induces the apex growth by stimulate cellular elongation at the basal border of the TZ. Particularly, it would seem that NO_3^- promotes rapid cell elongation of root apex by inducing, in the TZ, the xyloglucan endotransglycosylase (XET) enzyme (Fig. 10). This enzyme is involved in cell wall loosening during cell expansion (Pritchard *et al.* 1993; Vissenberg *et al.* 2000), leading root apex growth (Manoli *et al*., 2016) (Fig. 19).

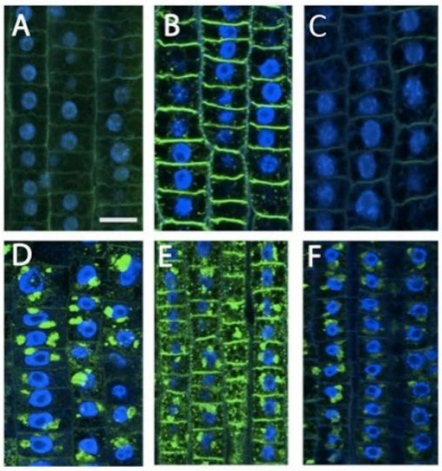


Fig. 9 *Immunolocalization of xyloglucans (green staining) in cells of root TZ (Manoli et al., 2016).*

Overall, Trevisan *et al.* (2015) demonstrated a clearly connection between several phytohormones (auxin, SLs and BRs) and nitrate in the growth adaptation of maize primary root. Basing on these findings, Manoli *et al* (2016) tried to better understand the specific role of SLs and auxin in maize root after 2h of NO_3^- 1mM supply.

Regarding auxin, PR length showed a positive correlation with IAA (Tian *et al.* 2008) and data suggested that nitrate led to increase the IAA-fluxes by involving PIN1 relocalization, likely by affecting endocytosis and vescicular trafficking. Recently, the involvment of auxin in LR growth regulation by nitrate was demonstrated, since *Arabidopsis* transporter NRT1.1 seems to be able to move both auxin and nitrate (Krouk *et al.* 2010a; Krouk *et al.* 2010b).

The expression of several genes involved in SL biosynthesis, signalling and transport was determined in all the zones of the maize primary root, as reported in the figure below (Fig.10):

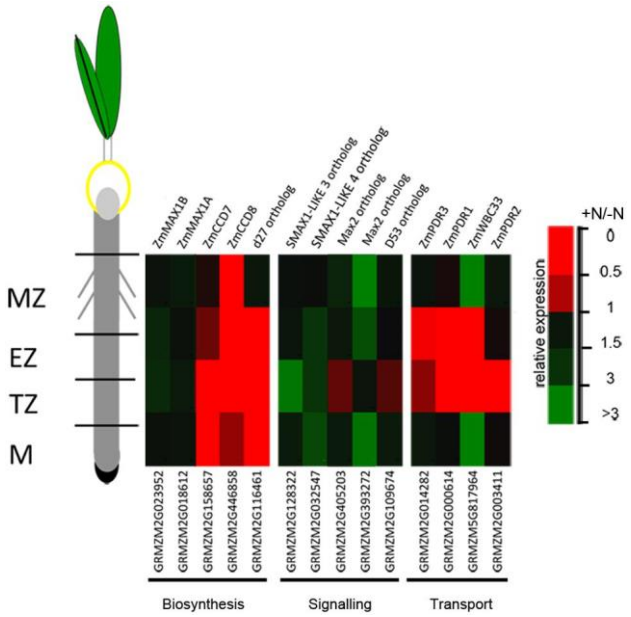


Fig.10 *Nitrate modulates SL-related gene expression (Manoli et al., 2016)*

Interesting, the expression of the greatest part of the genes belonging to biosynthesis and transports resulted inhibited after nitrate provision, and this was particularly evident in the TZ. As known, SLs regulate PINs localization at the plasma membrane (Crawford *et al.* 2010; Koltai 2015), for this reason the authors hypothesized that auxin re-localization observed after nitrate supply in the TZ cells of maize root apex could depend on the down-regulation of the SLs biosynthesis. By adding a nitric oxide scavenger (cPTIO) to seedlings

growing in normal conditions of nitrate supply, the authors observed the restored expression of several of the down-regulated genes (*ZmCCD7 ZmPDR3*, *ZmWBC33*, *ZmPDR2)*, suggesting the involvement of NO in SLs upstream pathway, playing as a negative modulator of SLs action.

In conclusion, the hypothetical scenario of how nitrate signaling affects PR growth through the cooperation of NO, auxin and SLs is summarize in the figure below (Fig.11) (Manoli *et al.*, 2016).

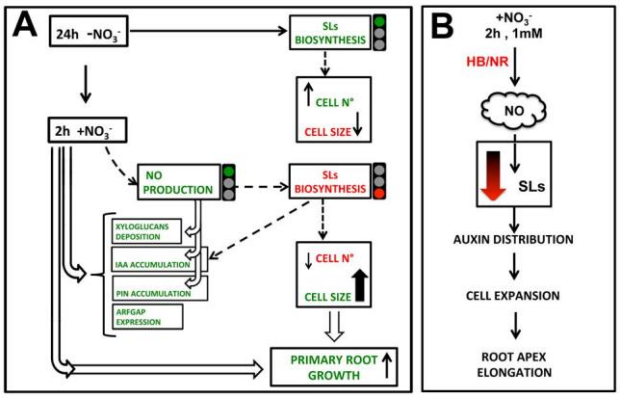


Fig. 11 *NO is early synthesized specifically in the TZ of root apex after the nitrate supply (Manoli et al., 2014), which inhibits SL biosynthesis and transport, affecting auxin polar transport and localization. This chain of events promotes cell growth, affecting xyloglucans deposition and cytoskeleton.*

2.AIMS

Nitrate (NO_3^-) and ammonium (NH_4^+) represent the most abundant source of nitrogen (N) in the available form for plants. Besides their roles as nutrients they also act as signals, able to regulate the transcription of a huge number of genes and to modulate the root system architecture and development.

Recent studies underlined the central role of the maize root transition zone in the perception of nitrate and in the following activation of molecular pathways that lead to changes in root architecture in response to different condition of nitrate availability. Particularly, it has been shown that NO homeostasis guaranteed by the cooperation of NR and nsHb is implicated in the nitrate signalling pathway, thus NO is early synthesized in the TZ after nitrate provision and influences primary root elongation, depending on NO_3^- concentration, by affecting the rearrangement of cytoskeletal.

Besides NO, also strigolactones and ROS (reactive oxygen species) seem to be involved in the signalling pathway governing the response to NO_3^- at the level of the TZ cells.

