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The maize root response to nitrogen fluctuations: signalling crosstalk with strigolactones, auxin and transcriptional regulation

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

November 4th, 2019

Laura Ravazzolo

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SUMMARY

Nitrogen (N) plays a vital role for plant life, but more than 50% of the available N is lost due to the low Nitrogen Use Efficiency (NUE) of crops. Over the past five decades, the massive employment of synthetic ammonia fertilizers allowed intensive agriculture to increase crop yields but, at the same time, has also given rise to many side effects. To limit the economic and environmental impact of excessive nitrogenous fertilization, improving crop NUE is essential for an efficient sustainable agriculture. Maize (*Zea mays* L.) is one of the world's major crops, important for its contribution as both feed and food. The food security issue is particularly relevant, since it has been estimated that the world population would rise to 10 billion by 2050.

Plants can uptake N in the soil in different N-containing forms, but nitrate (NO_3^-) and ammonium (NH_4^+) are the most common inorganic compounds in aerobic and anaerobic environments, respectively. In particular, nitrate acts both as nutrient and signalling molecule, regulating many developmental processes in plants. The root represents the crucial plant organ for nitrogen perception and uptake. Considering the fundamental role of the root system architecture (RSA) to determine the plasticity of plants to explore the soil searching for water and nutrients, and the deep impact of this at whole plant level, understanding the effects of different N source on crop RSA is essential.

Strigolactones (SLs) are carotenoid-derived phytohormones, acting as both endogenous and exogenous signalling molecules to play multiple roles in regulating plant development in response to various environmental stimuli and in concert with many other regulators. Starting from the hypothesis that nitric oxide (NO), auxin and SLs could take part to complex pathway governing the maize root adaptation to different N availabilities, this PhD work mainly investigated the involvement of SLs in the maize root developmental response to both nitrate and ammonium, thanks to a combined physiological and molecular approach.

The research was initially focused on studying the effect of the two different N source on SL exudation and biosynthesis (Chapter 2). A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method was applied to try to identify and quantify the already known SLs in maize root exudates obtained by seedlings grown with different N availabilities. In this direct SL quantification on maize root exudates, a maize-specific SL called zealactone was detected at high level in N-starved samples, while nitrate-supplied samples contained no detected zealactone isomer and ammonium-supplied plants displayed a decreased SL content, but not as strong as for nitrate provision. These results indicate a clear inhibitory effect of nitrate on zealactone

production. The expression of genes encoding key SL biosynthesis (*ZmCCD8*) and transport (*ZmWBC33*) components was then measured in roots in response to 24-48-72 h of nitrate, ammonium and N-starvation. The results showed that *ZmCCD8* expression appeared greatly inhibited after 24 h of nitrate provision. On the contrary, the ammonium supply for 24 h did not induce any down regulation of *ZmCCD8* expression, and quite the opposite, it significantly stimulated its transcription. Regarding *ZmWBC33*, its gene expression was similar to those exhibited by *ZmCCD8* in response to the various N-supply, N-deficiency and SL analogue/inhibitor. To confirm a realistic meaning of the induction of *ZmCCD8* and *ZmWBC33* expression in response to N- starvation, a germination bioassay on *Phelipanche ramosa* seeds was also performed. *P. ramosa* belongs to the Orobanchaceae family, namely obligate root-parasitic plants whose seeds can be used to indirectly detect the SL exudation. Actually, *P. ramosa* parasitic seeds are induced to germinate only after the chemo-detection of particular germination stimulants in host-roots exudates, where SLs are the major stimulants. Therefore, an indirect method to detect SL exudation was developed. To confirm the exudation of SL in response to a more general nutrient-stress, phosphate-deficiency (-P) was also evaluated. As result, only nitrate provision induced a strong inhibition of SL exudation, while a massive germination was visible for those *P. ramosa* seeds treated with root exudates obtained from both N-depleted, P-starved and NH_4^+-supplied roots. These results further indirectly confirmed the presence of SLs in the exudates harvested from N-deprived plants, while a massive inhibition of SL exudation resulted as a specific response to nitrate provision. *In situ* hybridization (ISH) experiments were then performed to better characterize the pattern of expression of *ZmCCD8* and *ZmWBC33* both in roots and shoot. In primary root (PR), they both were predominantly expressed throughout the vascular parenchyma, in the root tip and in young lateral root primordia (LRP). In shoots, they were expressed in young leaves, with only slight differences between their patterns.

The effects of 24 h of N-deficiency, nitrate and ammonium supply in the presence of a SL biosynthesis inhibitor (TIS108) and of a synthetic SL analogue (GR24) on lateral root (LR) density and primary root (PR) length were then evaluated. The results showed that nitrate and ammonium clearly induced LR development, while they displayed shorter PR if compare to N-deprived seedling. On the contrary, seedlings grew with nitrate supplied with GR24 showed a lower LRP density and a slightly longer PR length, thus resembling to N-deprived plants, while those treated with TIS108 in a N-deficient conditions displayed LRP density and PR length similar to nitrate and ammonium phenotype. Taken together, these data suggested that the stimulation of LR

development might be linked to the complete or partial inhibition of SL production observed in response to nitrate and ammonium, respectively.

Since our previous results suggest that SLs and auxin might cooperate to regulate the response of maize primary root to nitrate, the hypothesis that the negative effect of nitrate on SL biosynthesis/exudation could depend on auxin was further studied with a SL quantification *in planta*, gene expression assessment and lateral root density evaluation (Chapter 3). SLs were so quantified in maize root tissues of maize seedlings treated for 24 h with a N-deficient solution, or a P-deficient solution or NO_3^- 1 mM. Results obtained are in accordance with our previous results on exudates (Chapter 2), thus confirming the role of zealactones production as a clear response to N-deprivation. In addition, zealactone could be the typical signature for N-deprivation, whereas carlactonic acid appears to be highly produced even in -P conditions. Furthermore, the production of both appeared strongly impeded in nitrate-supplied plants. Gene expression of some selected auxin-responsive genes (i.e. *Aux/IAA*, *ARF*, *LBD* and *PIN*) and cell cycle-related genes (*CYCB, CDKB*) were assessed in N-deprived or nitrate-provided seedlings and in the presence of various SL and auxin analogues (GR24 2 µM and NAA 0.01-0.05-0.1-1 µM, respectively) or inhibitors (TIS108 2 µM and PCIB 10 µM, respectively), clustering the genes in 4 different group according to their functions. The expression of the auxin-related genes evidenced peculiar trends in response to nitrate, auxin, SLs and specific inhibitors, thus allowing to select few of them as good candidates to better characterize and deepen the auxinic action involved in the nitrate signalling. LR density was also assessed in seedlings treated as for gene expression analysis with N-deprived or nitrate-provided nutrient solution and in the presence of various SL and auxin analogues or inhibitors. Our preliminary results suggest that SLs and auxin share overlapping and divergent pathways to regulates maize lateral root development in response to nitrate availability.

The maize root response to different N provision was then assessed, trying to outline the different signature between nitrate and ammonium (Chapter 4). Accordingly, a root transcriptome analysis using RNA-Seq technology was assessed to compare gene expression profiles in maize root apex of seedlings exposed to N-depleted solution or supplied with nitrate 1 mM or ammonium 1 mM for 24 h. In concert with this molecular untargeted approach, a physiological evaluation of plant development in response to nitrate and ammonium was also performed. Hence, the chlorophyll, flavonoids and anthocyanins index were measured in leaf epidermis of maize seedlings grown for 7 days in the different N treatment. In order to get a complete picture of the plant status in response to nitrate and ammonium, shoot and root fresh weights were also

obtained, together with shoot and root area, and PR length determination. In addition, the amino acid profiles of root and leaves was obtained by means of HILIC-MS/MS quantification. Indeed, ammonium induced a transcriptomic profile characterized by a strong alteration of the hormonal balance and of transcript factors RNA regulation, while nitrate mainly repressed the response related to transmembrane transport and cell wall. In addition, they both have negative effect of cytoskeleton-related and peroxidase genes. On the other hand, ammonium induced a toxic accumulation of amino acids at leaf level, showing reduced total biomass and impaired leaf pigment content, while nitrate positively stimulate the plant growth, in particular at root level. The results provided new insight to better characterize how the early sensing of N-deficiency or nitrate/ammonium provision by root could impact on the overall plant growth and physiology.

Chapter 1

GENERAL INTRODUCTION

1. PREFACE

Current world population of 7.7 billion is expected to dramatically soar, reaching 8.6 billion in 2030 and 9.8 billion in 2050, with most of the increase in the developing regions (United Nations, 2017). At the same time, the number of undernourished people in the world has been on the rise since 2014, reaching an estimated 821 million in 2017, with the worst situations in South America and Africa (SOFI, 2018). Consequently, a resolute increase in food production is clearly needed to keep pace with the demand, but this must be done in an environmentally sustainable way. For instance, developing more land for agriculture or, even better, improving the employment of the land already dedicated to agriculture could be some ways to increase food production. On the other side, we also have to deal with the negative impact on climate change and global biodiversity of land erosion, increasing urbanisation, drought, floods and loss of soil fertility. In the previous century, the innovation in crop productivity has been extremely intense worldwide, thanks to the novel technological advances introduced during the Green Revolution. Most of the success was caused by the combination of high rates of investment in crop research – such as crop genetic improvement on producing high-yielding varieties for the major staple crops (wheat, rice, maize) – but also investment in infrastructure, market development and appropriate policy support (Pingali, 2012). In this scenario, a massive use of chemical fertilizers, with artificial nitrogenous fertilizers in the first place thanks to the Haber-Bosch process, has played a central role (Godfray *et al*., 2010). Nevertheless, environmental concerns about this high application of synthetizing ammonia products have emerged in most of the agroecosystems (Erisman *et al*., 2013; Shibata *et al*., 2015), along with the negative effect to human health and climate. In order to limits the economic and environmental impact of excessive nitrogenous fertilization, improving crop NUE (Nitrogen Use Efficency) is essential for an efficient sustainable agriculture.

Nitrogen (N) plays a vital role in plant life. Globally, during 1961–2010, maize, rice and wheat received a total of 1594 Tg (1 Tg = 1 million tonnes) of N-fertilizer (Ladha *et al*., 2016), but more than 50% of this available N was lost due to the low NUE of crops (Li *et al*., 2017). As the world's most widely cultivated crops, maize, rice and wheat have a combined annual harvest of 2.5 billion tonnes and represent the foundation of world food security (Save and Grow in practice: maize, rice, wheat; FAO, 2016). By 2050, world annual demand for maize, rice and wheat is expected to reach 3.3 billion tonnes (FAO, 2016). This extraordinary result on maize, rice and wheat yields was mainly due to the enormous growth of global cereal production, considering that

these three cereals provide an estimated 42.5% of the world's food calorie supply (Rich and Watt, 2013), with a contribution of 37% to the global supply of protein. Moreover, millions of tonnes of cereals are used to feed livestock that produce much of the world's meat, milk and eggs. A new study by FAO and the Organisation for Economic Cooperation and Development (OECD) estimates that global consumption of cereals will increase by 390 million tonnes between 2014 and 2024 (OCED and FAO, 2015). Accordingly, there will be an increasing demand for animal feed, with coarse grains accounting for more than half of the total. Notably, 70% of coarse grains is represented by maize alone. In addition, by 2024 developing countries will be consuming as food an additional 170 million tonnes of maize, rice and wheat.

Among cereals, maize (*Zea mays* L.) is one of the most important cereals both for human and animal consumption and its world production has grown from 205 million tons grain in 1961 to 1134 million tons grain in 2017, with a global area rising from 105 million ha in 1961 to 197 million ha in 2017 (FAOSTAT, 2017). Maize has a double employment: in the developed world it is mainly used to feed livestock and produce biofuel, while in many developing countries it is primarily grown as food crop and consumed directly as food. Actually, maize is particularly important in the diets of sub-Saharan Africa and Latin America (Shiferaw *et al*., 2011), and future prospects led to hypothesize its simultaneous rising demand and declining productivity that could lead, by 2050, to an annual cost of US$30 billion (Rosegrant *et al*., 2009). From these last few data, it is clear that important measures are needed to accelerate the growth of maize yield, but area harvested expansion is not the good answer due to the increasing land degradation. (Shiferaw *et al*., 2011). On the contrary, the major challenge is to improve maize yield without compromising environment and human health, and this could be achieved by improving our knowledge of maize biology.

Maize production doubled in the past 40 years as a result of increased yields that came from the use of improved crop varieties, along with greater inputs of fertilizer, irrigation, and pesticides (Evenson and Gollin, 2003). In fact, intensification of agricultural systems has also given rise to a series of negative environmental consequences, at both local and global level. Most of them are directly due to the large use in agriculture of synthetic ammonia fertilizers industrially produced by the Haber-Bosch process, one of the most important industrial innovations of the XX century that guided the Green Revolution. Under high temperature and pressure, natural gas (CH_4) is burned to produce hydrogen (H) that reacts with nitrogen (N_2) to form ammonia (NH_3), which is a highly reactive form of nitrogen. The discovery allowed to feed a rapidly growing

population and by the end of the century, worldwide ammonia production reached 80 million tonnes a year (Reay, 2015). Today, more than a third of all land is farmed and almost 50% of humanity is still dependent upon the Haber-Bosch process (Erisman *et al*., 2008). The sharp rise of synthetic N applied to the agricultural system was dramatic: from about 10 million tonnes N/year in the late 1950s to more than 120 million tonnes N/year in 2013 (Fowler *et al*., 2013). Another important issue is that the chemical synthesis of N fertilizers requires the use of a considerable amount of energy (Gellings and Parmenter, 2016). Remarkably, approximately 65% of globally N fertilizers are used only for cereal production (Garnett *et al*., 2009). Even though, it is estimated that less than 50% of the N applied is actually taken up by crops, while the remaining is lost in the soil causing serious pollution to the global biosphere (Kant *et al*., 2011).

Nitrogen (N) is one of the most abundant elements on Earth, but it is largely inaccessible for living beings because it is present predominately in its molecular unreactive and very stable triple-bonded form of dinitrogen gas (N_2). N enters the biosphere when N_2 is reduced through nitrogen fixation by very few specialized microorganisms (free-living or symbiotic nitrogen-fixing prokaryotes), or by physical processes such as lightning or by industrial nitrogen fixation (Fowler *et al*., 2013). On the contrary, reactive nitrogen (N_r) species are highly mobile and include all N species except N_2: inorganic reduced forms (ammonia [NH_3] and ammonium [NH_4^+]), inorganic oxidized forms (nitrogen oxide [NO_x], nitric acid [HNO_3], nitrous oxide [N_2O] and nitrate [NO_3^-]) and organic compounds. Agricultural sources of N_r produce atmospheric emission of NH_3, NO_x and N_2O to the air, and NO_3^- to groundwater (Erisman *et al.*, 2013). In the last decades, anthropogenic sources of newly created N_r has paired the global amount of natural terrestrial sources (Fowler *et al*., 2013) leading to an important change in the global nitrogen cycle (Galloway *et al*., 2013). Among the N_r species, the leaching of nitrate (NO_3^-) in groundwater can reach levels that can be detrimental to human health, causing methemoglobinemia in babies and probably cancer and heart disease (Espejo-Herrera *et al*., 2016). In addition, NO_3^- in concert with ammonia (NH_3) and ammonium (NH_4^+) that enter rivers or lakes contributes to both acidification and eutrophication, the last also called algal and cyanobacterial blooms that are toxic to humans and animals and that contribute to deadly hypoxic conditions (Grizzetti *et al*., 2011, and references therein). Finally, N_r can also be lost to the atmosphere as different kinds of polluting N-containing gaseous, contributing to air pollution and greenhouse gas formation when nitrate is oxidized to nitrogen oxides (NOx), including nitrous oxide (N_2O), nitric oxide (NO) and nitrogen dioxide (NO_2). Nitrous oxide is a very strong greenhouse gas with nearly 300 times the global warming potential per unit

weight of carbon dioxide (CO_2) and has also implicated in stratospheric ozone depletion (Mulvaney *et al*., 2009). N_r accumulation in the environment has also troubling effects on climate change (Erisman *et al*., 2011; Cameron *et al*., 2013), as shown in **Figure 1**.

In order to feed the almost 10 billion people projected for the world population in 2050, it will be necessary to increase agricultural production by 70–100% (McKenzie and Williams, 2015). The environmental and economic costs of this population growth and consequent increased of fertilization to meet a rising food demand could be lowered by increasing plant Nitrogen Use Efficiency (NUE), defined as the yield obtained/unit of available N in the soil. Genetic manipulation and breeding strategies to improve crop NUE, along with more sustainable agricultural practice should be the new means to reach a second Green Revolution, able to increase crop yield while decreasing N application (Han *et al*., 2015; Hirel and Lea, 2018).

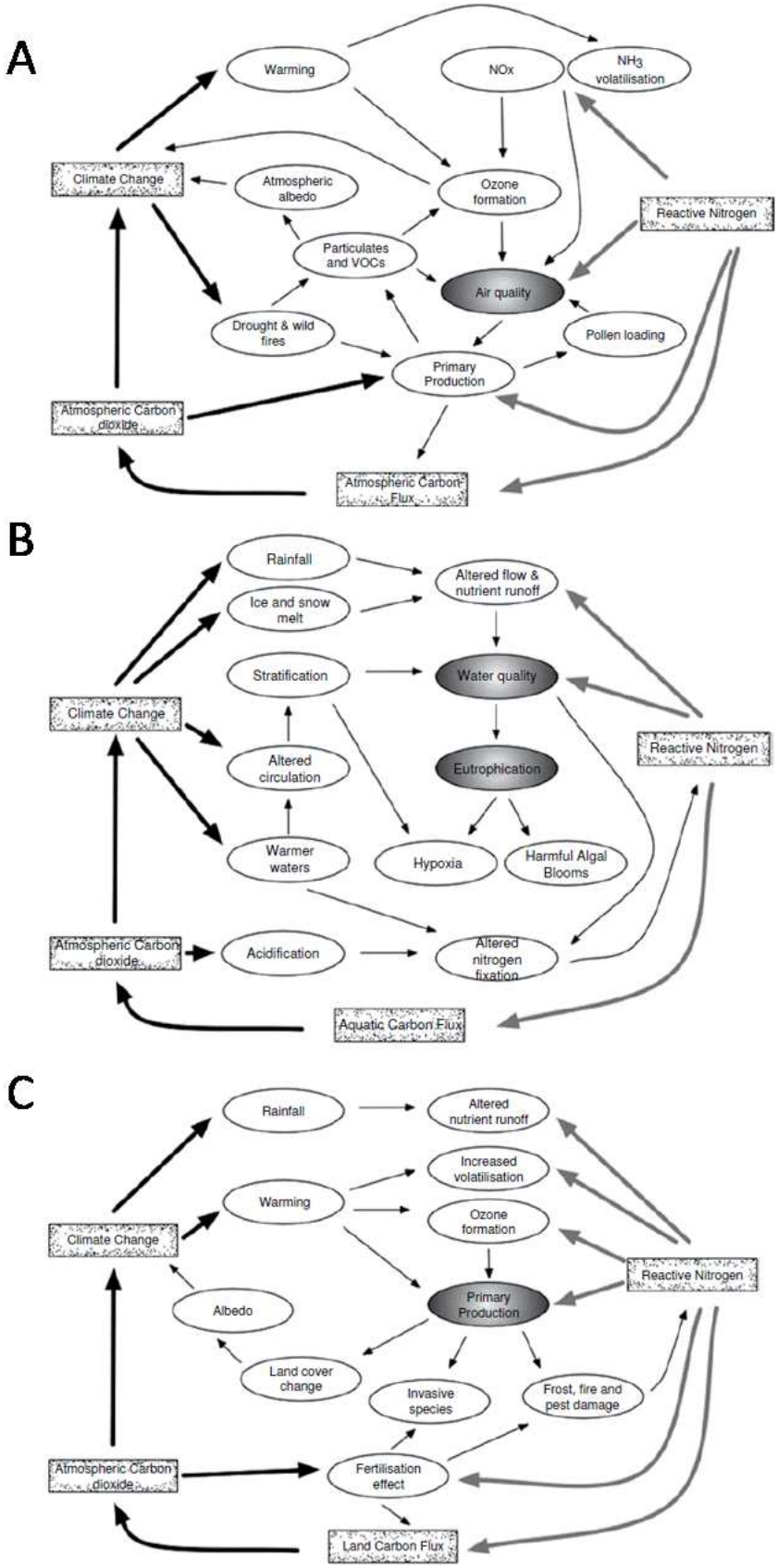

Figure 1. The web of nitrogen and climate change interactions in our atmosphere (A), in aquatic environments (B), and on land (C), from Reay, 2015.

2. NITROGEN IN AGRICULTURE

1.1 Nitrogen in plant life and Nitrogen Use efficiency (NUE)

Nitrogen (N) is the essential macronutrient required in the greatest amount by plants. It is a constituent of nucleic acids (DNA, RNA), proteins, enzymes and even important metabolic components such as chlorophyll, ATP and phytohormones (Andrews *et al.*, 2013; Gojon, 2017). It comprises almost 2% of plant dry matter and approximately 16% of total plant protein (Frink *et al*., 1999), so its availability, or lack as well, limits plant growth and development, crop yield and primary production at a planetary scale (Gutierrez, 2012). In addition to its relevance as an essential plant nutrient, N acts also as a signalling molecule that regulates a large number of plant processes including dormancy, seed germination, flowering time, leaf expansion, root development, resistance to biotic and abiotic stress, mediation of hormone signalling and expression of multiple N-responsive genes (Bouguyon *et al*., 2012; O'Brien *et al*., 2016; Guan, 2017). Most terrestrial plants adsorb nitrogen from the soil, where it can be found as two types of sources: inorganic compounds, such as for example nitrate (NO_3^-) and ammonium (NH_4^+), and organic compounds, such as amino acids, peptides, urea and proteins (Miller *et al.*, 2007). Among the different forms of nitrogen, nitrate (NO_3^-) is the preferred N source in aerobic agricultural soils, especially for cereals, while ammonium (NH_4^+) is important in acidic and anaerobic environments.

Under most cropping situations, plant productivity is limited by the availability of nitrogen in the soil (Dechorgnat *et al*., 2018). In addition, once N is provided to the soil through massive synthetic N fertilization, only half of the applied N fertilizer is effectively taken up by plants, while the remaining N may have detrimental environmental consequences and worrying effects on human health (Gruber and Galloway, 2008). Therefore, a deep understanding of the physiological, genetic and molecular mechanisms regulating Nitrogen Use Efficiency (NUE) in plants is urgently needed.

Nitrogen Use Efficiency (NUE) is an important but complex term that allows defining the total biomass or yield of grain, forage or fruit produced per unit of N fertilizer applied in the soil according to different plant species (Xu *et al.*, 2012). The complexity is due to the presence of multiple interacting genetic and environmental factors that govern NUE in every steps of N metabolism, such as soil type and management, interactions with microorganisms, nature of N source, climate (Moll *et al.*, 1982) and N uptake, translocation and assimilation (Hirel *et al*., 2007). The overall NUE of plants comprises both uptake (N uptake/N available from the soil) and

utilization (dry matter or protein yield/N uptake) efficiencies (Good *et al*., 2004; McAllister *et al.*, 2012; Xu *et al*., 2012) and can be calculated as:

$$NUpE * NUtE = \frac{Nt}{Ns} * \frac{Gw}{Nt} = \frac{Gw}{Ns}$$

Where:

- NUpE = N uptake efficiency;
- NUtE = N utilization efficiency;
- Nt = total N transported to the seeds;
- Ns = total N supplied to the plant;
- Gw = total grain-seed weight.

In addition to Nitrogen Use Efficiency, NUE term can also mean N Uptake Efficiency (NUpE), N Utilization (assimilation) Efficiency (NUtE), Apparent N Recovery rate (ANR), Agronomy Efficiency of fertilizer N (AE), N physiological Use Efficiency (NpUE), N Transport Efficiency (NTE), and N Remobilization Efficiency (NRE). Accordingly, a crop plant optimized for NUE would have a high rate of uptake of N from the soil, a high rate of N incorporation into organic forms, and high efficiency of N use, recycling, and remobilization into grains. Thus, several different approaches in improving NUE have been developed, in order to increase crop productivity using sustainable agriculture methods and preserving the environment (Zeigler and Mohanty, 2010; Li *et al*., 2017). Some good sustainable agriculture methods include crop rotation, no-till practices, rationalized using of fertilizer and establishment of ground cover (Hirel and Lea, 2018). At the same time, the combined approach of molecular biology and physiology through genetic manipulation and breeding strategies are important ways to obtained crops with enhanced NUE (Han *et al*., 2015). For instance, one breeding strategy could involve the selection of varieties able to establish a more efficient symbiosis with AMF (arbuscular mycorrhizal fungi) (Verzeaux *et al*., 2017), or to induce higher root colonization of N_2-fixing bacteria (Pamell *et al*., 2016). Simultaneously, the study of the genetic basis of plant nutrition and the root physiology and morphology through the integration of transcriptomics, proteomics and metabolomics allowed the identification of key elements of nutrient uptake, transport, and assimilation (Li *et al*., 2017). Nevertheless, the complex mechanisms underlying NUE regulation and signalling are still far from been completely understood, and the transfer from theory to practice is still difficult. Accordingly, other studies are necessary to better comprehend the crop NUE, both at molecular and morphological level.

1.2 Human impact on the nitrogen cycle: the environmental issue

The nitrogen cycle is a complex issue, involving both natural ecosystems and agricultural land and sharing connections between atmosphere, freshwater/marine and terrestrial nitrogen (**Fig.2**). The particularity of N for crop system is that, in the majority of soils, there are no available N source pools, so N must reach the plant-soil system through fertilization. Unfortunately, most of the N added to crops is lost in the environment as a dangerous pollutant (Galloway *et al*., 2008; Erisman *et al*., 2013). Among the reactive N species, nitrate (NO_3^-) is a very soluble source of N that undergoes leaching and runoff to represent a major hazard to both human health and environment (Cameron *et al*., 2013). Concerning human health, contamination of drinking water by an excess of nitrate can cause the "blue baby syndrome", namely a methemoglobinemia in babies that reduces the ability of red blood cells to release oxygen to tissues (Fewtrell, 2004). Moreover, excess of nitrate in drinking water has been also linked to cancer, due to the potential production of carcinogenic nitrosamines (Bedale e*t al*., 2016), and heart disease (Grizzetti *et al*., 2011). On the other way, some recent studies outline that nitrate and nitrite perform positive biological NO-like functions, for instance by protecting against gastric ulcers (Ma *et al*., 2018), so the question is far from being completely deepened.

Relating to the environmental problem, nitrate in concert with ammonia (NH_3) and ammonium (NH_4^+) can enter in both freshwater and marine ecosystem and contribute to eutrophication, namely a natural process in which increasing N inputs lead to surface water hypoxia. The explosive growth of phytoplankton and algal species that derive from eutrophication leads to the release of toxic compounds, can reduce biodiversity and increase the growth of toxic algal species (Grizzetti *et al*., 2011, and references therein; Reay *et al*., 2015). In addition to nitrate leaching in water ecosystem, N forms can also be lost to the atmosphere as N-containing gaseous, namely nitrous oxide (N_20), nitric oxide (NO) and nitrogen dioxide (NO_2), collectively known as NO_x, and ammonia (NH_3). NO_x emissions in the atmosphere are directly linked to the generation of secondary pollutants, such as tropospheric ozone and other photochemical oxidants and aerosols. These so-called secondary pollutants are responsible for both severe damages to human health and vegetation (Erisman *et al*., 2013; Fowler *et al*., 2013), but also for N_r deposition on the environment by acid rain (Robertson and Vitousek, 2009; Galloway *et al*., 2013). Finally, acid rain and dry deposition of N_r due to ammonia volatilization are the causes of acidification of natural ecosystems (Cameron *et al*., 2013) and production of harmful small airborne particles, such as

ammonium nitrate, that can enter into the human respiratory system (Schwartz *et al*., 2002). In conclusion, the modification of the global N cycle by human activities has risen multiple side effects on environment and human health. As before stated, it is thus necessary to reduce the amount of N fertilizers and to improve NUE to increase the sustainability of agriculture and preserve our planet ecosystems.

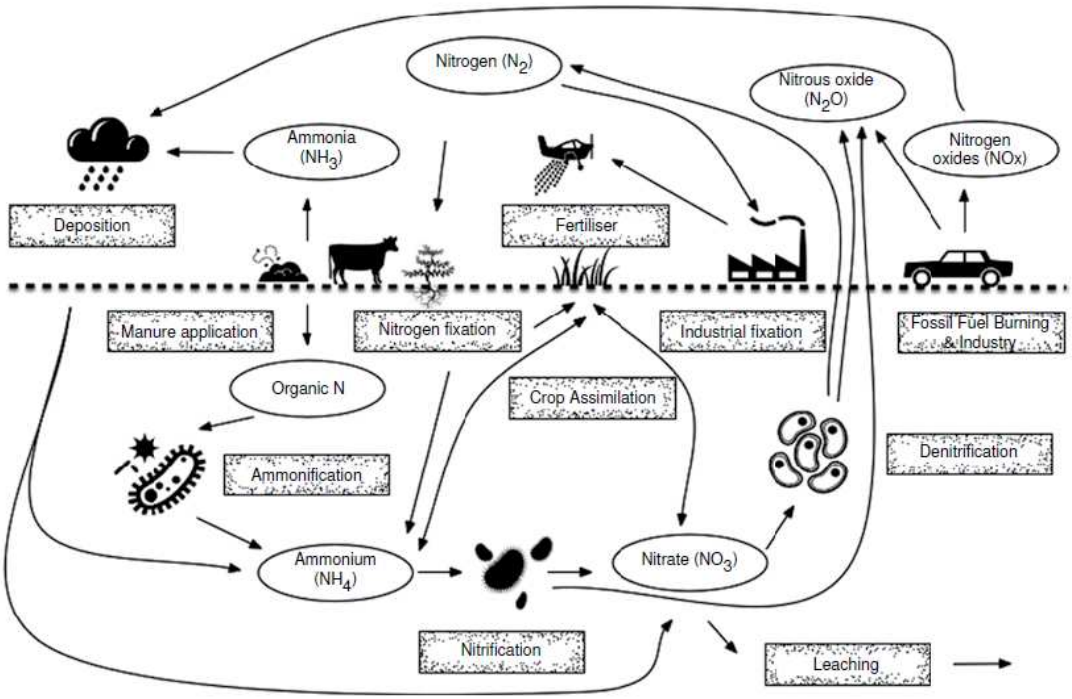

Figure 2. Human disturbance and the terrestrial nitrogen cycle. The dotted line represents the land surface with the boxes showing the key processes by which nitrogen is cycled and the ovals showing the changing form of nitrogen as it undergoes these processes. From Reay, 2015.

1.3 Nitrogen metabolism: uptake, assimilation and remobilization

Regulation of plant N-metabolism is complex and influenced by several physiological and metabolic processes, such as circadian rhythms, sugars synthesis and transport, key N metabolite levels and NO_3^- itself (McAllister *et al*., 2012). Plants take up N through four main routes, two concerning inorganic forms (uptake as nitrate or uptake as ammonium), one involving organic N compounds (uptake of amino acids, peptides, proteins) and the last regarding fixation of gaseous N_2 thanks to nitrogen-fixing bacteria in root nodules (Tegeder and Masclaux-Daubresse, 2018). Nitrate and ammonium represent the two major N source for crops in aerobic and anaerobic environments, respectively (Gojon, 2017). They both act as nutrients and signalling molecules, regulating many developmental processes in plants (Bouguyon *et al*., 2012; Undurraga *et al*., 2017; Liu and von Wirén, 2017).

In order to properly react to N fluctuations in the soil, the plant root has evolved many inorganic and organic N transporters with different substrate affinities and specificities (**Fig. 3**). For what concerns the root uptake of nitrate and ammonium, they are both realized through low- and high-affinity transporter systems (LATS and HATS, respectively). LATS operates and becomes significant at external NO_3^- concentration above 1 mM, while HATS prevails in the micromolar range (< 250 µM), (Tegeder and Masclaux-Daubresse, 2018; Dechorgnat *et al*., 2019).

Nitrate transporters belong to four different families:

- **NRT1/PTR**, recently renamed as Nitrate Trasporter1/Peptide Transporter Family (**NPF**) (Léran *et al*., 2014) and that mostly belongs to LATS. The exception is NPF6.3 (CHL1/NRT1.1) which has a dual high-and low-affinity for nitrate.
- **NRT2**, which belongs to HATS;
- **CLC** (chloride channels, with affinity also for nitrate), which encodes two tonoplast H^+/NO_3^- antiporters;
- **SLAC/SLAH** (slow anion channels), which encodes two guard cell anion channels.

Among these, only some nitrate transporters belonging to the *NPF* and *NRT2* gene family are directly involved in the acquisition of NO_3^- from soil, namely NRT2.1, NRT2.2 and NRT2.4 acting as HATS, NRT1.2 as LATS and the dual-affinity transporter NRT1.1/NPF6.3 To be active, NRT2.1 forms a functional unit with the membrane protein NITRATE ACCESSORY PROTEIN2.1 (NAR2.1), which plays a key role in regulating HATS by enhancing nitrate uptake (Laugier *et al*., 2012). On the other side, the *CLC* and the *SLAC1/SLAH* families, are responsible, together with the remaining *NPF* and *NRT2* genes, for the efflux, transport, and allocation of nitrate within the plant (Kant, 2018; Wang *et al*., 2018a).

Among ammonium transporters, there are two main systems:

- Saturable high-affinity uptake system (HATS), such as AMT1 (Ammonium Transporters). In Arabidopsis, 95% of NH_4^+ is imported by AMT1;1, AMT1;2 and AMT1;3 (Gazzarrini *et al*., 1999; Loque *et al*., 2006). On the contrary, the maize *AMT* gene family contains 8 members divided into four classes, *ZmAMT1* to *ZmAMT4* (Gu *et al*., 2013).
- Non-saturable and no ammonium specific low-affinity uptake system, such as aquaporins and cation channels. In addition, an Ammonium Facilitator 1 (AMF1) protein has been identified in soybean and yeast, thus probably being a NH_4^+ LATS (Chiasson *et al*., 2014).

Since ammonium is toxic to plant cells in high concentrations, in both cases uptake and assimilation are closely regulated.

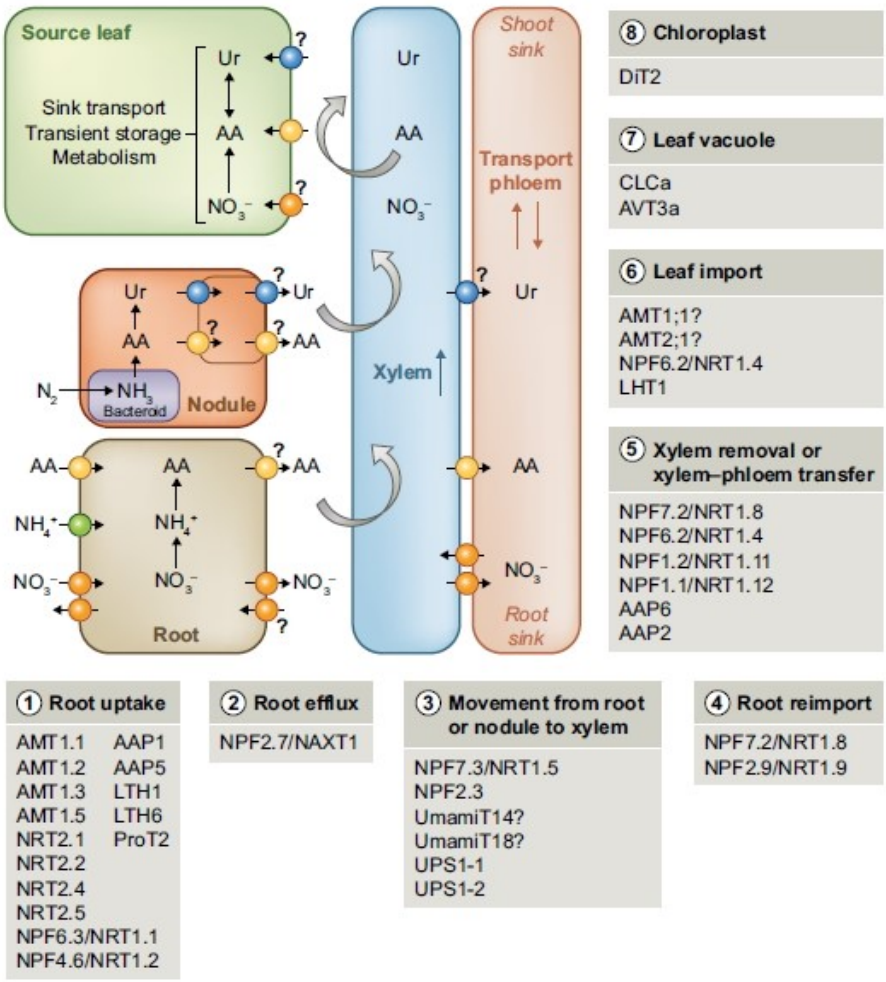

Figure 3. Schematic representation of nitrogen root uptake and partitioning from root to leaves. Inorganic N transporters for nitrate (NO_3^-; arrow with orange circle) and ammonium (NH_4^+; green circle), as well as organic N transporters for amino acids (AA; yellow circle) and ureides (Ur; blue circle) are shown at root, nodule, xylem, phloem and source leaf levels. From Tegeder and Masclaux-Daubresse, 2018.

Once nitrate and ammonium are absorbed, they are exposed to a complex process of assimilation, transformation and mobilization within the plant (Krapp, 2015) (**Fig. 4**).

For what concerns nitrate, it is taken up from roots and generally assimilated into ammonium and amino acids in the shoot, with the relocation usually occurring via the xylem vessels (Kant, 2018). The first assimilation step is cytosol reduction in nitrite (NO_2^-) by the enzyme nitrate reductase (NR). Since nitrite is fairly toxic to plant cells, it is rapidly imported into plastids and further reduced to ammonium thanks to the enzyme nitrite reductase (NiR). Indeed, ammonium can be provided directly from the soil, or it can come from nitrate reduction or secondary metabolism, such as amino acid catabolism and photorespiration (Andrews *et al*., 2013). Despite its origin, once ammonium ions enter the cell, they are incorporated into organic molecules of amino acid glutamine in the presence of glutamate thanks to the enzymatic cycle GS/GOGAT (Glutamine

Synthetase/Glutamate synthase) (**Fig. 4**). Generally, GS exists as two major isoforms: the cytosolic called GS1 and the plastidic form GS2, while GOGAT could be ferredoxin-dependent or NADH-dependent. GS2 in mainly involved in primary N assimilation of photorespiratory ammonium in leaves, while GS1 is important for primary N assimilation in roots (Tegeder and Masclaux-Daubresse, 2018). On the other side, the NADH-dependent GOGAT is located predominantly in non-photosynthesizing tissues, while the Fd-GOGAT activity is much greater in chloroplasts (Masclaux-Daubresse *et al*., 2010). Besides GS/GOGAT, other enzymes involved in N assimilation and metabolism are glutamate dehydrogenase (GDH), aspartate aminotransferase (AspAT) and asparagine synthetase (AS) (Li *et al*., 2017). Once N has been assimilated into organic forms, they are used as amino groups donors for the biosynthesis of others amino acids, which in turn can be directly used to form proteins and nucleotides (Forde and Lea, 2007) or transported through phloem to provide N to sink organs, such as mesophyll (Yesbergenova-Cuny *et al*. 2016). Regarding the fine regulation of N assimilation, all the above-mentioned enzymes and associated pathways are controlled by several factors, including soil N availability, plant N status, external and internal C status and changes in plant hormones (Krapp, 2015).

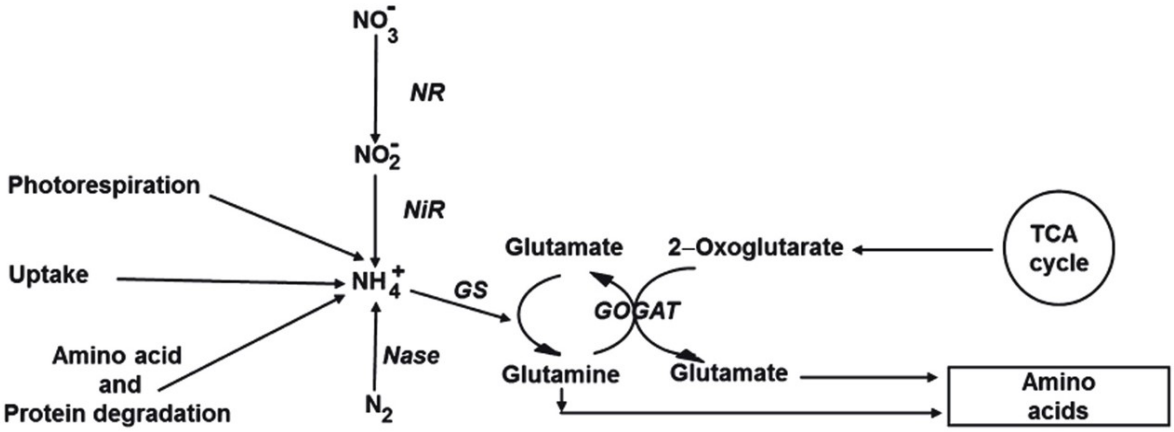

Figure 4. The assimilation of nitrogen (N) in higher plants. The main enzymes involved are indicated in italics: *NR*=nitrate reductase; *NiR*=nitrite reductase; *Nase*=nitrogenase; *GS*=glutamine synthetase; *GOGAT* =glutamate synthase. The ultimate source of inorganic N available to the plant is ammonium, which is incorporated into organic molecules in the form of glutamine and glutamate through the combined action of the two enzymes *GS* and *GOGAT* in the plastid or chloroplast. From Andrews *et al*., 2013.

1.4 Nitrate sensing and signalling in plants

As previously stated, nitrate is not only a nutrient as N source, but it acts also as a signalling molecule that regulates many developmental processes in the plant, including gene expression,

shoot and root development, seed germination and flowering (Wang *et al*., 2018a). Indeed, nitrate *per se* induces and regulates the so-called Primary Nitrate Response (PNR), a transcriptional response that does not require de novo protein synthesis, but only nitrate itself (Medici and Krouk, 2014). PNR genes are rapidly induced when nitrate-depleted plants are supplied with this anion. In particular, the up-regulation of nitrate own transporters and nitrate/nitrite reductase genes have been shown (Crawford and Glass, 1998). Other gene categories involved in the PNR encode proteins involved in ion transport, primary and secondary metabolism, biosynthesis of nucleic acids, transcription and RNA processing, hormone homeostasis (Bouguyon *et al*., 2012).

Regarding nitrate sensing, it seems to be directly mediated by some nitrate transporters, namely the dual-affinity NRT1.1/NPF6.3 and the high-affinity NRT2.1 (Ho *et al*., 2009; Bouguyon *et al*., 2015). As a dual-affinity transporter, NRT1.1/NPF6.3 can switch between high and low affinities through phosphorylation (low concentrations of external nitrate) and dephosphorylation (high concentrations of external nitrate) at the Thr101 site. In addition, NRT1.1/NPF6.3 differently interacts with two CPIK proteins (CALCINEURIN B-LIKE (CBL)-INTERACTION PROTEIN KINASES) to regulate nitrate signalling, namely CIPK8 for low-affinity response and CIPK23 for high-affinity response (Ho *et al*., 2009). Furthermore, NRT1.1/NPF6.3 is involved in regulating the N root-foraging process, for instance by up-regulating *ANR1* (ARABIDOPSIS NITRATE-REGULATED 1) to promote lateral root growth in Arabidopsis when high external nitrate is perceived (Remans *et al*., 2006a). Moreover, NRT1.1/NPF6.3 is able to regulate *NRT2.1* expression in response to different external concentrations of nitrate, and both these sensors play a role in lateral root development and root system architecture (RSA) regulation in response to nitrate (Krouk *et al*., 2010b).

Downstream of the nitrate sensing, the complex signalling cascade triggered by nitrate includes several transcription factor and kinases (**Fig. 5**), such as **NLP7** (Nodule Inception-like protein 7), **SLP9** (Squamosa Promoter-binding-like Protein 9), **TGA1/TGA4** (TGACG-BINDING FACTOR 1 and 4), **TCP20** (TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1-20), **NAC4** (NAM-ATAF-CCUC DOMAIN-CONTAINING PROTEIN) and **LBD37/LBD38/LBD39** (LATERAL ORGAN BOUNDARY DOMAIN family) in *Arabidopsis*.

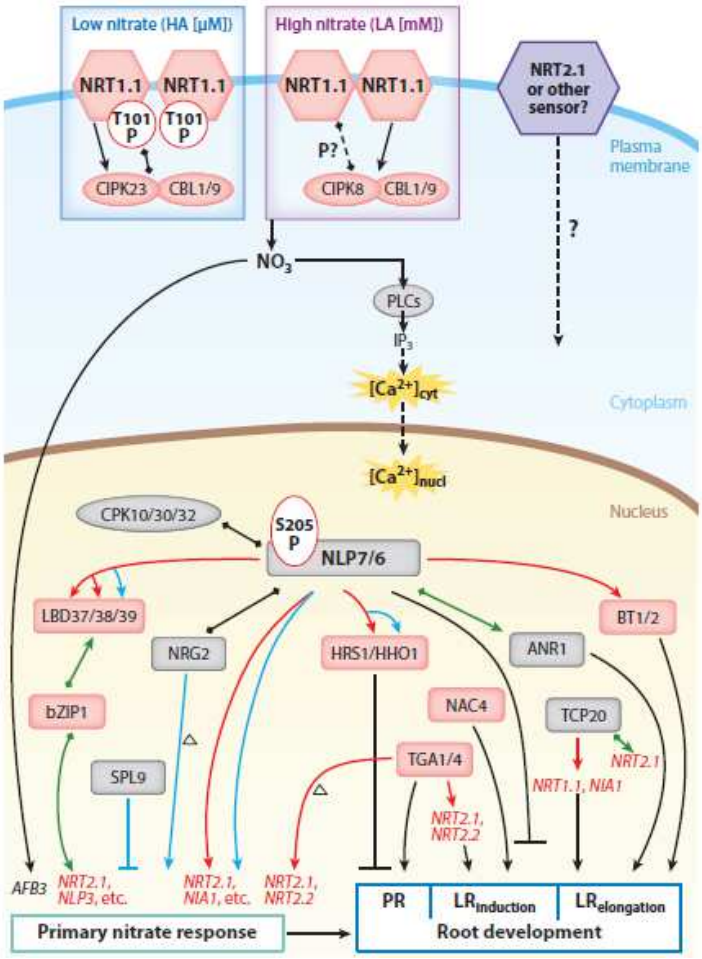

Figure 5. The nitrate signalling pathways in *Arabidopsis*. From Wang *et al*., 2018a.