To better discriminate the nitrate from the more generic nitrogen effects, in this work a treatment with ammonium was introduced and its effect on root development and gene regulation were analysed in comparison to those observed upon nitrate provision. Furthermore, a treatment with cPTIO, which is a NO scavenger, was also included in the experimental set, to allow us to better decipher the NO-dependent and NO-independent nitrate signalling.

To this aim the impact of both nitrate and ammonium on root and shoot fresh weights were measured, together with their effects on root length and lateral root development.

Furthermore, a set of previously identified genes were selected, and their expression was measured in N-starved, nitrate-, ammonium- and cPTIO- supplied roots.

Finally, a more detailed transcriptomic picture was gained by means of a RNA sequencing analysis.

Our results allowed to deepen the molecular regulation of the root response to these to nutrients, and to highlight common and specific signalling pathways involved in the root adaptation to their different availabilities.

3. MATERIALS AND METHODS

3.1 Maize growth conditions

The seeds used in the experiment have been originated from the maize inbred line B73. To get the seedlings, the germination of the maize seeds was occurred in darkness for 2 days at 25°C, by using paper rolls soaked with distilled water. After germination, the seedlings were moved to a hydroponic system in a growth chamber with a day/night cycle of 14/10 h at 25/18° C air temperature, 70/90 % relative humidity, and 280 μmol m-2·s-1 photon flux density.

Seedlings were grown for 24 hours in a N deprived solution and then were transferred for additional 24 h to a nitrate depleted solution (control -N) or to the following treatments:

- +NO_3^-, 1 mM
- +NH_4^+, 1 mM
- +NO_3^-, 1 mM + 1 mM cPTIO (2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide)

For each treatment three biological repetitions were conducted (15 seedlings per repetition). In nitrate depleted solution KNO_3 was replaced by KCl 1mM. The concentration of macro- and microelements presented in the growing solutions are reported in table below (Table 2):

Macroelements	**μM**	**Microelements**	**μM**
KNO_3 / KCl/ NH_4Cl	1000	FeNaEDTA	10
$MgSO_4$	200	H_3BO_3	4,6
$CaCl_2$	200	$MnCl_2$	0,9
KH_2PO_4	40	$CuCl_2$	0,036
		$ZnCl_2$	0,086
		$NaMoO_4$	0,011

Tab. 2 *Concentration of macro- and micro-elements used for the growing solution.*

For the gene expression analysis, primary roots of seedlings for each biological repetition were cut (1,5 cm root apex), frozen in liquid nitrogen and maintained at -80°C for the subsequent RNA extraction.

All chemicals were obtained from Sigma Chemicals (Sigma, St Louis, MO, USA) unless otherwise stated.

3.2 Growth measurements

3.2.1 Roots and shoots fresh weight

To extrapolate the effect of the four different treatments on roots and shoots fresh weight, the 2-days-old singles seedlings of each biological repetition (3 biological repetition for each treatment, 15 seedlings per repetition) were collected separately after the growth for 24h in a N deprived solution, then for other 24 hours in a -N (control)/+NO_3^-/ +NH_4^+/ +NO_3^- +cPTIO solutions. Roots and shoots of all maize seedling were separated and individually weighed. The average weight of root and shoot was calculated for every biological repetition and then was averaged between the three biological repetition order to obtain the average data of root and shoot weights for each tested treatment.

3.2.2 Lateral root primordia density and Primary root length

In order to measure the frequency of sites of lateral root primordia (LRP), the maize seedlings were firstly grown for 24 hours in a N deprived solution (T0) and then transferred for other 24 hours in different nutrient solutions, as described in the first paragraph. Each seedling was grown in single glass tube, filled with 30 ml of nutrient solution and covered with aluminium foil.

The number of root mitotic sites were visualized by applying haematoxylin staining solution supplied with ferric ammonium sulphate as explained by Canellas *et al*. (2002). Root images were collected using a flatbed scanner.

Image J Image Analysis (Rasband 2011) was used as the software of choice to extrapolate the primary root length and the lateral root density. An ANOVA statistic test was performed including three biological replicates for each treatment and (n=30).

3.3 RNA Extraction and cDNA synthesis

Total RNA was extracted from the root tissues by using TRIzol reagent (Invitrogen, San Giuliano Milanese, Italy) and treated with RQ1 RNAse-free DNAse (Promega, Milano, Italy) as described by Trevisan *et al.* (2011). Total RNA was then quantified with a

Nanodrop1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA) and cDNA was synthesized from 500 ng of total RNA, as described by Manoli *et al.* (2012).

3.4 Quantitative reverse transcription PCR (qRT-PCR)

The investigation of the gene expression in root apex was conducted by using the StepOne Real-Time PCR System (Applied Biosystems, Monza, Italy) as described by Nonis *et al*. (2007). In the experiment SYBR Green reagent (Applied Biosystems, Monza, Italy) was used, according to the manufacturer's instructions. For each reaction, 2.5 ng retrotranscribed RNA was used as template and three technical repetitions were performed on three independent biological repetitions. The absence of multiple products and primer dimers was investigated and confirmed by the melting curve analysis. According to the Livak and Schmittgen (2001) method and to Manoli *et al*. (2012), data were exported and target gene relative expression was determined using *MEP* as reference gene (GRMZM2G018103, membrane protein PB1A10.07c, primers: forward 5'-TGTACTCGGCAATG CTCTTG-3' and reverse 5'- TTTGATGCTCCAGGCTTACC-3'). The analysed genes and the sequences of the relative primers used in qRT-PCR are reported in the table below (Table 3). Primers sequences were designed using Primer3 web tool (Rozen and Skaletsky 2000) and further verified with the PRATO web tool (Nonis *et al.* 2007).

Maize GDB Accession ID	Name	Fw	Rv
Zm00001d037569	UPB1	TGCAGGATTCCATTCTTCATTTC	TTCTGAGGAACATGCCCGAG
Zm00001d042961	rbohA	CAGCGCACACAAGAACTCTC	CCCCGCATACATCAAAACTT
Zm00001d043543	rbohB	TTGGGTTACACGTGAGCAAG	AATGGAGCAAAGGCAACTGT
Zm00001d038762	rbohC	GGCACAGGAACTAAGCAAGC	AAACTCATCGCCAAGAAAGC
Zm00001d052653	rbohD	TGTCTCTGGTCGTTCTCAGC	CTCATCGCCCTCTAGAACCC
Zm00001d036215	MPK7	CACACCCTTACTTGGCATCA	ATCACCGGCTGAAATTGAAC
Zm00001d031908	SOD	CAGCGCACACAAGAACTCTC	CCCCGCATACATCAAAACTT
Zm00001d027511	CAT2	TAGCACGCAAACTGAATCGC	TGGAAACCCAGCCAAACATT
Zm00001d024119	PRX112	GCCTCCGTATTCTTCCAGGA	GGAAGAGATCGTCGGTCTGT
Zm00001d043442	CCD8	AGAAAGGTGTCTCTGCTGCT	CTATGGGCTCGCTCACATGA
Zm00001d038718	Hb2	GGCTGTTGATGCTTCCTAGC	ATGACGGGCCTTTTCTGAAT
Zm00001d049995	NRa	ATGATCCAGTTCGCCATCTC	GTCCGTGGTACGTCGTAGGT
Zm00001d043179	Pin9	CACCGTCGCCTCGCTCTCCATGCTCC	GGAGCATGGAGAGCGAGGCGACGGTG

Tab 3 *List of primer used for the qRT-PCR.*

3.5 Sequencing library preparation for RNAseq experiments

Three biological replicates were used for all RNA-seq experiments from each treatment. Tissues from primary roots were ground in liquid nitrogen, and total RNA was isolated with the TRIzol reagent (Invitrogen, San Giuliano Milanese, Italy). RNA quality was assessed via agarose gel electrophoresis and a Bioanalyzer (Agilent RNA 6000 Nano Chip; Agilent Technologies, Santa Clara, CA, United States). For all samples, an RIN (RNA integrity number) $\geq$ 8.0 was detected.

cDNA libraries for Illumina sequencing were constructed according to the instructions of the manufacturer (TruSeq RNA Sample Preparation; Illumina, San Diego, CA, United States).

3.6 Processing and mapping of sequencing reads

Base calling was performed using the Illumina Pipeline, and sequences were trimmed with ERNE (Vezzi *et al.* 2011). Quality trimming removed low quality and ambiguous nucleotides of sequence ends and adapter contamination. TopHat was used to map and annotate the sequences on the B73 reference genome (RefGen_v4;

ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/plant/Zea_mays/latest_assembly_versions/GCA_000005005.6_B73_RefGen_v4, accessed August 22, 2018). Only reads mapping with at least 80% of their length with 90% sequence identity to unique positions of the genome were considered for further analyses. The counting of the mapped reads is performed with Cufflinks, a software program freely available at http://cufflinks.cbcb.umd.edu/ (Schnable *et al.* 2009; Trapnell *et al.* 2012). Cufflinks counts the expression of each gene and reports it in "fragments per kilobase of transcript per million fragments mapped" or FPKM. The FPKM value is a measure of the expression of a transcript, normalized by transcript length and the total number of fragments. As such, the FPKM value can be used to compare the expression of the genes in the analyzed samples. A single unified assembly from each individual Cufflinks assembly was created thanks to Cuffmerge. For the analysis of differential gene expression, only active genes which show an expression median greater than a threshold of 1 were included. The differentially expressed genes in the analyzed treatments were determined by CuffDiff (Trapnell *et al.*, 2012). The raw sequencing reads were normalized by sequencing depth and log2-transformed. A gene was declared as significantly differentially expressed if its adjusted p-value (controlling the False Discovery Rate) was less than 0.05.

Sample name	NGS ID	Treatment	Comparison code
R1	CQP	-N	A
R2	CQQ	-N	A
R3	CQR	-N	A
R4	CQS	$+NO_3^-$	B
R5	CQT	$+NO_3^-$	B
R6	CQU	$+NO_3^-$	B
R7	CQV	$+NH_4^+$	C
R8	CQW	$+NH_4^+$	C
R9	CQX	$+NH_4^+$	C
R10	CQY	$+NO_3^-$ + cPTIO	D
R11	CQZ	$+NO_3^-$ + cPTIO	D
R12	CRA	$+NO_3^-$ + cPTIO	D

Table 4 *Sequenced samples*.

4. RESULTS

4.1 Effect of different nitrogen sources on maize seedlings growth

In order to better appreciate the morphological specific effects of different sources of nitrogen, 2-days-old maize seedlings were grown for 24 h in N-depleted solution, before being transferred for other 24 h to a N-free (control)/+NO_3^- / +NH_4^+/ (+NO_3^-) +cPTIO supplied medium.

4.1.1 Roots and shoots fresh weight

Root and shoot fresh weights showed a significant increase after NO_3^- provision, compared to control (-N), equal to 20% and 25%, respectively (Fig.12, A). On the other side, only a little increase was displayed by plants fed with ammonium, while a strong decrease was observed, compared to seedlings growth in + NO_3^- medium, in shoot and root weights when NO scavenger (cPTIO) was added (-28 % and -12 %, respectively).

4.1.2 Primary root lenght

Concerning root length (Fig.12, B), results confirmed what is already reported in previous studies (Manoli *et al*., 2016). Particularly, maize seedlings supplied with 1mM NO_3^- for 24 hours displayed a decline of primary root growth in comparison to control (-N), equal to 7%, underlying the inhibition role of prolonged exposure to nitrate on root elongation. The same trend was noticed when ammonium was added as nitrogen source (- 4%). However, the strongest (-15%) inhibition of elongation was noticed when the NO scavenger cPTIO was supplied together with nitrate, confirming the already hypothesised central role of nitric oxide in root elongation molecular pathway (Manoli *et al*, 2014).

4.1.3 Lateral roots

By using a haematoxylin staining solution supplied with ferric ammonium sulphate, it was possible to define the number of root mitotic sites of the 2-days-old maize plants fed on the four different growing solutions (Fig.12, C). In comparison to seedlings growth in the N-free medium, both nitrate and ammonium treatments seemed to have a stimulatory effect on the induction of lateral root, showing an increase equal to 20% and 14%, respectively. On the contrary, when cPTIO was supplied to the +NO_3^- growing solution a diminished LRP density (-8%), was observed, suggesting that nitric oxide could also participate to the nitrate regulation of lateral root.