First of all, *NPL7* has been suggested to be a master regulator in nitrate signalling, thus regulating other transcription factors. Accordingly, it has been proposed that nitrate inhibits, through a yet unknown mechanism, the export of NLP7 from the nucleus, leading to its rapid nuclear accumulation and to the transcription of many genes taking part in the nitrate pathway (Marchive *et al*., 2013). On the other side, SLP9 modulates the expression of several nitrate transporters and assimilatory genes including NRT1.1/NPF6.3, NRT2.1, NRT2.2 and NIR (Krouk *et al*., 2010a), while TGA1 and TGA4 transcript accumulation in roots in response to nitrate was observed (Alvarez *et al*., 2014). TCP20 has been shown to be involved in lateral root development by redirecting root growth to nitrate-rich patches (Guan *et al*., 2014), whereas NAC4 contribute increasing lateral root density together with auxin-related gene *AFB3* (AUXIN SIGNALING F-BOX PROTEIN 3) (Vidal *et al*., 2013). Finally, LBD37, LBD38, and LBD39 act as negative regulators of anthocyanin biosynthesis and are involved in regulating many genes of nitrate uptake and assimilation (Rubin *et al*., 2011).

Concerning ammonium, it is possible that it could modulate PNR thanks to putative ammonium sensors like AMT1;3 (Lima *et al*., 2010), while gene expression modulation by ammonium has been

shown through its downstream products, such as glutamine (Kamada-Nobusada *et al*., 2013), but many other studies are needed to better understand its involvement in N-related signalling.

3. MAIZE: ONE OF THE WORLD'S MOST IMPORTANT CROPS

3.1 The origin and the lifecycle of maize

Together with rice and wheat, maize (*Zea mays* L.) is one of the most important crops used for human food, animal feed and also as ethanol feedstock, producing more grain globally than any other crop (Dechorgnat et al., 2018). It is also called corn and it belongs to the *Poaceae* (*Gramineae*) family, namely herbaceous monocotyledon plants with, in case of maize, an annual cycle. Nowadays it appears like a tall plant with an extensive and complex root system, but in origin it was a wild and smaller grass called *teosinte* (Beadle, 1939). Historically, the cultivation of maize originated in Central America about 10000 years ago, probably in current Mexico. From there, maize spread to Canada and Argentina, and by the end of the 15th century, it arrived in Europe through Spanish *conquistadores*. Even if maize is well suited to hot and dry climates, it is so adaptable that can grow over a range of agroclimatic zones, such as from 58°N to 40°S, from below sea-level to more than 4000 m in, and from 250 mm of rainfall/year to more than 5000 mm (Chaudhary *et al*., 2014).

Botanically, maize has a fine root system (that will be deeply discussed in the following section), 8 to 20 leaves arranged spirally and alternate on the stem, a cylindric thick stem with 8 to 21 internodes and separated inflorescence, namely tassels as male and ear as female inflorescences (du Plessis, 2003). Maize growing cycle ranges from 3 to 13 months and, according to the Iowa System, the leaf staging is quite complex, dividing the maize development into vegetative (V) and reproductive (R) stages (**Fig. 6**).

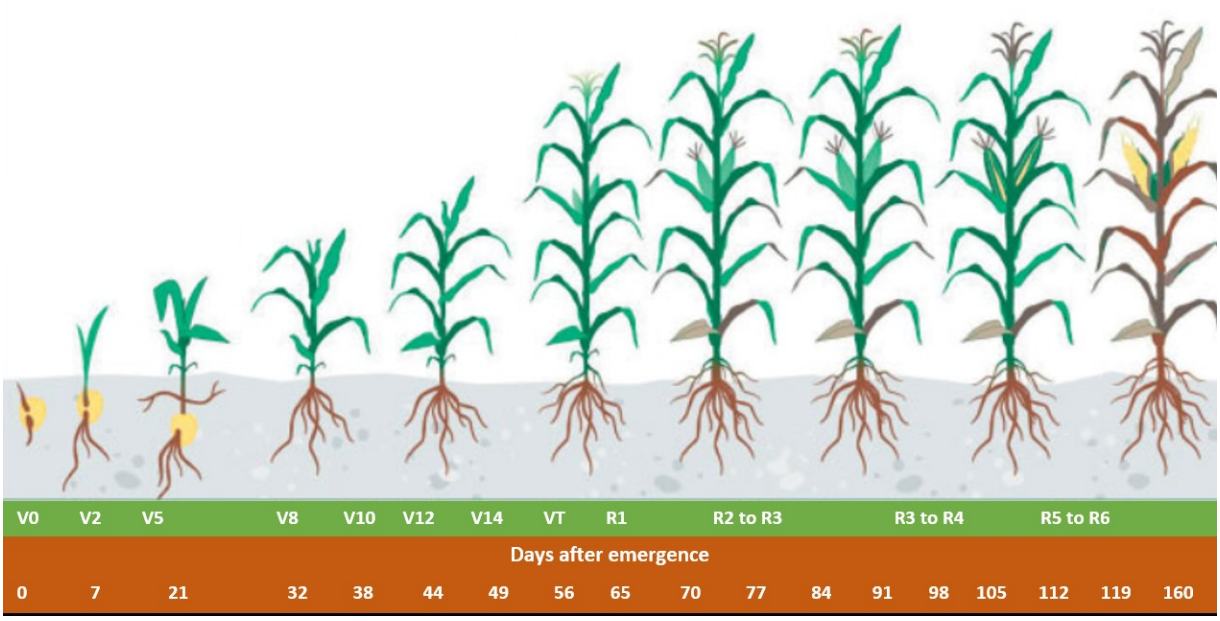

Figure 6: Maize growth stages. Adapted from Bondesio *et al*., 2016.

3.2 Maize as both feed and staple food

Despite being an important food crop, over the past decade maize has become a fundamental livestock feed too, with a global feed demand that grows 6% per year (Shiferaw *et al*., 2011). Accordingly, maize is the main staple food for people in 94 developing countries, including Africa, some region in Asia and South America, providing nearly 30% of food calories to more than 4.5 billion people (Chaudhary *et al*., 2014). In 2010, global maize production was 844 Mt, with USA, China, Brazil, Mexico, and Argentina as the top five maize producers in the world (FAO, 2012) (**Fig. 7**). Notably, maize production becomes higher and higher than the one of wheat and rice, the other major staple cereals.

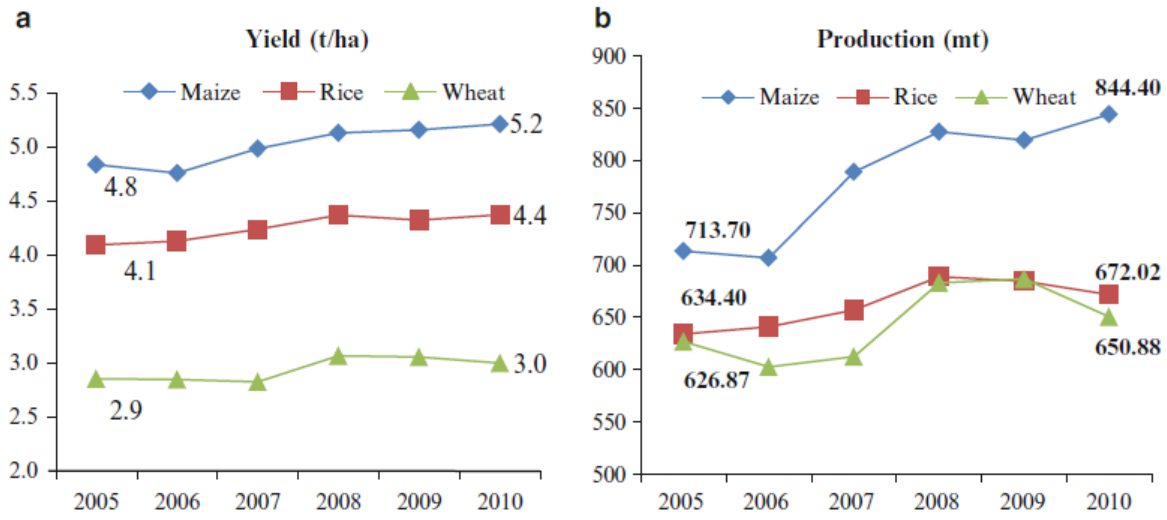

Figure 7: Maize productivity (a) and production (b) along with other world major cereals during 2010. From FAO, 2012.

Concerning its nutritional value, maize is very rich in carbohydrates, it provides vitamin E and A, but it lacks vitamins B and usable proteins (McCann *et al*., 2005). In addition, maize is rich in leucine which blocks the assimilation of niacin in the human body, thus causing protein deficiency. Nevertheless, maize global demand as livestock feed is about 63%, which becomes 70% in high-income countries and 20% in developing countries such as sub-Saharan Africa (**Fig. 8**). Furthermore, maize is important for biofuel production (Shiferaw *et al*., 2011). Since the demand for maize in developing countries is projected to double by 2050 (Rosegrant *et al*., 2009), understanding how to improve its productivity without damaging the environment with excessive fertilizers is urgently needed. Improvements to maize NUE will ultimately help deliver sustainable N fertilizer use in agriculture (Kant *et al*., 2011).

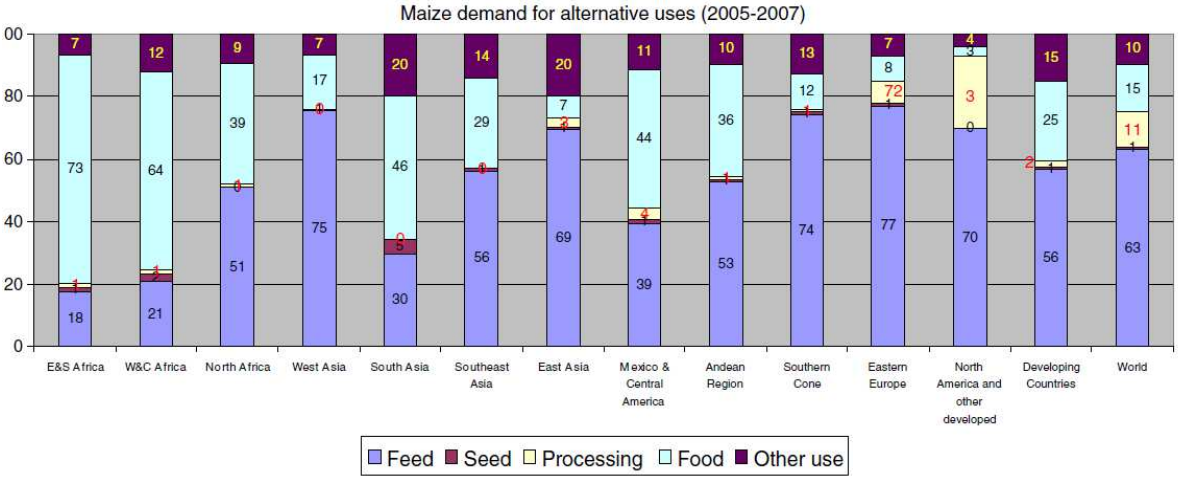

Figure 8: Maize differentiated demand in world regions. From Shiferaw *et al.*, 2011.

4. REGULATION OF ROOT DEVELOPMENT IN RESPONSE TO NITROGEN

The effects of nitrogen source on the root system are complex and depend on several factors, such as NO_3^- concentration in the soil, N endogenous status of the plant and the sensibility of the different species. Studies on root system architecture (RSA) and morphology are vital, considering that RSA determines the dramatic plasticity of plants to explore the soil for searching water and nutrients, including nitrate. Since maize is one of the most important crops studied to increase its NUE, an accurate description of the maize root system and how nitrogen sources regulate cereals root development is then provided.

4.1 Root branching in cereals: the maize root

The maize root system is complex and deserves an accurate description. Trying to simplify, the maize root development could be divided into two main groups (**Fig. 9**):

- Embryonic root systems: it derives directly from the seed to produce primary and seminal roots (PR and SR, respectively). PR elongates rapidly, forming many lateral roots (LR), even if usually it does not persist throughout maize's life (Feldman, 1994).
- Post-embryonic root system: also called the shoot-borne system, it derives from the shoot and originates crown roots (CR), if they are below the soil, and brace roots (BR), if they appear above the soil. Both CR and BR display many LR. Even if formerly LR are considered part of the post-embryonic system, they could represent the joining link between embryonic and post-embryonic system, being shared among PR, SR, CR and BR (Yu *et al*., 2015).

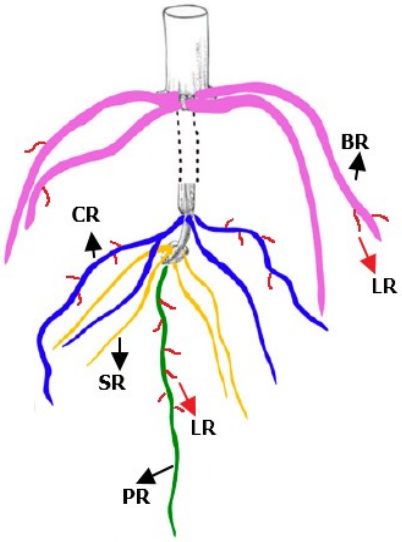

Figure 9. **Maize root system diagram**. Adapted from Yu *et al*., 2016.

Maize primary root (PR) and lateral roots (LR) are represented by a cylindric structure that ends at the distal extremity with the root apex (Alarcón *et al*., 2014) (**Fig. 10**). The root apex is the unlimited source of cells for the growing root and it consists of a meristem covered by a root cap. The root cap is the protective layer of cells that differentiate quickly, secreting a sort of mucilage and being rapidly discarded once they reach the root/soil surface. In addition, it is involved in gravity perception and other environmental stimuli (Sievers *et al*., 2002).

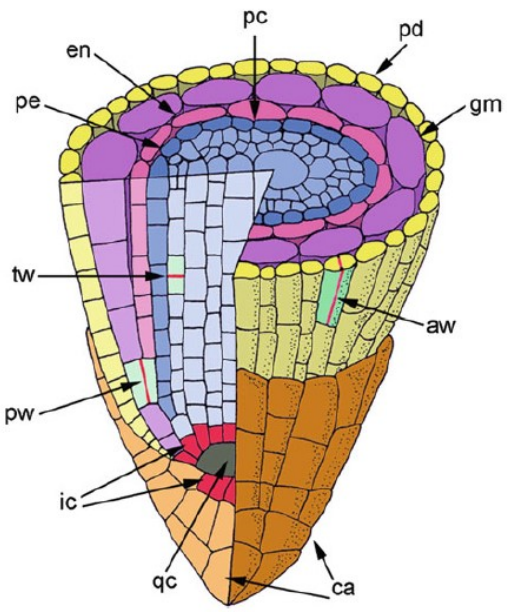

Figure 10. Schematic representation of maize root apex. From Alarcón *et al*., 2014. Abbreviations: *ca* root cap, *qc* quiescent centre, *ic* initial cells, *pw* periclinal wall, *aw* anticlinal wall, *tw* transverse wall, *pe* pericycle, *en* endodermis,*pc* procambium, *pr* protodermis,*gm* ground meristem.

Between the root cap and the meristem, the quiescent centre (QC) is located, namely a particular root region where cells divide infrequently but very important for the root organization and physiology. Accordingly, the cells surrounding above and below the QC are those initial cells with a high mitosis rate (Clowes, 1958): the cells below the QC divided to form the root cap, while the cells above the QC divided to form the rest of root tissues. In addition, a high rate of auxin release from the QC has been shown, together with a low ascorbic acid concentration, which in turn provides the characteristic to maintain quiescent the population of stem cells in the QC (Kerk *et al*., 2000). It is worthy of attention that *Arabidopsis* has 4 cells in the QC, while maize reaches 800 to 1200 cells (Hochholdinger *et al*., 2004).

Primary root (PR) is the first emerging root in both dicots and monocots (Smith and De Smet, 2012). The development of the PR includes 3 different processes: cell division, elongation, and differentiation (Alarcón *et al*., 2014). From the meristem, there are two types of cell division: formative, which follows anticlinal (perpendicular to root surface) or periclinal (parallel to root surface) plane, and proliferative divisions, which presents the equatorial plane transversely oriented with respect of the root's longitudinal axis.

Longitudinally, every plant root could be divided into 4 consecutive root zones (Baluška *et al*., 2010): the meristem (M, from the root tip to the above 2 mm), the transition zone (TZ, from

the M to the above 2 mm), the elongation zone (EZ, from the TZ to the above 4 mm) and the maturation zone (MZ, from the EZ to the above root until the seed). As before stated, the root apical meristem consists of undifferentiated meristematic cells in continuous cell division. Following the meristem, there is the elongation zone, namely a zone of rapid cell elongation, and the maturation zone, which shows cells undergoing differentiation and specialization to absorb water and mineral nutrients. In the MZ it is also possible to see root hairs (RH) and lateral root primordia (LRP). Between the M and the EZ, the transition zone is placed. The TZ is a unique zone responsible for the integration of various endogenous and exogenous signals, translating them into adaptative differential growth (Baluška and Mancuso, 2013; Trevisan *et al*., 2014). Accordingly, the TZ acts as a dynamic sensor, able to re-organize the root growth in response various stimuli, such as gravity (Masi *et al.*, 2015), touch and extracellular calcium (Ishikawa and Evans, 1992; Baluška *et al.*, 1996), osmotic stress (Baluška and Mancuso, 2013), oxidative stress and auxin (Mugnai *et al.*, 2014) and aluminium (Sivaguru *et al.*, 2013 ; Yang *et al.*, 2014; Kong et al., 2018). In addition, the TZ seems to represent the elected nitrate responsive zone in maize (Manoli *et al*., 2014; Trevisan *et al*., 2014). A further description of the nitrate sensing of the maize root TZ will be provided in the 4.3 *Paragraph*.

Transversely, the maize root presents epidermis, cortex and the vascular cylinder (Alarcón *et al*., 2014). The epidermis represents the uniseriate outermost layer of the root. On the contrary, the cortex comprises 6-10 layers of parenchymatous tissue, with the innermost called endodermis and the outermost called exodermis, both sharing highly specialized cells. In particular, endodermis cells present a characteristic U-shaped tertiary cell wall (Clarkson and Robards 1975). Finally, internally it is possible to recognize the vascular cylinder, with the first layer represented by the pericycle and the typical alternating organization of xylem and phloem poles. In maize, lateral roots originate from pericycle and endodermis cells located opposite the phloem poles, while in eudicots they are originated from pericycle cells opposite protoxylem poles (Hochholdinger *et al*., 2004; Casimiro *et al*., 2003, Jansen *et al*., 2012). If in *Arabidopsis* there are only two protoxylem poles, in maize there can be ten or more phloem poles, resulting in a highly radial branching root phenotype in maize (Smith and De Smet, 2012). Root branching and the proliferation of lateral roots increase the surface area of the root system, so enhancing the root capacity to detect and capture nitrate. Consequently, the control of LR development must be tightly regulated.

4.2 The control of lateral root development in response to nitrogen

Lateral roots (LR) create an extraordinary and extensive underground branching network (Atkinson *et al*., 2014), performing fundamental functions in soil exploration, water, and nutrient uptake and able to establish beneficial symbiosis. Generally, LR are more sensitive to variations in nitrogen source than PR (Tian *et al*., 2014; Hachiya and Sakakibara, 2017), but the control carried out by nitrate and ammonium remains complex and subjected to both genetic characteristic and environment (Sun *et al*., 2017; Forde, 2014; Yu *et al*., 2015; Xuan *et al*., 2017). Accordingly, in *Arabidopsis* nitrate and ammonium promote LR proliferation in a complementary way, with NH_4^+ increasing LR branching and hastening lateral root primordia (LRP) emergence, while NO_3^- promotes LR elongation (Remans *et al.*, 2006a, Lima *et al.*, 2010; Araya *et al*. 2016).

Therefore, LR development could be organized into four stages (Malamy and Benfey, 1997):

- LR initiation;
- LR primordia (LRP) formation;
- LR outgrowth and emergence;
- LR elongation.

In maize, LR initiation involves few pericycle cells at phloem poles, called founder cells, which are stimulated to de-differentiate and proliferate to produce the so-called LRP. This founder cell priming involves asymmetric cell division by cell cycle reactivation and auxin accumulation at the quiescent centre (Jung and McCouch, 2013). Indeed, LR initiation is positively regulated by auxin but negatively regulated by cytokinin (CK) and high concentrations of ethylene (ET). Regarding the regulation of LR initiation by nitrate in *Arabidopsis*, it has been shown that, under N-deficient conditions, NRT2.1 and NAR2.1 act as positive regulators of LR initiation (Remans *et al*., 2006b; Orsel *et al*., 2007).

LRP undergo nine different steps of anticlinal and periclinal cell division to emerge from the cortex (Malamy and Benfey, 1997). As for LR initiation, auxin is the most important signalling hormone also for LRP development together with cytokinin (CK), showing an antagonist effect with respect to auxin (Jung and McCouch, 2013). ANR1 (*ARABIDOPSIS* NITRATE-REGULATED1), a MADS-box transcription factor, and the NO_3^- "transceptor" NRT1.1/NPF6.3 were identified as key components governing LRP development in response to nitrate in *Arabidopsis* (Remans *et al*., 2006a). *ANR1* is specifically expressed in root, especially in LRP. Transgenic plants, with *ANR1* expression down-regulated or even suppressed, are less responsive to the localized NO_3^- signal.

Consequently, it has been suggested that *ANR1* acts downstream of the *NRT1.1/NPF6.3* in the signalling pathway stimulating LR development in response to nitrate (Remans *et al*., 2006a).

LR emergence and elongation are the final steps of LR development. Again, auxin is the master hormone, being both shoot-derived (Bhalerao *et al*., 2002) and LRP-derived (Swarup *et al*., 2008) to promote cell separation and upregulation of the cell wall-remodelling genes in the cell layers overlaying the LRP (Swarup *et al*., 2008). On the contrary, abscisic acid (ABA) acts as a negative regulator of LR outgrowth in Arabidopsis (Signora *et al*., 2001).

In cereals, such as maize, the LR genetic regulation in response to nitrate availability is complex (Bray and Topp, 2018 and reference therein). Indeed, only few lateral root mutants have been described in cereals, being generally related to auxin pathways (Hochholdinger and Tuberosa, 2009; Atkinson *et al*., 2014; Yu *et al*., 2018). For instance, the maize *rum1* encodes an Auxin/indole-3-acetic acid (Aux/IAA) protein called RUM1 (ROOTLESS WITH UNDETECTABLE MERISTEM 1) (von Behrens *et al*., 2011) that interacts with Auxin Response Factors (ARFs). Aux/IAA protein degradation leads to the release of ARFs which bind to the promoters of downstream auxin-responsive genes involved in lateral and seminal root formation (reviewed in Taylor-Teeples *et al*., 2016). *rul1* (*rum1-like1*) is the homolog of *rum1*, resulted from an ancient maize genome duplication (Zhang *et al*., 2016). Both RUM1 and RUL1 have the canonical four domain structure of Aux/IAA proteins and a nucleus localization, and they interact *in vivo* with ZmARF25 and ZmARF34 (Zhang *et al*., 2016), probably blocking LR formation in non-precursor pericycle cells (von Behrens *et al*., 2011). Moreover, RUM1 and RUL1 can form homo- and hetero-dimers *in vivo*, while *rul1* expression resulted independent from *rum1* expression. In addition, a direct binding of RUM1 to the *lrp1* (*lateral root primordia 1*) promoter has also been hypothesized (Zhang *et al*., 2015). In *Arabidopsis*, *AtLRP1* encoded a member of the SRS (Short Internodes-Related Sequence) family with a zinc-finger motif and it is involved in early lateral root formation (Smith and Fedoroff, 1995). In maize, the homolog *lrp1* is predicted to encode a transcription factor that acts as an auxin-inducible transcriptional activator. Genetically, *lrp1* expression is localized in root meristem and emerging lateral root primordia (LRP), and it is repressed by the binding with RUM1, thus suggesting an involvement of LRP1 in the maize auxin signal transduction downstream of *rum1* (Zhang *et al*., 2015). Furthermore, these data put in evidence the dual effect of RUM1 in regulating the initiation of lateral and seminal roots: ARF-mediated or LRP1-direct action as a transcriptional repressor of auxin-responsive genes. RUM1 can specifically interact also with RAP1 (RUM1 ASSOCIATED PROTEIN 1) (Zhang *et al*., 2016), while no interaction was observed

between RUL1 and RAP1. RAP1 is a homolog protein of AtSPR1, similar to a nitrilase-associated microtubule-localized protein in *Arabidopsis* (Nakajima *et a*l., 2004). The RAP1 family includes 6 other members called RAP1-like: RAL1, RAL2, RAL3, RAL4, RAL5, RAL6 (Zhang *et al*., 2016). Besides LR development, the control of shoot-borne roots is as well important in maize (Taramino *et al*., 2007). Accordingly, the LOB-domain paralogous maize proteins RTCS (ROOTLESS CONCERNING CROWN AND SEMINAL ROOTS) and RTCL (RTCS-Like) are involved in the crown root development in maize (Xu *et al*., 2015a). The LBD (LATERAL ORGAN BOUNDARIES Domain) protein family functions in defining organ boundaries and is involved in a variety of plant developmental processes (Majer and Hochholdinger, 2011). Maize LBD-dependent signalling in root development included *RTCS* and *RTCL,* which both share auxin-responsive elements and are preferentially expressed in roots. Auxin induces *ZmARF34* expression whose protein binds to the promoter of both *rtcs* and *rtcls*, thus activating their expression. Consequently, RTCL and RTCS bind to LBD downstream promoters by acting as transcription factors (Xu *et al*., 2015a). In maize, RTCS is the closest homolog of AtLBD29, and *rtcs* acts upstream of *rtcl*. Despite AtLBD19, AtLBD16 and AtLBD29 have redundant functions in LR emergence in *Arabidopsis* (Okushima *et al*., 2007), the maize *rtcs/rtcl* double mutants showed no reduction in LR density. Accordingly, LBD proteins in maize seem involved only in shoot-born root formation, while their closest homologs in *Arabidopsis* regulate LR initiation (Xu *et al*., 2015a).

As for *Arabidopsis*, the establishment of auxin response maxima is crucial in lateral root initiation in maize (Atkinson *et al*., 2014). Auxin transport is fundamental for the generation of this local auxin maxima and PIN transporters determine the polar auxin transport (PAT). It has been shown that where LR are developed, in phloem pole cells the monocot-specific *PIN9* can modulate auxin efflux to pericycle cells, activating the subsequent cell cycle (Yu *et al*., 2015). Furthermore, the inhibition of root growth by high NO_3^- supply was correlated with reduced auxin concentration in the roots (Tian *et al*., 2008), and it was found that the nitrate “transceptor” NRT1.1/NPF6.3 can transport both auxin and nitrate (Krouk *et al*., 2010b). Accordingly, NRT1.1/NPF6.3 seems to be involved in the repression of LR emergence at low NO_3^- concentrations by promoting auxin lateral basipetal transport out of the LR. Contrariwise, high NO_3^- levels seem to inhibit NRT1.1/NPF6.3-dependent basipetal auxin transport by inducing auxin accumulation in the LR tip and so stimulating their growth.

Moreover, other components were proposed in the regulation of LR initiation and LR outgrowth, namely miR167 and its target AUXIN RESPONSE FACTOR 8 (ARF8), and miR393 and the

auxin receptor AFB3 (AUXIN SIGNALING F-BOX PROTEIN 3) (Gifford *et al*., 2008). These miR/ARF couples were also studied for their modulation of both LR and PR growth in response to NO_3^- (Vidal *et al*., 2010) in *Arabidopsis*. AFB3 belongs to a group of F-box receptors for auxin and was found to be the unique auxin receptor transcriptionally induced by NO_3^-, suggesting that besides modulating auxin gradients in roots through the NRT1.1/NPF6.3 activity (Krouk *et al*., 2010b), NO_3^- also increases root auxin sensitivity by affecting *AFB3* expression (Bouguyon *et al*., 2015). Thus, according to these authors, stimulation of *AFB3* by NO_3^- appears to be only transient since the *AFB3* transcript is rapidly targeted by miR393 for degradation as soon as nitrate enters the assimilation pathway, showing an interesting nitrate-responsive mechanism in controlling root development in *Arabidopsis*.

There are also evidences that abscisic acid (ABA) could play a central role in mediating the regulatory effects of high NO_3^- levels on root branching in *A*. *thaliana* (Signora *et al*., 2001), as well as brassinosteroids and cytokinins (CK) (Kiba *et al*., 2011). Finally, a novel role for nitric oxide (NO) in regulating plant root growth is emerging in the last few years, with particular involvement of the transition zone in the root (Trevisan et al., 2011; Manoli et al., 2014; Trevisan *et al*., 2015).

4.3 Nitrate sensing in the root transition zone (TZ)

Recent studies performed in maize provided a new hypothesis about the participation of nitric oxide (NO) in root response to NO_3^- (Trevisan et al., 2011; Manoli et al., 2014; Trevisan *et al*., 2015). NO is a bioactive molecule considered as a general plant signal involved in many physiological and developmental processes in plants, regulating both biotic/abiotic stress responses and hormonal crosstalk (Domingos *et al*., 2015). NO has been reported to be required for primary root development (Fernández-Marcos *et al*., 2012; Manoli *et al.*, 2014), adventitious roots formation (Pagnussat *et al.*, 2003), lateral root development (Wang *et al*., 2010), and root hair formation (Lombardo and Lamattina, 2012). Moreover, it has been suggested the coordinated action of auxin and NO in the process of LR formation (Correa-Aragunde *et al.,* 2004; Guo *et al*., 2008) and in the regulation of stem-cell niche (Sanz *et al*., 2014).

Concerning the primary root development in maize and its early response to nitrate, NO appeared to be a key player in nitrate signaling (Trevisan *et al*., 2011). According to the authors, a nitrate reductase (NR) and a nonsymbiotic hemoglobin (nsHb) showed a coordinated spatio-temporal regulation of their expression in root epidermal cells after nitrate provision. NR and nsHb are involved in nitric oxide (NO) synthesis and scavenging. When external nitrate rapidly increases

high levels of nitrite are produced, so NR reduces nitrite to NO while nsHb activates detoxification of high intracellular NO concentrations, playing thus a protective role against NR-derived NO. The coordinated activity of NR and nsHBs to control NO after nitrate provision was deepened by Manoli *et al*. (2014), which demonstrated the *in vivo* NR-dependent NO production after nitrate supply. Interestingly, NO production showed to be preferably localized in the root transition zone (TZ), which appears to be the most nitrate responsive zone of maize root. Basing on these results NO homeostasis plays a key role in the complex signaling network involved in the root elongation stimulation after nitrate provision.

Afterward, the NO boost produced after the nitrate supply was shown to induce an auxin and PIN1 re-localization in the TZ cells, thus affecting cell division in favor of cell expansion and guiding the root apex elongation (Manoli *et al*., 2016). In addition to NO ad auxin, a contemporaneous study also hypothesized the involvement of the phytohormone strigolactones (SLs) in the scenario of the maize root response to nitrate (Trevisan *et al*., 2015). Given that the interplay of NO and auxin is important to control multiple aspects of root biology (Sanz *et al*., 2015) and considering the interplay between SLs and NO (Kolbert, 2019), the role of SLs in the pathway where NO act as coordinator of nitrate and auxin signalling to control the overall maize root response was then deepened studied.

5. STRIGOLACTONES

Strigolactones are a new class of phytohormones, firstly discovered in the 1960s as germination stimulators of the parasitic witchweed *Striga lutea* (Cook *et al*., 1966), but classified as plant hormones only in 2008 (Umehara *et al*., 2008). From 1966 until now, SLs have revealed to be positive inducers of Arbuscular Mycorrhizal Fungi (AMF) (Akiyama *et al*., 2005), inhibitors of shoot branching (Gomez-Roldan *et al*., 2008), regulators of root development (Koltai, 2011), inducers of longer internodes (de Saint Germain *et al*., 2013) and thicker stem (Agusti *et al*., 2011) and mediators of plant response to abiotic stresses such as nutrient deficiency (Mostofa *et al*., 2018). Moreover, SLs are involved in controlling the flowering time (Snowden *et al*., 2005), leaf shape and senescence (Lauressergues *et al*., 2015) and tuberization (Pasare *et al*., 2013), thus affecting almost every process in plant growth and acting as a fundamental signal in the rhizosphere too (**Fig. 11**).

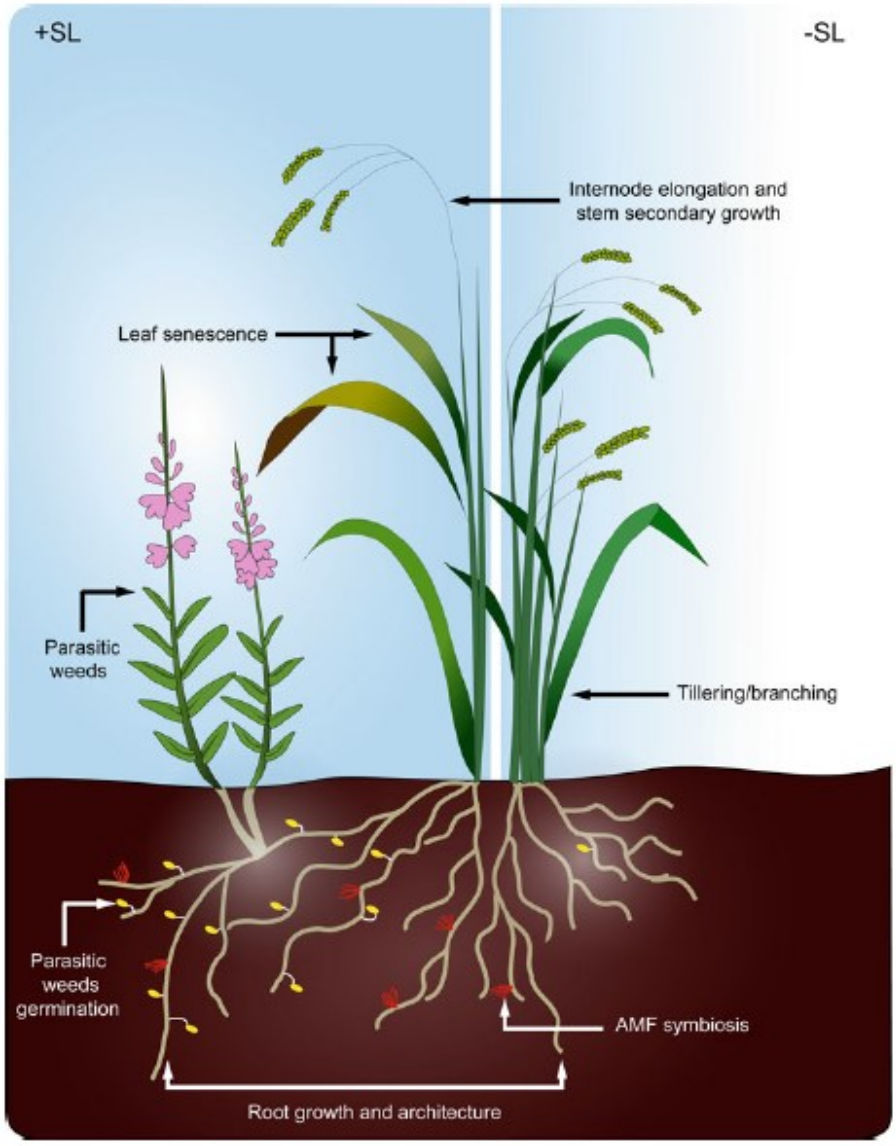

Figure 11. Graphical representations of some SL biological functions. From Jia *et al*., 2019.

5.1 Strigolactones chemistry and evolution

As stated before, the story of strigolactones started more than fifty years ago with the work of Cook and colleagues (1966), which found an unknown compound from cotton root exudates able to induce the germination of the redoubtable parasitic plant *Striga*. This genus and other root parasitic plants in the Orobanchaceae family have a strict germination dependency on particular rhizosphere chemicals released by a suitable and close host, and SLs are the major inducers (Bouwmeester *et al*., 2003). Cook had discovered the strigol, namely the first SL whose structure was elucidated (Cook *et al*., 1972) and twenty years later orobanchol also was isolated (Yokota *et al*., 1998), the latter being too able to induce the germination of *Orobanche* species.

Accordingly, these two firstly identified SLs became the references molecular structure that allow scientists to group what are now called canonical SLs in Strigol-type and Orobanchol-type (**Fig. 12**).

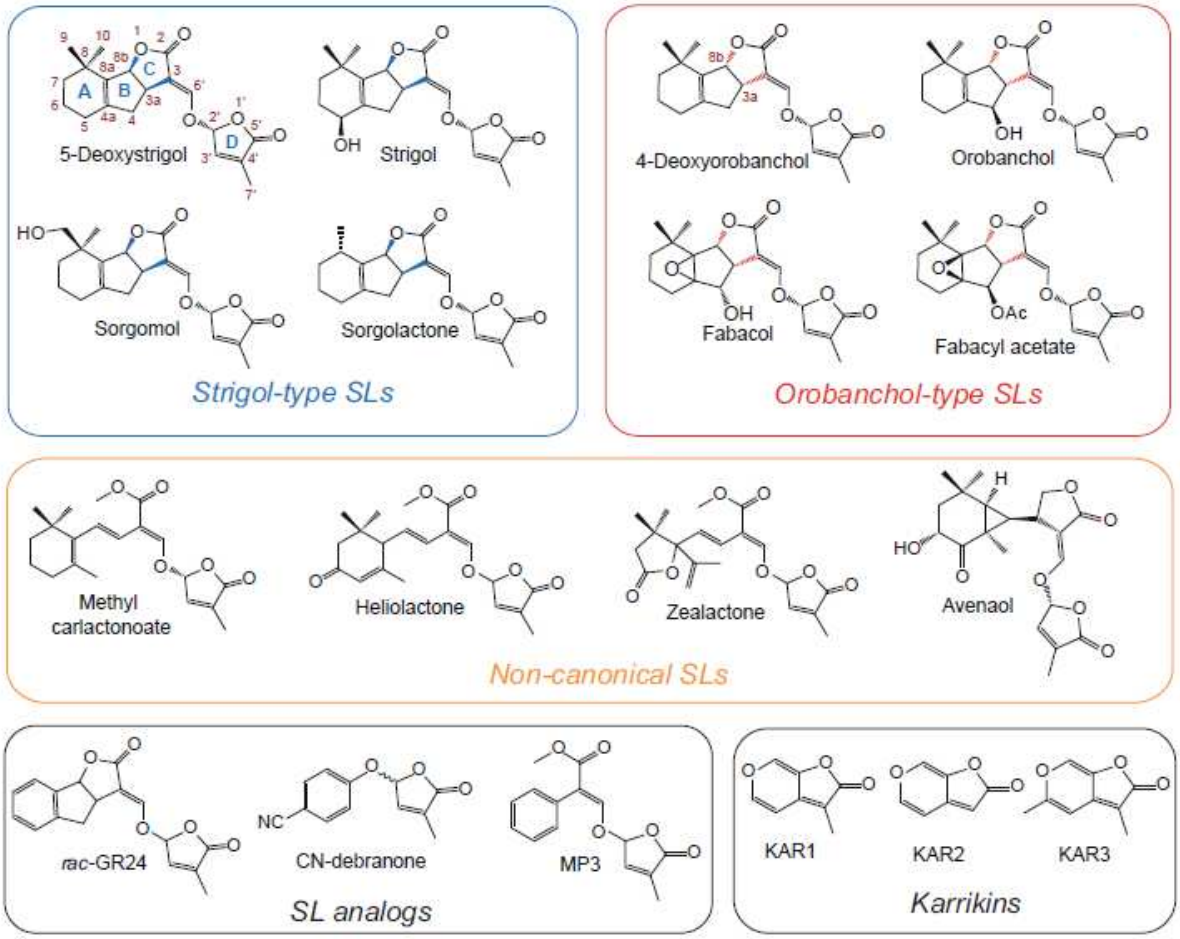

Figure 12. Structures and classification of SLs and analogues. From Jia *et al*., 2019.

Nowadays, around 25 natural SLs have been characterized and they all are carotenoid derivatives with a conserved butanolide D-ring (Xie, 2016). The D-ring possesses the biological property, has always an *R*-configuration and is connected to a variable moiety that allows distinguishing between canonical and non-canonical SLs. Indeed, canonical strigolactones present a tricycle lactone, called ABC-ring, linked to the D-ring. In addition, if the BC-ring junction has an α-orientation in the C-ring SLs are called orobanchol-type, while if there is a β-orientation they are classified as strigol-type. Instead of the tricycle lactone, non-canonical SLs are characterized by a variable second moiety connected to the D-ring (Jia *et al*., 2018). Some examples are zealactones (Charnikhova *et al*. 2017; Xie *et al*. 2017), methyl carlactonoate (Abe *et al*. 2014), avenaol (Kim et al., 2014) and heliolactone (Ueno *et al*. 2014). Plants release in the rhizosphere very tiny amounts of SLs (picomolar range), which are rapidly decomposed in the soil. Indeed, natural SL isolation and synthesis is challenging, thus the employment of synthetic analogues is widely used, such as

the universal standard used GR24 (Zwanenburg *et al*., 2016). In addition to SLs, other molecules that own the D-ring are karrikins (KAR). They are simpler molecules firstly identified from the smoke of burning plant material (Nelson *et al*., 2009) and they share some transduction components with SLs, but they do not induce parasitic plant germination (Waters *et al*., 2013).

From an evolutionary point of view, SLs and KAR are produced by all land plants, while the SL production in green algae remains unclear (Waters *et al*., 2017). In moss, SLs regulate radial expansion and branching (Proust *et al*., 2011), while Angiosperms probably firstly evolved the ability to exudate SLs to recruit AM fungi, thus enhancing the plant ability to adsorb nutrient from the soil (Gutjahr, 2014). At the same time, root parasitic plants in the Orobanchaceae family co-evolved with the host, using the SLs exudate as a consistent indicator for the host presence and thus allowing the seed germination close enough to it (Bonhomme and Waters, 2019).

5.2 Strigolactones biosynthesis

Many branching mutants allowed to outline the SL biosynthetic pathway, starting from 2005 when it was firstly hypothesized that SLs could derived from carotenoids (Matusova *et al*., 2005) and later in 2008 when it was confirmed that they actually are apocarotenoids (Umehara *et al*., 2008; Gomeze-Roldan *et al*., 2008). Accordingly, SLs are produced from carotenoids by consequently oxidative cleavages of specific double bonds operated by the non-heme iron enzymes CAROTENOID CLEAVE DIOXYGENASES (CCD7 and CCD8). Other enzymes involved in SL biosynthesis are a carotenoid isomerase called D27 (Lin *et al*., 2009) and a cytochrome P450 called MAX1 (Crawford *et al*., 2010), acting before and after CCDs respectively. Depending on the species where those SL biosynthetic enzymes were studied, a different nomenclature could be found:

- MAX for *Arabidopsis thaliana* (from *more axillary growth* mutants);
- D or HTD for *Oryza sativa* (from *dwarf* or *high-tillering* dwarf mutants);
- RMS for *Pisum sativum* (from *ramosus* mutants);
- DAD for *Petunia hybrida* (from *decreased apical dominance* mutants).

A simple table is then provided to simply find the correct function for every gene name (**Table 1**).

Protein	Arabidopsis	Rice	Pea	Petunia
9-cis/all-trans-P-carotene isomerase	*AtD27*	*D27*	-	-
Carotenoid cleavage dioxygenase7 (CCD7)	*MAX3*	*D17/HTD1*	*RMS5*	*DAD3*
Carotenoid cleavage dioxygenase8 (CCD8)	*MAX4*	*D10*	*RMS1*	*DAD1*
Cytochrome P450, cytochrome711 (CYP711)	*MAX1*	*OsMAX1*	-	*PhMAX1*

Table 1. Proteins and genes of the main plant species studied for SL biosynthesis. Modified from Pandey *et al.*, 2016.

According to the biosynthesis enzymes, the SL biosynthetic pathway could be divided into different steps, occurring mainly in the root tip, but also in shoot and root vasculature (**Fig.13**):

1. Inside the plastid, all-*trans*-β-carotene (C_{40}) is reversibly isomerized into 9-*cis*-β-carotene by the activity of the 9-*cis*/all-*trans*-β-carotene isomerase **D27**;
2. 9-*cis*-β-carotene is then cleaved at the C9'–C10' double bond into 9-*cis*-β-apo-10'-carotenal (C_{27}) and β-ionone (C_{13}) by the stereospecific carotenoid cleavage dioxygenase **CCD7**;
3. 9-*cis*-β-apo-10'-carotenal is then cleaved into carlactone (C_{19}) and the C_8 compound ω-OH-(4-CH_3) heptanal by the carotenoid cleavage dioxygenase **CCD8;**
4. Carlactone (CL) represents the common precursor of all SLs, its exported in the cytosol and then converted into carlactonoic acid (CLA) or canonical SLs by a cytochrome P450, such as **MAX1** in *Arabidopsis* and carlactone oxidase (**CO**) or orobanchol synthase (**OS**) in rice.
5. In *Arabidopsis*, carlactonoic acid (CLA) is methylated by a yet unidentified methyltrasferase into methyl CLA (MeCLA), which in turn is modified by a lateral branching oxidoreductase (**LBO**) in a yet unidentified SL-like compound that regulated shoot branching.