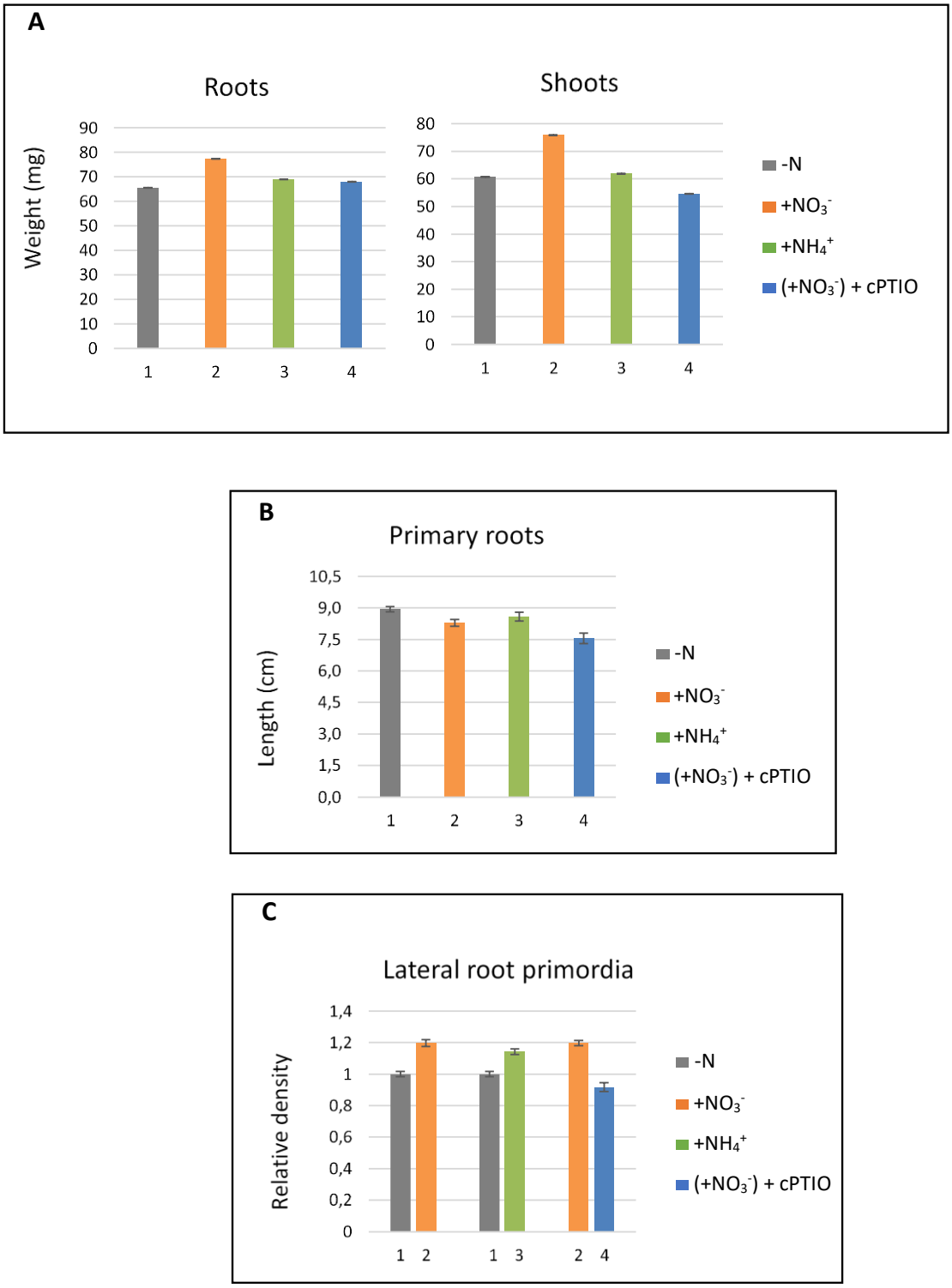


Fig.12 *Fresh weight of roots and shoots (A), primary roots length (B) and lateral root primordia density (C) of 2-d-old maize seedlings growth under different treatments.*

Treatment 1: grown for 24 h in -N (KCl 1 mM) solution.
Treatment 2: grown for 24 h in +KNO_3^- 1 mM solution.
Treatment 3: grown for 24 h in +NH_4 Cl 1 mM solution.
Treatment 4: grown for 24 h in (+KNO_3^-) + cPTIO solution.
Error bars represent the SEM for three biological replicates.

4.2 Gene expression

In order to better understand the molecular regulation of maize root growth, the expression profile of a set of genes previously identified as regulated in a N dependent-manner was analysed, by means of the Real Time PCR. The modulation of expression was evaluated on the total RNA collected from 1,5 cm root apex samples. The treatment with ammonium was conducted in order to discriminate the specific action of nitrate from the more generic nitrogen effects (Fig. 13, A) and cPTIO treatment was introduced to better understand the role of nitric oxide in regulation of gene expression (Fig.13, B), by adopting seedlings growth in N-free and $+NO_3^-$ medium as control, respectively.

4.2.1 Coordinated activity of *ZmNRa* and *ZmHB2* regulates the NO homeostasis in specific response to nitrate provision

The expression of two genes already identified for being expressed in the cells of transition zone in response to nitrate (Manoli *et al.*, 2014), namely *ZmNRa* and *ZmHb2*, was evaluated (Fig. 13, A). *ZmNRa* showed an induction of expression in response to nitrate provision, equal to 2-fold change (f.c.), while no significative differential regulation was observed when ammonium was supplied to seedlings. Concerning *ZmHB2*, the expression of this gene was induced by both nitrate and ammonium treatments, but the first one was able to trigger a higher expression level compared to the latter, showing 25,8- and 3,7- fold increase, respectively. Moreover, no noticeable differentially regulation was observed for both these genes, compared to control ($+NO_3^-$), when cPTIO was added (Fig.13, B).

4.2.2 Modulation of expression of ROS related genes

Reactive oxygen species (ROS) are important secondary messengers that play a central role in plant signalling pathways since they are implicated in a lot of different cellular functions (Jalmi and Sinha 2015), including regulation of root growth (Tsukagoshi *et al*., 2010).

In this work, 2-days-old seedlings were grown for 24h to N-starved, nitrate-, ammonium- and cPTIO- supplied medium, in order to better understand the involvement of ROS in root growth response after the exposure to different nitrogen source.

NADPH oxidases play a key role in root growth regulation, being involved in ROS production (Foreman *et al.* 2003). In plants, NADPH oxidases are named as Respiratory Burst Oxidase Homologs (RBOH), like their mammalian analogues RBO (respiratory burst oxidase). In the present work, the expression of four *Rboh* genes isoforms were analysed, showing different behaviour in response to the different growing conditions. *ZmRbohA* and

ZmRbohC showed a slight increase of expression after nitrate provision (1,8-2 f.c.), while *ZmRbohB* and *ZmRbohD* did not display a significative up- or- down regulation of expression, in comparison to control (-N) (Fig. 13, A). When ammonium was supply as nitrogen source, only *ZmRbohB* seemed to slightly be transcriptionally induced, showing a 1.8-fold up-regulation of expression level in comparison to control (-N). When nitrate-supplied seedlings were treated with cPTIO (Fig. 13, B), neither *ZmRbohA* nor *ZmRbohC* displayed a relevant differential regulation in comparison to control *(*+NO_3^-), while in the case of *ZmRbohB* and *ZmRbohD* transcriptional level, a slight induction was observed, equal to 1,9- and 2,9-fold increase, respectively.

Catalases and superoxide dismutase are two enzymes having a key role in ROS homeostasis. Both *ZmCAT2* and *ZmSOD* did not seem to be significative differentially regulated, in comparison to control (-N), neither by nitrate nor ammonium supply (Fig.13, A). Focusing on *ZmCAT2,* it showed a strong recovery of expression when NO scavenger was added to the +NO_3^- growing solution (12.8 fold higher than control) suggesting a possible inhibition role of nitric oxide in regulation of its expression (Fig.13, B). Conversely *ZmSOD* expression level was not differentially modulated by cPTIO treatment compared to control.

Basing on a transcriptomic approach, Trevisan *et al*. (2015) identified *ZmUBP1*, a maize orthologues gene of Arabidopsis, which is involved in root growth by regulating a set of peroxidases (Tsukagoshi *et al*., 2010). In the present study, the results showed an up-regulation of the *ZmUBP1* gene in response to ammonium provision which is equal to 2.9-fold increase (Fig.13, A). Conversely, nitrate supplied seedlings did not show a strong modulation of expression in comparison to control (-N) (1,7 fold up-regulation). The addition of nitric oxide scavenger did not significantly influence *ZmUBP1* transcriptional level compared to roots of seedlings that were grown in presence of NO_3^- (Fig. 13, B).

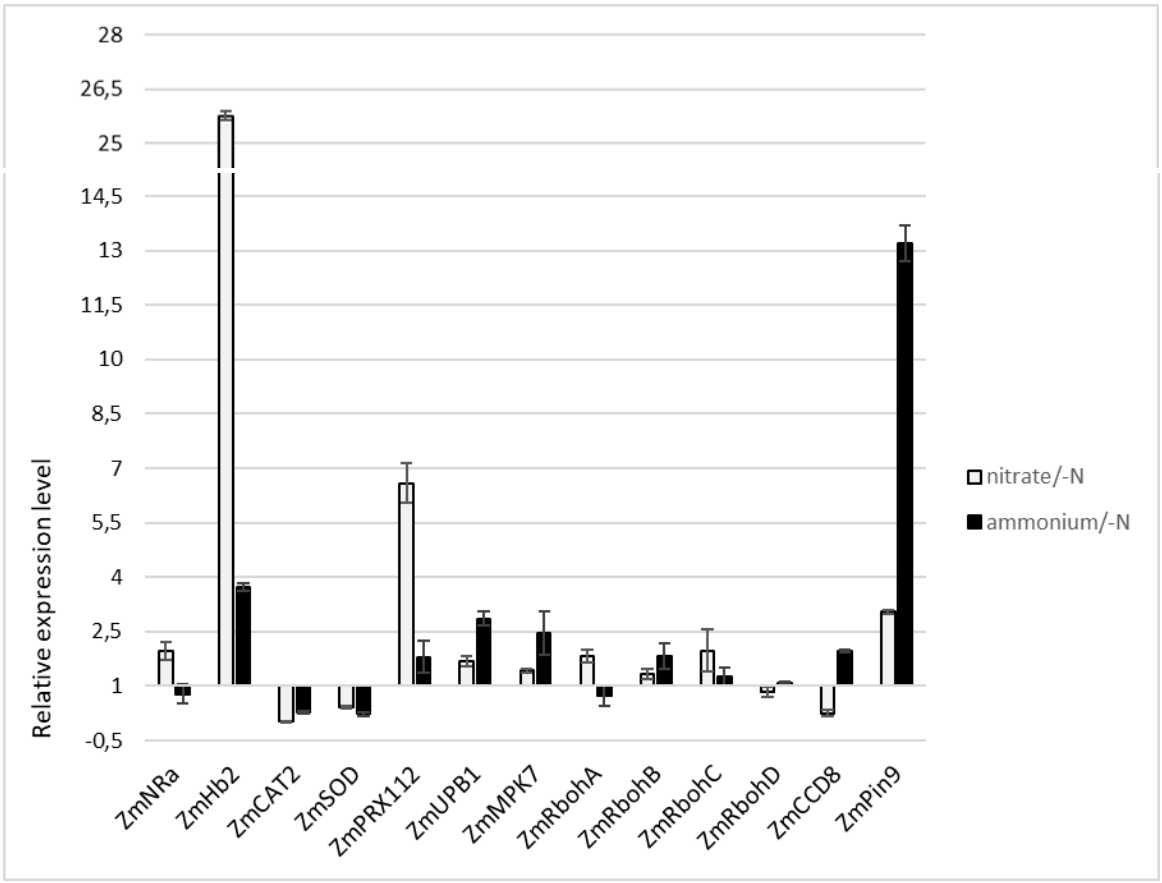
Amongst UBP1's target genes, *ZmPRX112*, a maize orthologues gene of *PER40* of *Arabidopsis* was analysed. The expression of this gene was strongly induced by 24 h of nitrate supply, since it showed a 6.6- fold up-regulation of transcriptional level compared to control (-N). (Fig.13, A). Conversely, such a strong induction has not been observed in case of ammonium provision (1,8-fold increase). When cPTIO was added to the nitrate supplied medium, no relevant stimulation of expression, compared to control (+NO_3^-), was observed (Fig.13, B).

In the present study it was also evaluated the transcriptional modulation of *ZmMPK7, a* maize orthologues gene of *MAPK6* of *Arabidopsis*. Particularly, a slight up-regulation was observed in ammonium-supplied root maize seedlings (2.5- fold increase of transcription level than control) (Fig.13, A). Conversely, no significative up-regulation was displayed when nitrate was provided as nitrogen source. When seedlings were growth for 24h h in contact with nitric oxid scavenger, no relevant regulation was observed in comparison to those fed on cPTIO-free medium (1,8 f.c.) (Fig.13, B).

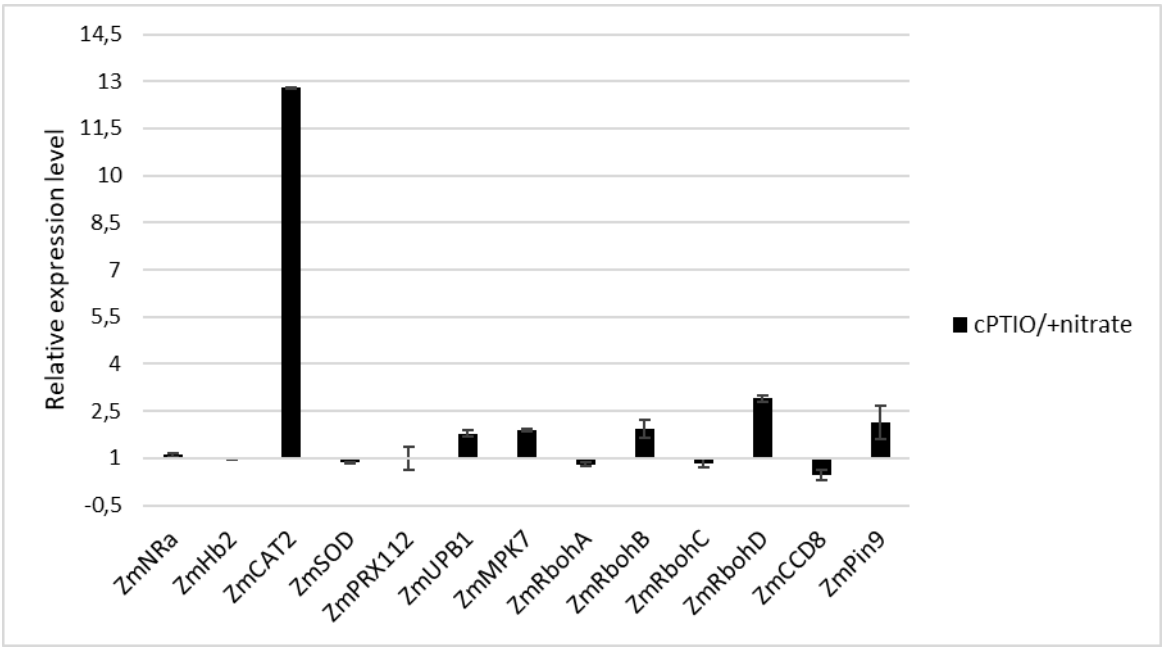
4.2.3 Expression profile of hormone related genes