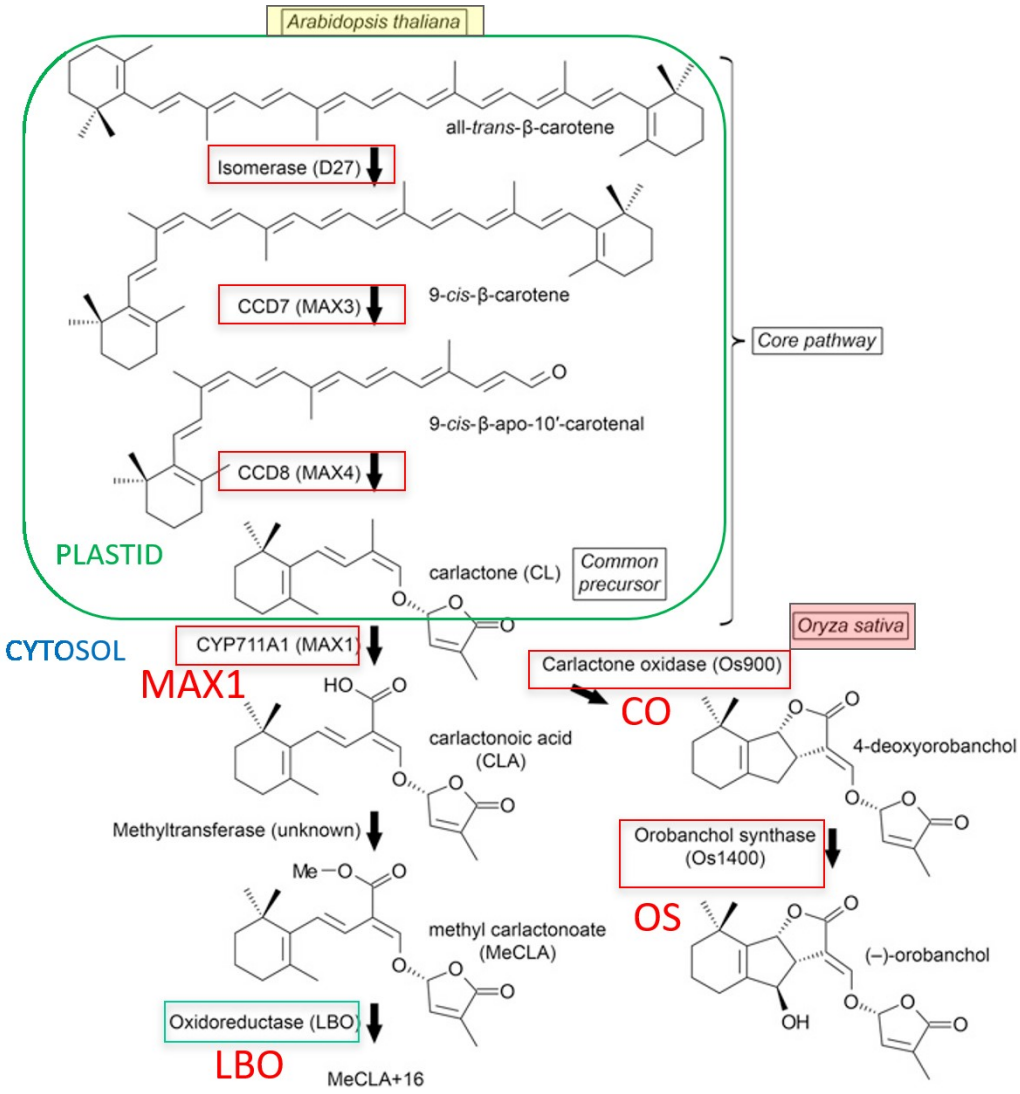

Figure 13. SL biosynthesis pathway. Modified from Brewer *et al*., 2016.

Since SLs take part in a wide number of processes involved in plant development and interaction with the environment, the control of SL production must be tightly regulated (Al-Babili and Bouwmeester, 2015). Accordingly, SL biosynthesis depends on the plant's nutrient status and soil nutrient availability, being induced with nutrient-deficiency conditions. Many evidences support the hypothesis that phosphate scarcity triggers SL production (Kohlen *et al*., 2011; Jamil *et al*., 2013; Umehar *et al*., 2010), while fewer studies are available in relation to the effects of nitrogen-deficiency (Yoneyama *et al*., 2007; Yoneyama *et al*., 2013; Sun *et al*., 2014). Moreover, SL exudation is also induced upon nutrient deficiency, especially in the case of phosphate, probably due to the need of attracting AMF thus improving phosphate uptake (Gutjahr, 2014). The reverse effect of this stronger SL exudation is the germination of root parasitic plants, if presents near the host, thus causing serious damage to a plant already suffering for nutrient lack (Brun *et al*., 2018).

Both increased SL biosynthesis and exudation are possible because of increased transcript levels of SL biosynthesis genes, such as *MAX3*-*MAX4* in Arabidopsis (Ito *et al*., 2016) and *D27*-*D10* in rice (Umehara *et al*., 2015). Contrariwise, DAD1 is downregulated in response to restored phosphate conditions in petunia (Kretzschmar *et al*., 2012).

Like any other hormones, also for SLs it is necessary to control its homeostasis through a negative feedback mechanism. For instance, the application of the synthetic SL GR24 to Arabidopsis led to a reduction of *MAX3* and *MAX4* transcript levels (Mashiguchi *et al.,* 2009), while SL biosynthesis mutants showed increased transcript levels for all biosynthesis genes, as shown for *Arabidopsis MAX3* and *MAX4,* rice *D10,* and petunia *DAD1* (Jia *et al*., 2019).

Besides this direct inhibition via negative feedback regulation, SL biosynthesis is also regulated by auxin and other phytohormones. Auxin acts as a positive regulator of SL biosynthesis genes, for instance, it increased *MAX4*/*D10* expression (Arite *et al.*, 2007). On the other side, if auxin is exogenously removed, for instance through decapitation or using auxin polar transport (PAT) inhibitor (i.e. NPA), fewer transcripts are available for *MAX3*/*RMS5* and *RMS1* in the stem (Hayward *et al*., 2009). Therefore, SL biosynthesis would presumably seem under the control of a long-distance feedback loop mediated by PAT, since higher SL levels inhibit SL biosynthesis by decreasing the auxin conductivity.

Abscisic acid (ABA) and gibberellins (GA) are other phytohormones involved in the regulation of SL biosynthesis. ABA is involved in the response to abiotic stresses, such as SL (Haider *et al*., 2018), and affects SL production. Accordingly, ABA biosynthesis mutants exhibit reduced SL levels in tomato and maize (López-Ráez *et al.*, 2008, 2010), suggesting a positive role of ABA in regulating SL biosynthesis. On the contrary, GA seems to have a negative role in SL biosynthesis. Indeed, it was recently reported that treatments with synthetic GA reduced the transcript levels of *D27*, *D10*, *D17*, *Os900*, and *Os1400*, resulting in decreased SL exudation in rice (Ito *et al*., 2017).

5.3 Strigolactones perception and signal transduction

In plants, SL actions are possible because their signals can be perceived and properly transmitted downstream to activate SL-related genes. Accordingly, many genes are involved in both SL perception and signal transduction (**Table 2**).

Protein	Arabidopsis	Rice	Pea	Petunia
α/β-Hydrolase	*AtD14*	*D14/D88/HTD2*	*RMS3*	*DAD2*
F-box component of the SCF complex	*MAX2*	*D3*	*RMS4*	*PhMAX2a, PhMAX2b*
Repressor of SL signalling	*SMXL6, SMXL7, SMXL8*	*D53*	-	-
Corepressor	*TPR2*	*TPL/TPR2*	-	-
Transcriptional factors	*BRC1, BRC2*	*IPA1, FC1/OsTB1*	*PsBRC1*	-

Table 2. Proteins and genes of the main plant species studied for SL signalling. Abbreviations: SCF, Skp1–Cullin–F-box; *SMXL*, *SUPPRESSOR OF MAX2 1-LIKE*; *TPL/TPR*, topless/topless-related protein; *BRC*, branching; *IPA*, ideal plant architecture. Modified from Waters *et al*., 2017.

The SL receptor is an α/β-hydrolase called D14 (DWARF14), which binds and catalyses the hydrolysis of SL thanks to the conserved Ser/His/Asp catalytic triad in the receptor active site pocket (Hamiaux *et al*., 2012, Yao *et al*., 2016). As a result, the 5-hydroxy-3-methylbutenolide (D-ring) and a tricyclic lactone (ABC-ring) are formed. Nevertheless, the production of these two products is not the final purpose for SL signalling, since they are biologically inactive. Instead, the conformational change occurring in the receptor D14 after hydrolysis is the critical step that allows the downstream signal transduction, as well as the formation of a covalently linked intermediate molecule (CLIM) with the D-ring linked to D14 (Yao *et al*., 2016). This process is accompanied by a simultaneous open-to-closed state conformational change in D14 that facilitates its interaction with the F-box protein called D3 or MAX2 (**Fig. 14**). MAX2/D3 is the F-BOX part of the SCF complex (SKP1, CULLIN and F-BOX), namely an E3 ligase that attaches multiple units of ubiquitin to its substrate, making it suitable for degradation via 26S proteasome (Shu and Yang, 2017). In SL signalling, the couple D14-D3 can interact with the repressor protein SMXL6-7-8/D53 and its corepressor TPL/TPR2 (TOPLESS/TOPLESS RELATED PROTEIN 2) to form a complex that will be ubiquitinated and degraded. This degradation will, in turn, switch off SL signalling cascades and activate the SL-related genes expression (Wang *et al*., 2015). Accordingly, when there are no SLs perceived, SMXL6-7-8/D53 and TPL/TPR act as transcriptional repressors of downstream SL target genes. For instance, in rice *IPA1* (*Ideal Plant Architecture 1*), encoding a SPL (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE) transcription factor, is involved in a loop feedback regulation with *D53* (Song *et al*., 2017). Another class of transcription factors (TF) acting downstream of *SMXL6-7-8/D53* and involved in shoot branching is represented by the TCP family (TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1), with examples in rice (*FC1/OsTB1*),

maize (*TB1*), Arabidopsis (*BRC1*, *BRC2*) and pea (*PsBRC1*) (Takeda *et al*., 2003; Guan *et al*., 2012; Aguilar-Martinez *et al*., 2007; Braun *et al*., 2012). Nevertheless, rice *FC1/OsTB1* and *TB1* expressions are not induced by SLs, so it is possible that SL signalling and TB1 expression are uncoupled in cereals (Guan *et al*., 2012). Indeed, more studied are needed to better characterize the downstream effect of SL.

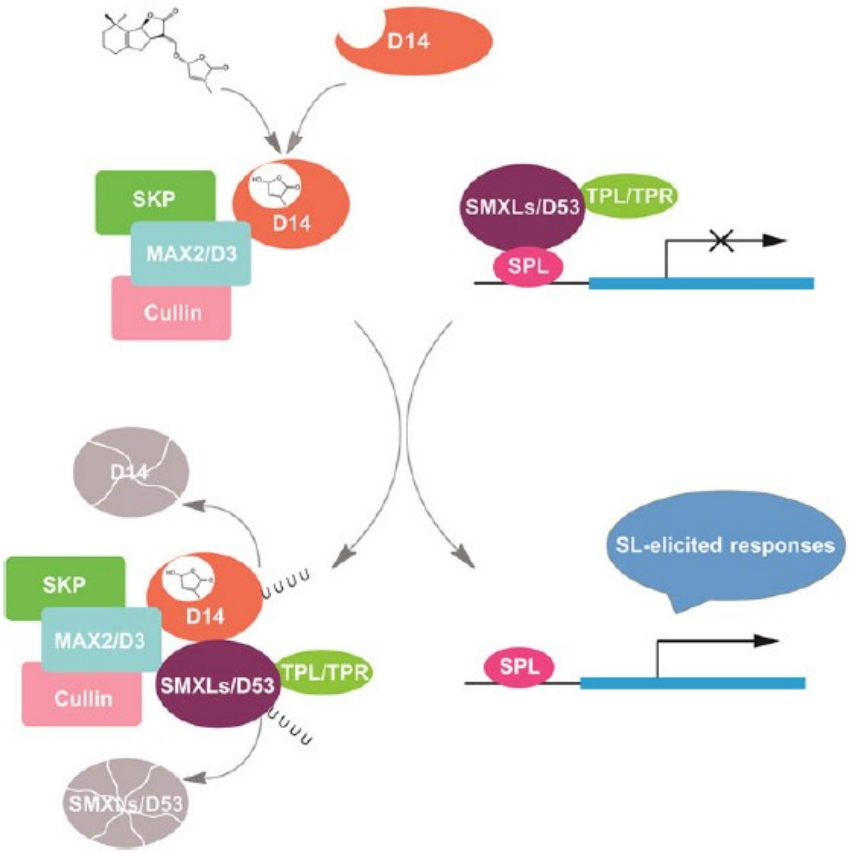

Figure 14. SL signal transduction. From Jia *et al*., 2019.

5.4 Strigolactones transport and exudation

As already stated, SL biosynthesis genes are mainly expressed in the root tip, but also in root vasculature and shoot (Abuauf *et al*., 2018; Booker *et al*., 2004; Sorefan *et al*., 2003). Accordingly, SL biosynthesis occurs in different tissues and SLs themselves or their precursors are transported from root to shoots. Even if this SL transport was confirmed by grafting experiments with SL-deficient mutants (Kohlen *et al*., 2011), only a poor knowledge of how and where this SL transport takes place has been achieved. For instance, orobanchol and strigol were detected in the xylem sap in tomato and Arabidopsis (Kohlen *et al*., 2011), but it was not possible to replicate the detection in a later study in rice (Xie *et al*., 2015b), thus how the root-to-shoot transport actually occurs is still without an answer. A hypothetic answer is that SLs could be transported to

shoot as some yet unidentified precursor that was not possible to detect in the xylem. Furthermore, SL transporters have been only partially identified and characterized. However, the first SL exporter was characterized by Martinoia and colleagues in 2012 from petunia (PDR1, PLEIOTROPIC DRUG RESISTANCE 1) (Kretzschmar *et al*., 2012). It showed a cell-to-cell transport for SL at root level and it seems to be involved in the SL exudation in the rhizosphere. According to these authors, PDR1 is an ABCG transporter (ATP-binding cassette type G transporter) asymmetrically localized in specialized root cortex cells called Hypodermal Passage cells (HPCs) and in root tip cells (Sasse *et al*., 2015). HPCs are non-suberized cells located in the root hypodermis (the outer cortex layer) that have been studied for their involvement in being the entry point for AMF colonization (Sharda and Koide, 2008). The particular outer-lateral localization of PDR1 in HPCs supports the idea that PDR1 might be involved in the SL exudation to the rhizosphere, while the apical-localization in root tips suggests that PDR1 could be also involved in the SL unload from biosynthetic tissues and eventually transported through the shoot (Borghi *et al*., 2016). In addition, a very recent study showed that PDR1 appeared also to be involved in the loading of SL into dormant buds in the main stem (Shiratake *et al*., 2019) (**Fig. 15**).

The identification of new putative SL transporters candidates induced by low nutrient conditions and/or exogenous GR24 provision and located in the plasma membrane of root cells is necessary to support the results obtained with PDR1 (Borghi *et al*., 2016). For instance, PDR1 close homologues, as PDR6 from *Nicotiana tabacum,* were identified (Xie *et al*., 2015a). Contrariwise, no SL transporters have been isolated yet from Monocots or even from *Arabidopsis*. Indeed, in Arabidopsis the closest PDR1 homolog has been found to be an ABA transporter not involved in SL transport (AtABCG40), demonstrating that not always the sequence information correctly correlates with the transporter's substrate (Borghi *et al*., 2016).

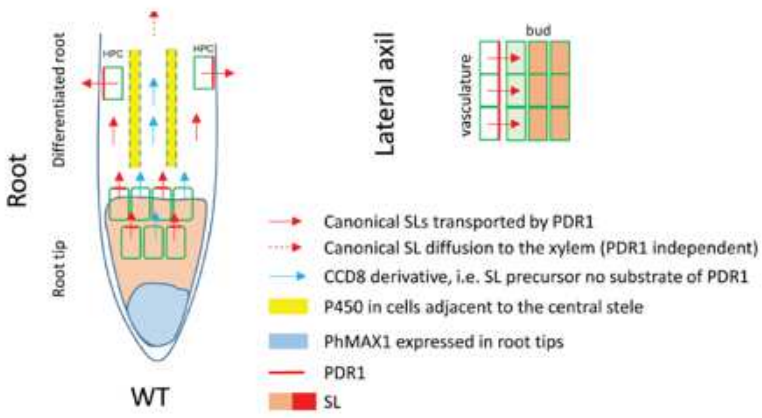

Figure 15. SL transport. Transport model of canonical SLs (red arrows), and of hypothesized SL precursors/SLs that are not substrates to PDR1 (blue arrows) in petunia roots and shoots. (A) SL transport in WT. (B) SL transport in pdr1 ko mutants. (C) SL transport in dad1 mutants. (D) SL transport in PDR1 OE plants. From Shiratake *et al*., 2019.

5.5 Strigolactones roles as hormones in plant development

As phytohormones, SLs are involved in controlling many developmental traits in plant, including shoot branching (Gomez-Roldan *et al*., 2008), root development (Koltai, 2011), internode growth (de Saint Germain *et al*., 2013), stem secondary growth (Agusti *et al*., 2011), flowering time (Snowden *et al*., 2005), leaf shape and senescence (Lauressergues *et al*., 2015) and tuberization (Pasare *et al*., 2013). In the next paragraphs, the attention will be focused on the two main plant organs: root and shoot.

5.5.1 Strigolactones in the regulation of shoot branching

The first function of SLs identified in 2008 and that made them part of the phytohormones was their suppression of branch outgrowth at shoot level (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Accordingly, shoot branching is the term that refers to the emergence of axillary buds at the axils of a leaf. Generally, these buds remain dormant since auxin accumulates in the shoot tip determining the well-known apical dominance (Kebrom, 2017). As previously said, auxin has a positive effect on SL biosynthesis, while it negatively regulates cytokinin levels (CK), thus SLs and CK could be considered as second messengers through which auxin acts (Rameau *et al*., 2019). Regarding SL interaction with auxin (IAA) to regulate the inhibition of lateral bud growth, two models of action, not mutually exclusive, were proposed:

- The direct-action model (**Fig. 16A**): SLs directly acts as a second messengers of auxin, by entering the dormant bud and inducing the *BRC1* (*BRANCHED1*) expression, namely a transcriptional regulator of bud activation potential that is also repressed by CK (Seale *et al*., 2017; Dun *et al*., 2012);
- The canalization model (**Fig. 16B**): SLs impede auxin canalization out of the dormant bud by the inhibition of PIN1 activity, thus maintaining the bud in the dormancy state (Shinohara *et al*., 2013).

In both models, the nutrient availability in the soil deeply influences the production of SLs and CK in the root, thus regulating shoot and root coordinated growth.

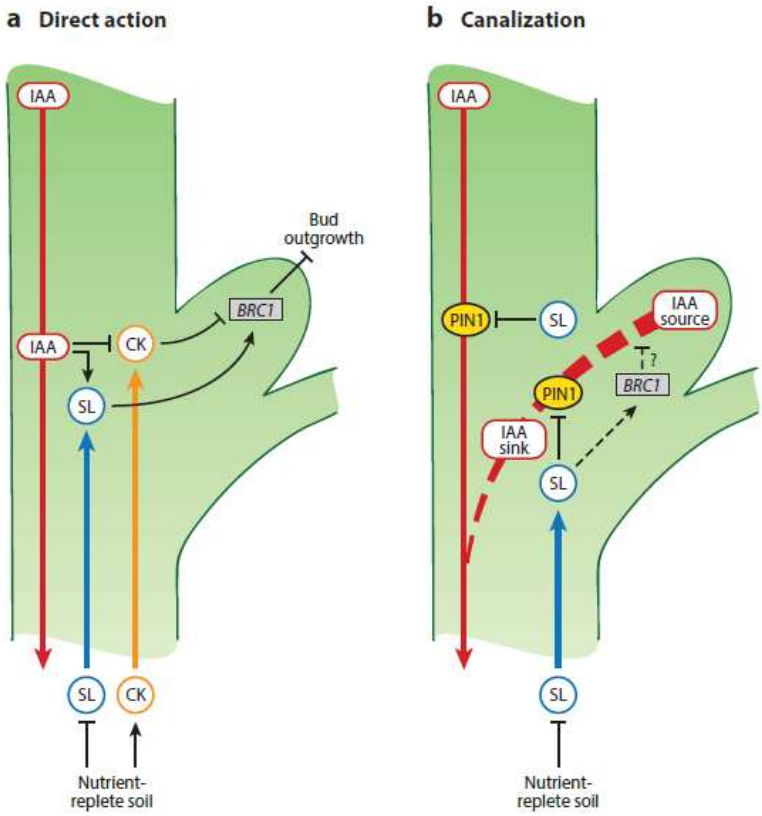

Figure 16. Two models for the regulation of shoot branching by SL interplay with auxin. From Waters *et al*., 2017.

5.5.2 Strigolactones in the regulation of root architecture

SLs are involved in the development of every component of the root architecture system (RSA), including functions in primary root (PR), lateral roots (LR), adventitious roots (AR) and root hair (RH) development (**Fig. 17**).

Regarding the involvement in PR development, the SL effect is dependent on the growth conditions and the plant species (Matthys *et al*., 2016). Studies in Arabidopsis showed that *rac*-GR24 treatments increase PR length, probably acting in the meristem (M) and transition zone (TZ) by increasing the number of cells in this region (Ruyter-Spira *et al*., 2011). Accordingly, it was hypothesized that SLs act as negative regulators of the polar auxin transport (PAT) (Koltai *et al*., 2010), for instance by depleting the auxin-efflux carrier PIN1 from the membrane of xylem parenchyma cells, thus affecting auxin levels in both M and TZ and involving LR development too. Nevertheless, this inhibitory effect of SLs on PIN1 is much slower than the one observed in the shoot and it is probably specifically located in the endodermis cells of TZ, thus challenging to detect (Shinohara *et al*., 2013). However, the positive effect of SL on the PR elongation is not general for all species. For instance, in rice this effect is strongly influenced by the nutrient status of the medium where the plant grows (Sun *et al*., 2014), while in tomato and Medicago this effect

was absent (De Cuyper *et al*., 2015), and in *Lotus japonicus* is was even opposite, showing a phenotype displaying a longer PR in the mutant with *CCD7* silenced (Liu *et al*., 2013).

On the other hand, the effect of SLs is clearly visible on lateral root (LR) development in many species, including Arabidopsis and rice. In both of them, a lower number of lateral root primordia (LRP) was observed in plants treated with *rac*-GR24 (Ruyter-Spira *et al*., 2011; Sun *et al.,* 2014), thus underlying the negative regulation of LR development by SLs. In addition, Jiang *et al*. (2016) showed that in Arabidopsis this GR24 effect on LR development is dual, since it results in both a minor effect on LR priming and a major effect on LR outgrowth, especially in the upper part of the root, while no effects were shown to affect LR initiation. It is noteworthy that the LR emergence inhibition by GR24 near the root-shoot junction spatially coincides with the downregulation of *PIN1*. Furthermore, this study revealed an involvement of the cytokinin (CK) signaling with the AHK3/ARR1/ARR12 module in the SL-mediated inhibition of LR development, while the reduction in auxin reflux could be the other mechanism involved as showed for PR growth (Cheng *et al*., 2013). Finally, it has to be highlighted that all these experiments were carried out with *rac*-GR24, namely the equimolar mixture of two enantiomers $GR24^{5DS}$ and $GR24^{ent\text{-}5DS}$, where only $GR24^{5DS}$ is perceived as a natural SL, while $GR24^{ent\text{-}5DS}$ activates the karrikins (KAR) signalling pathway through a yet unknown ligand (Scaffidi *et al*., 2014). Indeed, the results above mentioned could be due not only to SL signalling, but also KAR signalling, so both phytohormones could have a role in LR development.

Regarding the adventitious rooting capacity, SLs have an opposite effect: they positively regulate crown root (CR) development in rice by stimulating cell division (Sun *et al*., 2015), but they negatively affect AR in Arabidopsis and pea (Rasmussen *et al*., 2012). Unlike in PR and LR development, no links between SLs and auxin or CK has been found with respect to AR development (Rasmussen *et al*., 2012).

Finally, SLs are involved in the development of root hairs (RH), namely tip-growing epidermis outgrowths that help to anchor roots in the soils and to extend the nutrient uptake area (López -Bucio *et al*., 2003). In both Arabidopsis and tomato, *rac*-GR24 induces longer RH (Kapulnik *et al*., 2011; Koltai *et al*., 2010), while this effect was not visible in Medicago (De Cuyper *et al*., 2015). In this case, further analysis was made to better elucidate the involvement of SL or KAR in HR development, showing stronger effects for SLs but, in a lower extent, also for KAR-dependent signalling. As observed for LR, SLs and KAR might cooperate to regulate the development of HR. Regarding SLs, the effect seems to depend on both auxin and ethylene (ET) pathways, with ET

acting downstream of SLs with a direct effect on RH elongation and on auxin pathways (Kapulnik *et al*., 2011).

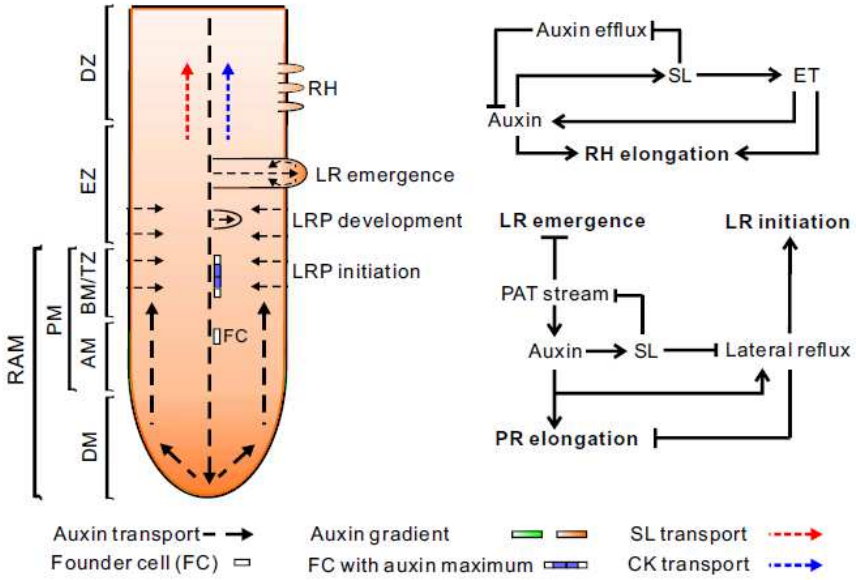

Figure 17. SLs and other phytohormones interplay in the root development. Abbreviations: P, primordium; DM, distal meristem; PM, proximal meristem; AM, apical meristem; BM, basal meristem; TZ, transition zone; EZ, elongation zone; DZ, differentiation zone; FC, founder cell. From Cheng *et al*., 2013.

5.6 Strigolactones in the rhizosphere

At the base of their role of signal molecules in the rhizosphere, the instability in the soil and the formation of a gradient made SLs as short-living molecules. In the rhizosphere, they can be perceived only by organisms sufficiently close to the SL-exuding root (De Cuyper and Goormachtig, 2017). The first identified function of SLs was their ability to induce the germination of *Striga* parasitic seeds (Cook *et al*., 1966). However, on an evolutionary point of view, their first function appeared on land plants was their capacity to attract the colonization of beneficial symbiotic arbuscular mycorrhizal fungi (AMF) (Akiyama *et al*., 2005). In addition, SLs may have a positive role in the rhizobia-legume symbiosis (Soto *et al*., 2010; Liu *et al.,* 2013) and in plant-pathogen interactions (López-Ráez *et al*., 2017), even if information about these roles is still scarce.

Since discovered, the dual activity of SLs has always been fascinating for scientists, showing how the interactions of the plant with the rhizosphere ecosystem could be in one hand positive and desirable, on the other hand dangerous and frightening (**Fig. 18**). A rapid overview of the two mains positive (AMF colonization) and negative (germination inducers of root parasitic plants) effects of SLs in the rhizosphere is then proposed.

5.6.1 Strigolactones and arbuscular mycorrhizal fungi

Evolved 400 million years ago and known since 1900, plant-arbuscular mycorrhizal fungi (AMF) symbiosis is known as the beneficial plant-symbiont interaction that helps to feed the world (Marx, 2004). Indeed, almost 80% of vascular plants establish a positive symbiosis with these AM fungi that belong to the Glomeromycota phylum. As obligate symbiont, AMF penetrate the host plant roots, by developing in root cortical cells highly branched structures called arbuscules and obtains from the host plant sugars and lipids to complete their development (Smith and Smith, 2011). In return, thanks to their excellent mineral uptake capacities, AMF enhance the host plant ability to uptake water and nutrients (especially inorganic phosphate) which would otherwise be unreachable to the roots.

A stated above, SLs are important symbiotic signals exuded by host roots to attract AMF colonization (Genre *et al*., 2013). Upon host recognition, SLs stimulate the AMF hyphal branching, thus allowing a stronger contact with the host root. In addition, SLs enhance AMF spore germination, hyphal growth and mitosis (Lanfranco *et al*., 2018; Rochange *et al*., 2019).

5.6.2 Strigolactones and parasitic plants

As mentioned above, SLs were firstly identified in 1966 as stimulants for the tiny seeds of parasitic *Striga* spp. (witchweed) (Cook *et al*., 1966). In addition, they also stimulate the germination of *Orobanche* spp. and *Phelipanche* spp. (broomrapes), all root parasitic plants belonging to the Orobanchaceae family and considered extremely dangerous pests in major crops (Vurro *et al*., 2019). Among *Striga* spp., which parasitize mainly maize, sorghum, rice and millet with a terrible impact on the Africa agriculture, the main weeds are *S. hermonthica*, *S. asiatica*, *S. aspera* and *S. gesnerioides.* Among broomrapes, seven species are the most serious weeds in Europe, North Africa and Asia: *P. ramosa*, *P. aegyptiaca*, *O. crenata*, *O. cumana*, *O. foetida*, *O. cernua* and *O. minor* (Parker, 2013). Broomrapes are dangerous pests for crops under a temperate climate, such as tomato, tobacco, carrot, sunflower, rapeseed, cabbage and hemp.

All these parasitic weeds are obligate parasites, since they have a slight *(Orobanche* spp. and *Phelipanche* spp.) or even absent (*Striga* spp.) photosynthetic capacity, a scarce storage reserve in their thousands of tiny seeds (Ø 200 µm) and require to rapidly attack the root host with their invasive organ, called haustorium, after germination in order to survive. Consequently, haustorium binds to the host vascular system and withdraw all water and nutrients from the host to the

parasite, thus affecting the host growth and eventually the host itself could die (Cardoso *et al*., 2011).

Given that if the germinated parasitic seed fails to connect to the proper host it will die within a few days, the survival of the parasite lean on its ability to perceive even small concentrations of the stimulants exuded from the root host and to germinate only in the presence of these stimulants (Vurro *et al*., 2019). Thus, witchweeds and broomrapes germinate already in the presence of nanomolar concentrations of *rac*-GR24. Moreover, in root exudates, there could be found different classes of SLs, such as strigol-type SLs from sorghum, orobanchol-type SLs from tomato and non-canonical SLs from both maize and sunflower (Wang and Bouwmeester, 2018).

Finally, it has to remember that plants use different strategies to adapt their development to challenging environmental conditions, such as nutrient-depleted soil. In case of nutrient deficiency, especially low phosphate and nitrogen, it was shown that roots exude more SLs, thus triggering improved AMF colonization, but also increasing the infestation by parasitic plants (López-Ráez *et al*., 2017). Therefore, the involvement of SLs in the abiotic stress response has to be analysed.

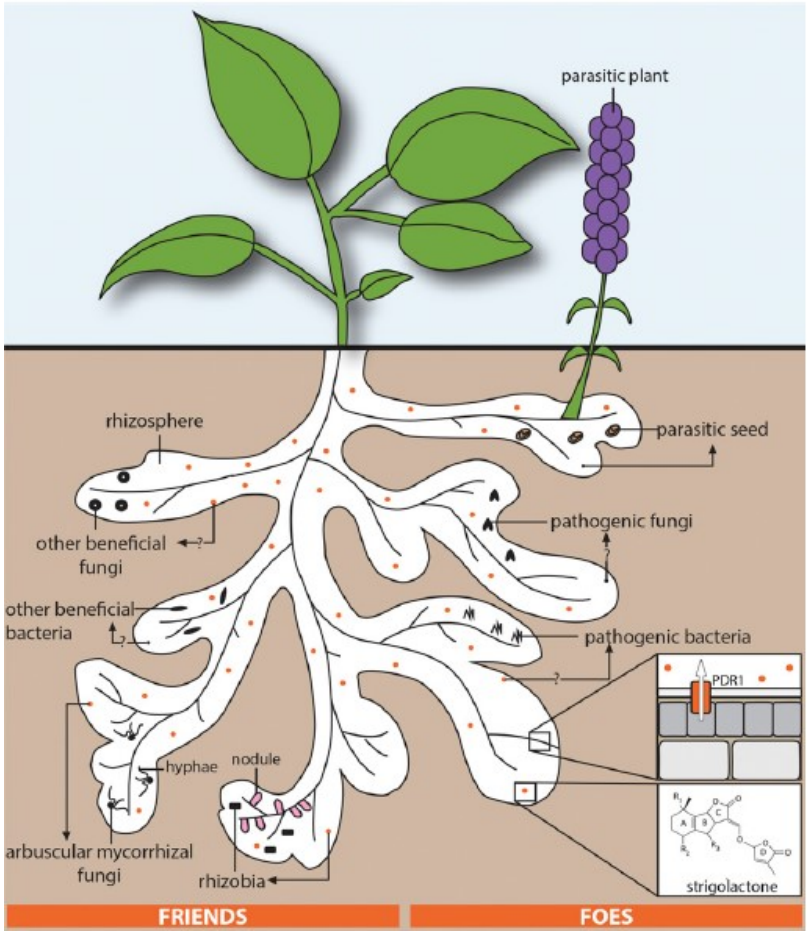

Figure 18. SLs and their dual effect on the rhizosphere. From De Cuyper and Goormachtig, 2017.

5.7 Strigolactones and abiotic stress

Being both phytohormones and unstable signal in the rhizosphere active at picomolar concentrations, SLs are fundamental molecules for a sessile organism such as a plant, able to rapidly adapt the growth and development in response to a more and more changing environment. Between the most challenging problems that a plant has to face, abiotic stress represents a major constraint to plant development and yield, with nutrient and water deficiencies in first place (Mostofa *et al*., 2018). Accordingly, SL involvement in abiotic stress response was revealed by the higher exudation rate observed in roots suffering of phosphate and/or nitrogen deprivation and by different studies on mutants grew under different nutritional stress (Marzec *et al*., 2013). In addition, several studies on the acclimatization responses to water scarcity outlined the involvement of SLs (Ha *et al*., 2014; Cardinale *et al*., 2018). Therefore, a brief overview of osmotic stress and nutrient starvation link to SLs is then presented.

5.7.1 Strigolactones in plant responses to osmotic stress

In Arabidopsis, *Lotus japonicus* and tomato, among the studies performed on the function of SLs in response to osmotic stress, drought and mild salinity were used as stress conditions to test SL mutants in response to *rac*-GR24 on stomata action and ABA involvement (Lv *et al*., 2018; Liu *et al*., 2015; Visentin *et al*., 2016). Under drought, SL-deficient tomato leaves show a lower level of ABA and high of SLs (Visent et al., 2016), while the same type of mutants in Arabidopsis and *Lotus japonicus* has no variations in ABA contents (Rameau *et al*., 2019). Concerning SL levels and biosynthetic genes in tomato and *Lotus* roots*,* they were decreased in response to drought, while in rice they appeared increased (Haider *et al*., 2018), thus showing opposite trends between monocots and dicots. According to the positive role of SLs in osmotic stress response in Arabidopsis and tomato, treatments with *rac*-GR24 induces stomata closing and reduced water loss. Nevertheless, this *rac*-GR24 effect seems to operate in an ABA-independent way, while a similar treatment with ABA induces a response due to both SLs and KAR (Cardinale *et al*., 2018). Accordingly, a model of action of SLs in tomato has been proposed, showing a negative effect of SLs on osmotic-induced ABA levels in roots, which must be promptly counteracted by the inhibition of SL synthesis genes in roots and a decreased shootward SL flow in case of drought stress. As a consequence, SL levels resulted derepressed in shoot, thus inducing stomata closing

and water saving (Cardinale *et al*., 2018). Finally, a SL-dependent action on ABA positive effect was shown also under salinity stress (Ren *et al*., 2018).

5.7.2 Strigolactones and nutrient starvation

Nutrient starvation is one of the biggest abiotic stress that influence root morphology and root/shoot biomass ratio in a plant that needs to adapt and cope with the stress itself in order to survive. Since SL production and exudation are increased under phosphorus (P) and nitrogen (N) starvation, it is evident how nutrient availability has a deep impact on SL metabolism and distribution (Rameau *et al*., 2019). In the soil, P is available as its inorganic form (Pi), while nitrate (NO_3^-) is the most common N source in aerobic conditions, but both these nutrients are subjected to the acquisition by roots (Saeed *et al.,* 2017). Indeed, plants adapt to low P and N levels in the soil by changing root architecture to enhance their uptake and slowing down the shoot growth. For instance, in Arabidopsis P-starvation induces inhibition of primary root (PR) in favour of higher lateral root (LR) density and length (Lopez-Bucio *et al*., 2003). Accordingly, in Arabidopsis during P-deficiency, treatments with *rac*-GR24 induce emergence of LRP, while in ideal growth conditions SLs reduce LR density (Ruyter-Spira *et al*., 2011). On the contrary, in rice low P and N levels lead to increased PR length and decreased LR density, a result obtained also by treating rice plants with *rac*-GR24 (Sun *et al*., 2014). According to these authors, P and N deficiency enhance the productions of SLs by upregulating the expression of SL biosynthesis genes *D10*, *D17* and *D27*, while SL signalling genes *D14* and *D3* appeared downregulated for negative feedback. Moreover, a role for NO in the regulation of rice root development by SLs was also hypothesized (Sun *et al*., 2016). According to these authors, low N and P treatments trigger NO production at root tip level, thus showing how SL and NO act as positive regulators of meristem activity. Besides that, no alterations on SL levels and SL biosynthesis gene expression were shown to be induced by exogenous NO provision, while it induces the expression of *D14* and *D53*, both SL signalling genes (Sun *et al*., 2016). Accordingly, in this study NO appears to act as an upstream positive regulator of SL signalling by inducing the degradation of the repressor D53. Besides that, a contrasting result was obtained in maize, where it seems that NO acts instead as an upstream negative regulator of SL biosynthesis during nitrate-induced root development (Manoli *et al*., 2016).

As stated before, nutrient starvation induces higher SL exudation rate in the rhizosphere to attract symbiotic partners as AMF (Lanfranco *et al*., 2018). Nevertheless, studies on many species show that even non-mycorrhizal plant, such as Arabidopsis, have greater SL exudation levels under

P- and N-deficient conditions, so the positive effect of nutrient starvation on SL biosynthesis and exudation could not be only correlated with the colonization by beneficial symbionts (Yoneyama *et al*., 2012; Mostofa *et al*., 2018). In addition, in *Petunia hybrida* it was shown an up regulation in the transcript levels of SL transporter *PhPDR1* during P starvation (Kretzschmar *et al*., 2012), thus confirming the enhanced SL exudation in the soil, but also showing that nutrient starvation influences also the transport of SLs from root to shoot and consequently the shoot architecture.

Taken together, the available data on SL involvement in root response to nutrient availability show that low P, and even the less studied low N signal, activate SL biosynthesis, transport and metabolism, leading to reduced growth in shoot to optimize the nutrient allocation and enhanced root development to improve nutrient uptake in the soil, with an important role in attracting the beneficial colonization of rhizosphere symbionts. Obviously, nothing of that could be possible without a deep crosstalk of SLs with other phytohormones, which is the focus of the next paragraph.

5.8 Strigolactones interactions with other phytohormones

Phytohormones are the essential molecules for every developmental stage in a plant. Until now, they are recognized as 9 groups: auxin (IAA), abscisic acid (ABA), cytokinins (CK), gibberellins (GA), ethylene (ET), jasmonic acid (JA), salicylic acid (SA), brassinosteroids (BR) and the last identified strigolactones (SLs) (Cheng *et al*., 2013). To better understand the SL functions *in planta* and in the rhizosphere community, the understanding of how SLs interface with other phytohormones pathways a central request is (**Fig. 19**).

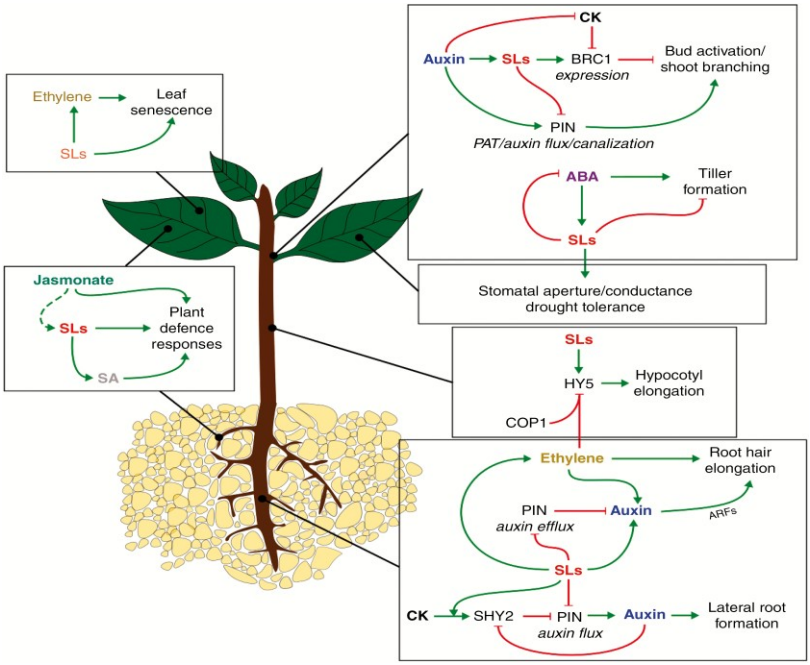

Figure 19. SLs and their interactions with other phytohormones. From Omoarelojie *et al*., 2019.

5.8.1 Auxin

Considered the involvement of SLs in regulating both shoot and root branching, their interaction with auxin is one of the best characterized. As stated before, the impact of auxin on SL signalling is mainly via up regulation of SL biosynthesis genes both in shoots and roots, with the derived SLs that could act as a second messenger for auxin-related responses (Rameau *et al*., 2019). On the other hand, SLs regulate some auxin transport proteins, namely the PIN auxin efflux carries family, thus interfering with the polar auxin transport (PAT) and auxin canalization both in shoot and root (Shinohara *et al*., 2013; Ruyter-Spira *et al*., 2011). Generally, SLs seem to negatively regulate both the transcription of *PINs* genes and the relative proteins polar localization on plasma membrane, even if these effects are dependent on the tissue considered. For instance, SLs induce endocytosis of PIN1 in stem vascular tissues, thus hampering auxin efflux from buds and resulting in shoot branching repression (Shinohara *et al*., 2013). Accordingly, SL-biosynthesis mutants show higher PINs levels and increased auxin transport, while SL-signalling mutants display lower PIN levels and decreased auxin transport (Liang *et al*., 2016). In addition, it was recently proposed that SLs may regulate auxin biosynthesis in the shoot, with a direct regulation on the expression of auxin biosynthesis genes, thus probably explaining the high levels of auxin in SL mutants (Ligerot *et al*., 2017). In roots, SLs and auxin interact to regulate root development through regulation of auxin transport and sensitivity (Ruyter-Spira *et al*., 2011; Koren *et al*., 2013). For instance, *rac*-GR24 treatments induce a long-term developmental effect on PIN levels of the root meristem

(Ruyter-Spira *et al*., 2011), while it induces root hair (RH) elongation by promoting exocytosis of PIN2 at epidermis levels (Pandya-Kumar *et al*., 2014) and *PIN1* downregulation at the root-shoot junction, thus affecting lateral root (LR) emergence in this upper part of the root (Jiang *et al*., 2016). Notably, it has to be reminded that all these studies used *rac*-GR24 applications, thus its effect could be dependent on both SL and KAR.

5.8.2 Cytokinins

Generally, SLs and CK act antagonistically to regulate many developmental processes. For instance, SLs inhibit shoot branching, while CK promote the bud activation (Dun *et al*., 2012), or SLs induce leaf senescence, while CK delay it (Yamada and Umehara, 2015). On the other way, SLs and CK act independently to regulate adventitious root (AR) development (Rasmussen *et al*., 2012), while they act synergistically to regulate lateral root (LR) and primary root (PR) development via SHY2 (SHORT HYPOCOTYL2) (Jiang *et al*., 2016; Koren *et al*., 2013). Moreover, it was hypothesized that they cross-regulate each other's biosynthesis genes, since pea SL-mutants show reduced CK levels in vascular tissue and are hypersensitive to CK treatments (Beveridge *et al*., 2000; Dun *et al*., 2012), while in rice CK treatments induce downregulation of SL biosynthesis genes (Xu *et al*., 2015b). Nevertheless, their cross-regulation may also derive from a long-term feedback regulation, instead of a direct effect (Young *et al*., 2014). In addition, being both synthesized in roots, SLs and CK transfer information about the nutrient status of the plant from the root to the shoot, thus their ratio may represent and indicator of the soil's nutritional quality (Rameau *et al*., 2019).

5.8.3 Abscisic acid

SLs and ABA share the biosynthetic derivation, being both carotenoid-derived signals, and are both involved in the plant response to abiotic and biotic stress, for instance in response to osmotic stress (López-Ráez, 2016). Nevertheless, their direct crosstalk still needs more studies, even if some information has been achieved (Osmoarelojie *et al*., 2019). Up to now, it is believed that ABA acts upstream of SLs, while SLs pathways are somehow involved in the ABA-mediated responses to drought and salt stress (López-Ráez et al., 2010; Visentin *et al*., 2016; Liu *et al*., 2015; Lv *et al*., 2018). On the other hand, SLs and ABA antagonistically act in regulating tiller formation in barley (Wang *et al*., 2018b) and in grapevine berries maturation (Ferrero *et al*., 2018). In addition,

SLs are involved in the feedback regulation of ABA signalling, since GR24 treatments induce upregulation of ABA catabolic genes but not ABA biosynthesis gene (Ferrero *et al*., 2018), and ABA levels result high in rice SL mutants with stronger tolerance to drought (Haider *et al*., 2018). Finally, one link between ABA and SL interaction could be represented by HY5 (ELONGATED HYPOCOTYL 5), namely, a transcription factor involved in the integration of light and ABA responses (Chen *et al*., 2008) whose expression and accumulation was shown to be under control of SLs (Jia *et al*., 2014).

5.8.4 Gibberellins

SLs and GA share analogy in the signalling mechanism, with both the receptors being α/β hydrolase proteins (D14 for SLs and GID1 for GA, respectively) that, after the bond with the related hormone, activate the degradation of repressor proteins (SMXLs/D53 for SLs and DELLAs for GA) by ubiquitination via proteasome 26S (Wallner *et al*., 2016). Nevertheless, very few evidences support a direct interaction within SLs and GA, rather they appear to act independently to regulate distinct processes, such as internode elongation (Bennet *et al*., 2016; de Saint Germain *et al*., 2013). Accordingly, even if some authors proposed a SL-dependent degradation of SLENDER RICE 1 (SLR1, a DELLA protein) (Nakamura *et al*., 2013) or a long-term GA-dependent negative regulation of SL biosynthesis and exudation (Ito *et al*., 2017), these results need to be confirmed with further experiments.