ZmCCD8, a gene specifically involved in stringolactones biosynthesis, showed a slight down-regulation of transcriptional level in comparison to control (-N) (Fig.13, A). In the case of ammonium supplyon the contrary it displayed a 2 -fold up-regulation of expression level. *ZmCCD8* transcription in our experimental conditions was un-affected by cPTIO supply (Fig.13, B).

ZmPIN9 is a monocot-specific PIN family member recently discovered, and it is specifically expressed in the root endodermis and pericycle (Forestan *et al.* 2010). The expression of this gene was measured in all the four treatment conditions, showing a 3-fold up-regulation of transcription in response to nitrate application (Fig.13, A). However, the stronger induction of transcription was observed when ammonium was added as nitrogen source, showing an expression level 13.2-fold higher than control (-N). When nitric oxid scavenger was added to the growing solution, an increase of transcriptional level was observed in comparison to control ($+NO_3^-$) equal to 2-fold change (Fig.13, B).



A



B

Fig. 13 *Expression profiles of selected genes in response to 24h nitrate and ammonium provision (A) and to nitrate in association with cPTIO (B). Total RNA was isolated from 1,5 cm root apex samples (starting from the root tip). For each gene, the expression level in the control plants (-N and +NO_3^- for A and B, respectively) was equal to 1. Error bars represent the SEM for three biological replicates.*

4.3 RNAseq analysis on maize root apex: preliminary results

In the present work, in order to deepen understand signalling pathways involved in the root adaptation to different nitrogen source availabilities, the maize RNA samples described above were used for Illumina Genome Analyzer deep sequencing. In total, 0.3 billion reads, each 75 nucleotides long, were generated, with approximately 32 million reads from each sample. The reads mapping to the genome (B73v4) were first categorized into two classes (Fig.14). Uniquely mapped reads are those that map to only one position in the genome. Multimapping reads are those that map to more than one position in the reference genome.

Sample	Sequenced Reads	Unique Mapping Reads	Multimapping Reads	Total Alignments
A	38,720,518	29,006,929	3,334,737	32,341,666
B	32,396,207	21,765,134	2,202,487	23,967,621
C	38,617,367	29,275,556	3,885,381	33,160,937
D	38,362,194	28,779,699	4,245,354	33,025,053

Fig.14 *Reads generated from the RNAseq analysis.*

Among the transcripts detected in total, 2189 were identified for being significantly responsive to the four treatments *(p-values* < 0.05) (Fig.15). Particularly, 536 transcripts (24,49%) seem to be differentially regulated between seedlings fed on N-free and $+NO_3^-$ growing solution (AvB), while 71 DGEs (3,24%) were identified for being differentially modulated among seedlings growth in N-deprived and ammonium supplied medium (AvC). 1498 genes (68,43%) were detected for being modulated by the addition of nitric oxid scavenger to $+NO_3^-$ solution (DvB), and only 84 (3,84%) were differentially expressed between maize seedlings growth in NO_3^- and NH_4^+ supplied medium (BvC).

Furthermore, differentially expressed genes were classified in up-regulated and down-regulated according to their $-N/+NO_3^-$, $-N/+NH_4^+$, $(+NO_3^-)$ + cPTIO/$+NO_3^-$, $+NO_3^-/+NH_4^+$ value ratio (Fig. 16). Among the 536 differentially expressed genes identified for being differentially modulated between A and B treatments, 462 (86,19%) were down-regulated and 74 (13,81%) were up - regulated in maize seedlings cultivated in N-free growing solution in comparison to those fed on + NO_3^- medium. Following the addition of cPTIO to nitrate supplied medium, 1057 (70,56%) genes resulted down-regulated, while 441 (29,44%) showed an up-regulation, referred to control $(+NO_3^-)$ (DvB). In comparison to root maize deriving from the seedlings fed on ammonium supplied medium, 69 (97,18%) genes seem to be down-regulated and 2 (2,82%) up - regulated in those growth in N-

deprived growing solution (AvC). Finally, among the 84 genes identified for being differentially modulated between $+NO_3^-$ and $+NH_4^+$ treatments (BvC), 61 (72,62%) and 23 (27,38%) resulted down- and up-regulated, respectively, by nitrate provision in comparison to seedlings fed on $+NH_4^+$ supplied medium.

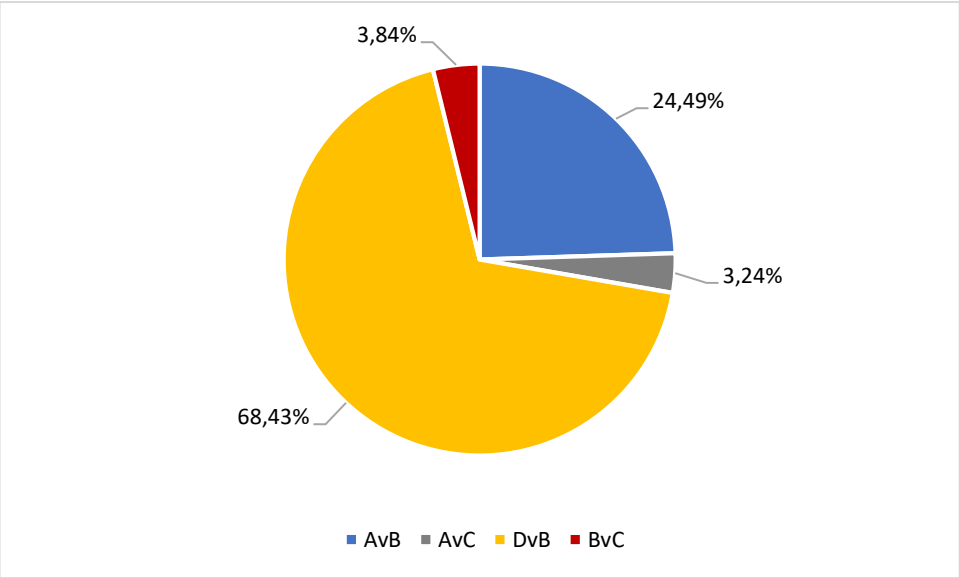


Fig.15 *Graphical distribution of Differentially expressed genes (DGEs) identified from the comparison between the maize seedlings growth in the four different treatments.*

A: Treatment 1(grown for 24 h in -N medium).
B: Treatment 2(grown for 24 h in $+NO_3^-$ medium).
C: Treatment 3(grown for 24 h in $+NH_4$ medium).
D: Treatment 4 (grown for 24 h in ($+NO_3^-$) + cPTIO medium).