5.8.5 Ethylene

SLs and ET are involved in regulating hypocotyl and root hair (RH) elongation, *Striga* seed germination and leaf senescence (Omoarelojie *et al*., 2019), with ET generally acting downstream of SLs. For instance, it was shown that, before germinating, SLs activate ET biosynthesis in *Striga hermonthica* seeds (Sugimoto *et al*., 2003), and that SLs are involved in the induction of leaf senescence through ET-mediated pathways (Ueda and Kusaba, 2015). Regarding hypocotyl growth, the complex COP1-HY5 integrates light and phytohormones signalling, with an opposite action of SLs and ET (Jia *et al*., 2014; Yu *et al*., 2013). Indeed, in light conditions, SLs positively induce *HY5* expression, whereas ET promote HY5 degradation via COP1. For what concerns root hair (RH) development, both a SL-ET-auxin crosstalk (Kapulnik *et al*., 2011) and an ET-dependent

mechanism were studied (Feng *et al*., 2017), thus showing a complex network for RH development.

5.8.6 Jasmonate

Up to now, the crosstalk between SLs and JA has not been sufficiently studied and only very speculative observations have been made, especially regarding their positive involvement in plant defence to biotic stress. For instance, Torres-Vera *et al*. (2014) showed that tomato SL-biosynthesis mutant displays a reduction in JA levels and downregulation of *PINII* expression, namely a gene involved in tomato resistance to *Botrytis cinerea.* On the other hand, no alterations in JA levels were visible in Arabidopsis after both GR24 treatments and inoculation with the endophytic fungus *Mucor* sp. (Rozpądek *et al*., 2018).

5.8.7 Salicylic acid

SLs and SA are both involved in the regulation of osmotic stress, senescence and defence against pathogens (Omoarelojie *et al*., 2019). Nevertheless, the only available result about SLs and SA interaction shows that, unlike JA, SLs induce increased SA levels in response of the mutualistic interaction between *Arabidopsis thaliana* and the fungus *Mucor* sp., while the *max2* mutant showed lower SA levels (Rozpądek *et al*., 2018).

5.8.8 Brassinosteroids

At the beginning of SL story, it was proposed that SLs could act in BR responses by targeting the degradation of BES1 via SCF^{MAX2} complex signalling during shoot branching (Wang *et al*., 2013). BES1 is a transcription factor involved in BR signalling that initially appeared stabilised in the gain-of-function *bes1-D* mutant, which in turn displayed an increased branching phenotype and altered SL sensitivity. Nevertheless, a following analysis showed no connections between SL and BES1 activity, since in this assay *bes1-D* mutant appeared fully sensitive to *rac*-GR24 and without alterations in the shoot branching (Bennet *et al*., 2016), leading to hypothesize that BES1 could not really be a target of SL signalling in shoot branching. According to these authors, these controversial results may be due to an involvement of KAR in BES1-dependent signalling, instead of SLs, but other analysis are required to better elucidate the BR-SL issue.

5.9 Strigolactones employment for crop enhancement applications

As seen until now, SLs possess so many biological functions that a more and more interest in their application in agriculture have risen in the last years. Accordingly, to their key role in regulating shoot and root architecture, the chance to modulate the all plant architecture to increase its yield is really promising, as well as the possibility to increase the productivity by modulating the plant resistance to both abiotic and biotic stress. In addition, a better knowledge of SL pathways would also mean the capacity to find a new way to reduce the infestations by root parasitic weeds of Orobanchaceae family, and at the same time increasing AMF colonization and improving the nutritional status of the plant (Jia *et al*., 2019). To reach that, synthetic SL have been studied, since natural SLs are too complex for synthesis on a large scale and need difficult, time-consuming and expensive methods (Prandi and McErlean, 2019). Synthetic SLs can be divided within analogues (i.e. GR24, GR7, Nijmegen) and mimics (i.e. debranones), with the first having a structure very similar to natural SL, and the latter having a simpler structure but with the maintenance of the D-ring, which is essential for the biological activity. SL inhibitors have also been identified and applied, such as the SL biosynthesis inhibitor TIS108 (Ito *et al*., 2013; Nakamura and Asami, 2014). Despite the promising results by applying SLs for agricultural purpose, no studies have yet investigated their impact on the indigenous soil microbial community, nor the biodegradability, photostability and production of by-products have been tested yet (Prandi and McErlean, 2019). Moreover, no legislative requirements have been defined yet, so SLs-containing products could be registered as plant protection products (PPPs), or plant strengthens (PSs), or even plant biostimulants (PBs). Nevertheless, SLs could be used to control the infestation of parasitic weeds, or as biofertilizers to increase AMF colonization, or directly as plant hormones, thus representing an interesting and attractive tool for a sustainable agriculture (Vurro *et al*., 2016).

To control parasitic weeds, the first method studied was the “suicidal germination”, namely the direct application of SLs in an infested field with parasitic seeds but without a host, thus causing the death of the just germinated parasitic weeds and the reduction of the parasite seed bank in the soil. Unfortunately, parasitic seeds germinate to a greater extent with natural SLs, which are too complex and expensive to synthesize chemically, so studies are directed in testing on field some SL analogues (Vurro *et al*., 2019). Instead of suicidal germination, another approach would be the SL degradation as soon as they are exuded in the soil by the host plant. For instance, borax was used as a chemical approach, giving positive results, but further studies are needed to

better understand how to prevent the excessive accumulation of boron in the soil (Kannan and Zwanenburg, 2014).

Thanks to their ability to induce colonization by AMF, SLs could be indirectly considered biofertilizers. As previously stated, AMF are fundamental to improve the nutrient uptake of their host plant, so SLs released in the soil represent a positive signal for their colonization (Vurro *et al*., 2016). Finally, SLs are now widely recognized as important phytohormones, with a strong regulation of their biosynthesis and exudation in response to adverse environmental conditions, such as nutrient deficiency and osmotic stress. Indeed, understating how SLs modulate the plant architecture and shape in response to both endogenous and environmental stimuli is of great interest for both scientists and agronomists, in order to stimulate the plant fitness and to gain its best productivity in the given environment. Furthermore, a possible application of SLs in medicine and pharmacology has also been achieved (Hasan *et al*., 2018).

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7. AIM OF WORK

The central role of strigolactones (SLs) in the overall process controlling the plant adaptation to nutritional stresses in a changing and challenging environment has been universally acknowledged in the last years. Furthermore, a specific involvement of SLs in the pathway underlying the nitrate response in maize root has also been recently hypothesised. In particular, it was proposed that nitric oxide (NO), auxin and SLs could take part to the complex pathway governing the maize root adaptation to different N availabilities.

The main scope of this work is to better understand the involvement of SLs in the maize root developmental response to nitrogen availability, using both physiological and molecular approaches. To this aim, firstly a LC-MS/MS analysis was applied to identify and characterized SLs in maize root exudates obtained by seedlings grown with different N availabilities, together with an indirect SL detection thanks to a germination bioassay. Furthermore, the transcriptomic regulation of SL-related genes and their mRNA localization were characterized together with the phenotypic response in terms of lateral root (LR) development and primary root (PR) length in response to N availability and in the presence of a SL inhibitor or a SL analogue.

In the light of the well-known involvement of auxin in the LR development, the interplay between SLs and auxin in the response to nitrate in LR development was then deepened thanks to a multiple approach based on LR density evaluation and gene expression assessment. A set of genes associated with the auxin signalling leading to LR development were selected and their expression were assessed in maize roots of seedlings grown in N-starvation or supplied with nitrate and different SLs and auxin analogues and inhibitors. The LR density was also evaluated in response to the same treatments used for gene expression analysis.

Since it was previously shown that nitrate and ammonium induce common but also specific response in maize lateral root development, the main components of the signalling leading to the global response to the two N-sources were then identified. For this purpose, an RNAseq-based untargeted approach was applied to investigate the global transcriptomic profiles displayed by –N, NO_3^- -supplied or NH_4^+-supplied maize root for 24 h. In concert with this molecular untargeted approach, a physiological evaluation of plant development in response to N-deprivation, NO_3^- and NH_4^+ supply was also performed from 24 h to 7 days of treatment. Hence, the chlorophyll, flavonoids and anthocyanins index were measured with a leaf-clip device, leaves and roots fresh weights and area were also obtained, and metabolic profiling of amino acids was performed to better complete the picture gained through transcriptomic analyses.

Chapter 2

THE CONTROL OF ZEALACTONES BIOSYNTHESIS AND EXUDATION IS INVOLVED IN THE RESPONSE TO NITROGEN IN MAIZE ROOT

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FOCUS: This chapter is focused on better understanding the involvement of strigolactones (SLs) in the maize root response to nitrogen availability. To this aim, an integrated approach based on the spectroscopic detection of SLs in root exudates and on multiple physio-molecular measurements was utilized. Results obtained allowed to clearly demonstrate the key role of SLs in the regulation of root response to nitrogen in this species and evidenced the existence of common and different elements between the response to nitrate or ammonium supply.

ABSTRACT

Nitrate acts as a signal in regulating plant development in response to environment. In particular nitric oxide (NO), auxin and strigolactones (SLs) were supposed to cooperate to regulate the maize root response to this anion. In this study, a combined approach based on LC-MS/MS and on physiological and molecular analyses was adopted to specify the involvement of SLs in the maize response to N.

Our results showed that N deficiency strongly induces SL exudation, likely through stimulating their biosynthesis. Nitrate provision early counteracts and also ammonium lowers SL exudation, but less markedly. Exudates obtained from N-starved and ammonium-provided seedlings stimulated *Phelipanche* germination, whereas when seeds were treated with exudates harvested from nitrate-provided plants no germination was observed. Furthermore, our findings support the idea that the inhibition of SL production observed in response to nitrate and ammonium would contribute to the regulation of lateral root development. Moreover, the transcriptional regulation of a gene encoding a putative maize WBC transporter, in response to various nitrogen supplies, together with its mRNA tissue localization, supported its role in SL allocation. Our results highlight the dual role of SLs as molecules able to signal outwards a nutritional need and as endogenous regulators of root architecture adjustments to N, thus synchronizing plant growth with nitrogen acquisition.

Key words

Ammonium, LC-MS/MS, Maize, Nitrate, Root, Strigolactones

1. INTRODUCTION

Nitrogen (N) plays a vital role in plants. Globally, during 1961–2010, maize, rice and wheat received a total of 1594 Tg of N-fertilizer (Ladha *et al*., 2016), but more than 50% of the available N was lost due to the low Nitrogen Use Efficiency (NUE) of crops (Li *et al*., 2017). Improving crop NUE is essential to limit the impact of nitrogenous fertilization and to improve sustainability. Plants can uptake N in the soil in different forms, but nitrate and ammonium are the most common inorganic compounds. However, soluble nitrate (NO_3^-) is the major N source for crops in aerobic environments (Wang *et al*., 2012). It acts both as nutrient and signal, regulating many developmental processes (Bouguyon *et al*., 2012; Undurraga *et al*., 2017).

In maize primary root, NO_3^- early perception seems to involve the fine-tuning control of NO production and scavenging (Manoli *et al*., 2014; Trevisan *et al*., 2014), which likely regulates auxin levels and its transporter PIN1 re-localization in the transition zone (TZ) cells (Manoli *et al*., 2016). The TZ, which is located between the meristem and the elongation zone, plays a key role in sensing the external environment and in translating it into suitable developmental responses (reviewed by Baluška *et al.,* 2010). Furthermore, a subsequent study hypothesized that besides NO and auxin also strigolactones (SLs) could take part to the complex pathway governing the maize root adaptation to different N availabilities (Trevisan *et al*., 2015). Since NO and auxin act synergistically to control multiple aspects of root biology (Fernández-Marcos *et al*., 2011; Sanz *et al*., 2015) the role of SLs in the pathway where NO could act as coordinator of nitrate and auxin signalling to control the overall root response should be further studied, even in light of the existing interplay between SLs and NO (Kolbert, 2018).

SLs are a new class of carotenoid-derived phytohormones which act as both endogenous and exogenous signaling molecules in response to various environmental stimuli (Matusova *et al*., 2005; Pandey *et al*., 2016; Waters *et al*., 2017). They were identified as stimulants for germination of parasitic weeds in the *Orobanchaceae* family (Cook *et al*., 1966) and for mycorrhization initiation (Akiyama *et al*., 2005), but they also play multiple roles in regulating plant development (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008; Brewer *et al*., 2013). Moreover, soil nutrient deficiencies trigger enhanced SL biosynthesis, which in turns seem to influence root architecture (Kapulnik and Koltai, 2014; Kohlen *et al*, 2012, Koltai, 2015; Ito *et al.*, 2016). In fact, strigolactones have been described to have an impact on lateral root and root hair formation (Kapulnik *et al*., 2011; Mayzlish-Gati *et al*., 2012). On the whole, the effect of SLs on modifying RSA (Root System

Architecture) in response to nutrient deprivation would appear dependent on auxin levels in the root (Waters *et al*., 2017).

SLs occur in very small concentrations, both in plant tissues and in their exudates and they may be unstable, thus making not easy their detection and purification (Boyer *et al*., 2012). Recently Boutet-Mercey *et al.,* (2018) developed a method for SL quantification by LC-MS/MS in root tissues. SLs are synthesized via all-trans-β-carotene isomerization, sequential oxidative cleavage of 9-*cis*-β-carotene by two carotenoid cleavage dioxygenases (CCD7 and CCD8), carlactone oxidations by cytochrome P450 monooxygenases MAX1, and yet to be characterized downstream conversions (Ruyter-Spira *et al*. 2013) giving birth to two sub-groups, strigolactones strictly speaking and strigolactones-like. Strigolactones-like are non-canonical SLs that do not include the classical ABCD skeleton, but still contain the D-ring, which mediates strigolactones perception and activity (de Saint Germain, 2016).

The functional annotation of transcripts isolated by RNA-sequencing in the TZ of nitrate-supplied maize root identified a set of genes likely involved in SL biosynthesis and transport (Trevisan *et al*., 2015). Transcriptomic data demonstrated that 2h of nitrate are enough to strongly inhibit the expression of *ZmCCD7* and *ZmCCD8* in TZ cells. Moreover, three genes encoding ABC (ATP-binding cassette) transporter proteins, (*ZmPDR1*, *ZmPDR3* and *ZmWBC33*), isolated from accessions classified among the term “drug transporter activity” were highly co-expressed with the carotenoid cleavage dioxygenase genes, suggesting that they could putatively take part to the SL transport and/or exudation.

The present study was aimed at better understanding the involvement of SLs in the maize response to N availability. A LC-MS/MS method was applied to try to identify already known SLs in maize root exudates obtained by seedlings grown with different N availabilities and to characterize their profile and the extent of their exudation in response to the nutrient treatment.

The transcriptional regulation of genes encoding SL biosynthesis components and putative SL transporters was also evaluated and *in situ* hybridization experiments were performed to study their mRNA localization. Finally, phenotypical analyses, based on both *Phelipanche* germination assays and lateral root (LR) development assessment were performed to gain new insights into the regulation of SL’s endogenous and exogenous effects mediated by N availability in this crop.

2. MATERIALS AND METHODS

2.1 Maize growth conditions

Seeds of the maize inbred line B73 (*Zea mays* L.) were germinated as described by Manoli *et al*. (2014). After germination seedlings were grown for 24h in a N-deprived solution and then transferred to: -N (negative control), NO_3^- 1 mM or NH_4^+ 1 mM. The expression analyses were performed after 24 h (T1), 48 h (T2) or 72 h (T3). To test the effect of phosphate availability a second experiment was performed by growing seedlings in a P-deprived solution (-P) for 24 h and then transferring them for further 24 h in a similar –P solution or in a PO_4^{3-}supplied medium (40 µM). 6-phenoxy-1-phenyl-2-(1H-1,2,4-triazol-1-yl) hexan-1-one (TIS108) and *rac*-GR24 (Strigolab, Torino, Italy) were used at a 2 µM concentration as inhibitor of SL biosynthesis (Ito *et al*., 2011) and as synthetic SL analogue, respectively.

Lateral root primordia (LRP) analysis and exudates collection were carried out at T1. For exudates collection seedlings were transferred to a renewed solution and exudates were collected after 24 hours.

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 $m^{-2}{\cdot}s^{-1}$ photon flux density was used.

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

2.2 SL identification and quantification in exudates

Exudates were obtained by two independent experiments in three biological replicates. The extraction of root exudates was based on the protocol of Gomez-Roldan *et al*. (2008). Each exudate was prepared with at least 1 g of root fresh weight (each sample had an accurate weight of root). All volumes of root exudates and the corresponding blank samples were extracted with an equivalent volume of ethyl acetate and 10 ng of GR24 were added as an internal standard. All the extracts were evaporated to dryness and finally dissolved in 100 µL of acetonitrile before LC-MS/MS analysis. Chromatographic conditions were similar as in Boutet-Mercey *et al.* (2018).

Ninety transitions MRM (Multiple Reaction Monitoring) of the literature were monitored using Waters Xevo TQ-S equipped with an ESI source in positive or negative mode. The **Supplementary Table S1** (available online at https://doi.org/10.1093/pcp/pcz108) shows the monitored transitions for 31 SLs including 20 canonical SLs, 5 non-canonical SLs et 6 unknown, according to

bibliography. The source parameters for the MRM mode were similar as in Boutet-Mercey *et al*. (2018). The relative quantification of the putative SL was carried out by a ratio between area of the chromatographic peak of the putative SL and area of internal standard GR24 (MRM transition 321 > 224) multiplied by the amount of added internal standard, relative to the mass of exuding roots.

Experiments with three biological replications were repeated twice (two cultures) to confirm the results. The data are presented as means ± standard errors (n = 3) from a typical single experiment. Exuded amounts of SL were compared statistically by using Student's t-test ($P < 0.05$).

2.3 RNA extraction and cDNA synthesis

One cm of root apices from the root tip cap was sampled from 15 to 20 pooled seedlings, in three independent biological repetitions, and immediately frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) as previously described by Trevisan *et al.* (2011). RNA was quantified with a Nanodrop1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA) and reverse transcribed to cDNA as described by Manoli *et al.* (2012).

2.4 Quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed using the StepOne Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) as described by Nonis *et al*. (2007). SYBR Green reagent (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) was used in the reaction, according to the manufacturer's instructions. Melting-curve analysis confirmed the absence of multiple products and primer dimers. Target gene relative expression was determined according to the Livak and Schmittgen (2001) method, using *MEP* (membrane protein PB1A10.07c, Zm00001d018359) as reference gene, according to Manoli *et al.* (2012). Primers were designed using Primer3 web tool (version 4.0.0; http://bioinfo.ut.ee/primer3/; Rozen and Skaletsky, 2000) and further verified with the PRATO web tool (Nonis *et al*., 2011). The list of genes and of the primers used are reported in **Table 1.**

Three technical replicates were performed on three independent biological repetitions.

Table 1: List of primers used in qRT-PCR experiments. Primers used to amplify ISH probes are evidenced in bold.

PRIMER	SEQUENCE	DESCRIPTION
Zm00001d002736_T01_For	AGTCCACACCCGTCTACCTG	***ZmCCD7***
Zm00001d002736_T01_Rev	GGTCCAGCTTCTTGTTCAGC	***ZmCCD7***
Zm00001d043442_T01_For	AGAAAGGTGTCTCTGCTGCT	***ZmCCD8***
Zm00001d043442_T01_Rev	CTATGGGCTCGCTCACATGA	***ZmCCD8***
Zm00001d043598_T01_For	GGAAACCCGATCAGCAGGT	***ZmPDR1***
Zm00001d043598_T01_Rev	GCAGTAAAGCCAGCCAACAC	***ZmPDR1***
Zm00001d019398_T01_For	CGCTAACACGGTCTCATCAA	***ZmWBC33***
Zm00001d019398_T01_Rev	ATCATCATCAGCCCTTCGAC	***ZmWBC33***

2.5 RNA *In situ* hybridization of *ZmCCD8* and *ZmWBC33*

In situ hybridization of maize primary root with digoxigenin (DIG)-labeled probes was performed as described by Trevisan *et al.* (2011). *ZmWBC33* and *ZmCCD8* antisense probes were amplified in PCR using the primers listed in Table 1. The fragment was cloned into the T-easy vector (Madison, WI, USA) for labelling. The sense and antisense probes were synthesized in vitro using T7 and SP6 RNA polymerases (Roche, Basel, Switzerland) and labelled with digoxigenin RNA labelling mix (Roche) following the manufacturer's protocol. Roots were fixed, dehydrated, infiltrated with paraffin and sectioned (7 µm) as described by Trevisan *et al*. (2011). Histo Clear II (National Diagnostics, Atlanta, GA, USA) was used to remove paraffin from sections. Slides were hydrated in a decreasing ethanol series. Hybridization was conducted as described by Trevisan *et al*. (2011). After staining, slides were observed with an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan). Images were captured with an Axiocam Zeiss MRc5 color camera (Carl Zeiss, Oberkochen, Germany), and processed with Adobe Photoshop 6.0.

2.6 Maize root exudate collection and *Phelipanche ramosa* germination bioassay

Parasitic seeds (provided by prof. Antonio Elia, University of Foggia) were pre-conditioned under sterile conditions as reported by Pouvreau *et al*. (2013). After the preconditioning period, the GFFP (Glass Fiber Filter Paper) disks with parasitic seeds were treated with 50 µL of root exudates and

incubated in darkness at 25°C for 6 days. To better contrast the radicle, seeds were also stained using 40 µL of Neutral Red solution (1:4000, w/v) for each disk (Guillotin *et al*., 2016). Germinated seeds were then counted using a stereomicroscope (Olympus BX50 microscope, Olympus Corporation, Tokyo, Japan). Images were captured with an Axiom Zeiss MRc5 colour camera (Carl Zeiss, Oberkochen, Germany), and processed with Gnu Image Manipulation Program (GIMP).
Three biological replicates for each treatment and an ANOVA statistic test were performed (n=30).

2.7 Lateral root density analysis

Seedlings were grown for 24 h in the N-deficient solution and then transferred in different nutrient solutions for 24 h, as described in the first M&M paragraph. The effect of NO_3^- was evaluated also after only 2 h of treatment.
To better visualize LRP an haematoxylin staining solution supplied with ferric ammonium sulphate was used, as described by Canellas *et al*. (2002). Root images were collected using a flatbed scanner. The lateral root and the primary root length were measured using the Image J Image Analysis Software and the LR density was expressed as percentage compared to the value observed for N-deprived roots. Three biological replicates for each treatment and an ANOVA statistic test were performed (n=30).

3. RESULTS

3.1. N-starvation, nitrate and ammonium provision differently affect SL exudation

To assess the effective presence and content of SL in the exudates obtained by plants grown without N or with NO_3^- or NH_4^+, a LC-MS method was developed.
SLs are usually screened by the LC-MS/MS in precursor ion scan mode, searching for ions undergoing the specific loss of Cycle D (-97 Da) (Xie *et al.*, 2010), but this strategy lacks sensitivity. MRM mode, being the most sensitive mode in LC-MS/MS, was then used for screening and quantification purposes, listing every MRM of SLs from literature. This allowed us to check the presence or absence of 31 SLs (20 canonical SLs, 5 non-canonical SLs and 6 unknown) in root exudates from different nutritional conditions and blanks and quantify them if they were present. All chromatographic peaks with an area above 1000 were integrated. However, the common peaks between medium blank and exudate samples were considered as false positives and

ignored. A peak was also ignored if it was found in one experiment (in one transition and one retention time) but not in the other. In order to obtain positive control samples (e.g. expected to contain SLs produced by maize), some maize roots were let to exudate in P starvation conditions, e.g. ideal conditions for SL production (Lopez-Raez *et al*., 2008a). Finally, one putative zealactone isomer eluting at the retention time of 10.8 min was detected as a SL, quantified and confirmed in both experiments (**Supplementary Table S1,** available online at https://doi.org/10.1093/pcp/pcz108). We found a number of additional signals but none except this compound could be confirmed matching our criteria. This compound exhibited typical characteristics of strigolactones. It was detected at MRM channels *m/z* 399>302 and 345>248, with characteristic losses of cycle D. Five MRM transitions with precursor ions *m/z* 399, 377 and 345 (**Table 2**) showed a response at that retention time, suggesting the putative SL would have a mass of 376. Accordingly, *m/z* 399 would correspond to the sodium adduct, *m/z* 377 would be the proton adduct and *m/z* 345 could be a fragment produced in the source of the mass spectrometer from a neutral loss of methanol. The two main MRM arising from *m/z* 399 et 377 corresponded to expected or published transitions for zealactone 1a and 1b (**Supplementary Fig. S1**), the 3 other arising from *m/z* 345 had been putatively attributed to didehydroorobanchol isomers (Lopez-Raez *et al*., 2008b) or didehydrostrigol isomers (Xie *et al*., 2007), suggesting that this *m/z* 345 fragment still bears strigolactone structure. The mass of the putative M376 SL corresponds to the mass of zealactones 1a and 1b as presented in Charnikhova *et al.* (2017). However, no signal was confirmed at the main MRM transition (377>97) presented for zealactones 1a and 1b in Charnikhova *et al,* (2017), and no standard was available to confirm the zealactone identity. So, the compound was hereafter referred to as putative zealactone isomer.

In the quantification of strigolactones in maize exudates, this putative zealactone isomer was detected at a significant level (1.2 ng equivalent GR24 per g exuding root) in samples obtained from phosphate-starved seedlings, which were utilized as a positive control for SL exudation (**Fig. 1**). Surprisingly this compound was detected at a much higher level (13.2 ng eq GR24/g root) in nitrogen-starved samples. In contrast, nitrate-supplied samples contained no detected zealactone isomer, indicating a clear inhibitory effect of nitrate on zealactone production. However, the effect of ammonium supply on SL content in exudates showed a decrease of the SL level but weaker than with nitrate supply.

Table 2: **LC-MS/MS parameters for putative zealactone isomers detected in the samples**

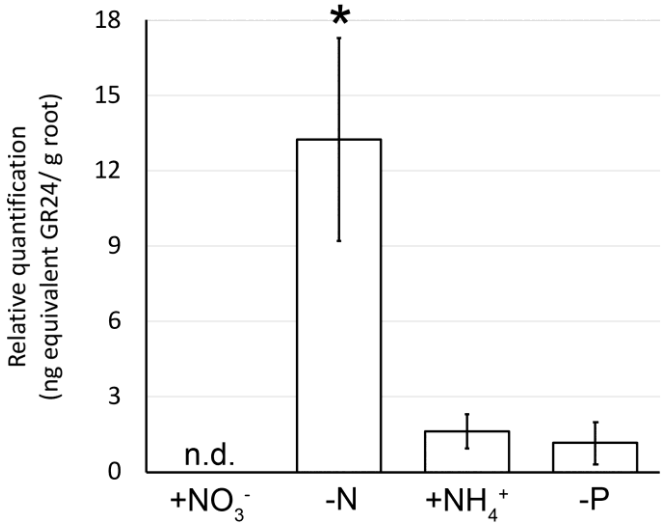
Compounds	RT (min)	MRM transitions	CV (V)	CE (eV)	Q/C
Putative SL like Zealactone isomer [M+ Na]+	10.86	399 > 302[a]	20	20	Q
Putative SL like Zealactone isomer [M+H]+	10.86	377 > 345[b]	20	15	C
Putative SL like Zealactone isomer [M+H-CH3OH]+	10.86	345 > 248[c]	20	15	C
Putative SL like Zealactone isomer [M+H -CH3OH]+	10.86	345 > 203[d]	20	15	C
Putative SL like Zealactone isomer [M+H -CH3OH]+	10.86	345 > 175[d]	20	15	C

RT (min): Retention time in minutes
Diagnostic transition MRM: characteristic precursor and product ions for multiple reaction monitoring
CV (V): cone voltage
CE (eV): collision energy
Q /C: transition used for quantification (Q) or confirmation purpose (C)
a. Putative specific MRM transition for D ring-containing ions [M+Na - D ring]+ (Xie et al., 2010).
b. Loss of a methanol group (Charnikhova et al., 2017).
c. Putative specific MRM transition for a D ring-containing ion (Xie et al., 2010) after in source loss of a methanol group.
d. Putative MRM transitions after loss a methanol group, analog to didehydro-Orobanchol transitions (Lopez-Raez et al., 2008)

Figure 1: LC-MS/MS, MRM quantification of SL in maize root exudates

Quantitative analysis of the relative amounts of putative zealactone forms in maize root exudates [ng*(g root FW)$^{-1}$] of seedlings exposed to additional 24h of nitrate (NO_3^-), ammonia (NH_4^+) or N starvation (-N) after a 24h-pre-incubation under N-deficient conditions. Quantification in root exudates of phosphate-starved seedlings (-P) was included as positive control. The root exudates were collected after the treatment and then shock-frozen in liquid nitrogen immediately afterward. Following extraction, the analytes were quantified by analysis using of LC-MS/MS, MRM mode. The experiments were repeated twice, and data were from a typical single experiment. Values are mean ± SE of three replicates. Asterisks indicate significant differences in SL levels between -N and N fertilization conditions according to Student's t test ($P < 0.05$).

nd: non-detected.

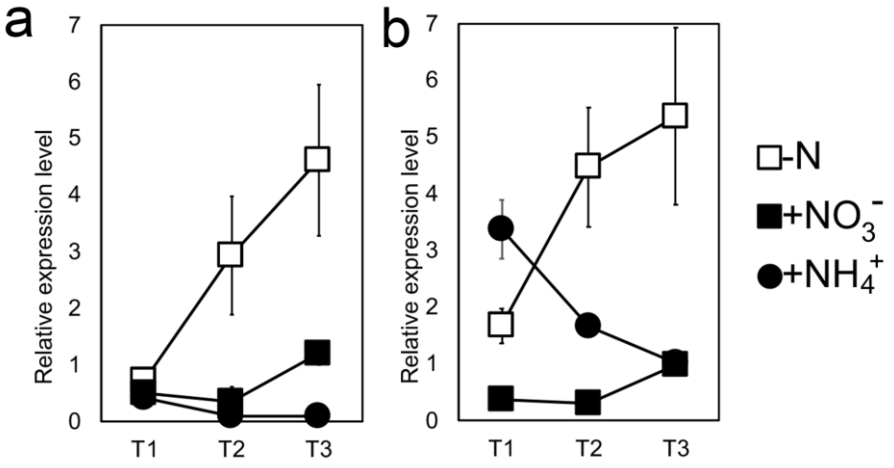


3.2. N and P regulation of SL-related gene transcription in the primary root

The TZ of the root apex is very responsive to a short (2 h) nitrate treatment, which rapidly triggers the downregulation of genes involved in SL production and action (Trevisan *et al*., 2015). Here the transcript levels of *ZmCCD7*, *ZmCCD8*, encoding components of SL biosynthesis were measured in the first cm of the root apex (including M, TZ, EZ and MZ) of seedlings grown without N for 24 hours and then transferred to a similar solution (negative control) or to two different solutions containing NO_3^-, 1 mM, NH_4^+, 1 mM for additional 24 (T1), 48 (T2) and 72 (T3) hours (**Fig. 2**) The transcription of both *ZmCCD7* and *ZmCCD8* was clearly up-regulated by N-deficiency, with an increasing trend with the increase of time of permanence in –N. On the contrary, when NO_3^- was supplied the level of their expression didn't change during the experiment and it was always significantly lower (1,5-4,5; 8-15 and 4-6 fold changes for *ZmCCD7* and *ZmCCD8* at T1, T2 and T3 respectively). As far as the NH_4^+ supply was concerned, a different trend was observed for *ZmCCD7* and *ZmCCD8* expression. In fact, *ZmCCD7* was transcribed at very low levels in all the time-points, while *ZmCCD8* expression was still up-regulated after 24 hours of ammonium supply (T1) and decreased thereafter (T2 and T3) to levels lower than those measured for N-depleted roots.

Figure 2: Real-time qRT-PCR expression profiles of SL biosynthesis *ZmCCD7*, *ZmCCD8* genes in maize roots.

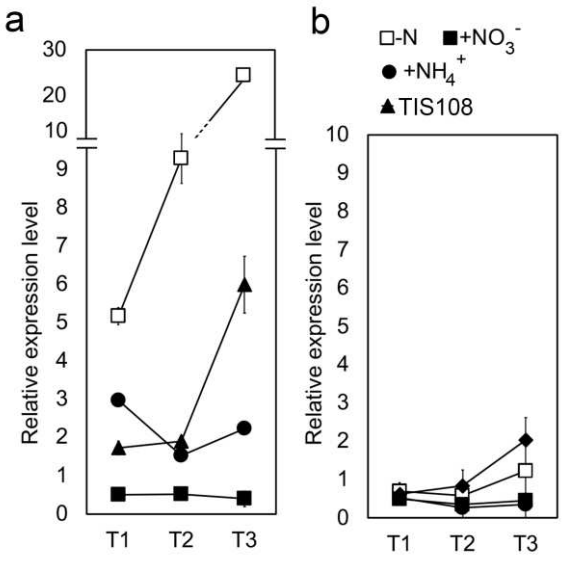
Maize seedlings were grown in hydroponics media either under N-deprivation (-N) or subjected to 1 mM N-fertilization (NO_3^- or NH_4^+) after a 24h-pre-incubation period in N-deficient conditions. After 24 hours (T1), 48 hours (T2) and 72 hours (T3) of treatment 1 cm of root apices from the root tip cap were collected from every pool of plants (n=15 to 20) to detect relative mRNA levels for ZmCCD7 (a) and ZmCCD8 (b) by means of qRT-PCR analysis. Expression levels were normalized to MEP (Zm00001d018359, Manoli et al. 2012). Data are mean ± SE for three biological replicates.



The expression of two genes putatively involved in SL transport (*ZmPDR1* and *ZmWBC33*) was then assessed in the same nutritional condition and also in the presence of TIS108 (**Fig. 3**). *ZmPDR1* was expressed at very low levels in almost all treatments, with a significant increase of its expression observed only after 72 hours (T3) both in N-starved and TIS108-supplied roots. *ZmWBC33* displayed a different profile with a significantly higher expression in N-deprived roots (10, 17 and 61 fold changes at T1, T2 and T3 respectively) in comparison to NO_3^--supplied plants. NH_4^+ provision also clearly down-regulated *ZmWBC33* expression, even though less rapidly in comparison to NO_3^-. In fact, after 24 hours of NH_4^+ supply *ZmWBC33* transcription was still six times higher than that observed in the presence of NO_3^-. The provision of TIS108 to N-starved roots negatively affected the transcription of *ZmWBC33*.

Figure 3: Real-time qRT-PCR expression profiles of *ZmPDR1* and *ZmWBC33* genes in maize roots

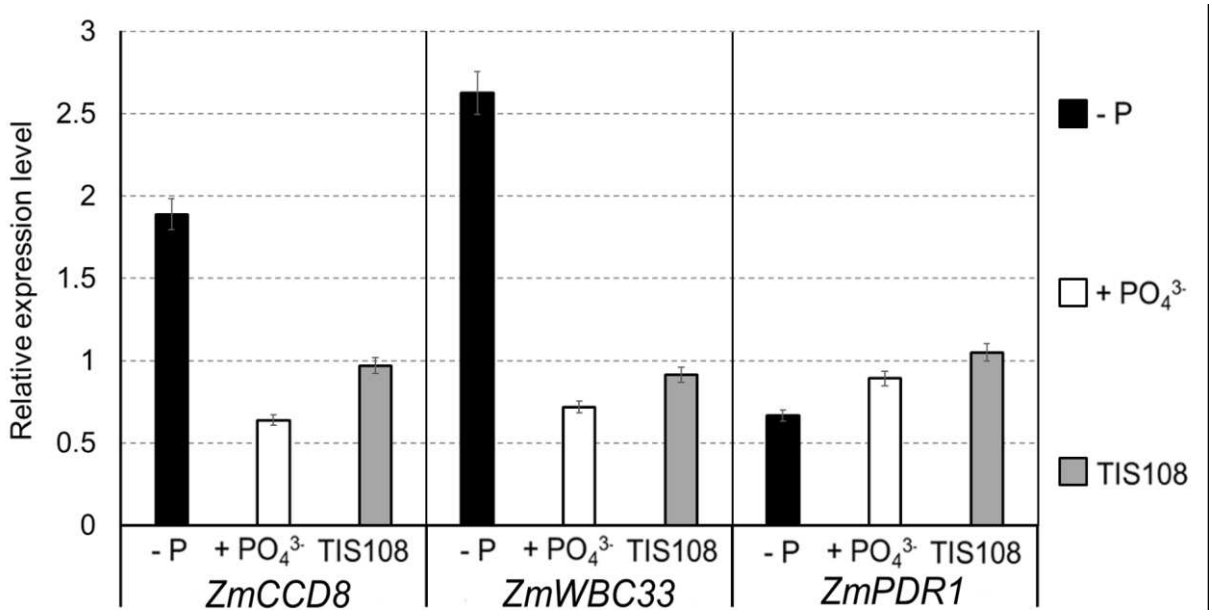
Maize seedlings were grown 24 hours in a N-deprived nutrient solution and then transferred to a 1 mM N-supplied media (NO_3^- or NH_4^+), to a N-deprived solution (-N) or to a N-deprived solution supplied with 2 µM TIS108, for additional 24 hours (T1), 48 hours (T2) and 72 hours (T3). At each time point 1 cm of root apex from the root tip cap was collected from every seedling (n=15 to 20) and the relative mRNA levels for ZmWBC33 (a) and ZmPDR1 (b) were evaluated by means of qRT-PCR. Error bars represent the SEM for three biological replicates.



The expression of *ZmCCD8*, *ZmPDR1* and *ZmWBC33* was measured also in P-depleted and Pi-supplied maize root after 24 hours of treatment (T1) for comparison. The expression of both *ZmCCD8* and *ZmWBC33* was significantly induced by P-starvation, but no differences were observed for *ZmPDR1*. Moreover, when TIS108 was supplied to P-starved seedlings an appreciable decrease of *ZmCCD8* and *ZmWBC33* transcription was noticed (**Fig. 4**). All together these results

seem to suggest that *ZmWBC33*, and not *ZmPDR1*, could take part in the transport of SL. Structural and phylogenetic analysis of ZmWBC33 is reported in the supplementary data (**Supplementary Fig. S2, Supplementary Table S2** available online at https://doi.org/10.1093/pcp/pcz108).

Figure 4: Real-time qRT-PCR expression profiles of *ZmCCD8*, *ZmWBC33*, *ZmPDR1* genes in maize roots.
Seedlings were grown 24 hours in a P-deprived nutrient solution and then transferred to a 40 µM PO_4^{3-} ($+PO_4^{3-}$) solution, to a P-deprived solution (-P) or to a P-deprived solution supplied with 2 µM TIS108 (TIS108) for additional 24 hours. At the end of the treatment 1 cm of root apex from the root tip cap was collected from every seedling at each time point (n=15 to 20) and the relative mRNA levels for ZmCCD8, ZmPDR1, ZmWBC33 were evaluated by means of qRT-PCR. Error bars represent the SEM for three biological replicates.



3.3. Spatial pattern of *ZmWBC33* and *ZmCCD8* in primary root and in shoot

To further identify the particular tissues in which SL-related transcripts accumulate, *ISH* experiments were performed for *ZmWBC33* and *ZmCCD8* in root and shoots. *ISH* allowed detection of target mRNAs in both tissues (Fig. 5). A reliable expression was consistently observed for both the antisense probes, but no labelling with the sense probe was recorded (**Fig. 5A-B and Supplementary Fig. S3**). A relatively uniform distribution of signals was observed for the transcripts of these two genes, revealing that both are predominantly expressed throughout the vascular parenchyma of the primary root, even though *ZmWBC33* showed a higher mRNA accumulation than *ZmCCD8*. In root apex longitudinal sections, comprising the root cap and meristematic area, a clear hybridization signal for *ZmWBC33* and *ZmCCD8* was detected also in the

epidermis and in 1–2 longitudinal files of cells immediately inside of it (hypodermis) (**Fig. 5A-B, panels I-II**). A more diffuse signal was also detected in the outermost layers of the stele, including the pericycle. Moreover, expression was detected in the initials of the epidermis and cortex, in the potential metaxylem tissues, in cortex cells surrounding lateral root primordia (LRP). (**Fig. 5A-B, panels I-II**). Apart from hypodermis, faint staining was seen in root tip cells. This was particularly evident for *ZmCCD8* probe, which signal was completely absent in the quiescent centre cells, but it started to accumulate in their immediate daughters and in the proximal meristem (**Fig. 5B, panel I**). As cellular differentiation progressed, mRNAs of these SL-related genes accumulated in the region where the xylem would develop (Fig. **5A-B, panels I-II**), with the expression domain of *ZmWBC33* larger than those of *ZmCCD8*.

ZmCCD8 transcript levels appeared to decrease in all distal tissues as they progressively elongated and/or differentiated (**Fig. 5B, panels I-II**), while *ZmWBC33* transcripts accumulated also in elongation zone and in the closest part of differentiated root tissue (**Fig. 5A, panels I-II**). At the late stage of vascular development, when cellular differentiation was being completed, expression of these genes continued in the cells between the metaxylem elements (**Fig. 5B, panels II)**. Cross-sections of roots gave the same patterns of *ZmWBC33* and *ZmCCD8* signals throughout the root apex (**Fig. 5A-B, panels VI-VII**).

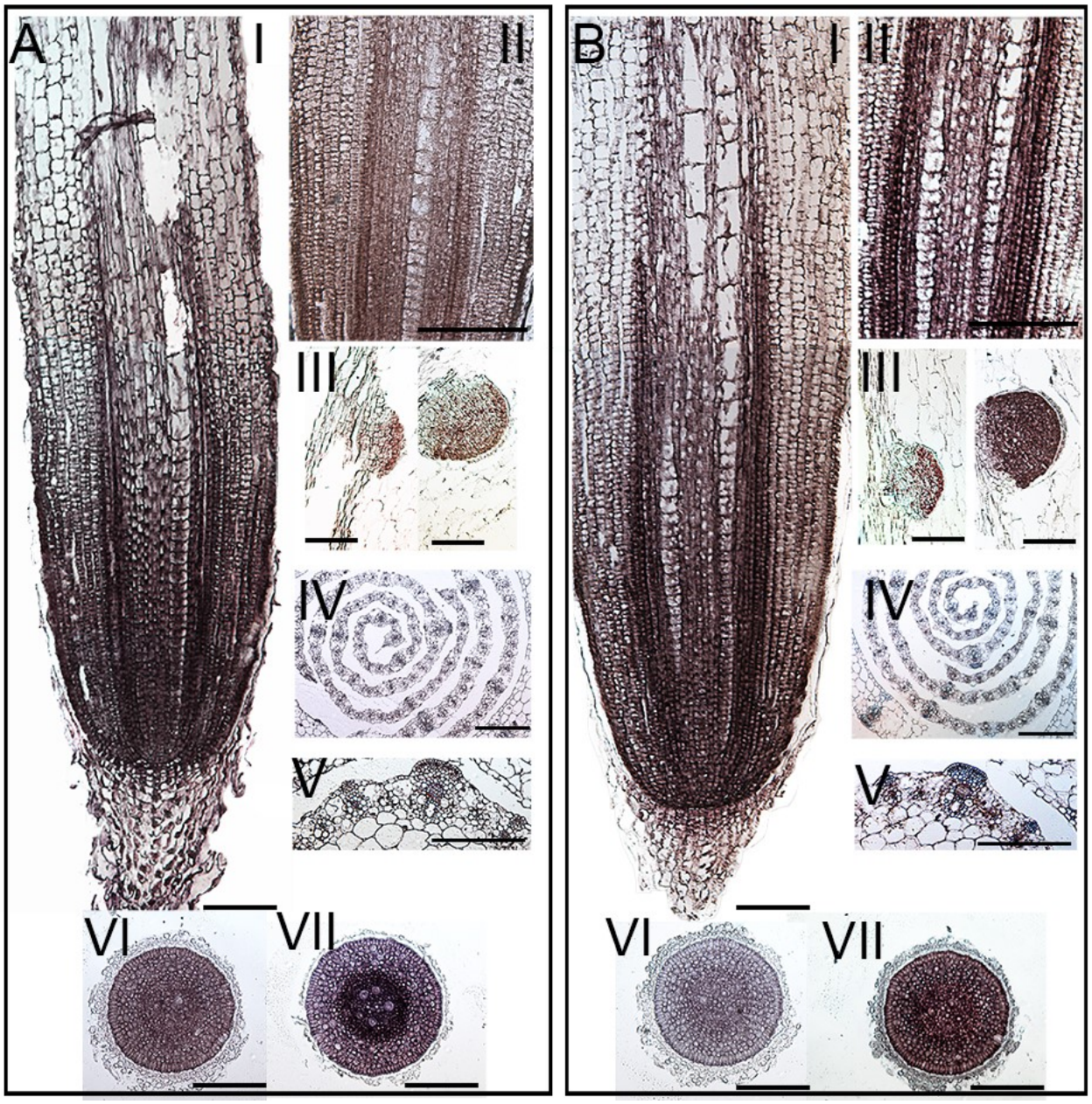
Interestingly, *ZmWBC33* and *ZmCCD8* expression was patchy detected in young lateral root primordia and became evident as the lateral root tips start to emerge from the primary root (**Fig. 5A-B, panel III**). The signal was not present at detectable levels in lateral root founder cells or in lateral root initials.

As already mentioned, SL biosynthesis is not restricted to the roots, thus the tissue specific distributions of *ZmWBC33* and *ZmCCD8* were carried out also in aerial tissues (**Fig. 5A-B, panels IV, V**). The cross-sections of the vegetative shoot apexes show that they are expressed also in young leaves, with only slight differences between their patterns. Their expression was limited to the adaxial surface and the vascular bundles of young leaves (**Fig. 5A-B, panels IV, V**). In the aerial tissues, probe signal for *ZmCCD8* (**Fig. 5B, panels IV, V**) is more intense than *ZmWBC33* (**Fig. 5A, panels IV, V**), but *ZmWBC33* seems to be more localized in the phloem, xylem and vascular bundles. Hybridization signal was not detected in epidermis and mesophyll cells.

Figure 5: *In situ* hybridization of *ZmWBC33 (A) and ZmCCD8 (B)* gene in primary root and emerging lateral roots of 3 days old maize seedlings exposed to nitrate depletion (72h).

Hybridization signal is visible as red – purple precipitate. Longitudinal (panels I-III) and transversal (IV-VII) sections from the primary root region (panels I-III, VI and VII) and shoot apex (panels IV and V) were reacted with antisense digoxigenin-labeled probe for *ZmWBC33* (A) and *ZmCCD8* (B). The expression of *ZmWBC33* and *ZmWBCCD8* in emerging lateral root primordia (longitudinal section of primary root) are reported in panels III, A and B respectively. Hybridization with *ZmWBC33* and *ZmCCD8* gene-specific sense probes (negative control) are included in supplementary materials (**Supplementary Fig S3**).