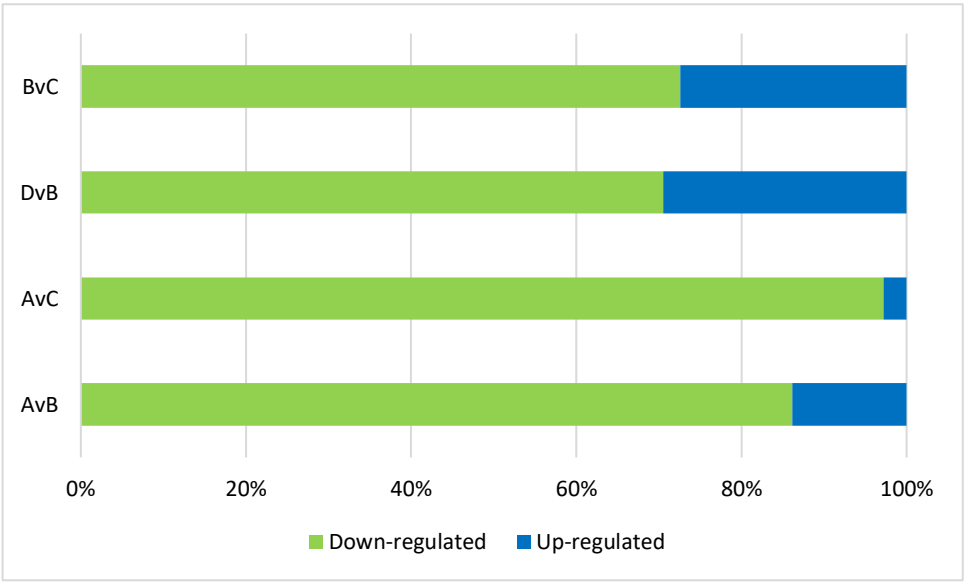


Fig.16 *Graphical representation of up- and down- regulated genes detected from the pairwise treatments comparison (-N/+NO_3^- -N/+NH_4^+, (+NO_3^-) + cPTIO/+NO_3^-, +NO_3^-/+NH_4^+).*

5. DISCUSSION AND CONCLUSIONS

The root system is essential for plant growth indeed it plays a key role in water and nutrient acquisition, but it also significantly contribute to anchorage to the soil (Kochian 2016). Nitrogen is a very important element requested by plant, and basing on its availability in the soil, plant root system is able to adapt its architecture.

Nitrate (NO_3^-) is one of the most abundant sources of available nitrogen forms for plants (Bouguyon *et al.* 2012) and besides its role as essential nutrient, it also acts as a signal, able to regulate plant roots development (Kant 2018). Recent studies focused on the specific effect of nitrate as the nitrogen source on maize root development, highlighted the importance of its availability in the regulation of root architecture. Particularly, in maize in function of its concentration and time of exposure it seems to play a doubtful role in regulating roots development (Trevisan *et al*., 2014). The effects observed in this species seem to partly rely on the production of nitric oxide by nitrate reductase (NR) observed as a consequence of nitrate provision (Manoli *et al*., 2014). Moreover, also ROS (Reactive Oxigen Species) signalling and SLs (strigolactones) metabolism seem to be involved in the nitrate regulation of maize root architecture (Trevisan *et al*, 2015; Manoli *et al*, 2016, Trevisan *et al*., 2018). The main scope of this work was to try to discriminate the effects deriving specifically by nitrate from those more commonly dependent by nitrogen. To this aim the root growth measurements and the transcriptional regulation of a few of previously selected genes were performed on maize seedlings grown either in the presence of nitrate or ammonium.

In fact, besides nitrate, also ammonium (NH_4^+) represents an important ionic form of nitrogen available for cultivated plants (Cameron *et al.* 2013). Differences in root morphology development were reported following the provision of ammonium or nitrate as nitrogen source (Bloom *et al.* 2002; Vatter *et al.* 2015).

Concerning root length, results confirmed what is already reported by Manoli *et al*. (2016), who showed that 2 hours of permanence in the presence of nitrate are sufficient to induce a significant elongation of primary roots. However, maize seedlings fed for 24 hours on nitrate supplied medium displayed a clear decline of primary root growth, underlining the inhibition role of prolonged exposure to nitrate on root elongation. The 24 hours treatment with ammonium gave rise to the same phenotype, thus both ions during the 24 h-treatments lead to a decrease of primary root growth. Analogous behaviour was showed for lateral roots development, both for ammonium and nitrate 24 h-treatments, even if this could rely

to different molecular mechanisms. In fact, nitrate and ammonium seem to promote LR proliferation in a different and complementary way, with ammonium increasing lateral root branching and nitrate promoting lateral root elongation (Remans *et al.* 2006; Lima *et al.* 2010). More recently thanks to a mathematical modelling approach, Araya *et al.* (2016) demonstrated that ammonium hastens lateral root primordia (LRP) emergence.

Furthermore, to discriminate the nitrate effects mediated by NO and those NO-independent, a treatment with cPTIO (an NO scavenger) was also included in our experimental set up.

Nitric oxide produced as a consequence of nitrate provision is toxic and it must be kept at at steady-state level by the involvement of haemoglobin (HB) that is also regulated transcriptionally by nitrate (Trevisan *et al.* 2011). Several others authors reported the key role of nitric oxide in the signalling pathways leading to root development (Pagnussat *et al.* 2002; Pagnussat *et al.* 2003; Correa-Aragunde *et al.* 2004; Lombardo *et al.* 2006).

In this work, both primary root growth and lateral root development resulted inhibited by cPTIO, leading to the suggestion that these nitrate effects could likely be mediated by nitric oxide.