Bars = 200 µm

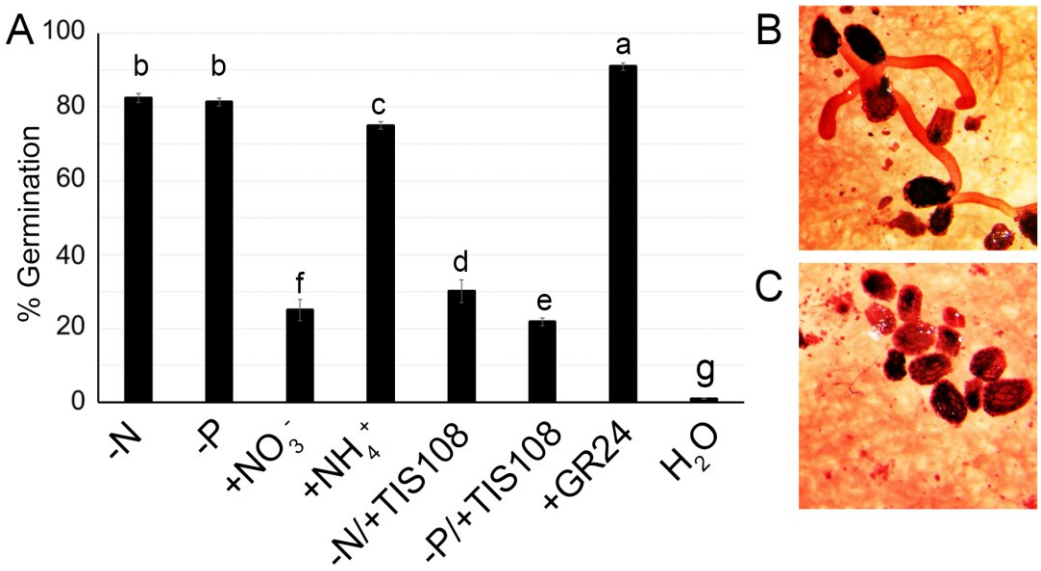


3.4. Exudates differently affect *Phelipanche ramosa* seed germination

To evaluate the effects of SL exuded by root on the rhizosphere an indirect assay was performed. Root exudates obtained from N-depleted, P-starved and ammonium-supplied roots triggered an appreciable induction of the germination of *P. ramosa* seeds (85%, 80% and 75% higher than in the negative control, respectively) **(Fig. 6A and B).** A similar effect was observed when seeds were supplied with GR24 (positive control). In contrast, when seeds were treated with exudates obtained from nitrate supplied plants only a slight (less than 25%) germination rate was observed with respect to the control. Furthermore, when TIS108 was provided to both N- and P-depleted roots, thus presumably inhibiting SL biosynthesis and exudation, only a weak germination (20%) of *P. ramosa* seeds was observed. As expected, no spontaneous germination could be observed when *P. ramosa* seeds were incubated only with the nutrient solution as a control (–data not shown).

Figure 6: ***Germination of*** **P. ramosa** ***seeds induced by root exudates of maize seedlings.***

Maize seedlings were grown 24 hours in a N-deprived nutrient solution and then transferred to a 1mM N-supplied media (+NO_3^- or +NH_4^+), to a N-deprived solution (-N) or to a N-deprived solution supplied with 2 µM TIS 108 (-N/TIS108), to a nitrate-supplied media plus GR24 (GR24) or water (H_2O) for additional 24 hours. Another pool of seedlings was grown in a phosphate deprived solution (-P) for 24 h and then transferred for additional 24 h in –P media (-P) or in a -P solution supplied with TIS108 2 µM (-P/TIS108). Root exudates were collected as reported by Pouvreau *et al*., (2013) and used to test the induction of germination in *Phelipanche ramosa* seeds. Each disk was treated with root exudates in triplicate. Germinated seeds were evidenced by Neutral Red staining and counted using a stereo microscope. The germination rate was expressed as mean percentage. Letters above the bars indicate different significance groups (P<0.05).

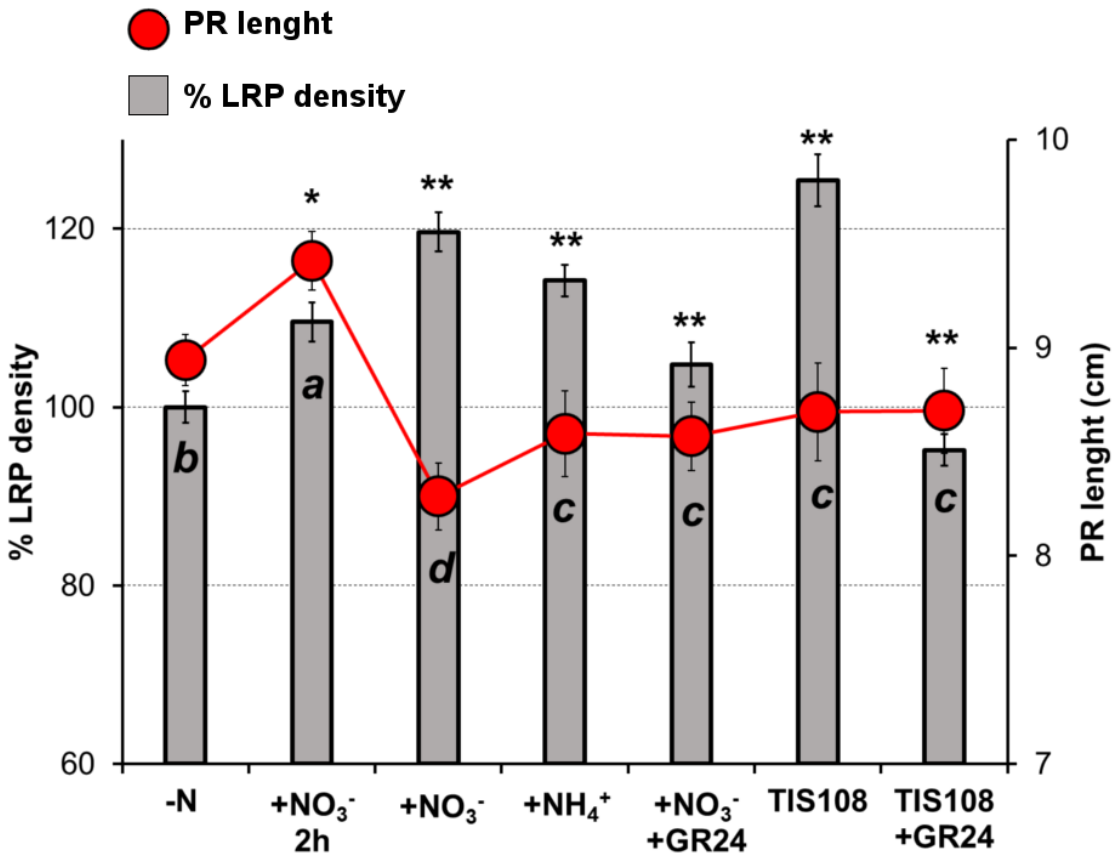


3.5. N-deficiency inhibition of LR development seems to involve SL signalling

The effects of N-deficiency, nitrate and ammonium supply in the presence of a SL biosynthesis inhibitor (TIS108) and of a synthetic SL analogue (GR24) on lateral root density (number of LRP/mm primary root) were evaluated (**Fig. 7**). When seedlings were moved from a N-free solution to a nitrate-supplied medium, the LRP density showed a significant increase (+10% already after 2 h and +20% after 24 h of nitrate supply, respectively). The length of primary roots (PR) showed an increase in the first 2 hours of nitrate supply (+15%) and a decrease in response to a more prolonged treatment (24 hours, -12%). Moreover, when TIS108 was supplied to N-deprived seedlings a significant increase of LRP density (+25%) and a slight decrease of primary root length (-7.5%) were observed, likely re-establishing the phenotype observed for nitrate supplied plants. A pattern similar to that observed after 24 hours of nitrate provision was noticed in response to ammonium, with a reduction of PR length (-8.5%) and a parallel increase of LRP density (+15%). On the contrary, seedlings supplied with a synthetic analogous of SL (GR24) (in the presence of nitrate) showed a lower LRP density (-15%) and a slightly longer PR length (+5%), thus resembling to N-deprived plants.

Finally, seedlings grown without N and supplied with both TIS108 and GR24 also displayed a phenotype similar to that observed for –N plants, thus supporting previous results.

Figure 7: Lateral root primordia (LRP) density and primary root length of maize seedlings exposed to different nitrogen provision. Maize-seedlings were grown in a N-depleted solution for 24 h and then transferred for: 24h in a nitrogen depleted solution (-N), or 2 h in a 1 mM nitrate supplied solution and for the remaining 22 h in nitrogen depleted solution ($+NO_3^-$ 2h), or 24 h in a 1 mM nitrate supplied solution ($+NO_3^-$), or for 24 h in 1 mM NH_4^+ (NH_4^+), or for 24 h in a 1 mM NO_3^- supplied solution plus 2 µM GR24 ($+NO_3^-$ +GR24) or 24h in a N-depleted media plus 2 µM TIS108 (TIS108) or 24h in a N-depleted media plus both 2 µM TIS108 and 2 µM GR24 (TIS108 + GR24). An hematoxylin staining was used to evidence the mitotic sites associated with the earliest stages of lateral root development. Data are expressed as increment of LRP density respect to the control (grey blocks, left axis). For every thesis, -N treatment was the control (100%). Results are presented as mean ± SE from three biological replicates for each treatment and an ANOVA statistic test was performed (* indicates significant differences with P<0.05; ** indicate significant differences with P<0.01). Red circles (right vertical axis) represent the primary root length recorded after each treatment. Results are presented as mean ± SE from three biological replicates for each treatment and an ANOVA statistic test was performed. Letters above the bars indicate different significance groups (P<0.05).



4. DISCUSSION

Nitrogen is a key element for crops but its availability in agricultural soils is limited and plants have developed strategies to adapt to its fluctuations. Nitrate represents the principal N form for crop, and it acts both as nutrient and signal, regulating many aspects of plant metabolism and development. Previous work led to the hypothesis of a coordinated action of NO, auxin and SLs in regulating the early response of maize root apex to nitrate (Trevisan *et al.,* 2015; Manoli *et al*., 2016). In this paper, further evidences on SL involvement in the signalling pathway governing root maize response to N were gained.

Phosphate deficiency has been demonstrated to be the optimal condition for the stimulation of SL production (Kapulnik and Koltai, 2016). However, in our growth condition, nitrogen deficiency is much more effective than phosphorous deficiency in stimulating SL accumulation in the exudates and triggers the exudation of significantly higher amounts of these compounds, if compared with either nitrate or ammonium supplied plants (**Fig. 1, Table 2, Supplementary Table S1, Supplementary Fig. S1**).

However, while 24 hours of nitrate supply are sufficient to totally switch down SL exudation, ammonium is less effective, or it needs more time to inhibit this process. This different behaviour of roots in response to different N forms could be motivated by the evidence that plants take advantage of mycorrhizal associations for NH_4^+ acquisition more than it does in the case of nitrate (Chalot *et al.,* 2016; Guether *et al*., 2009).

The trend of expression levels observed for Zm*CCD7* and Zm*CCD8* in response to N-starvation, or in the presence of either NO_3^- or NH_4^+ (**Fig. 2**) globally suggest that the accumulation of transcripts encoding *ZmCCD8* could represent a reliable and useful marker for SL biosynthesis also in maize, in accordance with the results obtained by Arite *et al*. (2007) in rice.

Until now, the only characterized SL transporters are the ABCG protein PDR1 from *Petunia axillaris* (Kretzschmar *et al*., 2012) and its close homologue PDR6 from *Nicotiana tabacum* (Xie *et al*., 2015). In contrast, no SL transporters have been isolated yet from Monocots or even from Arabidopsis. Among the transcripts expressed in TZ of maize roots and down-regulated by 2h of nitrate provision two genes encoding a maize homolog of PDR1 and a WBC transporter (*ZmWBC33*) respectively were identified (Trevisan *et al*., 2015). ZmWBC33 (**Supplementary Fig. S2, Supplementary Table S2**) is a member of the WBC subfamily of maize ABCG transporters (Pang *et al.*, 2013), named after the identification of the canonical WHITE-BROWN complex of *Drosophila melanogaster* (Ewart *et al*., 1994). The present

results show a marked induction of *ZmWBC33* transcription by nitrogen and phosphate deprivation, and a clear downregulation of its expression in the presence of nitrate and ammonium, whilst only slight regulation of the expression of *ZmPDR1* was observed in response to N supply or deprivation (**Fig. 3**). The expression profiles observed for *ZmCCD7*, *ZmCCD8* and *ZmWBC33* are consistent with the pattern of SL exudation detected by LC–MS/MS, and support the hypothesis that N deficiency triggers SL production and exudation, and that both nitrate and ammonium act as a negative signal to inhibit or reduce SL exudation. Moreover, according to the expression pattern observed for *ZmWBC33*, nitrate is more effective and rapid, while roots seem to require a more prolonged presence of ammonium to down-regulate its transcription, in accordance with the previously described trend of SL exudation. The transcription of both *ZmCCD8* and *ZmWBC33* was also strongly induced by P deprivation, which, in turn, did not affect *ZmPDR1* transcription.

SLs are synthesized in both the roots and shoots and are transported outside as exudates or acropetally, presumably in the xylem, to repress bud activity (Borghi *et al*., 2016). In shoots like in roots, SL biosynthetic tissues are spread along the vasculature or localized in specific organs. In the present work a detailed *in situ* localization of *ZmWBC33* and of *ZmCCD8* mRNA has been performed (**Fig. 5**). *ZmWBC33* mRNAs were detected in all surveyed tissues but preferentially in roots. In primary root *ZmWBC33* was shown to be lightly expressed in the meristem of the root tip, and it starts to accumulate in the epidermis and in cortical cells along the vasculature, included the stele of the transition and elongation zones. The same localization pattern was observed for PaPDR1 in Petunia (Sasse *et al*., 2015) and for NtPDR6 in Nicotiana (Xie *et al*., 2015). PDR1 exhibits an asymmetrical localization in petunia root tips leading authors to suppose that at least in this region of the root active cell-to-cell transport occurs. An analogous hypothesis was supported by recent work using fluorescent-tagged SL (Prandi *et al*., 2014; Fridlender *et al*., 2015). In our experiments *ZmWBC33* transcripts co-localizes with those of the SL-biosynthesis gene *ZmCCD8* in the stele, in the cortex and in the epidermis. Sasse and co-authors (2015) also showed that *PDR1* co-localizes with *CCD8* in the root tip of Petunia, and a similar pattern was observed also for *CCD8/MAX4* in *Arabidopsis thaliana* (Sorefan *et al*., 2003). These findings support our hypothesis that ZmWBC33 could be involved in the SL cell-to-cell flux in maize root.

In differentiated zone, *ZmWBC33* transcript was observed in root vascular tissues and in the apical meristem of LRP at different stages. *ZmCCD8* and *ZmWBC33* were localized in the vasculature also in shoots, confirming that SL synthesis takes place also in the aerial part (Lopez-Obando *et al*., 2015) and

supporting the hypothesis that *ZmWBC33* could be involved in the transport of SL out of the leaves, either to the lateral buds or to the main stem.

In the root, *ZmWBC33* could regulate SL accumulation in the meristem, which was suggested to be highly sensitive to alterations in strigolactones concentration (Ruyter-Spira *et al*., 2011). Out of the root tip, the *ZmWBC33* localization in the vasculature seems to suggest that it might contribute to loading SL into the xylem thus contributing to translocation to shoot and to coordination of shoot and root growth. Moreover, *ZmWBC33* specific localization in the epidermis of the root tip and root elongation zone may suggest its involvement in the root secretion processes.

In the LRP and the aerial tissues ZmWBC33 could redistribute the SL produced *in loco* by ZmCCD8, changing SL homeostasis and participating in lateral root and shoot development.

Major questions for the future concern ZmWBC33 structure-function relationships, which will require the elucidation of its three-dimensional structures and multisubstrate binding properties. Specifically, it will be interesting to investigate if ZmWBC33 directly transports SLs, identify the specific substrate(s), recognize the identity of a putative dimerization partner and test how ZmWBC33 activity is regulated in response to environmental conditions that prompt changes in growth.

To try to better decipher how N availability affects few SLs-mediated functions the effects of root exudates on the germination rate of *Phelipanche ramosa* were observed (**Fig. 6**). Exudates obtained by N-deprived and NH_4^+-supplied roots significantly induced this species to germinate, signifying that even if ammonium considerably constrain SL production (**Fig. 1, Table 2, Supplementary Table S1, Supplementary Fig. S1)**, the amount of these compounds is still sufficient to exert their stimulatory action on *Phelipanche* germination and conceivably on mycorrhizal partners, confirming the already known extraordinary sensibility for SLs of parasitic species (Boari *et al.,* 2016). On the contrary, upon nitrate supply, that drastically inhibits SL exudation, and when TIS108 was supplied to N-deprived roots, very low or no germination was observed, further indirectly confirming the presence of SLs in the exudates harvested from N-deprived plants.

SLs are crucial molecules not only for root-soil communication but also as endogenous signals in regulating whole plant development (Koltai and Beveridge, 2013; Agusti *et al*., 2011; Ueda and Kusaba, 2015). Lateral root development has considerable biological and agronomical relevance in the overall response to nitrogen (York *et al*., 2016). Few discoveries highlighted the complexity of the molecular networks modulating the plasticity of LR formation in response to nutrients in cereals (reviewed in Yu *et al*., 2016). SLs seem to control lateral root development (Kapulnik and Koltai, 2014, Koltai *et al*.,

2010) depending on auxin levels (Ruyter-Spira *et al*., 2011) and possibly also through a cytokinin-auxin feedback loop (Jiang *et al*., 2016). However, until now no evidence on the SL participation to the pathway through which N influence LR development have been reported. In Arabidopsis 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).

The present results indicate that nitrate and ammonium supply to maize seedlings previously grown in a minus N solution noticeably stimulate LR development (**Fig. 7**). Nevertheless, when GR24 was supplied together with nitrate, the -N phenotype was re-established. This phenotype could not be attributed entirely to an SL effect, because rac-GR24 is known to activate responses that are specific to naturally occurring SLs and responses that are not, such as KAI2 pathway (Scaffidi *et al*., 2014). However, provision of TIS108 (which inhibits SL biosynthesis) to N-starved plants, completely restored the +N phenotypes. In addition, GR24 was also used to complement the root phenotype with TIS108, showing that the complementation assay led to a phenotype similar to N-deprived plants. Even if maize produces in large amount non-canonical SLs (Charnikhova *et al*., 2017) that can contribute to different responses *in vivo*, while GR24 is a canonical SL, GR24 itself is still the most widely used synthetic SL for bioassay (Zwanenburg *et al*., 2016). Nevertheless, non-canonical SLs such as zealactones would be the most appropriate choice, even though they are still scarcely available. Taken together, these data seem to suggest that the stimulation of lateral root development could be linked, at least in part, to the complete or partial inhibition of SL production observed in response to nitrate and ammonium respectively.

In a recent study Koltai and co-authors (2015) reported that the strigolactone-signalling pathway affects auxin transport, cellular trafficking and PIN polar localization in the plasma membrane. Moreover, PDR1 overexpression was demonstrated to influence auxin transport/allocation in several tissues of Petunia (Liu *et al*., 2018). In maize roots of seedlings grown in a N-deprived solution for 24 hours a reallocation of PINs by cytoskeleton remodelling was observed already after two hours of nitrate provision (Manoli *et al*., 2016). This study suggests that nitrate induces fast NO burst, which impairs SLs levels resulting in both PIN-dependent auxin re-distribution and cell elongation, thus providing a hypothetical model of how NO, auxin and SLs may cooperate in regulating the early response of maize root apex to nitrate.

The interplay existing between SLs and NO has been deeply reviewed by Kolbert (2018) and many papers reported the link between auxin and nitrate in the control of root development in

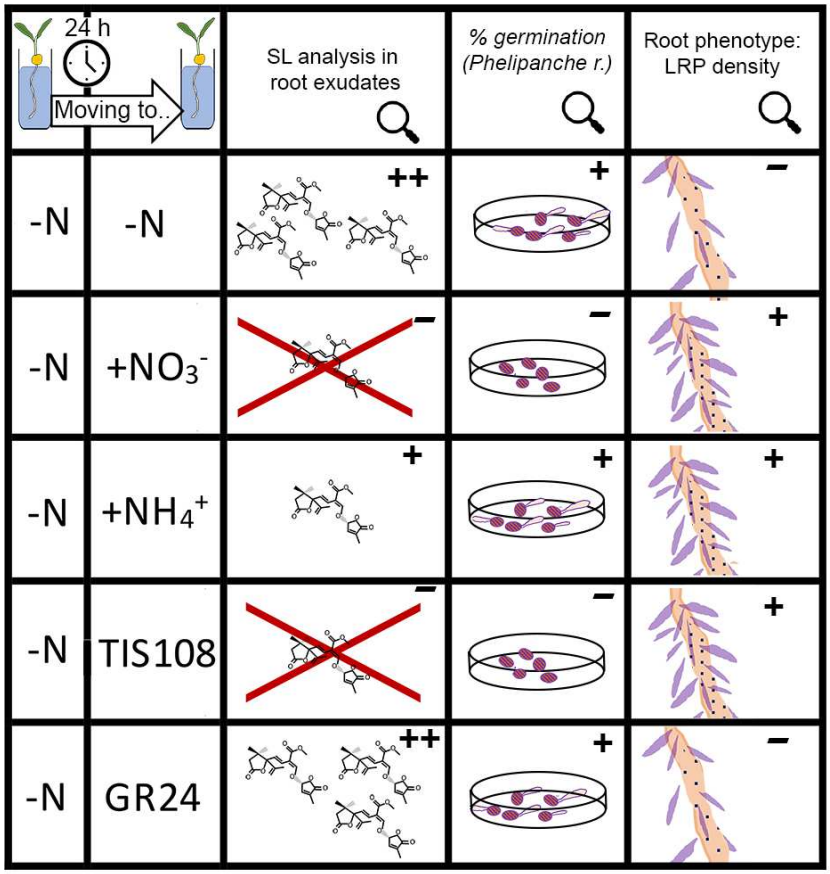
Arabidopsis (for example Krouk *et al*., 2010; Mounier *et al*., 2014), but only few information is available for maize (Sun *et al*., 2017). The present results allow to include also SLs among the key components of the response of maize root to N, but further evidence needs to be provided to precisely clarify the exact interaction among N, auxin and SLs in the regulation of lateral root development.

In conclusion (**Fig. 8**), this study demonstrates that N-deficiency strongly induces SL exudation in maize roots and that nitrate rapidly switches off SL exudation. Moreover, ammonium reduces SL exudation by roots but less markedly in comparison to nitrate, thus likely allowing root to continue to establish mycorrhizal associations. However, the decrease of SL production observed in response to both these ions would seem to contribute to the signalling pathway underlying lateral root development in response to N.

Furthermore, a putative novel component of the maize SL transport machinery has been identified, even though further functional studies are mandatory to gain new insight into the WBC33 actual role.

A more precise knowledge of the SL involvement in the integration of information on N availability and hormonal signalling to regulate the maize root plasticity to nutritional stresses could be of great interest both for root biology research and for the possible applications of these molecules in agriculture.

Figure 8: Scheme of the proposed model for the role of SLs in the response of maize seedlings to different nitrogen sources. Maize seedlings were grown for 24h in a nitrogen depleted solution, and then they are moved to a different media, according to the absence (-N) or the presence (+NO_3^- or NH_4^+) of nitrogen sources. To better decipher the role of strigolactones, an SL biosynthesis inhibitor (TIS108) or a synthetic SLs (GR24) were added to the growth media (N-depleted or NO_3^- -supplied respectively). LC-MS/MS, MRM SLs quantification showed a significantly higher content of a putative maize zealactone in exudates obtained by N-deprived roots, whilst nitrate and ammonium provision switches off SLs exudation, even though the ammonium effect appeared less incisive respect to nitrate. The presence of a minimal amounts of SLs in exudate of ammonium-treated roots would seem to enable plants to establish a relationship with their neighbours, as confirmed by the *P. ramosa* germination rate, which was on the contrary almost completely inhibited in the presence of exudates derived from nitrate-supplied roots. Furthermore, LRP density in response to N-deprivation, nitrate or ammonium supply, TIS108 or GR24 provision led to the hypothesis that the decrease of SLs content observed in response to both nitrate and ammonium would contribute to the signalling pathway underlying lateral root development in response to N.

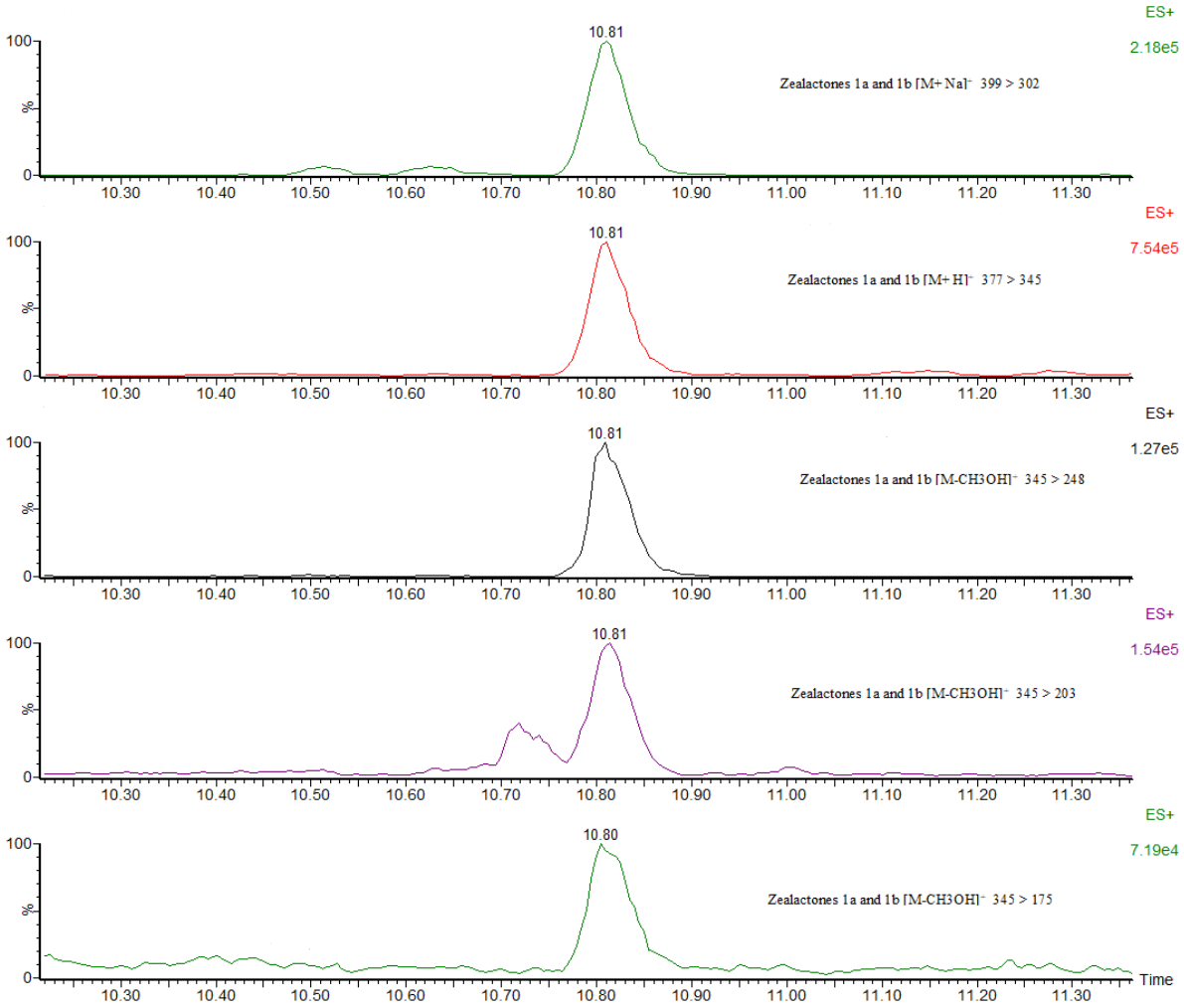


5. SUPPLEMENTARY DATA

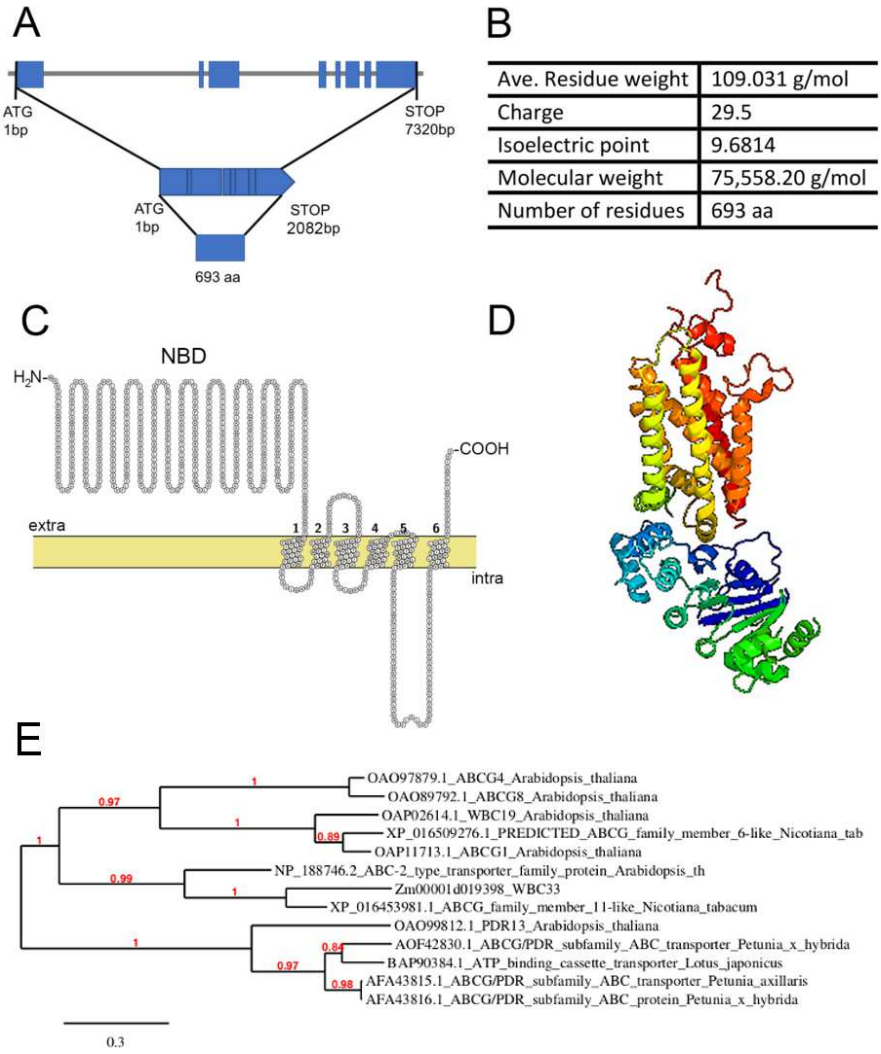
Supplementary Table S1: **MRM transitions monitored for the SLs screening according to bibliography or deduced of literature.** (Available online at https://doi.org/10.1093/pcp/pcz108)

Supplementary Table S2: Bioinformatics analysis of *ZmWBC33* promoter. (Available online at https://doi.org/10.1093/pcp/pcz108)

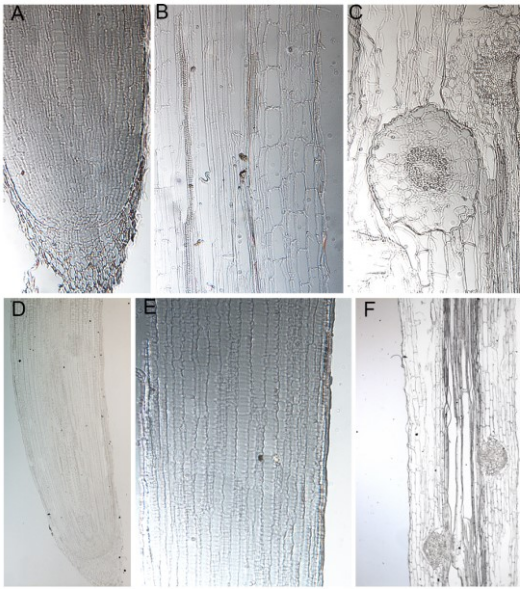
Supplementary Figure S1: MRM chromatogram of root exudate from maize seedlings.



Supplementary Figure S2: Nucleotide and deduced amino acid sequence information about ZmWBC33 gene and protein, with a phylogenetic tree of PDR1 protein sequence homologs.



Supplementary Figure S3: *In situ* hybridization of primary root and emerging lateral roots of 3 days old maize seedlings exposed to nitrate depletion (72h) with sense digoxigenin-labeled probe for *ZmWBC33* and *ZmCCD8.*



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Disclosures

Conflicts of interest: No conflicts of interest declared

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Chapter 3

THE MAIZE ROOT RESPONSE TO NITROGEN FLUCTUATIONS INVOLVES A COORDINATED ROLE FOR STRIGOLACTONES AND AUXIN

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FOCUS: Results reported in chapter 2 led us to hypothesise the existence of a link between the stimulation of lateral root (LR) development, and the strong inhibition of strigolactones (SLs) production observed in response to nitrate. Then, the new question: where and how is auxin involved in this LR pathways? To deepen the involvement of auxin in the SL-mediated regulation of maize root development in response to nitrate availability, some experiments were performed.

1. INTRODUCTION

Nitrogen (N) acts as a signal in regulating plant development in response to the environment. Plants can uptake N in the soil in different N-containing forms, but nitrate (NO_3^-) represent the major N source for crops in aerobic environments (Miller and Cramer, 2004; Gojon, 2017). Nitrate acts not only as a nutrient but also as a signalling molecule, regulating many developmental processes in plants (Bouguyon *et al*., 2012; Undurraga *et al*., 2017).

The root represents the crucial plant organ for N perception and uptake (Kiba and Krapp, 2016). In monocots cereals, such as maize, a complex root system is developed, including, besides primary root (PR) and lateral root (LR), a shoot-borne system of crown and seminal roots (CR and SR, respectively) (Smith and De Smet, 2012). PR is the first root to emerge, deriving from embryonically derived meristematic tissue, while LR formation is a post-embryonic event. Generally, LR are more sensitive to variations in nitrate levels than PR (Tian *et al*., 2014), but the pathway regulating LR development in response to this nutrient is quite complicated (Sun *et al*., 2017 and references therein), differing between plant species, genotype and being also subject to environment (Yu *et al*., 2015a; Xuan *et al*., 2017).

The modulation of LR development in response to nutrient availability in cereals is highly complex (Bray and Topp, 2018 and references therein) and, up to now, only a few lateral root mutants have been described in this group of plants and they are generally auxin-related (Hochholdinger and Tuberosa, 2009; Atkinson *et al*., 2014; Yu *et al*., 2018). For instance, the maize *rum1* encodes the monocot-specific Aux/IAA10 protein (von Behrens e*t al*., 2011). RUM1/IAA10 specifically interacts with RAP1, a homolog of AtSPR1 which is similar to a nitrilase-associated microtubule-localized protein (Nakajima *et al*., 2004), and probably also with the six maize members of its homolog protein family RAL (Zhang *et al*., 2016). *rul1/IAA29* is the homolog of *rum1/IAA10*, resulting from an ancient maize genome duplication (Zhang *et al*., 2016). Accordingly, the expression of auxin-responsive genes is mediated by two types of proteins: the Auxin Response Factors (ARFs) that can activate or repress transcription by a direct binding to DNA, and the transcriptional repressors Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) (Guilfoyle and Hagen, 2007; Ludwig *et al*., 2013). Aux/IAA protein interacts *in vivo* with ARFs protein, regulating lateral and seminal roots formation probably blocking LR formation in non-precursor pericycle cells (Taylor-Teeples *et al*., 2016). Downstream, the control of LR initiation via the Aux/IAA-ARF-dependent auxin signalling module could involve the transcription factor NAC1 (Zhang *et al.,* 2014), the heat shock protein of

101 kDa (HSP101) (Martínez-de la Cruz *et al*., 2015), and a group of three PLETHORA transcription factors (PLT1, BBM1 and HSCF1), which belong to the AP2/EREBP (Apetala2/Ethylene-Responsive Element Binding Protein) family (Aida *et al.*, 2004). Besides, maize auxin-dependent signalling in root development included *rtcs1 (rootless concerning crown and seminal roots 1),* also called *LBD2*, which encodes a member of the plant-specific LATERAL ORGAN BOUNDARIES DOMAIN (LBD) transcription factor family (Tai *et al*., 2017). The LBD protein family acts in defining organ boundaries and is involved in a variety of plant developmental processes (Majer and Hochholdinger, 2011).

Formation of LR primordia (LRP) is driven by the coordinated division of cells and controlled by the careful regulation of cell cycle genes in pericycle cells, such as the cell cycle activators of the *CDKB* and *CYCB* classes (de Almeida Engler *et al*., 2009). Accordingly, it was suggested that in maize nitrate supply induces early pericycle cell divisions by modulating mitosis-specific cell cycle progression, thus increasing the number of emerged LR in an auxin-dependent way (Yu *et al*., 2015b).

A correct polar auxin transport (PAT) is important to control auxin concentration in the root tip, regulating root morphology and development (Habets and Offringa, 2014). Basipetal auxin transport is facilitated by PIN auxin efflux carriers in response to local nitrate supply (Yu *et al*., 2016). For instance, monocot-specific *ZmPIN9* gene in phloem pole cells of shoot-borne roots modulates auxin efflux to pericycle cells and subsequent cell cycle activation (Yu *et al*., 2019), while ZmPIN1a, ZmPIN1b, and ZmPIN1c are orthologs of AtPIN1 (Forestan *et al*., 2012). In addition, in Arabidopsis the auxin influx carriers AUX1 and LAX3 are involved in LR initiation (Marchant *et al*., 2002), as was proposed for the maize LAX1 and LAX2 (Zhang *et al*., 2014).

Recently, it was hypothesized that auxin and strigolactones (SLs) could take part to the complex pathway governing maize root adaptation to different N availabilities (Trevisan *et al*., 2015; Manoli *et al*., 2016). Moreover, it was demonstrated that N deficiency triggers the exudation of maize typical SLs, namely zealactones, while nitrate strongly and early prevents the process (Ravazzolo *et al*., 2019). In the same paper, it has also been hypothesised that the reduction of SL levels observed in response to both these ions would participate in the complex signalling pathway leading to LR developmental response.

In the present work, the involvement of auxin in the SL-mediated regulation of maize LR development in response to nitrate availability was evaluated. LR density was measured in the presence of various SL and auxin analogues or inhibitors, and the expression of several genes encoding key components

of auxin signalling were measured upon nitrate supply and in the presence of the same auxin and SLs analogues or inhibitors. The results suggest that SLs and auxin share overlapping and divergent pathways to regulate maize LR development in response to nitrate availability and future work will be aimed at better investigate all these aspects.

2. MATERIALS AND METHODS

2.1. Maize growth conditions

Seeds of the maize inbred line B73 (*Zea mays* L.) were germinated as described by Manoli *et al*. (2014). After germination, seedlings were grown for 24h in a N-deprived solution (-N) and then transferred to 14 different treatments, as reported in **Table 1**: N-deprived solution (1), 1 mM nitrate-supplied media (2), nitrate-supplied solution with 10 µM PCIB (3), N-deprived solution supplied with 0.01 µM NAA (4); N-deprived solution supplied with 0.05 µM NAA (5); N-deprived solution supplied with 0.1 µM NAA (6); N-deprived solution supplied with 1 µM NAA (7); N-deprived solution supplied with 0.01 µM NAA and 10 µM PCIB (8); nitrate-supplied solution with 2 µM GR24 (9); nitrate-supplied solution with 2 µM GR24 and 0.01 µM NAA (10); nitrate-supplied solution with 2 µM GR24 and 0.05 µM NAA (11); nitrate-supplied solution with 2 µM GR24 and 0.1 µM NAA (12); N-deprived solution supplied with 2 µM TIS108 (13), and N-deprived solution supplied with 2 µM TIS108 and 10 µM PCIB (14).

Only for the SL quantification on root tissue, the effect of phosphate availability was performed as positive control by growing seedlings in a P-deprived solution (-P) for 48 h.

Table 1: List of treatments used in the study

ID treatment	Treatment description	Aim of the treatment
1	**-N**	nitrogen-deprived nutrient solution (**negative control**)
2	**+NO_3^-** (1 mM)	nitrate-supplied nutrient solution (**positive control**)
3	**+NO_3^-** (1 mM) **+PCIB** (10 µM)	inhibition of auxin signalling
4	**-N+NAA** (**0.01** µM)	provision of a synthetic auxin
5	**-N+NAA** (**0.05** µM)	provision of a synthetic auxin
6	**-N+NAA** (**0.1** µM)	provision of a synthetic auxin
7	**-N+NAA** (**1** µM)	provision of a synthetic auxin
8	**-N+NAA** (0.01 µM) **+PCIB** (10 µM)	provision of a synthetic auxin but inhibition of auxin signalling
9	**+NO3-** (1 mM) **+GR24** (2 µM)	provision of a synthetic strigolactone analogue
10	**+NO3-** (1 mM) **+GR24** (2 µM) **+NAA** (**0.01** µM)	provision of a synthetic strigolactone analogue and of a synthetic auxin
11	**+NO3-** (1 mM) **+GR24** (2 µM) **+NAA** (**0.05** µM)	provision of a synthetic strigolactone analogue and of a synthetic auxin
12	**+NO3-** (1 mM) **+GR24** (2 µM) **+NAA** (**0.1** µM)	provision of a synthetic strigolactone analogue and of a synthetic auxin
13	**-N +TIS108** (2 µM)	inhibition of strigolactones biosynthesis
14	**-N +TIS108** (2 µM) **+PCIB** (10 µM)	inhibition of strigolactones biosynthesis and of auxin signalling

6-phenoxy-1-phenyl-2-(1H-1,2,4-triazol-1-yl) hexan-1-one (TIS108) and $GR24^{5DS}$ (Strigolab, Torino, Italy) were used as inhibitor of SL biosynthesis (Ito *et al*.,2011) and synthetic SL analogue, respectively. p-chlorophenoxyisobutyric acid (PCIB) and 1-naphthaleneacetic acid (NAA) were used as auxin signalling inhibitor (Oono *et al*., 2003) and synthetic auxin analogue, respectively. Unless stated otherwise, all chemicals were obtained from Sigma Chemicals (Sigma, St Louis, MO, USA).
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 was used.

2.2. SL identification and quantification in root tissue

After germination, seedlings were grown for 24h in a N-deprived solution (-N) and then transferred for 24 h to -N (negative control) or 1 mM NO_3^-. The effect of phosphate availability was also performed by growing seedlings in a P-deprived solution (-P) for 48 h. All root system was sampled from 10 seedlings for every treatment, in three independent biological repetitions, and immediately frozen and powdered in liquid nitrogen. Following extraction, the analytes were quantified by analysis using LC-MS/MS, MRM mode, as described by Ravazzolo *et al*. (2019). Data are reported as mean ± SE of three replicates.

2.3. Lateral root analysis

Seedlings were grown for 24 h in the N-deficient solution and then transferred in 14 different nutrient solutions for 24 h, as described in **Table 1**.
To better visualize LRP a haematoxylin staining solution supplied with ferric ammonium sulphate was used, as described by Ravazzolo *et al*. (2019). Root images were collected using a flatbed scanner. The lateral root density was measured using the Image J Image Analysis Software and the LR density was expressed as percentage compared to the value observed for nitrate-provided roots (positive control; treatment ID = 2). Three biological replicates for each treatment and an ANOVA statistic test were performed (n=30).

2.4. RNA extraction and cDNA synthesis

After 24 h in the equivalent treatment, each whole root was sampled from 15 to 20 pooled seedlings for treatment, in three independent biological repetitions, and immediately frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific,

Waltham, MA USA) as previously described by Trevisan *et al.* (2011). RNA was quantified with a Nanodrop1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA) and reverse transcribed to cDNA as described by Manoli *et al.* (2012).

2.5. Quantitative reverse transcription PCR

qRT-PCR was performed using the StepOne Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) as described by Nonis *et al*. (2007). SYBR Green reagent (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) was used in the reaction, according to the manufacturer's instructions. Melting-curve analysis confirmed the absence of multiple products and primer dimers. Target gene relative expression was determined according to the Livak and Schmittgen (2001) method, using *MEP* (membrane protein PB1A10.07c, Zm00001d018359) as reference gene, according to Manoli *et al.* (2012). Primers were designed using Primer3 web tool (version 4.0.0; http://bioinfo.ut.ee/primer3/; Rozen and Skaletsky, 2000) and further verified with the PRATO web tool (Nonis *et al*., 2011). The list of genes and of the primers used are reported in **Table 2.**

These genes were divided into four subgroups according to their main functions (**Table 2**):

- Group 1: regulators of cell cycle connected with auxin-signalling **(Fig. 3);**
- Group 2: Aux/IAA-ARF-dependent auxin signalling module **(Fig. 4);**
- Group 3: regulators of auxin response in root development **(Fig. 5)**;
- Group 4: auxin carriers **(Fig. 6).**

Three technical replicates were performed on three independent biological repetitions.

Table 2: List of genes and primers used in the study

Group	Name	Accession B73v3	Accession B73v4	Primers Sequence 5´-3´	Description	References
GROUP 1	***CYCB1;1***	GRMZM2G310115	Zm00001d012560	CGTCTATGTTTGCTTGTCGGG	Cyclins, cell cycle activators of CYCB class involved in auxin-dependent cell progression	Yu et al., 2015
				TGAAACACAACATGCCATCACA		
	CYCB1;4	GRMZM2G034647	Zm00001d010656	TACGCGACGGAGCAGTTT	Cyclins, cell cycle activator involved in nitrate cell cycle progression during lateral root initiation, also called cyc1	Yu *et al.*, 2016
				AGCGAACTCAACCAAATCCC		
	CYCB2;3	GRMZM2G073671	Zm00001d036360	TCCGCTCCTCCTCCATCATA	Cyclins, cell cycle activator involved in nitrate cell cycle progression during lateral root initiation, also called cyc3	
				GATTCTCCATTGCCGACGC		
	CDKB1;1	GRMZM2G495626	Zm00001d044672	CAAGTCCCAGTTCTAGCCGT	Cyclin Dependent Kinases, cell cycle activators of CDKB class involved in auxin-dependent cell progression	Yu et al., 2015
				TCATGAACCAGTTGCCTAGATG		
	CDKB2;1	GRMZM2G068193	Zm00001d031485	GTGTCGTGTGTGCTGTGAAC	Cyclin Dependent Kinases, cell cycle activators of CDKB class involved in auxin-dependent cell progression	
				AGAGTGACGAACCTTGCCTG		
GROUP 2	***IAA5***	GRMZM2G004696	Zm00001d039624	GGGAACTCTGCTGGTCTTGA	Auxin Aux/IAA transcriptional repressors of downstream auxin-regulated genes with high expression in maize roots	Ludwig *et al.*, 2013
				GTTCGGTGCTGTCTGGTCTT		
	IAA10	GRMZM2G037368	Zm00001d043878	TGCTGTTCTTGTGCCCTTTG	Auxin Aux/IAA transcriptional repressors, also called RUM1 (Rootless with Undetectable Meristem 1)	von Behrens *et al.*, 2011
				GGGCAGCTCTCTTGACATCA		
	IAA14	GRMZM2G077356	Zm00001d013302	GTGCAAGAACAAGAGCTGAAGA	Auxin Aux/IAA transcriptional repressors of downstream auxin-regulated genes with high expression in maize roots	Ludwig *et al.*, 2013
				TCTGGTCCATGAACGAGTTGA		
	IAA15	GRMZM2G128421	Zm00001d013707	GGGATCGGCGAGACAATGAA		
				CACCCACCAAGCTCATCCTT		
	IAA19	GRMZM2G079200	Zm00001d036918	CGGCAGTGATTGTTGTTGGT		
				CGGTGTATGACGTGTGCATG		
	IAA29	GRMZM2G163848	Zm00001d011588	ACAAACAAGGGACGGTGAGC	Maize Aux/IAA protein, also called RUL1 (RUM1-like)	
				TGGGCGAGAGAGAGAAGAGG		

	ARF8	GRMZM2G034840	Zm00001d001945	GAAACGGCAACATCTCGCTT	Auxin response factors (ARFs) repressed by Aux/IAA binding; their closest homologue in Arabidopsis is *MP/ARF5*, which regulates vascular development in root	Zhang *et al*., 2014
				CCCCGGCGAATCTATCCAAT		
	ARF37	GRMZM2G086949	Zm00001d026540	TGCGTCAAATTCTCTGGTCG		
				CACGTCCGTTAGCTCGATTC		
GROUP 3	***RAP1***	GRMZM2G124317	Zm00001d047282	ACACTGGCAACTTCCTTACG	RUM1 Associated Protein 1 (Protein SPIRAL1), interacts with RUM1	Zhang *et al*., 2016
				CGGTTGTTCACGATTCAGAC		
	RAL3	GRMZM2G165461	Zm00001d048795	GCCAACCGCATCTGTAATTT	RAP1-like protein (Protein SPIRAL1)	
				ACTAGTCCAGCAGGCGAAAG		
	LBD2	GRMZM2G092542	Zm00001d027679	AGAGAGATGACGGGGTTCG	Lateral Organ Boundaries Domain-containing protein; also called RTCS1	Xu *et al*., 2015
				CCTCGTAGGAGATGGTGACG		
	NAC1	GRMZM2G081930	Zm00001d003052	CACCAACTGCAACAACGCC	Maize NAC domain-containing TF, homologue of *AtNAC1* which controls lateral root initiation via Aux/IAA-ARF-dependent signalling	Zhang *et al*., 2014
				ACCTCACACTACGTACCTACTG		
	HSP101	GRMZM2G360681	Zm00001d038806	TGAATAAGCGTGTAGATAAGC	Heat Shock Protein 101 that mediates auxin responses in maize crown root	Martínez -de la Cruz *et al*. 2015
				TATGAGAGCATTCGTCCAT		
	PLT1	GRMZM2G141638	Zm00001d042492	CTGAAGGGTAGGGTGATGGC	AP2/EREBP TF, orthologues of *AtBBM* which control adventitious root formation	Zhang *et al*., 2014
				GCTTGCCGGTTCAATTTCCT		
	BBM1	GRMZM2G366434	Zm00001d017207	GTCATCGTTGTCGTGTTT		
				ACAGCACATCAGGATAACT		
	HSCF1	GRMZM2G139082	Zm00001d046913	CCACTACTACAACTATCTCTTC	Heat Shock Complementing Factor 1, AP2/EREBP TF, homolog of *AtPLT3/AtPLT7* involved in PIN1 regulation	
				CGTGAATAGCAAGCAGAA		
GROUP 4	***PIN1A***	GRMZM2G098643	Zm00001d044812	GGTTTCGGCTTCAGATCAGGCGGCAAG	Maize PIN-formed (PIN) auxin efflux carriers mainly involved in root polar auxin transport (PAT)	Forestan *et al*., 2012
				GCCACGACCGCCGGCATGGAGATGTG		
	PIN1B	GRMZM2G074267	Zm00001d018024	ACGAAGCTGCGGGAGCCGTTC		
				CTCCGCCTGGCTGCCGCACAGGGACA		
	PIN1C	GRMZM2G149184	Zm00001d052269	CCAGGGGGGACGTCGAGCTCGAGGCC		
				CCTCGCCGTCGTCCTTGGGGTAC		

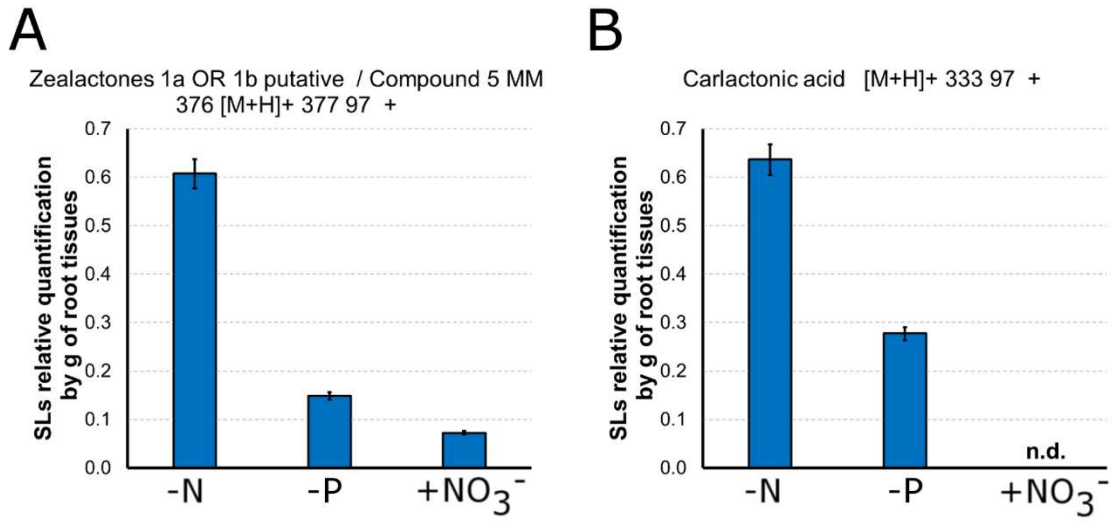
	PIN2	AZM5_105354 AZM5_3988	Zm00001d046893	AGGTGGCCAACAAGTTCGCGTCTGGG	Maize PIN-formed (PIN) auxin efflux carriers mainly involved in root polar auxin transport (PAT)	Forestan *et al*., 2012
				CCTTCTTGCGCGGGGCCACGTACG		
	PIN5C	GRMZM2G040911	Zm00001d006082	GCGGCGGCAAGGGCAAGGACAGAG		
				TGTGACCAGCGGCCATAACGACTGAGCC		
	PIN8	GRMZM5G839411	Zm00001d043660	GCACCATAACATCTTGCCGAGACTCCT		
				AGGAGTCTCGGCAAGATGTTATGGTGC		
	PIN9	GRMZM5G859099	Zm00001d043179	CACCGTCGCCTCGCTCTCCATGCTCC		
				GGAGCATGGAGAGCGAGGCGACGGTG		
	LAX1	GRMZM2G129413	Zm00001d030310	CGAGTTTGCTCCACAGTAGTT	Like-Aux1, maize auxin influx carrier, probably involved in lateral root development	Zhang *et al*., 2014
				TACAACAGGGCATTTGACGA		
	LAX2	GRMZM2G149481	Zm00001d028401	ATGAGAGACGATGAAATGAAC		
				TAGAGGAGAGAACGAAGATG		

3. RESULTS AND DISCUSSION

3.1 N-starvation specifically increases the amount of SLs detectable in root tissue

To confirm the hypothesis that N-deficiency triggers the biosynthesis of SL in maize root tissues, the content of SLs in roots of N-deprived, or nitrate-supplied or P-deprived maize seedlings was measured by means of LC-MS/MS (**Fig. 1**). The putative zealactone isomer was detected at a significant level (0.15 ng equivalent GR24 per g root tissue) in samples obtained from phosphate-starved seedlings, which were utilized as a positive control for SL exudation. Not surprisingly, this compound was detected at a much higher level (0.6 ng eq GR24/g root) in nitrogen-starved samples. In contrast, nitrate-supplied samples contained very low zealactone isomer (0.07 ng eq GR24/g root), indicating a clear inhibitory effect of nitrate on zealactone production (**Fig. 1A**). Accordingly, zealactone could be the typical signature for N-deprivation, whereas carlactonic acid appears to be highly produced even in -P conditions (**Fig. 1B**). Nevertheless, the production of both appeared strongly impeded in nitrate-supplied plants. These results clearly confirm the hypothesis that zealactone production is a clear response to N-deprivation, indicating that the increase of SLs exudation observed in **Chap. 2** mainly depends on an increase in their biosynthesis and further support the role of *ZmCCD8* as a reliable marker for SL biosynthesis (Ravazzolo *et al*., 2019).

Figure 1. SL quantification on root tissue. Quantitative analysis of the relative amounts of putative zealactone forms (**A**) and carlactonic acid (**B**) in maize root tissues [ng*(g root FW)$^{-1}$] of seedlings exposed to additional 24h of nitrate (+NO_3^-) or N-starvation (-N) after a 24h-pre-incubation under N-deficient conditions. Quantification in root tissues of phosphate-starved seedlings (-P) was included as positive control. The root tissues were collected after 24 h of each treatment and immediately shock-frozen in liquid nitrogen. Following extraction, the analytes were quantified by analysis using of LC-MS/MS, MRM mode, as described by Ravazzolo *et al*. (2019). Values are mean ± SE of three replicates. n.d.: non-detected



3.2 SLs and auxin share overlapping and divergent pathways to regulates maize lateral root development in response to nitrate availability

Lateral root (LR) density (number of LRP/mm primary root) was measured in the presence of various SL and auxin analogues (GR24 2 µM and NAA 0.01-0.05-0.1-1 µM, respectively) or inhibitors (TIS108 2 µM and PCIB 10 µM, respectively) for 24 h (**Fig. 2; Table 1**). The effects of N-deficiency and NO_3^- provision were also assessed as negative and positive control, respectively.

Globally, results obtained support the hypothesis that the response of maize roots to nitrate availability could depend on a sophisticated interplay between auxin and SLs.

Accordingly, when seedlings were moved from a N-free solution to a nitrate-supplied medium (treatment 2), the LRP density showed a significant increase (+22%), thus confirming the results obtained in **Chap. 2**. On the contrary, when nitrate-fed seedlings were supplied with the auxin signalling inhibitor (PCIB, treatment 3), a significant reduction in LRP number was observed if compared to nitrate-provided plants alone (-13%), thus mimicking the phenotype of N-deprived plant. Therefore, the LR development in response to nitrate provision appeared to involve auxin

signalling. In particular, when N-deprived seedlings were supplied with increasing NAA concentrations (treatments 4 to 7) the phenotype of nitrate-supplied plants was partially re-established, and the lowest NAA concentration (0.01 µM NAA, treatment 4) was sufficient to increase the LRP density of 15% if compared to N-deprived plants (treatment 1). Nevertheless, when N-deficient seedlings were provided with both auxin analogue (0.01 µM NAA) and auxin signalling inhibitor (PCIB) (treatment 8), the LR density dropped down of -32% if compared to the positive control of nitrate-supplied plants (treatment 2). Since PCIB was not able to completely inhibit LR development if provided to nitrate-fed plants (treatment 3), while it has a deep negative impact if provided together with NAA to N-deficient seedlings (treatment 8), it could be speculated that, besides auxin, another player might be involved in the LR development regulation in response to nitrate-provision and probably placed before auxin in the signalling pathway.

In **Chap. 2** we showed that SLs are involved in the negative regulation of LR development, and herein we confirmed those results by results for treatment 9 and treatment 13. Accordingly, seedlings supplied with a synthetic analogous of SLs ($GR24^{5DS}$, treatment 9) in the presence of nitrate showed the lowest LRP density (-36%), but when the SL biosynthesis inhibitor (TIS108, treatment 13) was supplied to N-deprived seedlings, a significant increase of LRP density (+24%) was observed, likely re-establishing the phenotype observed for nitrate supplied plants. To deepen if SLs could be the player involved together with auxin in the nitrate-dependent LR development, GR24 was provided together with increasing NAA concentrations to nitrate-supplied seedlings (treatment 10 to 12). The results showed an increase of 26% in LR density if compared to seedlings provided only with GR24 in a nitrate-supplied medium (treatment 9), but still 10% lower than the positive control (treatment 2). These results suggest that the nitrate-dependent induction of LR development could be partially independent of auxin signalling. Moreover, when N-deficient seedlings were provided with both TIS108 and PCIB (treatment 14), thus blocking SL biosynthesis and auxin signalling, the LR development was only partially inhibited (-8% if compared to nitrate-provided plants), while the negative control in -N (treatment 1) showed a reduction of -22%. These results led to hypothesize that the LR induction after SL inhibition (treatment 2 phenotype) could not depend entirely on auxin actions, thus strengthening the idea that the SLs specific inhibition and auxin positive effect in response to nitrate provision may be fundamental for LR development, but some divergent pathways excluding auxin may also be involved.

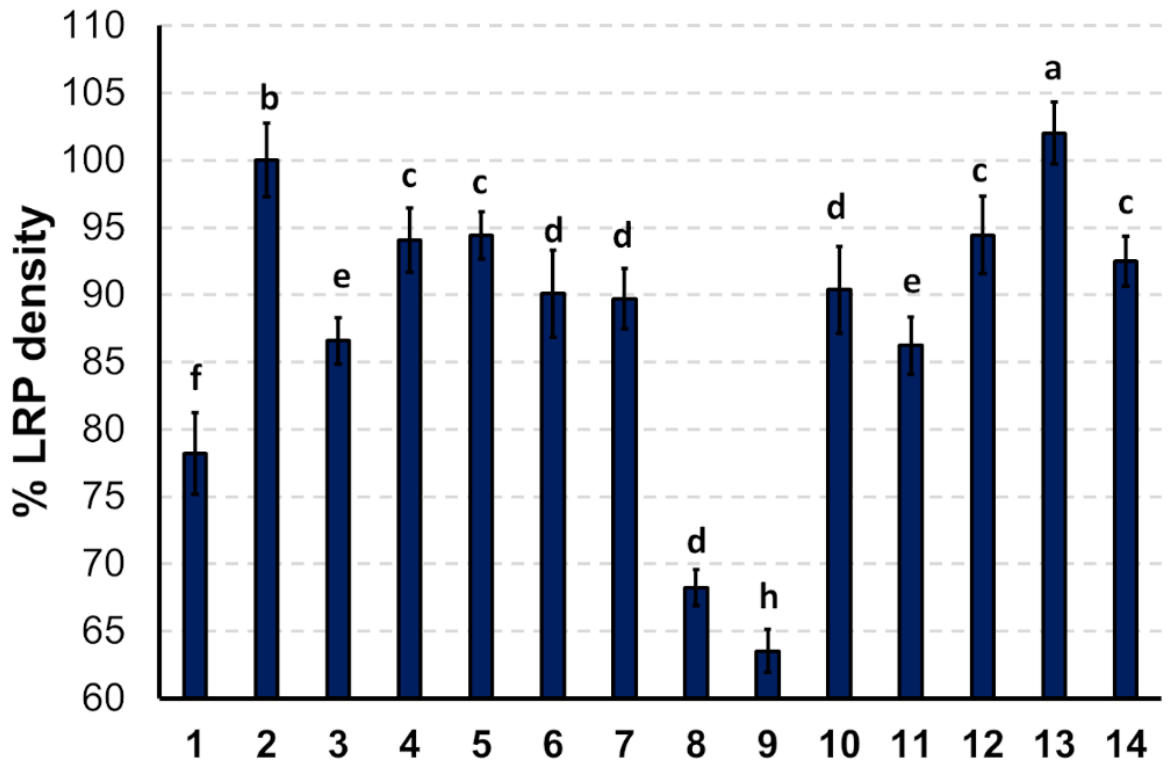


Figure 2. Lateral root primordia (LRP) density of maize seedlings exposed to different nitrogen provision and different auxin and SL inhibitors and analogue.

Maize seedlings were grown 24 hours in a N-deprived nutrient solution and then transferred for additional 24 hours to 14 different treatments, as reported in **Table 1**: N-deprived solution (1), 1 mM nitrate-supplied media (2), nitrate-supplied solution with 10 µM PCIB (3), N-deprived solution supplied with 0.01 µM NAA (4); N-deprived solution supplied with 0.05 µM NAA (5); N-deprived solution supplied with 0.1 µM NAA (6); N-deprived solution supplied with 1 µM NAA (7); N-deprived solution supplied with 0.01 µM NAA and 10 µM PCIB (8); nitrate-supplied solution with 2 µM GR24 (9); nitrate-supplied solution with 2 µM GR24 and 0.01 µM NAA (10); nitrate-supplied solution with 2 µM GR24 and 0.05 µM NAA (11); nitrate-supplied solution with 2 µM GR24 and 0.1 µM NAA (12); N-deprived solution supplied with 2 µM TIS108 (13); N-deprived solution supplied with 2 µM TIS108 and 10 µM PCIB (14). A haematoxylin staining was used to evidence the lateral root primordia (LRP) as described by Ravazzolo *et al.* (2019). Root images were collected using a flatbed scanner and analysed using the ImageJ Software. Data are expressed as increment of LRP density respect to the control as treatment 2. Results are presented as mean ± SE from three biological replicates for each treatment and an ANOVA statistic test was performed (n=30). Letters above the bars indicate different significance groups ($P<0.05$).

3.3 The molecular regulation of the LR development in response to nitrate involves SLs and auxin

To further study the coordinated role of auxin and SLs in the molecular regulation of the response to nitrate in LR development, the expression of a gene set was evaluated in roots of seedlings grown in N-deficiency or nitrate-supply and in the presence of different SL and auxin analogues or inhibitors (**Table 1; Table 2**).

Genes in group 1 (*CYCB* and *CDKB*) were chosen for their involvement in the auxin-dependent cell progression related to LR development (Yu *et al*., 2015b) (**Fig. 3**), Accordingly, they all showed significant up-regulation in response to NAA provision to N-deficient medium (treatment 4), in particular *CYCB1;1*, *CDKB1;1* and *CDKB2;1* with a 2.5 fold-change. A similar increased expression was also shown in response to nitrate provision (treatment 2), even if in a less extent, thus showing the positive effect of nitrate on cell cycle progression. Nevertheless, *CDKB1;1* showed a 15 fold-change in response to the concomitant provision of both NAA and PCIB to N-deprived seedlings (treatment 8), thus showing a possible auxin-repressible feedback effect on this gene. In addition, no interesting variations in response to SL were observed (treatments 9-10-13).

Aux/IAA proteins function as transcriptional repressors of downstream auxin-regulated genes, interacting with ARFs to control the expression of downstream auxin-responsive genes (Ludwig *et al*., 2013). Among the 31 *Aux/IAA* genes identified in maize (Wang *et al*., 2010), those involved in lateral root development were selected (**Fig.4**). *IAA14, IAA19* and *IAA29* were slightly up-regulated by nitrate (treatment 2) and more markedly by auxin provision (treatment 4). *IAA29* also showed specific up-regulation in response to TIS108 (treatment 13) and down-regulation in response to GR24 (treatment 9), thus suggesting that its positive regulation in response to nitrate supply may be linked with the inhibition of SL biosynthesis. *IAA29* is also called *RUL1* (*RUM1-like*) and is a paralogue of *IAA10/RUM1,* whose mutant *rum1* is deficient in the initiation of seminal roots and lateral roots at the maize primary root (von Behrens *et al*., 2011). Indeed, *IAA29* could be a good marker to study the interplay between auxin and SL in nitrate signalling. On the other hand, no interesting variations in response to SL were observed neither for others *IAA* nor for *ARFs*.

Among the genes in group 3 (**Fig. 5**), clustered for their involvement in the downstream auxin response in the root development as transcription factors, *LBD2* showed a significant down-regulation in response to PCIB (treatment 3), while it appeared 5 times up-regulated by NAA provision (treatment 4). However, *LBD2* showed up-regulation also in response to both GR24 and

TIS108 provision (treatment 9 and 13, respectively), thus showing no evident link with the SL pathways. On the contrary, *HSP101*, an Heat Shock Protein 101 that mediates auxin responses in the maize crown root (Martínez-de la Cruz *et al*., 2015), appeared auxin-repressible and a good marker of the positive effect of nitrate on auxin signalling, since it showed no significant variations in response to nitrate provision (treatment 2), but up-regulation when PCIB is added to nitrate (treatment 3), and even to a greater extent when PCIB is added to N-deficient medium provided with NAA (38 fold-change) (treatment 8).

NAC1 displayed a particular behaviour, being strongly down-regulated in response to both nitrate and NAA (**Fig. 3**). *AtNAC1* has been characterized as an intermediary in the auxin-signalling pathway that activates genes encoding molecules involved in the specification of LR initiation (Xie *et al*., 2000). Since it was shown that auxin activates *AtNAC1* transcription (Xie *et al*., 2000), while in the present results auxin provision as NAA displayed an inhibitory effect, further studies should be performed to better understand the *NAC1*'s role in the maize LR development.

Finally, among auxin carriers (**Fig. 6**), the more interesting genes appear to be *PIN8* and *PIN9*. They were both up-regulated by nitrate provision (treatment 2), but while *PIN9* showed no variations in response to NAA, *PIN8* displayed an up-regulation of 9 fold-change in response to NAA provision. (treatment 4). In addition, *PIN8* showed down-regulation in response to PCIB (treatment 3), and up-regulation in response to GR24 provision (treatment 9).

These expressions evidenced peculiar trends in response to nitrate, auxin, SLs and specific inhibitors, allowing to select few of them as good candidates to better characterize and deepen the auxinic action involved in the nitrate signalling. They would also represent a useful marker to decipher the reciprocal actions of SLs and auxin in this pathway.

In conclusion, these preliminary results suggest that SLs and auxin share overlapping and divergent pathways to regulate maize lateral root development in response to nitrate availability, but future work will be aimed at better investigate all these aspects.

Fig. 3 Real-time qRT-PCR expression profiles of GROUP 1 genes in maize roots. Maize seedlings were grown 24 hours in a N-deprived nutrient solution and then transferred for additional 24 hours to different treatments, as reported in Table 1: N-deprived solution (1), 1 mM nitrate-supplied media (2), nitrate-supplied solution with 10 µM PCIB (3), N-deprived solution supplied with 0.01 µM NAA (4); N-deprived solution supplied with 0.01 µM NAA and 10 µM PCIB (8); nitrate-supplied solution with 2 µM GR24 (9); nitrate-supplied solution with 2 µM GR24 and 0.01 µM NAA (10a N-deprived solution supplied with 2 µM TIS108 (13). After 24 h of each treatment, the complete root system was collected from every seedling (n=4) and the relative mRNA levels for each gene were evaluated by means of qRT-PCR. Data are mean ± SE for three biological replicates.

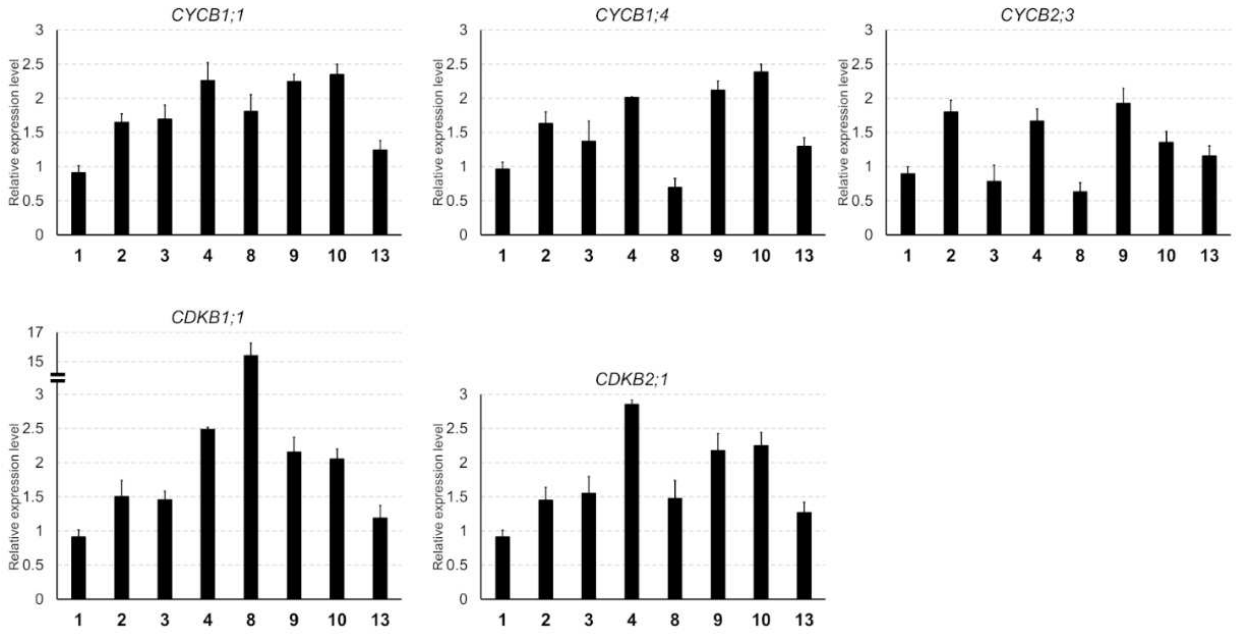


Fig. 4 Real-time qRT-PCR expression profiles of GROUP 2 genes in maize roots. Maize seedlings were grown 24 hours in a N-deprived nutrient solution and then transferred for additional 24 hours to different treatments, as reported in Table 1: N-deprived solution (1), 1 mM nitrate-supplied media (2), nitrate-supplied solution with 10 µM PCIB (3), N-deprived solution supplied with 0.01 µM NAA (4); N-deprived solution supplied with 0.01 µM NAA and 10 µM PCIB (8); nitrate-supplied solution with 2 µM GR24 (9); nitrate-supplied solution with 2 µM GR24 and 0.01 µM NAA (10a N-deprived solution supplied with 2 µM TIS108 (13). After 24 h of each treatment, the complete root system was collected from every seedling (n=4) and the relative mRNA levels for each gene were evaluated by means of qRT-PCR. Data are mean ± SE for three biological replicates.

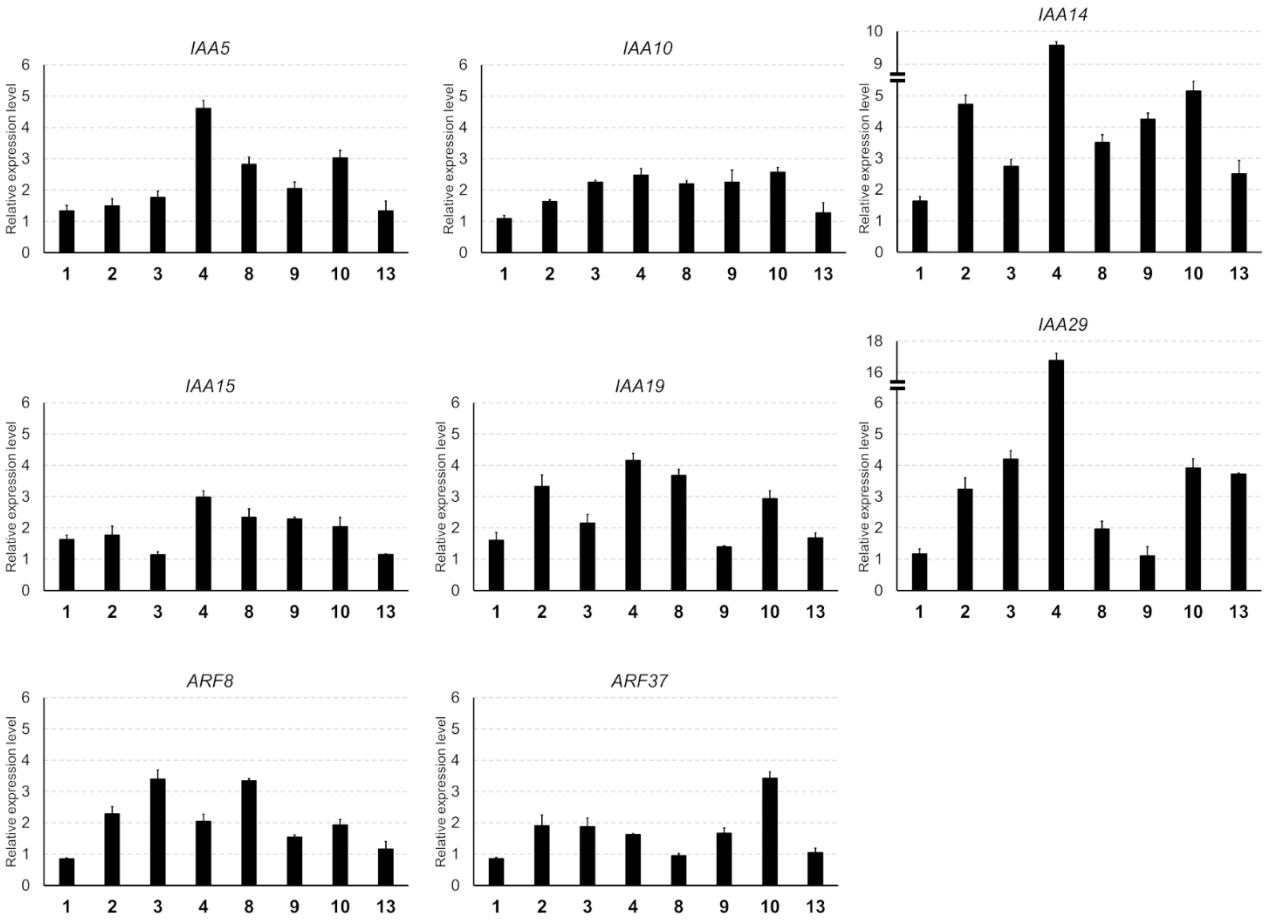


Fig. 5 Real-time qRT-PCR expression profiles of GROUP 3 genes in maize roots. Maize seedlings were grown 24 hours in a N-deprived nutrient solution and then transferred for additional 24 hours to different treatments, as reported in Table 1: N-deprived solution (1), 1 mM nitrate-supplied media (2), nitrate-supplied solution with 10 µM PCIB (3), N-deprived solution supplied with 0.01 µM NAA (4); N-deprived solution supplied with 0.01 µM NAA and 10 µM PCIB (8); nitrate-supplied solution with 2 µM GR24 (9); nitrate-supplied solution with 2 µM GR24 and 0.01 µM NAA (10a N-deprived solution supplied with 2 µM TIS108 (13). After 24 h of each treatment, the complete root system was collected from every seedling (n=4) and the relative mRNA levels for each gene were evaluated by means of qRT-PCR. Data are mean ± SE for three biological replicates.

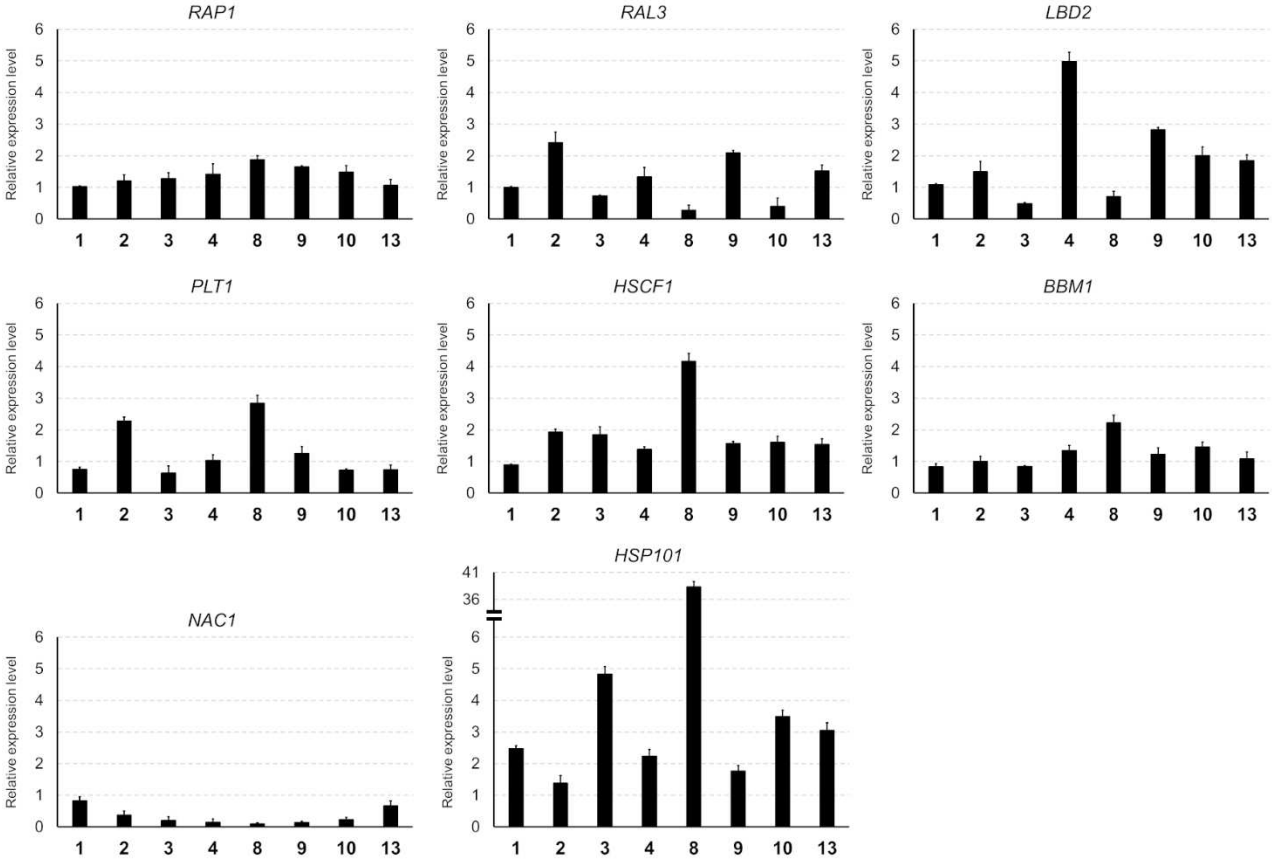
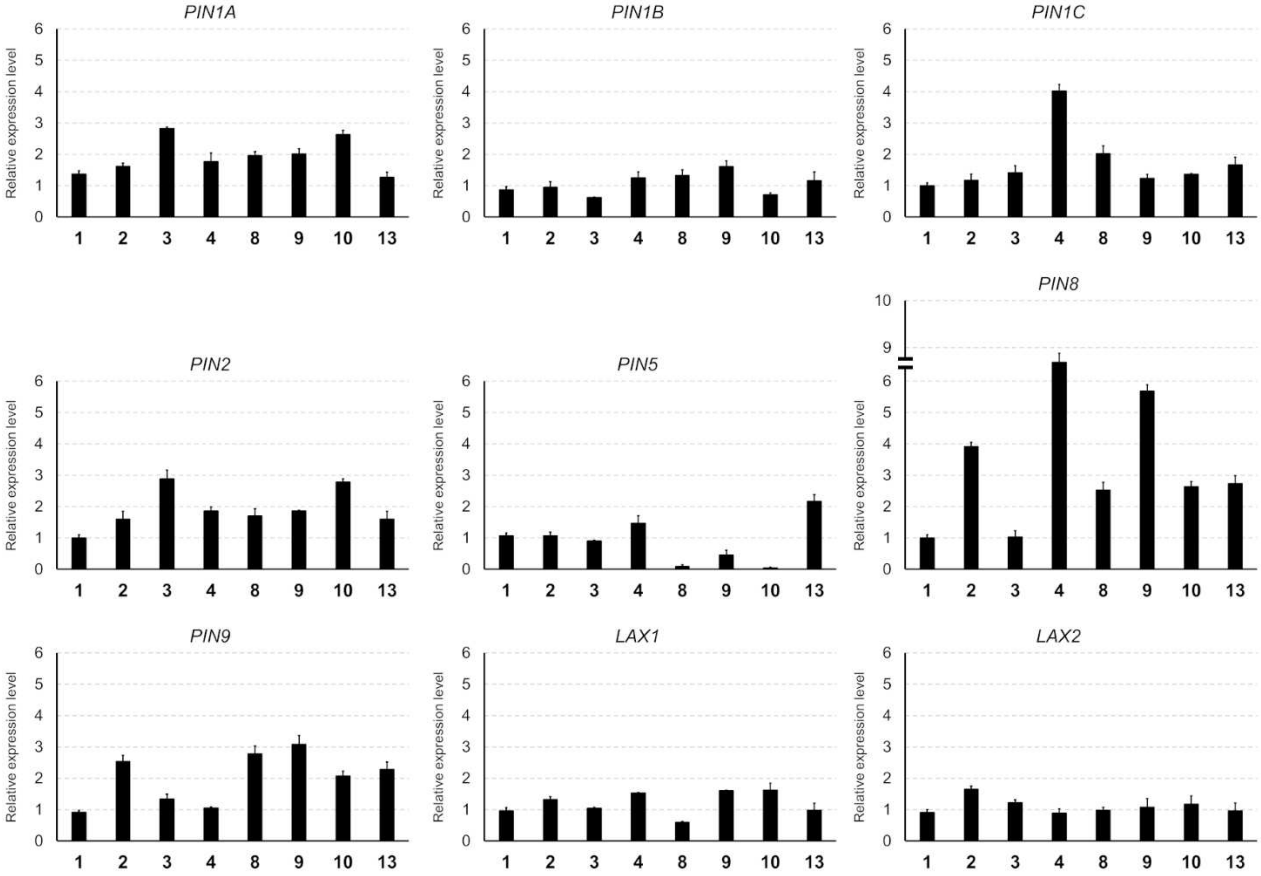


Fig. 6 Real-time qRT-PCR expression profiles of GROUP 4 genes in maize roots. Maize seedlings were grown 24 hours in a N-deprived nutrient solution and then transferred for additional 24 hours to different treatments, as reported in Table 1: N-deprived solution (1), 1 mM nitrate-supplied media (2), nitrate-supplied solution with 10 µM PCIB (3), N-deprived solution supplied with 0.01 µM NAA (4); N-deprived solution supplied with 0.01 µM NAA and 10 µM PCIB (8); nitrate-supplied solution with 2 µM GR24 (9); nitrate-supplied solution with 2 µM GR24 and 0.01 µM NAA (10a N-deprived solution supplied with 2 µM TIS108 (13). After 24 h of each treatment, the complete root system was collected from every seedling (n=4) and the relative mRNA levels for each gene were evaluated by means of qRT-PCR. Data are mean ± SE for three biological replicates.



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Chapter 4

NITRATE AND AMMONIUM DIFFERENTIALLY AFFECT MAIZE ROOT TRANSCRIPTOME AND SEEDLINGS PHYSIOLOGY

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FOCUS: In chapter 2, the study of the involvement of strigolactones (SLs) metabolism and signalling in the maize response to both nitrate and ammonium highlighted common and specific elements between these two nitrogen nutrients. Herein in chapter 4, an untargeted approach was used to more deeply characterize the molecular signatures for the perception of nitrate and ammonium by maize roots and to evaluate their impact on seedlings growth and metabolism.

ABSTRACT

Nitrogen (N) is an essential macronutrient for plant development and crop yield. Nitrate and ammonium are the main N inorganic forms by plants and act as both nutrients and signalling molecules. Nitrate is the major N form in aerated soils, while ammonium is predominant in anaerobic environments. Plants developed several physiological responses to better adapt to N availability fluctuations, leading to optimize their root architecture in response to the nitrogen form and distribution in soil. Nitrate and ammonium affect root development in a specific way by regulating the expression of several common and different genes.

In this study, an RNA-sequencing approach was applied to gain comprehensive information on the main pathways involved in the overall response to nitrate or ammonium in maize root. Furthermore, plant growth, leaf pigments content (through SPAD measurements) and amino acids tissue content were analysed in plants grown in N-deficiency or supplied with either nitrate or ammonium. Our results show that these two nutrients act on some specific and different molecular mechanisms. Accordingly, NH_4^+ provision activates the response related to biotic stress, while nitrate acts as a negative regulator of transmembrane transport. Both the N-source repress genes related to the cytoskeleton and reactive oxygen species (ROS) detoxification, whereas they have a positive impact on AP2/EREBP, LOB and WRKY transcription factors regulation. At a physiological level, ammonium particularly affects leaf pigments and plant growth, the latter being particularly slowed down, while both nitrate and ammonium induce marked differences in the global amino acid profiles in root and leaves.

KEY WORDS

ammonium; amino acids; growth; maize; nitrate; RNA-Seq; root.

1. INTRODUCTION

Nitrogen (N) availability strongly affects plant growth and crop productivity, being a structural constituent of proteins, nucleic acids and many secondary metabolites and representing about 2% of plant dry weight (Miller and Cramer, 2005).

Since in natural environments N availability is often limiting, plants have developed specific mechanisms to adapt to different N sources (Kiba and Krapp, 2016). Except for some species which are able to fixe atmospheric nitrogen through symbiotic associations with soil microbes, most of them need to directly uptake nitrogen from the soil. Nitrate and ammonium are the two inorganic dominant N forms in natural and cropland soils, accounting for 70% of anion and cation absorption (Lu *et al*., 2009). Nitrate represents the major N form in most aerated soils and ammonium in some acidic and/or anaerobic environments (Miller and Cramer, 2005).

Due to the soil complex properties in soil waters, the diffusion coefficient of nitrate is 10–100-fold higher than that of ammonium (Miller and Cramer, 2005). Consequently, nitrate is able to rapidly move toward root by mass flow, whereas cationic ammonium is adsorbed by soil particles (Giehl and von Wirén, 2014). To adapt to their fluctuations and different behaviour in soil and to optimize N uptake, plants need to adapt their root architecture by finely regulating primary root (PR) growth and lateral root (LR) development. Several studies performed in *A. thaliana* (Remans *et al.*, 2006; Lima *et al.*, 2010; Bisseling and Scheres, 2014; Li *et al.*, 2014; O'Brien *et al.*, 2016) led to hypothesise that ammonium stimulates LR branching, whereas nitrate stimulates LR elongation, thus highlighting a complementary effect of these two ions on LR development which would reflect the plasticity of LRs to the distinct mobilities of both these nutrients. However, even if these two nutrients affect plant growth and physiology, not always concordant results were obtained, probably depending on the plant species and genotype utilised (Tang *et al*., 2019).

From the physiological point of view many similar responses, as for example trehalose synthesis, glycolysis and sucrose degradation, are triggered by nitrate and ammonium (Wang *et al*., 2003; Scheible *et al*., 2004), but also different and specific signalling seems to exist in response to these nutrients. (Patterson *et al*., 2010). Indeed, due to the higher need of energy required to assimilate nitrate, these two ions trigger contrasting effects on cellular energetics and redox status (Bloom, 1997; Noctor and Foyer, 1998). Furthermore, the content of various metabolites and the activity of many enzymes are also clearly affected by the presence of either nitrate or ammonium

(Chaillou *et al*., 1991; Cramer and Lewis, 1993; Pasqualini *et al.*, 2001; Goodchild & Givan 1990; Frechilla *et al*., 2002; Escobar *et al*., 2006).

However, despite the different effect of nitrate and ammonium on plant metabolism and development, only a few studies have been performed on the molecular characterization of plant response to ammonium, in comparison to those focused on nitrate regulation of gene expression (Patterson *et al*, 2010).

The assimilation of ammonium and nitrate converge once that nitrate has been reduced to ammonium, leading to suppose that genes commonly regulated by both ammonium and nitrate presumably respond to ammonium or to a metabolite downstream of ammonium assimilation (e.g. glutamate or glutamine) and that the nitrate-specific gene regulation depends on nitrate ion itself or on nitrite (Wang *et al*., 2007) or on nitric oxide (Planchet and Kaiser, 2006).

Nitric oxide has been identified as a crucial component of the signalling occurring in the transition zone of maize root after nitrate provision being involved in the early developmental response of primary root to nitrate (Trevisan *et al*, 2011, 2014, 2015; Manoli *et al*., 2014, 2016).

Moreover, more recent results on maize root (Ravazzolo *et al.*, 2019) led to hypothesise that the regulation of LR development by nitrate and ammonium could partially depend on the inhibition of strigolactones (SL) biosynthesis observed in response to both these ions, but additional regulatory elements should be identified to better characterize common and specific responses to these two nutrients.

Untargeted study approaches could strongly improve our knowledge on the global pathways governing the physiological response to these two nutrients, allowing also to identify novel components of these responses. The identification of key genes underlying the response to nitrogen starvation and nitrate or ammonium provision may enable novel approaches to increase N use efficiency (NUE) and to improve plant resilience to stress.

Actually, metabolic and signalling responses to N provision also include redox homeostasis adjustments which in turn may influence the global response to abiotic stress (Trevisan *et al*., 2019; Tsukagoshi, 2016; Kagenishi *et al*., 2016; Wang *et al*., 2015). Moreover, the intensification of the application of N fertilizers over the past few decades, besides improving crop productivity (Kant *et al.*, 2011), it also triggered side and sometimes conflicting effects on plant–pathogen interactions (Tavernier *et al.*, 2007), and has been shown to boost disease development (Solomon *et al*., 2003). For these reasons, from now on breeding for resistance need also to carefully take into consideration interactions under different N regimes (Mur *et al*., 2017).

In this study, an RNA-sequencing approach was applied to gain comprehensive information on common and specific pathways regulating gene expression in response to nitrate or ammonium compared to N-deprived maize roots. Afterwards, Gene Ontology (GO) and MapMan enrichment analyses were performed to try to identify the main common and specific pathways that distinguish the maize root response to N-starvation, nitrate and ammonium provision.