Two genes encoding *ZmNRa* and *ZmHb2* respectively, and previously identified and characterized for their role in NO production and detoxification (Trevisan *et al*., 2011), were included among the list of genes chosen for the Real Time PCR. Our results showed an increase of expression of both of them, which is particularly evident in the case of *ZmHb2*, underling its fundamental role in the response to nitrate in maize root. As expected, the addition of NO scavenger to the growing solution inhibited its transcription. Likewise, also *ZmNRa* up-regulation resulted inhibited, suggesting that nitric oxide can have a positive effect on *ZmNRa* expression through a feedback up-regulation mechanism.

RNA-Seq technology was recently used to generate the transcriptome profiles of the genes in response to NO_3^- provision, particularly in the transition zone, after 2h of NO_3^- 1 mM supply (Trevisan *et al.,* 2015). This approach led to the identification of a huge number of nitrate-responsive genes among which several genes were annotated as ROS (reactive oxygen species)-related. More recently, Trevisan *et al*. (2018) demonstrated a different ROS localization and distribution in the cells of maize root apex in response to N deficiency or nitrate supply.

Here, to better decipher the role of ROS signalling in root growth development, the expression of several ROS-related genes was analysed under the four different tested treatments.

Superoxide dismutase and catalases are important enzymes since they have a crucial role in ROS homeostasis, catalysing the dismutation of superoxide to H_2O_2 and its consequent detoxification in H_2O. Our results brought to light an interesting profile of expression of *ZmCAT2*, which seemed to be specifically down regulated by the presence of nitric oxide.
The profile of expression of four genes encoding Rboh (Respiratory Burst Oxidase) (Jalmi and Sinha 2015) was also evaluated. *ZmRbohA* and *ZmRbohC* resulted to be specifically induced by nitric oxide, since when cPTIO was added to the growing solution, no up-regulation was observed. Conversely, *ZmRbohB* and *ZmRbohD* seemed to be down regulated by the presence of nitric oxide.
Reactive oxygen species produced by NADPH oxidases activity are known for being important messengers involved in signal transduction mechanisms, leading to the activation of Mitogen activated protein kinase (MAPK) cascade, which consequently activate specific set of downstream targets (Jalmi and Sinha 2015). Indeed, MAPKs represent one of the most important phosphorylation signalling cascade involved in oxidative stress response (Jalmi and Sinha 2015). In the present study it was evaluated the expression profile of *ZmMPK7,* a maize orthologues gene of *MAPK6* of Arabidopsis, which encodes a key component of MAPK signalling. Profile of expression did not show any particular modulation of transcription of this gene neither in response to nitrate supply nor consequently to the exposure to nitric oxide scavenger (cPTIO), while a slight up-regulation was observed when ammonium was added to the growing solution as nitrogen source.
Moreover, Trevisan et al. (2015) identified *ZmUBP1*, maize orthologues of the *UBP1* gene of *Arabidopsis*, as an interesting target for nitrate in maize root. UPB1 is a transcription factor belonging to the bHLH subfamily 14 (Toledo-Ortiz *et al.* 2003) and it is known for the ability to modulate the balance between cell proliferation and differentiation by negatively regulating the expression of a set of peroxidases, which are involved in the regulation of ROS levels between cell proliferation and cell elongation zones (Tsukagoshi *et al.* 2010). Consequently, ROS content and regulation in the transition zone by peroxidases UBP1-controlled represent a critical aspect in root growth. Trevisan *et al.* (2018) pointed out the role of nitrate provision in the transcription regulation of *ZmUPB1*, which in turn regulates the expression of the peroxidase PRX112, triggering to the regulation of maize root growth basing on nitrate availability.

The present results showed an up-regulation of *ZmUBP1* gene in response to ammonium provision. Interestingly, the addition of nitric oxide scavenger did not influence *ZmUBP1*

transcription level, compared to roots of seedlings grown in the presence of NO_3^-, suggesting that *ZmUBP1* transcriptional regulation is not nitric oxide-mediated.
ZmPRX112 is a maize orthologue gene of *PER40* of *Arabidopsis*, and it is particularly interesting since it is highly expressed at the boundary of the elongation and meristematic root zones (Tsukagoshi *et al.* 2010). Peroxidases have two functions: one is the reduction of H_2O_2, the second is the catalysis of the hydroxylic cycle, resulting in ROS formation, particularly $O_2^{\cdot-}$ (Tsukagoshi et al., 2010), thus playing a key role in the regulation of ROS production. Our results suggest that the induction of *ZmPRX112* transcription is specifically due to nitrate provision as nitrogen source, probably through NO-mediated signalling.

Manoli *et al.*, (2016) demonstrated an integrative action of auxin, SLs and nitric oxide in root apex nitrate-mediated response. Particularly, early response to nitrate supply lead to an inhibition of strigolactones biosynthesis and transport (Manoli *et al*., 2015), resulting in a consequent stimulation of primary root growth. An inhibitory effect of nitrate on the expression of *ZmCCD8*, which is specifically involved in SLs biosynthesis, was observed also in the present results, but the addition of cPTIO did not further modify its expression, indicating a NO-independent mechanism at the base of its down-regulation. Interesting, a stimulation of expression was shown when ammonium was directly added to the growing medium.

The biosynthesis and transport of auxin and its signalling play a crucial role in controlling root growth and development (Saini *et al.* 2013). Particularly, its differential distribution in plant tissues, established by a polar transport, can trigger a wide range of devel opmental processes affecting also root architecture (Blilou *et al.* 2005; Dubrovsky *et al.* 2008). In order to gain new insights in auxin-related response, in the present study the expression of *Zea mays PIN9* auxin carrier was evaluated and showed a strong up-regulation of expression in response to both nitrate and ammonium, giving further strength to the already hypothesised role of auxin in the root response to nitrogen.
Overall, the expression profile of the genes analysed in the present study showed different pattern of expression in response to the different tested treatments, which are not always clearly interpretable and suggest sophisticated molecular mechanism of regulation at the base of root architecture adjustments. Thus, further untargeted approaches seem to be necessary for the identification of the missing pieces involved in molecular pathway governing root growth and adaptation to nitrogen fluctuations. To this aim, a RNAseq analysis was conducted on tissues of maize root grown as described above (-N, NO_3^- 1mM,

NH_4^+ 1 mM and cPTIO). Preliminary results pointed out differential regulation of expression linked to 2189 genes in response to the different treatments. Particularly, more than 60% of the DGEs identified resulted differentially modulated between seedlings fed on nitrate and cPTIO supplied medium. This suggest that a consistent part of the transcriptional effect of nitrate depend on NO, underling the key role of nitric oxide in the signalling pathway governing the root development.

Regarding ammonium treatment, only 3,84% out of the total were identified for being differentially modulated between maize seedlings growth in NO_3^- and NH_4^+ supplied medium, likewise 3,24% DGEs were identified for being differentially expressed by comparing seedlings growth in N-free and ammonium supplied medium.

Future studies will be conducted in order to more deeply characterize putative targets involved in the molecular pathway governing root growth development.

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