Moreover, to complete the picture additional measurements were performed to depict the effects of these three nutritional treatments on biomass accumulation, chlorophyll, flavonoids and anthocyanins index and amino acid profiles of roots and leaves. Our results provide new insight on the maize root molecular regulation to N-deficiency and nitrate or ammonium supply thus allowing to identify key specific components of the responses to these different nutritional conditions. Furthermore, the analyses of physiological parameters above described allowed to better characterize how the early sensing of these three nutritional cues by root could impact on the overall plant growth and physiology.

2. MATERIALS AND METHODS

2.1 Maize seedlings growth

Seeds of maize (*Zea mays* L.) B73 inbred line were rinsed in distilled water and germinated on wet filter paper at 25°C in the dark, as described by Manoli *et al*. (2014). After germination seedlings were grown for 24 h in a N-deprived solution and then transferred to: -N (negative control), NO_3^- 1 mM or NH_4^+ 1 mM supplied nutrient solution. For each condition, three biological replicates were analysed. RNA extraction for RNA-Seq analysis was performed after 24 h (T1) in each treatment solution. Leaves and roots fresh weight and primary root (PR) length were obtained after both 24 h (T1) and 7 days (T7). At T7, the amino acid content of root and leaves were determined, and the area of leaves and roots was also measured. Chlorophyll, flavonoids and anthocyanins index were measured after 3 days (T3), 6 days (T6) and 7 days (T7) in each condition. The solutions were constantly aerated and changed every two days. Seedlings were grown 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 $m^{-2} \cdot s^{-1}$ photon flux density.

2.2 RNA extraction and libraries preparation for Illumina sequencing

1.5 cm of root apices from the root tip cap were sampled from 10 to 15 pooled seedlings at T1, in three independent biological repetitions, and immediately frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) and a subsequently DNAse digestion was performed with RQ1 RNAse-free DNAse (Promega, Milano, Italy) on an aliquot of total RNA as described by Trevisan *et al.* (2011). The extracted RNA was quantified using a Nanodrop 1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA) and its quality further validated by a Fluorometer analysis (Thermo Scientific, Qubit® 2.0, Wilmington, DE, USA). Libraries for Illumina sequencing were prepared accordingly to the manufacturer instructions using the TruSeq RNA Sample Preparation kit (Illumina, San Diego CA, USA). Sequencing was performed at the Centro di Ricerca Interdipartimentale per le Biotecnologie Innovative (CRIBI, Padova, Italy), on a NextSeq500 (Illumina) instrument.

2.3 Processing of sequencing reads and differential expression analysis

Base-calling was performed using the Illumina Pipeline. The resulting raw reads (23–35 million per library; **Table 1**) were processed for adapter clipping and quality trimming using Trimmomatic 0.33 (Bolger *et al*., 2014). The resulting high-quality reads were mapped to the maize B73 reference genome (RefGen ZmB73 Assembly AGPv4 and Zea_mays.AGPv4.38.gtf Gramene transcript annotation; Jiao *et al*., 2017) with Tophat 2.0.13 (Kim *et al*., 2013) using the following modifications from default parameters: maximum intron size, 60,000; minimum intron size, 5; up to three mismatches and gaps allowed. The sequence alignment files (BAM format) were then filtered using Samtools (Li *et al*., 2009) to remove alignments with MAPQ smaller than 1 (corresponding to multi-mapped reads assigned to more than 10 different genomic positions). Differential expression analyses were performed with Cuffdiff v2.2.1 (Trapnell *et al*., 2013) selecting the following options: --multi-read-correct, --compatible-hits-norm, --dispersion-method per-condition and --library-norm-method quartile. The genes showing a false discovery rate (FDR)-adjusted p-value ≤ 0.05 were considered as differentially expressed genes (DEGs). Hierarchical clustering of DEGs was performed using the Morpheus software (https://software.broadinstitute.org/morpheus/) and displayed as a heat map.

2.4 Gene Ontology (GO) enrichment and functional analysis

GO term enrichment was determined by comparing the number of GO terms in DEGs to the number of GO terms in the expressed genes with Ontologizer (Bauer *et al*., 2008) software using the term-for-term approach for overrepresentation statistical analysis and a Bonferroni correction for multiple testing. Maize GO annotation was retrieved from maize-GAMER project (Wimalanathan *et al*., 2018). Functional analysis of differential expression genes (DEGs) was done using MapMan (Thimm *et al*., 2004; Usadel *et al*., 2009): overrepresentation of categories was determined using Fisher's exact test and resulting p-values were adjusted according to Benjamini and Hochberg (Benjamini and Hochberg, 1995). A critical cut-off value of 0.05 (corresponding to a Z-score ≥ 1.96) was applied to select enriched categories.

2.5 Maize seedlings growth analysis

Roots and leaves from every treatment (-N or $+NO_3^-$ or $+NH_4^+$, 20 plants each) were separately sampled at T1 and T7, in three independent biological repetitions, and fresh weights were obtained. Root and leaves images were collected using a flatbed scanner. Roots and leaves area and the primary root (PR) length were measured by means of ImageJ software analysis. Data represent the average of three replicates (n = 20) ± standard error. For statistical analysis, data derived from the NO_3^- or NH_4^+ treatments were compared with those of control plants (-N) using ANOVA test; data were considered significant when $p < 0.05$.

2.6 Chlorophyll, flavonoids and anthocyanins optical measurements

Thanks to the fluorescence's properties of chlorophyll (CHL) and the characteristic of UV-A absorber of flavonoids (FLAV) and green light absorber of anthocyanins (ANTH), maize seedlings were evaluated for their epidermis absorbance in leaves using the optical sensor DUALEX SCIENTIFIC+^{TM} (Force-A, Orsay, France). In addition, the Nitrogen Balance Index (NBI) was directly obtained by the instrument as the ratio between CHL and FLAV content. At every time point (T3, T6 and T7), two lectures were performed on the first young leaf entirely developed for each plant in every treatment (-N or $+NO_3^-$ or $+NH_4^+$, 20 plants each). To assess FLAV content, readings were made on both adaxial and abaxial sides of leaves and their values were summed-up (Cerovic *et al*.,

2012). Three biological replicates for each treatment and an ANOVA statistic test were performed (n=30). Data represent the average of three replicates ± standard error.

2.7 Analysis of amino acids using Hydrophilic interaction chromatography-Tandem Mass Spectrometry (HILIC-MS/MS)

Roots and leaves from every treatment (-N, $+NO_3^-$ and $+NH_4^+$, 20 plants each) were separately sampled at T7, in three independent biological repetitions, weight and immediately frozen in liquid nitrogen. Each sample was grinded to fine a powder in liquid nitrogen and exactly weighted (100 mg). Samples were then extracted in an ultrasound bath with a solution of diluted HCl (0.5 M) for 10 minutes at room temperature. Standard solutions were prepared weighting the exact amount of each amino acid in diluted HCl solution (0.5 M) in four different concentrations from 10 µg/ml to 1 µg/mL. Solutions were centrifuged and used for analysis. For hydrolysis, samples were added of 15% Trichloroacetic acid (10 mL) and left at 80°C for one night. The pH of the solution was then adjusted with an ammonia solution (33%) to 2.0 and solutions were used for analysis. For analysis, an Agilent Z-HILIC Column was used as stationary phase (3 x 100 mm, 4 micron), eluents were ACCN (A) and water 0.5% formic acid (B). Gradient start with 1 minute to 95%A, then in 11 minutes to 70%A, then 14 minutes 40%A then at 14.5 back to 95%A. The flow rate was 0.450 µL/min. Each amino acid transition was optimized with the corresponding standard solution. Transitions are reported as follow:

Compound	Polarity	Q1	Q3	Capillary	Collision	Dwell Time
glycine	Pos.	76.00	30.30	40.000	8.000	0.100
alanine	Pos.	90.00	44.50	40.000	8.000	0.100
serine	Pos.	106.00	60.40	30.000	8.500	0.100
proline	Pos.	116.00	70.30	40.000	10.500	0.100
valine	Pos.	118.00	72.40	30.000	8.500	0.100
threonine	Pos.	120.00	74.40	30.000	8.000	0.100
cystine	Pos.	122.00	75.70	30.000	11.500	0.100
isoleucine	Pos.	132.00	86.50	40.000	7.000	0.100
leucine	Pos.	132.00	86.50	40.000	7.500	0.100
asparagine	Pos.	133.00	87.40	40.000	6.500	0.100
aspartic acid	Pos.	134.00	74.30	33.876	12.000	0.100
glutammine	Pos.	147.00	84.40	30.000	14.000	0.100
lysine	Pos.	147.10	84.40	30.000	13.500	0.100
glutamic acid	Pos.	148.00	83.70	30.000	13.000	0.100
methionine	Pos.	150.00	133.40	38.318	6.000	0.100
histidine	Pos.	156.10	110.00	40.000	9.500	0.100
phenylalanine	Pos.	166.10	119.60	40.000	9.500	0.100
arginine	Pos.	175.10	70.40	40.000	18.000	0.100
tyrosine	Pos.	182.10	135.60	40.000	10.000	0.100
tryptophan	Pos.	205.10	187.60	40.000	7.000	0.100

Data represent the average of three replicates (n = 20) ± Standard Error (SE). For statistical analysis, data derived from ANOVA test and are considered significantly different with $p < 0.05$.

3. RESULTS

3.1 Reads processing, transcriptome de novo assembly, and differential expression analysis

RNA-Seq technique was employed to analyze the transcriptomic profiles of maize root apexes in response to N-supply as nitrate 1 mM ($+NO_3^-$) or ammonium ($+NH_4^+$), compared to those grown in N-deprivation (-N). RNA was extracted from root samples (three biological replicates for each treatment) and used for libraries preparation for Illumina sequencing. After sequencing, adapters and low-quality reads were removed, resulting in 23 to 35 million high quality reads per biological replicate, with about 98% of them mapped on the maize B73 reference genome (**Table 1**). Cuffdiff v2.2.1 (Trapnell *et al.*, 2013) was then used for differential expression analysis after estimation of gene transcript abundances in the different conditions (expressed as RPKM - Reads Per Kb per Million).

Result	-N libraries					
	R1		R2		R3	
N° of total reads	26357768	100%	27157883	100%	30178515	100%
N° of high-quality reads	26254402	99.61%	27051232	99.61%	30045802	99.56%
N° of mapped reads	**25805014**	**98.29%**	**26614982**	**98.39%**	**29474225**	**98.10%**
Uniquely mapped reads	23890148	92.58%	24711441	92.85%	26654323	90.43%
Multi-mapped reads	1914866	7.42%	1903541	7.15%	2819902	9.57%
Unmapped reads	103366	1.71%	106651	1.61%	132713	1.90%
Result	**$+NO_3^-$ libraries**					
	R1		R2		R3	
N° of total reads	26061640	100%	32597592	100%	28820292	100%
N° of high-quality reads	25946454	99.56%	32459506	99.58%	28694329	99.56%
N° of mapped reads	**25477079**	**98.19%**	**31900192**	**98.28%**	**28073982**	**97.84%**
Uniquely mapped reads	23203264	91.08%	28856879	90.46%	24892495	88.67%
Multi-mapped reads	2273815	8.92%	3043313	9.54%	3181487	11.33%
Unmapped reads	115186	1.81%	138086	1.72%	125963	2.16%
Result	**$+NH_4^+$ libraries**					
	R1		R2		R3	
N° of total reads	35121464	100%	25513916	100%	23264596	100%
N° of high-quality reads	34934389	99.47%	25352921	99.37%	23145412	99.49%
N° of mapped reads	**34296996**	**98.18%**	**24704601**	**97.44%**	**22609271**	**97.68%**
Uniquely mapped reads	31201251	90.97%	22009628	89.09%	20703717	91.57%
Multi-mapped reads	3095745	9.03%	2694973	10.91%	1905554	8.43%
Unmapped reads	187075	1.82%	160995	2.56%	119184	2.32%

Table 1: Summary of reads obtained by RNA-Seq analysis. For each treatment ($+NO_3^-$, $+NH_4^+$) or control condition (-N), three biological replicates were processed (R1, R2, R3).

For each comparison between N-supply plants (+NO_3^- or +NH_4^+) vs plants grew in N-deprivation (-N), the genes showing a log2 fold change ratio >|0.58| (corresponding to a 1.5-fold change variation in expression level) and a false discovery rate (FDR)-adjusted p-value ≤ 0.05 were considered as differentially expressed genes (DEGs). Pairwise comparisons resulted in 1349 significantly responsive to NO_3^-, while 1264 were significantly regulated by NH_4^+ (**Fig. 1A**). DEGs were then classified as up-regulated or down-regulated accordingly to the direction of the recorded expression variation induced by the N-supply. The two applied N sources showed a different impact on transcriptional regulation of root apexes: among the 1349 NO_3^- DEGs, 41.9% was up-regulated and 58.1% down-regulated, while as much as a 92% of the 1264 NH_4^+ DEGs was up-regulated and only 8% resulted significantly down-regulated by the cation (**Fig. 1A**). The genes up- and down-regulated by each treatment were then grouped based on the magnitude of their transcriptional changes (**Fig. 1B-C**). Accordingly, the majority of DEGs regulated by nitrate showed a log2-fold change between 1 and 2.5 (32%), thus being up-regulated 2 to almost 6 times with respect to control (-N), while 21% of DEGs displayed a fold-change repression from 2 to almost 6 times (-2.5<log2<-1). Only 5.7% and 2.7% of transcripts showed stronger up-regulation or down-regulation, respectively. (**Fig. 1B**). On the other hand, the most represented log2-fold change category for ammonium-regulated DEGs was again the 1<log2<2.5, showing almost 70% of genes being up-regulated from 2 to almost 6 times by the cation, while 18% was up-regulated more than 6 times and only 5.6% down-regulated from 2 to almost 6 times (-2.5<log2<-1) (**Fig. 1C**).

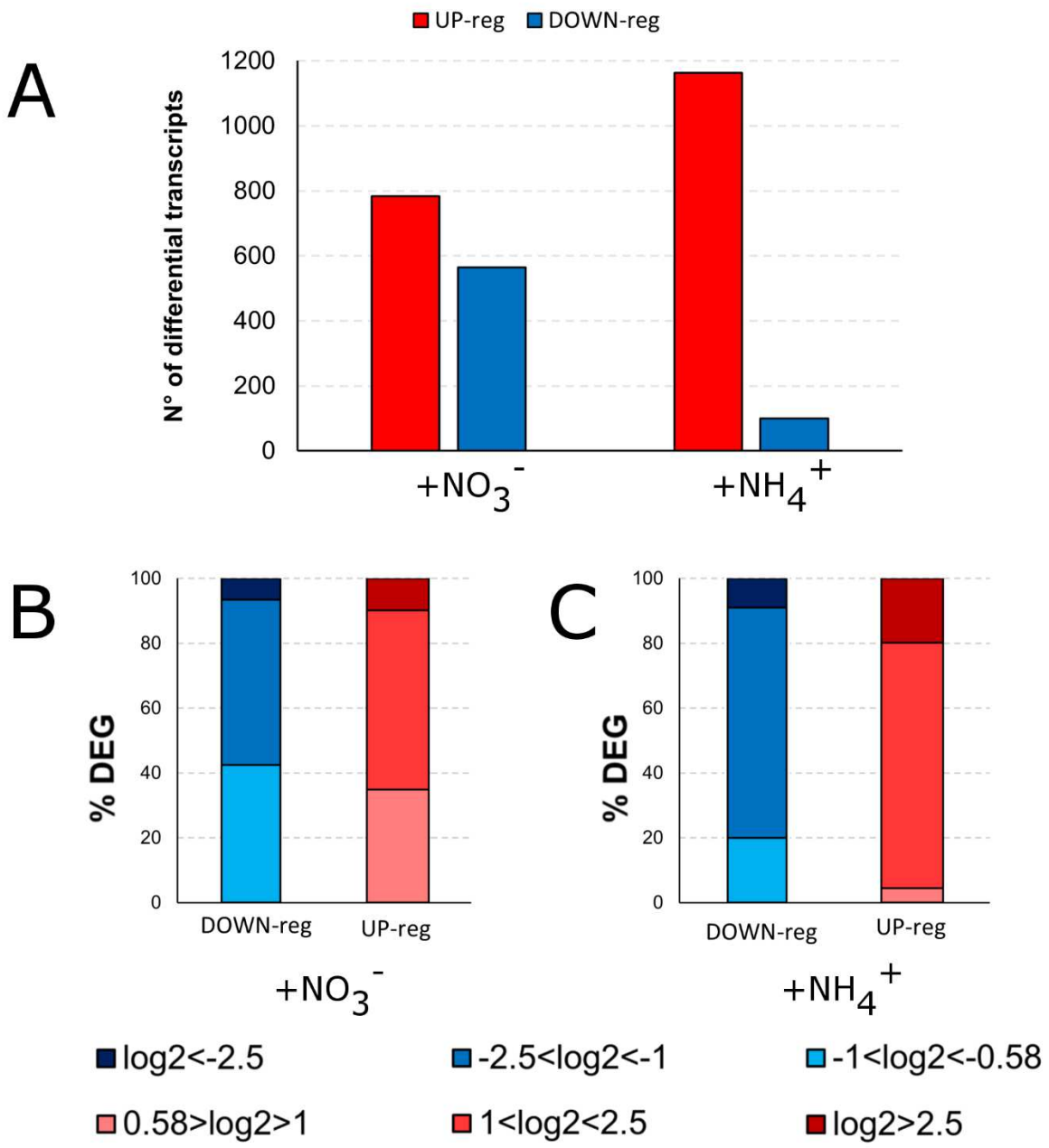


Figure 1. Distribution of differentially expressed genes (DEGs) identified (log2 FC >|0.58|; FDR≤0.05) by RNA-Seq analysis from the comparison between +NO_3^- or +NH_4^+ supplied maize seedlings for 24 h with respect to the control (-N, nitrogen deficient solution). Data are shown as number of genes differentially expressed in response to each treatments (**A**) and as percentages in +NO_3^- treatment (**B**) and +NH_4^+ (**C**) on the total amount of DEGs. Among the up- and downregulated DEGs, several ranges of induction or repression are shown as log2 of the gene expression fold changes (**B-C**). DEGs were classified as up-regulated (+NO_3^-/–N>0.58; +NH_4^+/–N>0.58) or downregulated (+NO_3^-/–N<-0.58; +NH_4^+/–N<-0.58) according to their log2 values (corresponding to a 1.5 fold change increase or decrease in expression).

By comparing the DEGs regulated by nitrate or ammonium (**Fig. 2)**, it was possible to identify four sub-groups of genes which expression was affected by both N sources. These groups only account for a small fraction of DEGs (289 genes, corresponding to the 12.4% of DEGs). Among them, 229 DEGs (9.9%) were up-regulated in response to both NO_3^- and NH_4^+, while 28 DEGs (1.2%) were commonly down-regulated in response to both NO_3^- and NH_4^+. DEGs showing opposite transcriptional changes in response to NO_3^- and NH_4^+ were identified as well: 31 DEGs (1.3%) were down-regulated by nitrate but at the same time up-regulated by ammonium, while only 1 gene was significantly up-regulated by nitrate and down-regulated by ammonium provision. On the other hand, the majority of DEGs (87.6% out of 2324 unique DEGs) resulted specifically and exclusively regulated by nitrate or ammonium if compared with –N-treated roots, as shown in the Venn diagram.

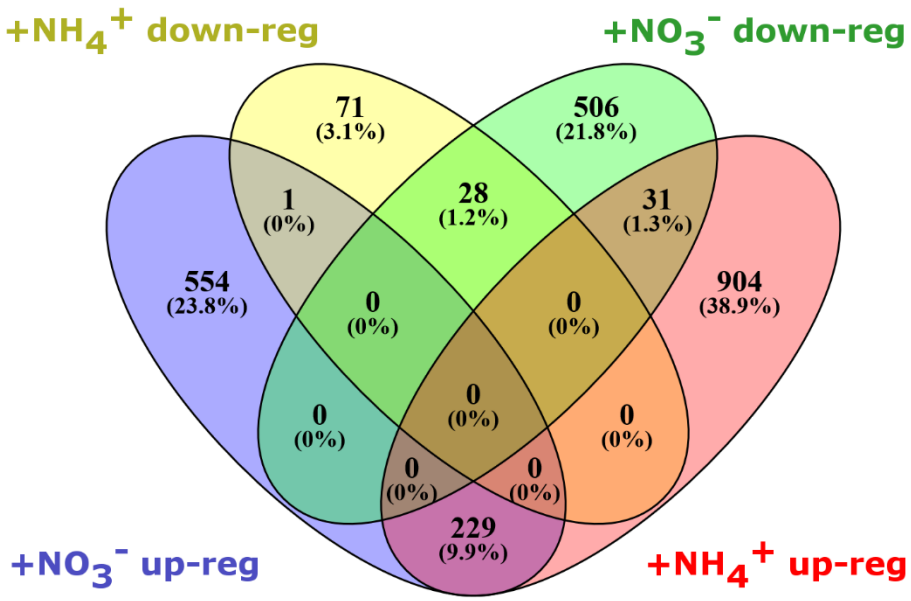


Figure 2. Venn diagram showing the numerical and percentage comparison of significant differential expressed genes that overlapped in $+NO_3^-$ and $+NH_4^+$ treatments with respect to the control (-N). The diagram shows the numerical and percentage comparison of all significant up- and down-regulated differential expressed genes following NO3- and NH4+ treatments. The no overlapping numbers represent the genes that are uniquely identified as DE in the corresponding treatment.

To better dissect the overlapping and unique root response to 24 h of nitrate or ammonium provision, hierarchical clustering of DEGs was then performed using their normalized expression values in the three samples. The DEGs being differentially expressed in at least one treatment were arranged into 8 clusters according to their expression dynamics, as displayed in the heat map (**Fig. 3**). As from the Venn analysis, the cluster analysis confirmed the existence of groups of genes commonly regulated by nitrate and ammonium provision (clusters 2, 3 and 8), while others are specifically regulated only by nitrate (clusters 5 and 7) or by ammonium (clusters 1 and 6). Cluster 4 instead contains the single gene up-regulated by nitrate and down-regulated by ammonium provision (Zm00001d015905). In addition, the heat map pointed out at different behaviour within the genes up-regulated by both nitrate and ammonium, revealing 183 DEGs that are weakly up-regulated by nitrate but strongly up-regulated by ammonium (cluster 2) and 237 DEGs with a strong up-regulation induced by nitrate and a weak up-regulation induced by ammonium provision (cluster 3). Only a subset of the DEGs included in these two clusters was classified as commonly up-regulated by N supply in the Venn analysis.

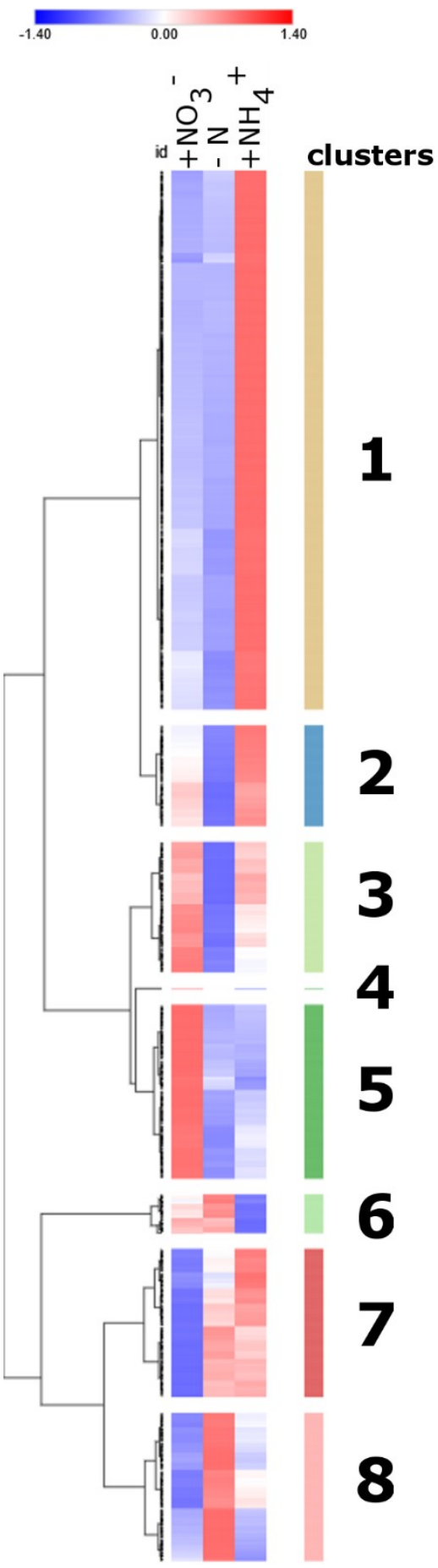


Figure 3. Clustering analysis of genes differentially expressed in $+NO_3^-$ and $+NH_4^+$ treatments with respect to the control (-N) in maize root tissues. z-score scaled RPKM values for all the 2324 genes resulted as DEGs in at least one treatment were used for hierarchical clustering analysis. The analysis reveals 8 different clusters with specific expression behaviours in response to different N-provision or deficiency. Re-scaled expression values for each gene in each sample are reported in a blue to red colour scale (blue: lower RPKM values, red: higher RPKM values). RPKM: Reads Per Kb per Million.

3.2 Annotation and classification of clustered DEGs into GO functional categories

In order to better understand the different and similar effect of NO_3^- and NH_4^+ provision for 24 h to the maize root apex, a GO enrichment analysis was performed to functionally characterize the DEGs included in the 8 different transcriptional profiles (**Fig. 4**).

Genes specifically up-regulated by ammonium (cluster 1) were enriched in GO terms related to 'hormone-mediated signaling pathway', in particular in response to ethylene, salicylic acid, jasmonic acid, karrikins and abscisic acid. It is interesting to note how these hormones could be linked for their functions to the other main term induced by ammonium such as 'response to biotic stimulus'. Indeed, ammonium up-regulated DEGs resulted also enriched in terms involved in the 'immune response' and 'regulation of programmed cell death'. These terms were also over-represented among DEGs included in cluster 2, which included those genes strongly up-regulated by NH_4^+ and weakly up-regulated by nitrate. In addition, another term unique for cluster 1 is 'response to water deprivation'. On the other hand, among the very few terms enriched in DEGs of cluster 6, 'cell proliferation' is unique for the DEGs down-regulated by ammonium application.

Among the GO terms associated with NO_3^- response, the terms 'polysaccharide binding' and 'apoplast' were over-represented among DEGs included in cluster 5 (up-regulated only by nitrate) and cluster 3 (strong up-regulated by nitrate and only weak induction by ammonium). Regarding the terms enriched in DEGs repressed by $NO3^-$ (cluster 7), many terms could be related to the transmembrane transport, such as 'integral component of plasma membrane', 'cation transmembrane transport' and 'active transmembrane transporter activity'. In addition, other enriched terms specific of this cluster are related to secondary metabolism, such as 'phenylpropanoid biosynthetic process' and 'secondary metabolite biosynthetic process'.

Investigating the common response to both nitrate and ammonium, similar terms regarding cytoskeleton organization, such as 'structural constituent of cytoskeleton', 'microtubule', 'microtubule motor activity' and 'kinesin complex', are enriched in cluster 8 (DEGs down-regulated both by NH_4^+ and NO_3^-) and also in cluster 6 (DEGs down-regulated by NH_4^+):. In addition, within cluster 8 and cluster 7 (DEGs down-regulated by NO_3^-) common terms are 'peroxidase activity', 'cellular oxidant detoxification' and 'response to oxidative stress'. Finally, the single gene induced by nitrate and repressed by ammonium (cluster 4) corresponds to the Zm00001d015905 accession, which encodes a SWEET sugar transporter (Sosso *et al*., 2015,2018).

From these results, it seems that NH_4^+ strongly affects the plant hormone balance in favour of hormones involved in the stress response, in particular by activating the biotic stress and defence response pathways. In addition, the only unique term for DEGs down-regulated by ammonium is 'cell proliferation'. On the other hand, nitrate repressed the expression of genes related to the transmembrane transport and secondary metabolism, while from this GO enrichment only few information appeared specific of nitrate-induced up-regulation, above all regarding the apoplast localisation and the polysaccharide binding. Moreover, genes down-regulated by both inorganic N sources are associated with reactive oxygen species (ROS) detoxification, thus showing how the root needs these radicals as signalling molecules, and negatively affect cell organization at the cytoskeleton level.

Therefore, the different number of genes in shared GO terms and the specific enriched GO terms in different gene ontologies categories may provide the basis for the understanding of the specific maize root responses to N supply.

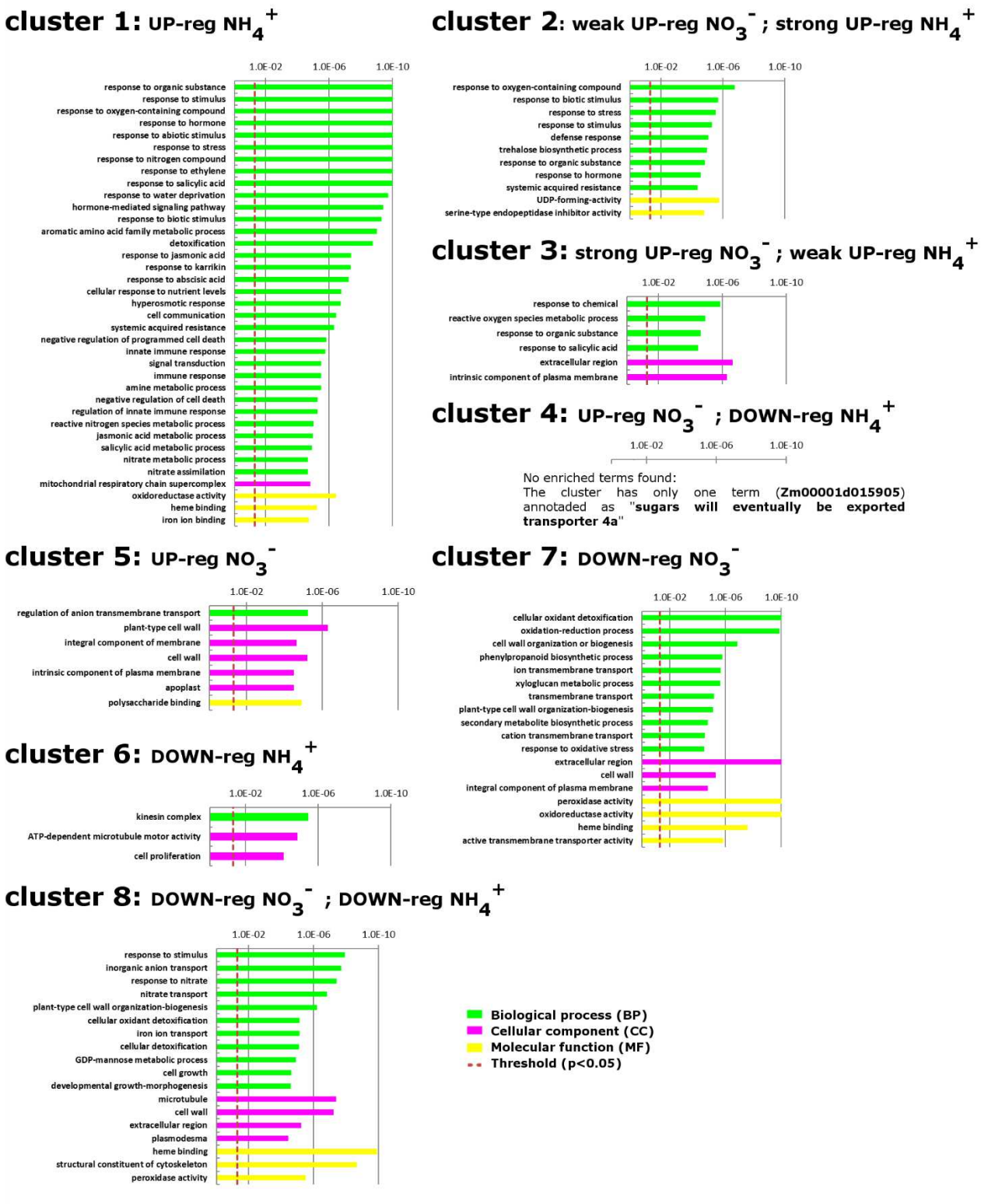


Figure 4. Enrichment analysis of DEGs clustered in 8 groups using Ontologizer. The figure shows the GO categories overrepresented among up and down-regulated gene sets in $+NO_3^-$ and $+NH_4^+$ treatments with respect to the control (-N) in maize root tissues.

3.3 Classification of DEGs into MapMan functional categories

In addition to GO enrichment, functional analysis of DEGs was performed by means of MapMan over-representation analysis. Several pathways were found enriched or depleted among genes differentially expressed in the two analysed N-treatments (**Fig. 5**). However, as expected, some gene categories were similarly enriched following the application of the two N-treatments. The MapMan categories 'N-metabolism' and 'cell organisation' were highly enriched among both NO_3^- and NH_4^+ DEGs, with the first bin showing significantly up-regulation of nitrate metabolism-related genes and the latter a strong down-regulation of genes involved in the cytoskeleton organisation through actin and tubulin. Moreover, the bin 'cell wall' was significantly repressed under NO_3^- provision, while genes belonging to this pathway were not significantly affected by NH_4^+.

The 'regulation of transcription' bin appeared mainly regulated by ammonium provision, while only the AP/EREBP transcription factor (TF) appeared up-regulated by both ammonium and nitrate. On the other hand, ammonium induced strong and exclusively up-regulation of also AS2/LOB and WRKY TFs genes.

In addition, genes involved in 'hormone metabolism and responses' bin were differentially regulated by both nitrate and ammonium, with this last being more effective, as already shown from GO enrichment. 'Ethylene' and 'salicylic acid metabolism' bins were over-represented among DEGs induced by NH_4^+, while 'gibberellin metabolism' and 'abscisic acid degradation' bins over-represented among genes repressed by ammonium and nitrate, respectively.

Moreover, NO_3^- was shown to induce the 'lipid degradation' and 'protein degradation' bins, thus adding new data to the few obtained with GO enrichment. Among the 'second metabolism' bin, NO_3^- positively regulated genes involved in 'sulphur metabolism' sub-category and NH_4^+ positively regulated the 'flavonoids' sub-category, while 'lignin metabolism' was significantly repressed by nitrate. In addition, NH_4^+ induced strong enrichment of terms in the 'biotic stress' bin, thus confirming what was observed also by GO enrichment.

These results provide new information about the importance of transcription factors (TFs) regulation exerted exclusively by ammonium (AS2/LOB and WRKY TFs) and ammonium together with nitrate (AP2/EREBP TFs). Moreover, a positive effect of nitrate on lipid and protein degradation, together with a negative effect on cell wall were shown. In addition, they confirm the negative effect of both N-sources on peroxidases and cytoskeleton organization, while they show a negative effect of nitrate on transport processes.

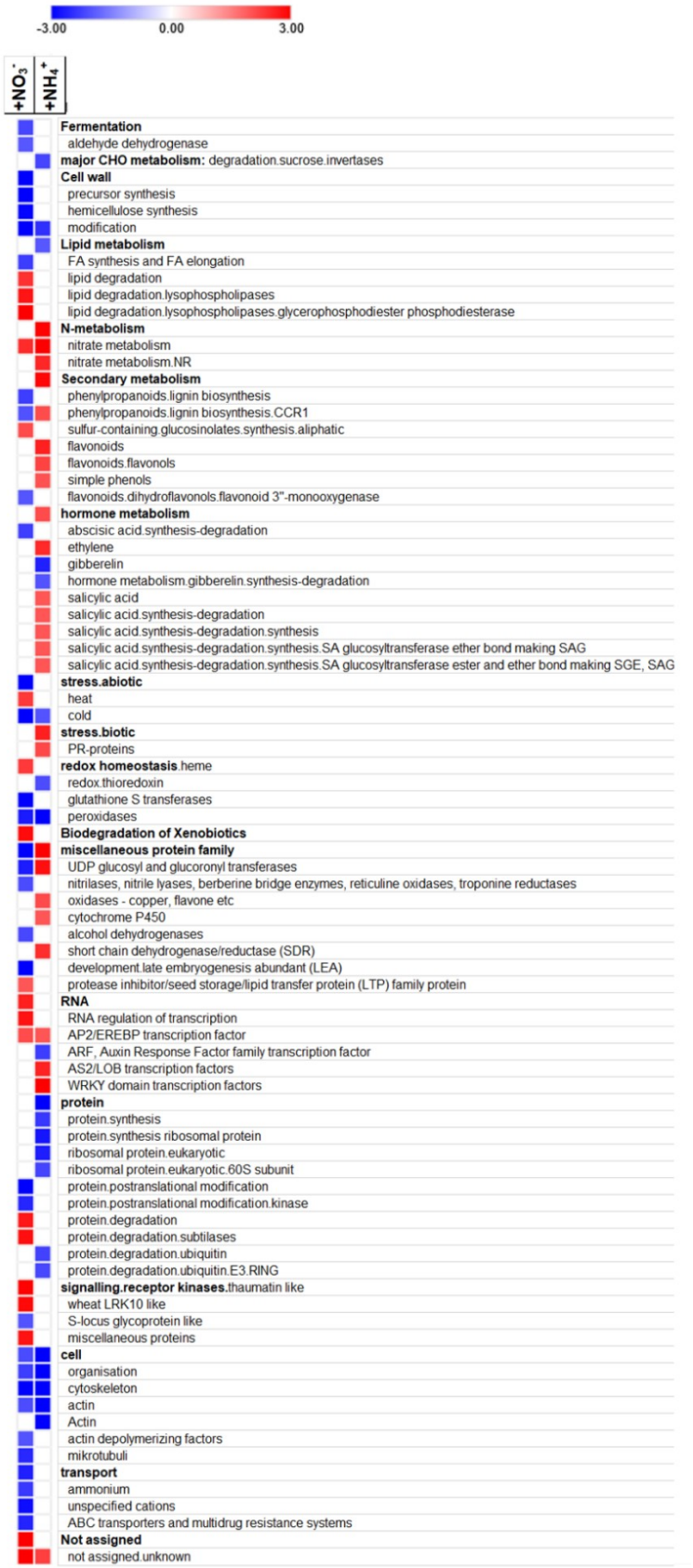


Figure 5. **MapMan functional categories enriched of DEGs identified in two treatments ($+NO_3^-$/–N; $+NH_4^+$/–N)**. Z-scores were deduced from p-values (i.e. 1.96 corresponds to a P-value of 0.05) and are mapped in a blue to red colour scale (blue: enrichment among down-regulated DEGs, red: over-representation among up-regulated DEGs).

3.4 Differences in biomass accumulation between ammonium and nitrate nutrition

The estimation of plant growth, measured as the biomass of roots and leaves during the different nutritional treatments, revealed that the two nitrogenous sources affect plant growth with a different dynamic (**Fig. 6**). After 24 hours of treatments (T1), no significant differences were detected among both shoot and root biomass allocation in nitrogen starved plants (-N, negative control) and nitrate-supplied plant ($+NO_3^-$), while ammonium-($+NH_4^+$) triggered a stronger reduction of total biomass accumulation with respect to both –N and $+NO_3^-$ plants (about -16%) (**Fig. 6C**).

After one week of nitrogen provision (T7), the total biomass accumulation was statistically differentiated in the three sets of plants (**Fig. 6C**). The highest accumulation was observed in $+NO_3^-$ plants that reached a total biomass 10% higher in comparison to that measured for -N plants. This increase was mainly due to a higher root biomass accumulation (+25% of the root biomass compared to -N-plants), while shoot biomass was similar to -N (**Fig. 6A-B**). Interesting ammonium application resulted in lower total biomass accumulation with respect to both nitrogen starved plant (-N; -35%) and nitrate-supplied plants ($+NO_3^-$, -40%), and the reduction was detectable in both leaves (about -39% with respect to both -N and $+NO_3^-$,) and roots (-28% if compared to -N, and -42% if compared to $+NO_3^-$).

Considering leaves and roots area at T7, NH_4^+-treated plants showed a 46% reduction of both parameters with respect to -N plants. On the contrary, nitrate significantly induced a higher leaf area (+10%) but a smaller root area (-17%) if compared to N-deprived plant (**Fig. 7B**). Primary root (PR) length showed a different trend between T1 and T7, with the longest PR in -N at T1, while at T7 a different behaviour of the two N-sources was observed. Indeed, after seven days the longest PR was induced by nitrate (+12% compared to -N), while ammonium provision induced a strong reduction in PR length (-30%) with respect to -N (**Fig. 7A**).

According to these results, ammonium seems to immediately and negatively affect biomass allocation even at T1, and this inhibition of root and shoot biomass accumulation becomes more evident at T7, where also a strong negative effect on primary root growth was shown. On the other hand, nitrate has a positive effect on increasing total biomass at T7, primarily thanks to the induction of longer and thinner roots.

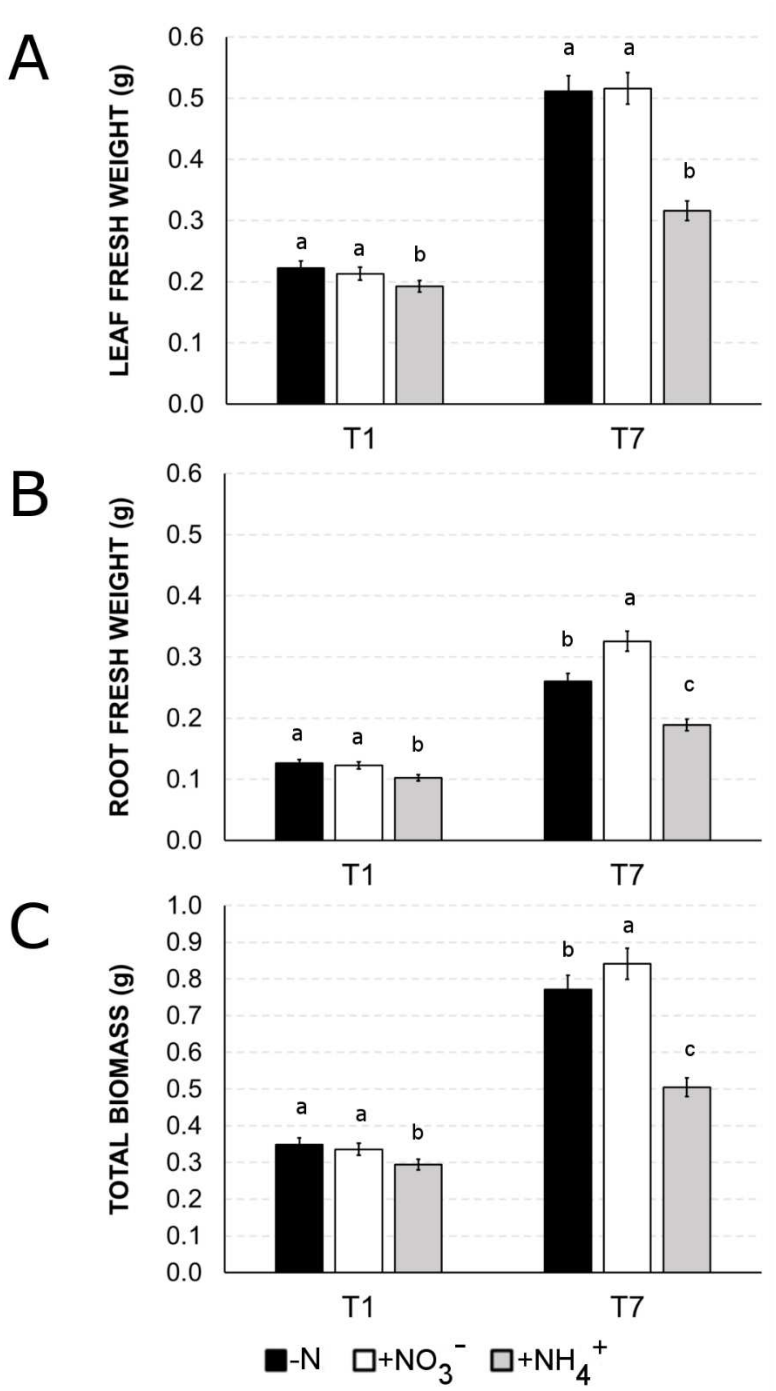


Figure 6: Effect of different N-source (NO_3^- or NH_4^+) or N-deficiency (-N) on leaf biomass (fresh weight, panel A) and root biomass (fresh weight, panel B). The distribution of total biomass was reported in panel C. T1 shows the effect after 24 h of treatment, T7 after 7 days. Similar letters at the top of the bars are not significantly different ($P < 0.05$) by an ANOVA test. Each value is designed as the mean of three biological replicates ± SE (n = 20).

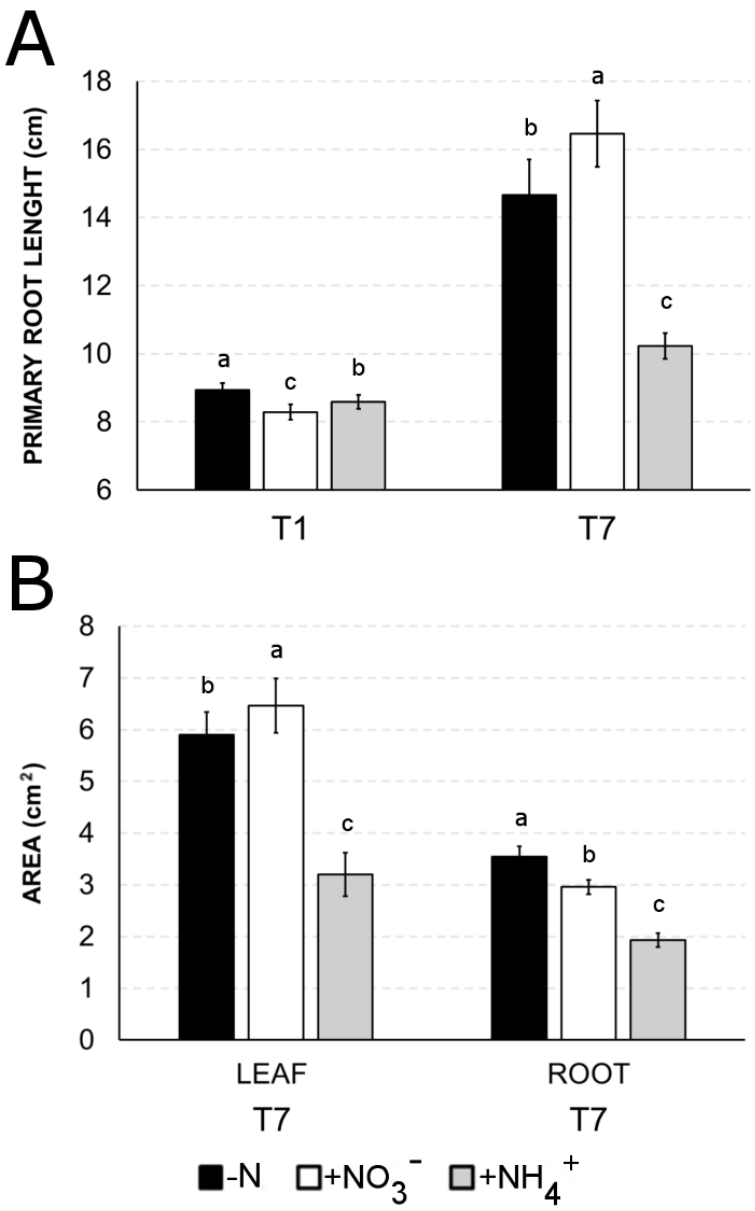


Figure 7: Effect of different N-source (NO_3^- or NH_4^+) or N-deficiency (-N) primary root length (cm, panel A) and surface area (cm^2, panel B). T1 shows the effect after 24 h of treatment, T7 after 7 days. Surface area was obtained for both roots and leaves only at T7. Similar letters at the top of the bars are not significantly different ($P < 0.05$) by an ANOVA test. Each value is designed as the mean of three biological replicates ± SE (n = 20).

3.5 Leaf pigments prediction with optical sensor

To deeper study the global effect *in planta* of the absence or supply of N as inorganic ions, the leaf pigment contents were indirectly obtained through the optical sensor Dualex. Three fluorescence indices, namely leaf chlorophyll (CHL), flavonoids (FLAV) and anthocyanins (ANTH), together with the ratio between CHL and FLAV which is called NBI (Nitrogen Balance Index), were

evaluated in the three sets of plants subjected to the three different N treatments at three times points: T3 (3 days), T6 (6 days) and T7 (7 days) (**Fig. 8**).

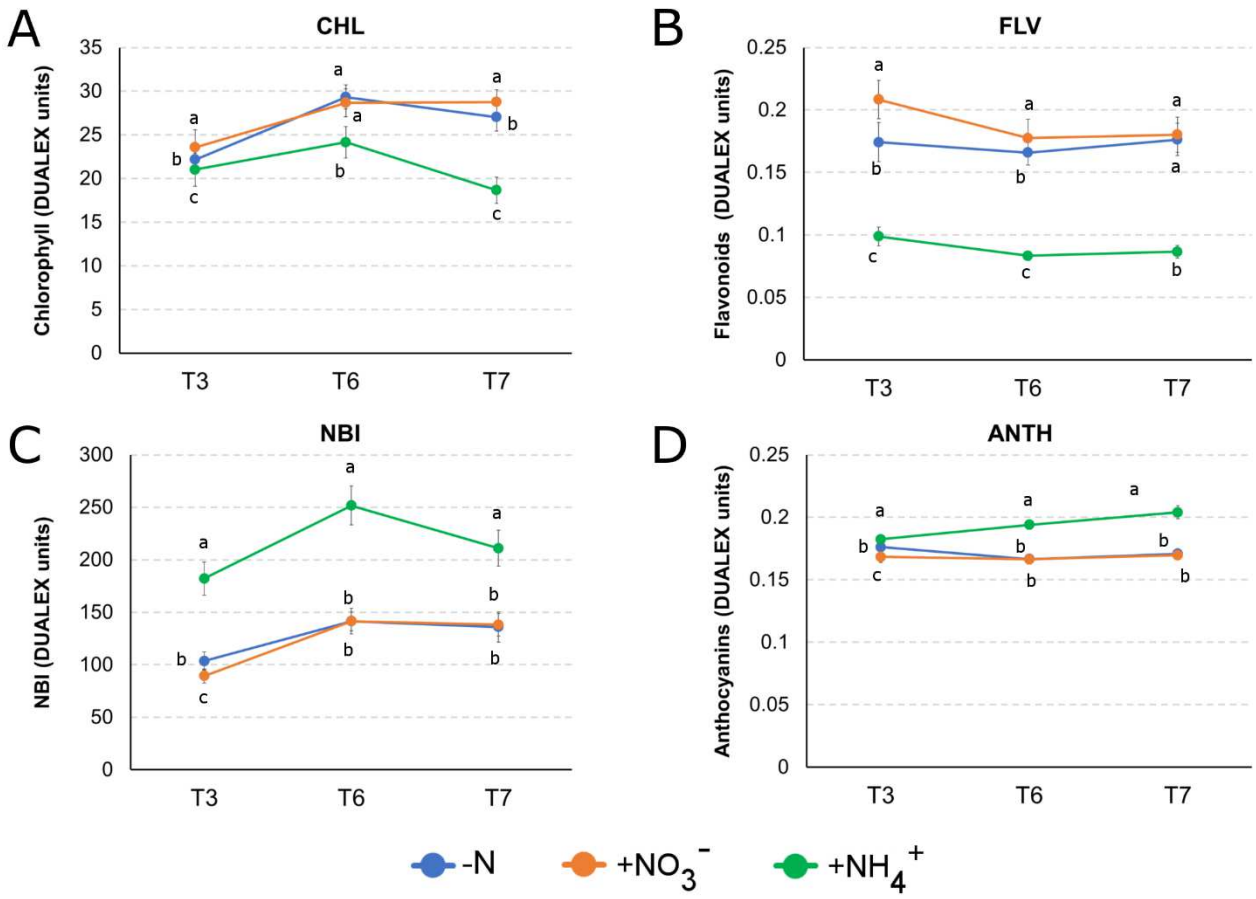


Figure 8. **Values of (A) leaf chlorophyll content, (B) leaf flavonoids content, (C) leaf anthocyanins content and (D) Nitrogen Balance Index**. Maize seedlings were grown 24 h in a N-deprived nutrient solution and then transferred to a 1mM N-supplied media (NO_3^- or NH_4^+) or to a N-deprived solution (-N) for additionally 3 days (T3), 6 days (T6) and 7 days (T7). Epidermis absorbance was obtained with the optical sensor DUALEX SCIENTIFIC+™ (Force-A) at the same hour of every time point. Values represents means of three replicates ± SE (n = 20). Similar letters at corresponding dot within treatments are not significantly different ($p < 0.05$) by an ANOVA test.

According to Chl content, nitrate ($+NO_3^-$) induced the strongest accumulation after 3 days of treatment, immediately followed by N-starved plants (-N) and ammonium supplied plants ($+NH_4^+$) which showed slight but significant differences (**Fig. 8A**). Both -N and $+NO_3^-$ plants showed a similar increasing trend of CHL accumulation, with a maximum at T6 where their values appeared statistically identical and stayed stable after additional 24h (T7). On the other hand, ammonium provision ($+NH_4^+$) determined only a moderate CHL accumulation at T6, while a drastic reduction was visible at T7.

Regarding flavonoids index (FLAV), a decreasing trend of accumulation was observed for nitrate-supplied plants, with the highest content at T3 (**Fig. 8B**). N-deficient plants showed a similar but significant lower level until T6, while at T7 FLAV index became identical between $+NO_3^-$ and -N. As for CHL, ammonium supplied plants showed a lower FLAV content compared to the other two sets of plants, but this pattern of accumulation remained stable at T6 and T7 too.

A different effect was exerted by N sources in anthocyanin accumulation (ANTH) (**Fig. 8D**), with a constant and increasing higher level in $+NH_4^+$ plants from T3 to T7, while $+NO_3^-$ and -N treatments seemed to not affect ANTH level at any time point.

NBI index was strongly enhanced by the provision of ammonium to the plants from T3 to T6, but the value dropped at T7(**Fig. 8C**). The same NBI positive trend was observed in both nitrate-supplied and nitrogen-starved plants from T3 to T6, but the value stayed stable and indistinguishable between the two treatments from T6 to T7. However, the NBI index exhibited by both $+NO_3^-$ and -N plants was lower than the one observed in $+NH_4^+$ plants at all the time points.

These results showed that the fluorescence indices values were generally comparable between nitrate-supplied and N-starved plants, while a peculiar behaviour was generally observed in $+NH_4^+$ plants. Indeed, ammonium-treated plants displayed a negative trend for chlorophyll accumulation from T6, a constant and positive trend in anthocyanins levels and a global non-variation in flavonoids level. On the other hand, -N and $+NO_3^-$ plants showed an increasing trend in the chlorophyll level, a decreasing trend in flavonoids level from T3 and T6, which became stable from T6 to T7, and a stable level of anthocyanins at every time point. Therefore, the indirect parameter of nitrogen balance index NBI (CHL/FLAV) showed how ammonium particularly affects the N status at the leaf level, while nitrate-supplied plants remain similar to the N-deficient ones.

3.6 Nitrate and ammonium differently regulate the amino acid profile in maize root and leaf

In order to appreciate how a different N source affected the plant N metabolism, total free amino acids and total hydrolysed amino acids were determined in root and leaf tissues of maize plants grown for 7 days in N-deficiency (-N) or provided with $+NO_3^-$ or NH_4^+ 1 mM (**Fig. 9; Fig. 10**).

The provision of a N source induced a significant lower global free amino acid level in the root, with -12,6% in NO_3^--treatment and -4.5% in NH_4^+-treatment with respect to the amount detected in N-deficiency (-N), respectively. On the contrary, they displayed higher concentrations in the leaves of both NO_3^-- (+28.5% compared to -N) and NH_4^+-supplied plants (+124.9% with respect to -

N) (**Table 2**). As far as total hydrolysed amino acids are concerned, their content in leaves and root was, instead, always lower in nitrate or ammonium-supplied plants if compared with N-deprived plants, especially in leaves of NH_4^+-supplied seedlings which showed a decrease of hydrolysed amino acids of about 70% compared to –N plants. (**Table 3**).

Looking at the free amino acids, alanine (Ala) was the most abundant amino acid, representing about 17-25% of the total amount of free amino acids found in leaves and roots (**Fig. 10**). In general N provision, either as nitrate or ammonium, generally triggers a higher Ala accumulation in leaves than in roots. This effect was particularly evident in the case of ammonium provision (**Fig. 9C-D**). Free Tyrosine (Tyr) and valine (Val) were also abundant in all three treatments both in roots and leaves, while leucine/isoleucine (Leu/Ile) resulted particularly conspicuous in roots, and asparagine (Asn) in leaves (**Fig. 10A**).

Contrariwise, the most present as hydrolysed amino acid was cysteine (Cys): in both tissues and three treatments it reached or even exceeded, 50% of total hydrolysed amino acids amount (**Fig. 10B; Table2**), followed by histidine (His), arginine (Arg) and Leucine/Isoleucine (Leu/Ile). Cys showed the greatest amount at root level treated with NH_4^+ (4927.9 µg/g), while the NO_3^- treatment induced no significant differences in Cys concentration compared with -N. Oppositely, Cys level was highest in leaves subjected to N-deficiency (4475 µg/g), while it was 40% lower in NO_3^- -supplied plants (2559 µg/g) and even 70% lower in +NH_4^+- supplied plants (1328 µg/g) (**Fig. 9A-B**).

Regarding glutamine (Gln) free level, which is the main compound for N translocation, no significant differences among treatments were detected nor in leaves or roots, while a significantly higher level in free glutamic acid (Glu) was observed both in roots and leaves treated with NO_3^- and NH_4^+ in comparison to –N-roots. Notably, in roots the free Glu level almost doubled in +NH_4^+ if compared to NO_3^-treatment, while only a very low amount of this amino acid was detected after hydrolysis.

Another free amino acid that showed a high level in response to NH_4^+ in the leaf is asparagine (Asn), while no significant difference between -N and NO_3^- treatments were detected. On the contrary, in roots Asn displayed no significant differences between -N and NH_4^+, while a huge drop in response to NO_3^- was observed.

As free amino acids, arginine (Arg) and histidine (His) showed a similar trend, since their levels were high in root treated with NO_3^-, while no amount of them was detectable at all in any treatment at leaf level. On the other hand, after hydrolysis both Arg and His showed the highest

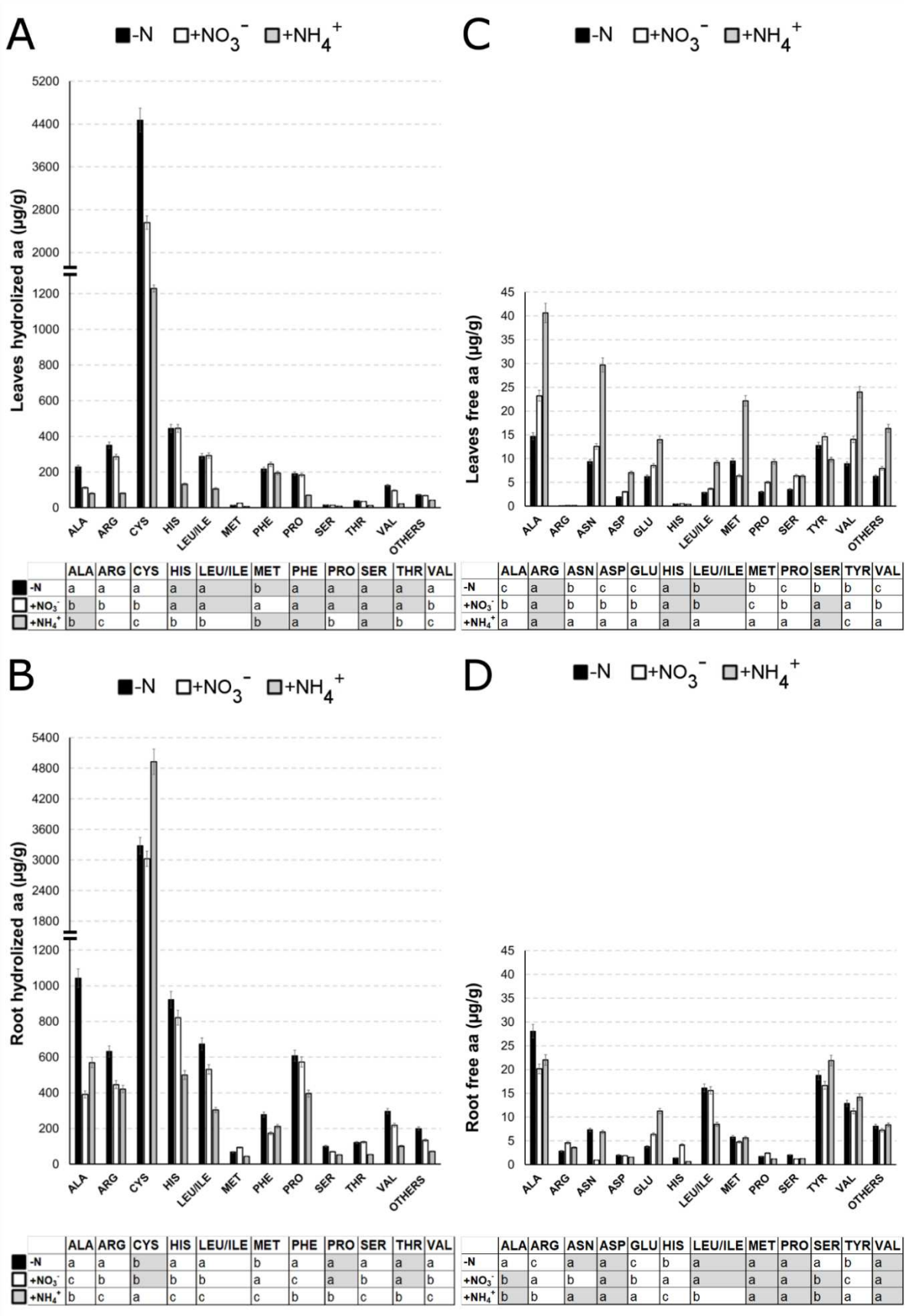
level in roots of -N seedlings, while in leaves no significant differences were detected between -N and + NO_3^- treatments.

The combination of leucine and isoleucine (Leu/Ile) as free amino acids showed an opposite trend in roots and leaves treated with NH_4^+, being significantly higher in leaves and lower in roots, while no differences were observed among -N and +NO_3^- treatments. After hydrolysis, the trend remained the same in roots, while in leaves the highest amount was again in -N and +NO_3^- supplied plants, whereas NH_4^+ treatment induced a significant fall in Leu/Ile level.

Furthermore, the content of free aspartic acid (Asp), methionine (Met), proline (Pro) and valine (Val) was significantly higher in leaves of NH_4^+-treated plants whereas no changes were evidenced in roots tissues in response to any treatment. On the contrary, serine (Ser) free level significantly increased in both NO_3^- and NH_4^+ supplied leaves, while it slightly decreased in roots. After hydrolysis, Met was the only amino acid that showed the highest amount in response to NO_3^- (and not to -N) both in leaves and roots, while Pro, Val and Ser displayed the highest amount in response to N-deficiency in roots.

Finally, among the free amino acids, only tyrosine (Tyr) level was lower in leaves in response to NH_4^+ and slightly higher in response to NO_3^-, while this trend appeared opposite in roots. After hydrolysis, Tyr level was not detected, while phenylalanine (Phe) showed a significantly higher level in response to -N in root, immediately followed by NH_4^+ treatment.

In conclusion, NH_4^+ supplied plants generally displayed higher levels of the free amino acids in leaves (above all for Ala, Asn, Glu, Met and Val), with the only exception of Tyr. In roots, nitrate and ammonium provision similarly affected the accumulation of free Ala, Asp, Met, Pro, Ser and Val levels, while Arg and His were higher in NO_3^--treated roots. On the other hand, Ala and Ser were the only free amino acids showing the highest level at root level in response to N-deprivation (-N). As far as hydrolysed amino acids are concerned, they displayed the lowest content in NH_4^+-supplied plants and were instead most abundant in tissues of N-deficient plants (-N). Met was the only hydrolysed amino acid that showed the highest amount in response to NO_3^- both in leaves and roots, while Ala and Phe were the only hydrolysed amino acid that displayed the lowest amount in response to NO_3^- in roots.



	ALA	ARG	CYS	HIS	LEU/ILE	MET	PHE	PRO	SER	THR	VAL
-N	a	a	a	a	a	b	a	a	a	a	a
+NO3-	b	b	b	a	a	a	a	a	a	a	b
+NH4+	b	c	c	b	b	b	a	b	a	b	c

	ALA	ARG	ASN	ASP	GLU	HIS	LEU/ILE	MET	PRO	SER	TYR	VAL
-N	c	a	b	c	c	a	b	b	c	b	b	c
+NO3-	b	a	b	b	b	a	b	c	b	a	a	b
+NH4+	a	a	a	a	a	a	a	a	a	a	c	a

	ALA	ARG	CYS	HIS	LEU/ILE	MET	PHE	PRO	SER	THR	VAL
-N	a	a	b	a	a	b	a	a	a	a	a
+NO3-	c	b	b	b	b	a	c	a	b	a	b
+NH4+	b	c	a	c	c	c	b	b	c	b	c

	ALA	ARG	ASN	ASP	GLU	HIS	LEU/ILE	MET	PRO	SER	TYR	VAL
-N	a	c	a	a	c	b	a	a	a	a	b	a
+NO3-	b	a	b	a	b	a	a	a	a	b	c	a
+NH4+	b	b	a	a	a	c	b	a	a	b	a	a

Figure 9. **Levels of the mostly abundant hydrolysed amino acid (A-B) and free amino acid (C-D) with respect to leaves (A-C) and roots (B-D).** For each panel the results of ANOVA test ($P < 0.05$) are reported. Each value is designed as the mean of three biological replicates ± SE (n = 20).

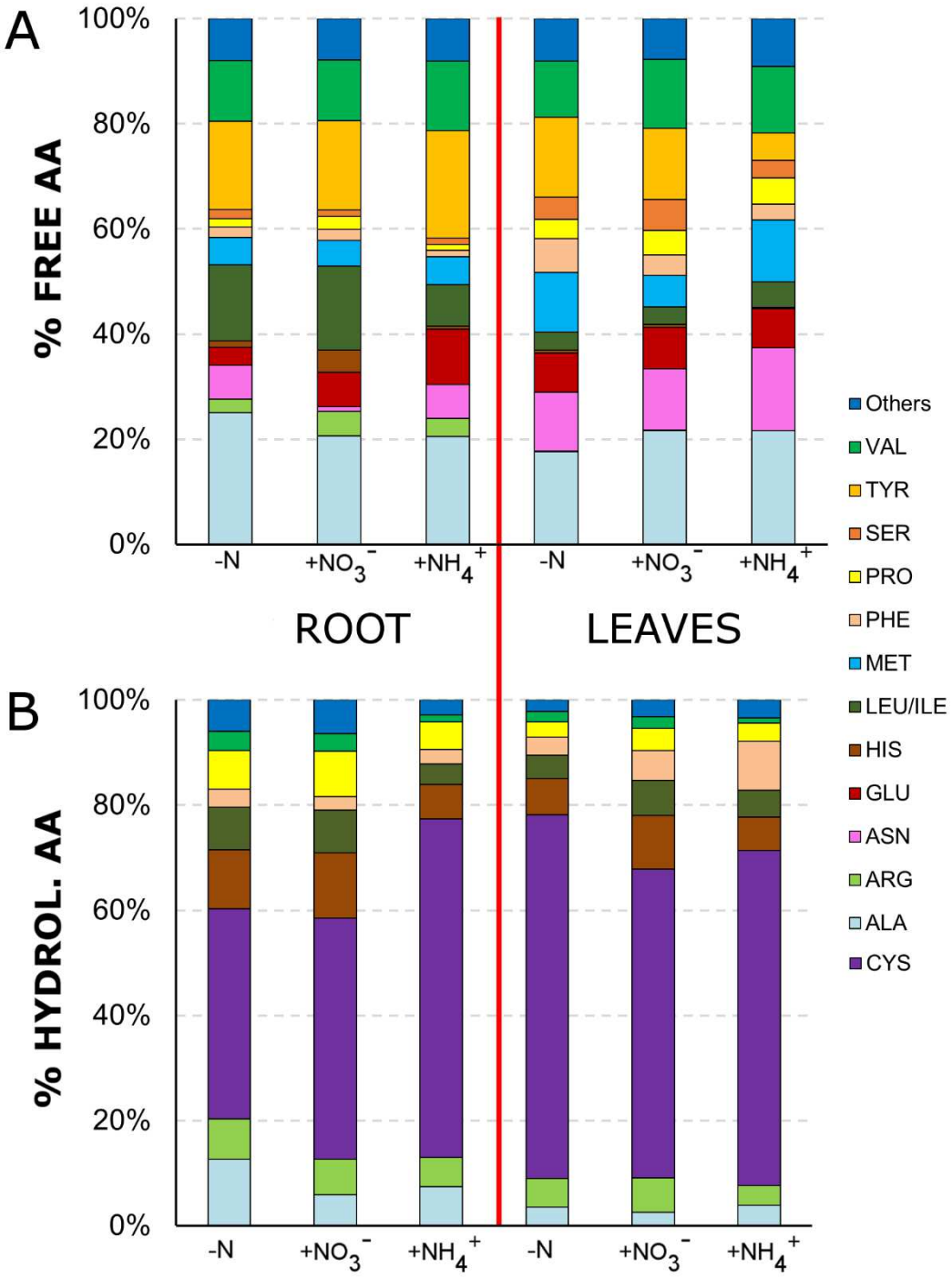


Figure 10. **Relative percentage (%) of the most present free amino acids (A) and the mostly present hydrolysed amino acids (B) with respect to the total amino acid composition for each treatment in each tissue**. Each value represents the mean of three biological replicates (n = 20).

Table 2. Complete quantification for all free amino acids detected as average of µg/g of weighted tissue ± SE and the proportion of total amino acids (%). **TOT**: calculated sum of total amino acids. **Δ% -N**: calculated percentage increase/decrease of total free amino acids detected in each treatment ($+NO_3^-$ or $+NH_4^+$) with respect to the N-deficient treatment (-N). The asterisk indicates significant differences with respect to -N treatments (Student's t-test with: ** p≤0.01, * p≤0.05).

	-N ROOT			$+NO_3^-$ ROOT				$+NH_4^+$ ROOT				-N LEAVES			$+NO_3^-$ LEAVES				$+NH_4^+$ LEAVES			
FREE AA	**µg/g**	**SE**	**%**	**µg/g**	**SE**	**%**	**p**	**µg/g**	**SE**	**%**	**p**	**µg/g**	**SE**	**%**	**µg/g**	**SE**	**%**	**p**	**µg/g**	**SE**	**%**	**p**
GLY	1.72	0.30	1.53	1.73	0.28	1.77		2.62	0.27	2.45		1.53	0.29	1.82	1.99	0.21	1.85		2.46	0.21	1.30	
ALA	28.08	0.19	25.06	20.15	0.15	20.57	*	22.01	0.21	20.56	*	14.72	0.15	17.58	23.23	0.19	21.58	*	40.65	0.37	21.58	*
SER	2.04	0.29	1.82	1.19	0.27	1.22	**	1.28	0.35	1.20	**	3.53	0.27	4.22	6.38	0.42	5.93	**	6.33	0.42	3.36	**
PRO	1.70	0.17	1.52	2.45	0.13	2.50	**	1.19	0.09	1.11	**	3.03	0.18	3.62	5.03	0.16	4.67	**	9.35	0.12	4.97	**
VAL	12.88	0.52	11.50	11.25	0.47	11.49	**	14.20	0.58	13.26	**	8.93	0.41	10.66	14.06	0.69	13.06	**	23.99	0.92	12.74	**
THR	2.05	0.12	1.83	2.09	0.12	2.14	**	2.46	0.19	2.30	**	1.21	0.18	1.45	1.56	0.19	1.45	**	4.23	0.23	2.24	**
CYS	0.76	0.06	0.68	0.44	0.06	0.45		0.12	0.00	0.11		0.43	0.03	0.51	0.42	0.03	0.39		0.75	0.09	0.40	
LEU/ILE	16.13	0.73	14.40	15.61	0.54	15.94	**	8.49	0.29	7.93	**	2.87	0.27	3.43	3.64	0.37	3.38	**	9.15	0.60	4.86	**
ASN	7.31	0.44	6.52	0.93	0.17	0.95	*	6.90	0.44	6.44	*	9.39	0.58	11.21	12.57	1.04	11.68	*	29.68	2.19	15.76	*
ASP	1.99	0.19	1.78	1.89	0.27	1.93		1.56	0.31	1.46		1.97	0.42	2.35	3.02	0.67	2.81		7.04	0.71	3.74	
GLN	0.91	0.18	0.81	0.49	0.14	0.50	*	0.21	0.04	0.19	*	0.46	0.06	0.55	0.37	0.06	0.35	*	0.66	0.19	0.35	*
GLU	3.79	0.39	3.38	6.38	0.48	6.51	**	11.28	1.21	10.54	**	6.24	12.90	7.45	8.56	12.77	7.95	**	14.00	0.55	7.44	**
LYS	0.60	0.08	0.53	0.24	0.07	0.25		0.53	0.08	0.50		0.50	0.08	0.59	0.22	0.06	0.21		1.40	0.08	0.74	
MET	5.83	0.54	5.20	4.77	0.80	4.87	**	5.64	1.42	5.27	**	9.53	1.06	11.38	6.39	0.39	5.94	**	22.15	1.52	11.76	**
HIS	1.44	0.08	1.28	4.19	0.21	4.28	**	0.63	0.17	0.59	**	0.48	0.10	0.57	0.50	0.13	0.47	**	0.42	0.06	0.22	**
PHE	2.26	0.32	2.02	2.04	0.45	2.08		1.35	0.24	1.26		5.37	0.69	6.41	4.19	0.78	3.90		5.65	0.98	3.00	
ARG	2.83	0.15	2.53	4.60	0.23	4.69	**	3.60	0.22	3.37	**	0.12	0.07	0.14	0.13	0.05	0.12	**	0.15	0.06	0.08	**
TYR	18.81	1.25	16.78	16.66	0.79	17.01	**	21.91	1.10	20.46	**	12.81	0.72	15.29	14.63	0.75	13.59	**	9.79	0.62	5.20	**
TRP	0.94	0.05	0.84	0.83	0.04	0.85	**	1.10	0.05	1.02	**	0.64	0.04	0.76	0.73	0.03	0.68	**	0.49	0.03	0.26	**
TOT	**112.06**	1.70	100	**97.94**	1.37	100		**107.06**	1.56	100		**83.75**	1.02	100	**107.62**	1.42	100		**188.34**	2.55	100	
Δ% -N						-12.6%				-4.5%							+28.5%				+124.9%	

Table 3. Complete quantification for all hydrolysed amino acids detected as average of µg/g of weighted tissue ± SE and the proportion of total amino acids (%). **TOT**: calculated sum of total amino acids. **Δ% -N**: calculated percentage increase/decrease of total hydrolysed amino acids detected in each treatment ($+NO_3^-$ or $+NH_4^+$) with respect to the N-deficient treatment (-N). The asterisk indicates significant differences with respect to -N treatments (Student's t-test with: ** p≤0.01, * p≤0.05).

	-N ROOT			$+NO_3^-$ ROOT				$+NH_4^+$ ROOT				-N LEAVES			$+NO_3^-$ LEAVES				$+NH_4^+$ LEAVES			
HYDR.AA	**µg/g**	**SE**	**%**	**µg/g**	**SE**	**%**	**p**	**µg/g**	**SE**	**%**	**p**	**µg/g**	**SE**	**%**	**µg/g**	**SE**	**%**	**p**	**µg/g**	**SE**	**%**	**p**
GLY	57.30	0.90	0.70	41.68	0.96	0.63	**	29.96	1.54	0.39	**	28.15	1.15	0.44	18.53	0.58	0.43	**	15.81	0.77	0.76	**
ALA	1042.81	2.50	12.68	390.80	1.73	5.92	**	569.89	1.35	7.45	**	228.59	1.15	3.54	112.12	1.54	2.57	**	80.18	1.54	3.85	**
SER	100.63	3.66	1.22	69.68	2.69	1.06	**	52.01	1.69	0.68	**	16.25	1.35	0.25	13.07	0.96	0.30	**	9.25	0.96	0.44	**
PRO	607.75	15.20	7.39	573.04	14.4	8.68	**	395.29	14.24	5.17	**	191.54	8.47	2.96	183.43	7.51	4.21	**	70.01	8.47	3.36	**
VAL	296.47	12.32	3.61	218.82	9.24	3.32	**	100.84	7.31	1.32	**	125.04	5.97	1.93	96.78	5.39	2.22	**	22.17	5.20	1.06	**
THR	122.03	6.74	1.48	123.47	6.16	1.87	**	53.92	2.89	0.70	**	39.82	1.44	0.62	34.67	1.35	0.80	**	12.03	1.54	0.58	**
CYS	3280.00	14.63	39.88	3024.74	13.4	45.83	*	4927.87	14.05	64.40	*	4474.61	7.51	69.22	2559.45	4.23	58.73	*	1327.59	5.39	63.71	*
LEU/ILE	673.41	11.74	8.19	530.62	12.3	8.04	**	303.73	7.89	3.97	**	288.61	4.62	4.46	291.66	3.27	6.69	**	106.46	2.12	5.11	**
ASP	36.47	1.15	0.44	29.97	1.15	0.45	**	9.93	0.77	0.13	**	6.89	0.58	0.11	10.43	0.64	0.24	**	1.59	0.13	0.08	**
ASP.a	23.04	0.58	0.28	16.13	0.31	0.24	**	4.85	0.12	0.06	**	3.91	0.27	0.06	5.56	0.25	0.13	**	0.93	0.06	0.04	**
GLU	44.65	0.60	0.54	20.05	0.77	0.30	*	9.51	0.44	0.12	*	17.13	0.50	0.27	18.89	0.38	0.43	*	13.08	0.31	0.63	*
LYS	8.05	0.17	0.10	4.28	0.17	0.06	**	2.18	0.12	0.03	**	3.97	0.13	0.06	3.87	0.06	0.09	**	2.67	0.10	0.13	**
MET	68.42	1.54	0.83	93.65	5.58	1.42	**	44.02	1.35	0.58	**	14.87	0.77	0.23	24.43	0.96	0.56	**	7.67	0.58	0.37	**
HIS	922.69	5.00	11.22	821.18	4.81	12.44	**	499.59	1.15	6.53	**	444.92	1.15	6.88	445.25	0.77	10.22	**	132.01	0.58	6.33	**
PHE	278.06	0.77	3.38	173.53	0.77	2.63	*	212.14	0.96	2.77	*	216.69	1.35	3.35	243.98	0.96	5.60	*	194.58	0.46	9.34	*
ARG	632.21	1.20	7.69	446.18	0.85	6.76	**	421.51	0.58	5.51	**	350.91	0.63	5.43	285.49	0.29	6.55	**	80.46	0.21	3.86	**
TRP	29.73	0.08	0.36	21.63	0.06	0.33	**	14.82	0.03	0.19	**	12.45	0.04	0.19	10.06	0.02	0.23	**	7.47	0.01	0.36	**
TOT	8223.72	187.3	100	6599.45	170	100		7652.066	275.3	100		6464.362	250.4	100	4357.65	143.1	100		2083.95	74.2	100	
Δ% -N						-19.7%				-6.9%							-32.6%				-67.8%	

4. DISCUSSION

Plant development is highly influenced by nitrogen (N) availability, so specific transport and signalling mechanisms were evolved in response to different N sources (Kiba and Krapp, 2016). Most of the studies have been focused on nitrate (NO_3^-) and ammonium (NH_4^+), since they are present in both natural and cropland soils at much higher levels than other sources (Miller and Cramer, 2005). Plants supplied with ammonium or with nitrate display many physiological differences, as for example contrasting effects on cellular energetics and redox status (Bloom, 1997; Noctor and Foyer, 1998). A better knowledge of mechanisms regulating nitrate and ammonium response in plants will help to develop a strategy to enhance inorganic N use efficiency (NUE) in plants, leading to increase crop yield and decrease the use of N fertilizers (Yang *et al*., 2017).

Previous results (Ravazzolo *et al*., 2019) led to hypothesise that nitrate and ammonium both inhibit strigolactone (SL) exudation by maize seedlings, even though to a different extent. In the same paper, it was also postulated that this inhibition of SL production could at least partially be involved in the induction of lateral root (LR) development observed in maize seedlings in response to both nitrate and ammonium. However, it is known that nitrate and ammonium trigger LR growth by activating different underlying mechanisms which are likely regulated by specific molecular events. To better characterize this response a physio-molecular comparative study was performed on maize seedlings grown in the presence of nitrate or ammonium or in N-starvation. To this aim, an untargeted approach was applied to deepen the transcriptomic signature of maize roots in response to N-depletion (24 h), nitrate provision (24 h, 1 mM) or ammonium provision (24 h, 1 mM).

Ammonium provision to N-starved roots predominantly up-regulates gene transcription, with only very few genes being down-regulated, whilst nitrate supply induces and repress transcription at a similar number of genes (**Fig.1**). Other studies performed in rice (Yang *et al*., 2015; Chandran *et al*., 2016) and in oilseed rape (*Brassica napus*) (Tang *et al*., 2019) showed instead a prevalent down-regulation of gene expression in response to ammonium, suggesting the existence of individual responses among species and genotypes.

Present results also point out that a huge percentage of genes are highly and specifically responsive to only one of these two nitrogen forms, suggesting that distinct and independent pathways are activated to optimize the plasticity of the response to these two ions, as already

shown in different species (Nacry *et al.,* 2013; Krapp *et al.,* 2014; Medici and Krouk, 2014; Krouk, 2016; O'Brien *et al.,* 2016). In particular, Gene Ontology (GO) (**Fig. 4**) and MapMan (**Fig. 5**) enrichments clearly indicate that nitrate provision to N-deprived roots negatively affect transmembrane transport and secondary metabolism, suggesting that 24 hours of nitrate provision are sufficient to down-regulate many root saturable transport systems (Krouk *et al*., 2010; Humbert *et al*., 2013) and to restore primary metabolism by slowing down the biosynthesis of secondary metabolites such as phenylpropanoids and flavonoids, which are known to be abundantly synthetized in the stress response signalling (Fritz *et al*., 2006; Francini *et al*., 2019; Isah, 2019). Nitrate also specifically induce the transcription of genes involved in polysaccharide binding and sugar transport, congruent with results on leaf area, biomass accumulation and chlorophyll index (**Fig. 6-8**) and with previous studies (Diaz *et al*., 2007; Meléndez *et al*., 2006; Zamboni *et al*., 2014).

Otherwise, ammonium provision seems to specifically activate genes involved in the hormonal signalling underlying defence responses, leading to suppose that it might positively affect the maize tolerance to biotic stress (**Fig.4-5**). Indeed, though N input increases plant defence, it also increases the amount of N compounds available for pathogens (Tavernier *et al.*, 2007), with the N form potentially affecting the extent of disease development and the degree of plant resistance (Borrero *et al.*, 2012, Gupta *et al.*, 2013, Cowley and Walters, 2002). Actually, a precise tuning of nitrogen fertilization, aimed at considering also the potential interplay between plants and pathogen could thus significantly impact on resistance to pathogens.

Current results also highlight specific and exclusive regulation of a unique family of transcription factors in response to ammonium (**Fig. 5**), which noticeably induces the expression of genes encoding WRKY transcription factors, playing crucial roles in the response to stress (Banerjee and Roychoudhury 2015; Phukan *et al*., 2016; Song *et al*., 2016). Moreover, ammonium triggers the activation of genes belonging to pathways related to water deprivation (**Fig. 4**). Water stress is a distinguishing feature of NH_4^+ toxicity, together with chlorosis, growth inhibition and increased shoot:root ratio (Miller and Cramer, 2005). The strong induction of the defence and stress pathways by ammonium in the B73 maize genotype could depend, therefore, on a kind of toxicity of this N form.

On the other hand, nitrate specifically down-regulates the transcription of genes related to hemicellulose synthesis and transporters (**Fig. 5**). Accordingly, similar expression profiles have been recently evidenced in Arabidopsis (Landi and Esposito, 2017), where it was suggested that a

cell wall remodelling might be important for the enhanced nitrate uptake and correct plant growth in maize. Nevertheless, the exact molecular mechanism by which the N status affects cell wall modelling is still unknown (Ogden *et al*., 2018).

Besides those characterizing specific responses to nitrate or ammonium, further groups of genes displayed instead common trend of regulation in response to both of them. Genes encoding key elements in the pathway leading to reactive oxygen species (ROS) detoxification were mainly down-regulated by both these N sources, as a consequence of the recovery of a balanced nutritional equilibrium that switches down the state of N-deficiency stress. Moreover, both nitrate and ammonium induce the expression of genes encoding transcription factors belonging to the AP2/EREBP family (APETALA2/Ethylene-responsive element binding protein). The identification of common elements belonging to shared signalling pathway similarly regulated upon nitrate and ammonium, confirms the existence of many processes responsive to the overall plant nitrogen status as observed also by Wang *et al*. (2003) and Scheible *et al*. (2004). Genes regulated in a coordinated way by both ammonium and nitrate presumably respond to a signal downstream of ammonium assimilation, as for example some amino acids as glutamate (Glu) or glutamine (Gln), which have been demonstrated to be powerful regulators of gene expression (Gutierrez *et al*., 2008). Levels of free glutamate therein obtained clearly confirm the recognized role of this amino acid (Forde, 2014; Toyota *et al*., 2018) as putative signal accumulating in both nitrate and ammonium provided roots and possibly involved in the downstream regulation of the expression of common genes (**Fig. 9-10**). However, glutamine was detected at very low levels in maize roots and displayed higher concentration in N-starved roots.

Biomass accumulation seems to be strongly compromised in response to ammonium, both in leaves and roots (**Fig. 6**), and chlorophyll accumulation also displayed a negative trend (**Fig. 8A**). Moreover, a strong negative effect on primary root growth was also noticed (**Fig. 7**), whereas nitrate positively influences the root biomass production (**Fig. 6**), primarily thanks to the induction of longer and thinner roots, which are needed to extend the root apparatus in the deeper layers of soil to seek for this nutrient (Gruber *et al*., 2013).

NH_4^+-provided plants also displayed significantly higher contents of anthocyanins in comparison to N-starved and NO_3^--supplied plants (**Fig. 8D**). Anthocyanins are secondary metabolites derived from the specific branch of the flavonoid pathway and key molecules in the defence against environmental stresses (Chen *et al*., 2019).

These results altogether further support the idea that ammonium nutrition could enhance biotic stress tolerance, perhaps as a side effect of its slight toxicity, even though the mechanism(s) underlying this toxicity is still not completely understood (Britto and Kronzucker, 2002; Sarasketa *et al*., 2016).

A higher accumulation of free amino acid in leaves of NH_4^+-provided plants was observed if compared with N-deprived and NO_3^- supplied seedlings (**Fig. 9C-D**). The increase of amino acids content in leaves in response to ammonium has been already documented and interpreted as a toxic symptom (Miller and Cramer, 2005), but it has also been correlated with an enhanced abiotic stress tolerance through the induction of protecting pathways (Flowers and Colmer, 2015). For instance, it was shown that ammonium provision induced higher amount of proline both in *Matricaria chamomilla* (Kováčik and Klejdus, 2014) and *Oryza sativa* (Sun *et al*., 2017) leaves. In the present results, the amount of free proline doubled in response to ammonium treatment in leaves, if compared to nitrate-supplied plants (**Fig. 9-C**). Proline accumulation is known to act as an osmolyte and chaperone that is accumulated under various stress conditions (Szabados and Savouré, 2010), with a positive correlation with an enhanced stress tolerance to high ammonium conditions in rice (Sun *et al*., 2017), but with a negative impact on chamomile plants treated with the same cation (Kováčik and Klejdus, 2014).

Compared to nitrate-provided plants, ammonium-supplied plants also displayed a higher level of asparagine in both leaves and roots, possibly due to the asparagine involvement in the mechanism of cell protection against NH_4^+-induced stress (Prinsi and Espen, 2015). Furthermore, asparagine has been suggested to play a role in the response to many abiotic stresses, for example sulphur- and phosphate-deficiency, together with drought and salt stress (Lea *et al.,* 2007; Curtis *et al*., 2018). Moreover, N can be redirected from glutamine to asparagine as a temporary measure to control nitrogen excess, such as excessive ammonium provision (Lean and Miflin, 2011).

Nitrate-supplied plants showed a significant increase of the content of histidine (His) in roots (**Fig. 9-D**). Thanks to the presence of an imidazole side group, histidine can be found in the active site of many enzymes. Moreover, histidine plays important roles in phosphoryl transfer and metal ion homeostasis (Stepansky and Leustek, 2006; Ingle, 2011), but the routes of its biosynthesis and catabolism in plants are still scarcely understood, so future research should be aimed at better deepen the link between nitrate-supply and increased histidine content in root.

Regarding the hydrolysed amino acid profile (**Fig. 9A-B**), ammonium induced a global lower hydrolysed amino acid level in roots if compared to N-deprived plants, with only cysteine being

increased of 50%. Cysteine can be used as an extra energy source for plant (Hildebrandt *et al*., 2015) but also to produce sulphur-containing defence metabolites such as glutathione and glucosinolates (Romero *et al*., 2014). These findings confirm the already hypothesised activation of pathways related to oxidative and biotic stress response upon ammonium provision.

On the other hand, nitrate led to higher accumulation of hydrolysed methionine both in roots and leaves (**Fig. 9A-B**). Methionine is a precursor of the primary methyl group donor S-adenosyl-methionine (SAM). SAM is the precursor of ethylene and glucosinolates, but it is also involved in the regulation of cell division and in the synthesis of cell wall, cell membrane and chlorophyll (Amir, 2008). This result, together with the positive effect on chlorophyll content in leaves (**Fig. 8**) and the up-regulation of the bin 'sulfur-containing glucosinolates', and the down-regulation of the cell wall-related bins (MapMan analysis in roots **Fig. 5**), led to hypothesize that nitrate-provided plants need to balance between cell wall loosening and thickening (Mu *et al*., 2016), leaf pigments content (Meléndez *et al*., 2006) and secondary metabolites production (Isah, 2019) to allow a correct plant growth in response to enhanced N uptake.

In conclusion, this study provides a detailed picture of the global transcriptomic response of maize roots to early nitrogen starvation or nitrate and ammonium provision, allowing to distinguish common and individual elements of the overall response, as for example hormonal pathways, transcription factors, primary and secondary metabolism and defence response, all contributing to accomplish the response to inorganic N. Besides, the biomass accumulation together with the leaf pigments results further supported the main hypothesis driven by the RNA-seq analyses and the profiling of amino acids contents in root and leaves gave us new insight into the metabolic regulation of this response.

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Chapter 5

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Nitrogen (N) is a key signal in regulating plant development in response to the environment but its availability for crops is limited and they need to cope with its fluctuations. Nitrate (NO_3^-) represents the principal N form for crop, and it acts both as nutrient and signal, regulating gene expression and several physiological and developmental processes. Roots are the fundamental organs to sense NO_3^- in their environment and to quickly rearrange the growth of all plant as a developmental response to it. In particular, it was shown that the root apex, which includes the fundamental transition zone (TZ), represents the key root zone to sense external fluctuations of nitrate and translates them into a signalling cascade to control the adaptive differential growth responses. Accordingly, previous work led to the hypothesis of a coordinated action of nitric oxide (NO), auxin and strigolactone (SLs) in regulating the early response of maize root apex to nitrate (Trevisan *et al*., 2014, 2015; Manoli *et al*., 2016). As reviewed in detail in Chapter 1, strigolactones are a novel class of phytohormones involved in many physiological and stress-induced plant responses to the environment, but their exact role in the response to nitrate starvation is still under investigation.

In this PhD dissertation, further evidence on SL involvement in the signalling pathway governing root maize response to N was gained (Chapter 2). Accordingly, during the first part of my PhD project we used a direct LC-MS/MS quantification and an indirect germination bioassay to demonstrate that N deficiency triggers zealactones exudation, while nitrate strongly prevents it within just 24 h of supply. On the other hand, also ammonium exerts a repression on SL exudation, but in a less effective and more time-consuming way. This effect on exudation by NO_3^- and NH_4^+ was then confirmed by the trend in transcripts levels observed for the SL biosynthesis gene *ZmCCD8*, which in turn appeared as a reliable and useful marker for SL biosynthesis in maize. Moreover, the results showed a marked induction in the transcription of the putative maize SL transporter ZmWBC33 by nitrogen and phosphate deprivation, while a clear downregulation of its expression in the presence of nitrate was observed. Currently, no SL transporters have been isolated yet from Monocots, but only PDR1 in petunia (Kretzschmar *et al*., 2012) and its close homologue PDR6 from *Nicotiana tabacum* (Xie *et al*., 2015). *WBC33* transcripts localization was visualized with *ISH* technique, showing a similar localization pattern observed for PDR1 in petunia (Sasse *et al*., 2015) and PDR6 in tobacco (Xie *et al*., 2015), together with a co-localization with *CCD8*, thus supporting our hypothesis that ZmWBC33 could be involved in the SL cell-to-cell flux in maize root. Nevertheless, a deeper analysis of ZmWBC33 structure-function relationships and of a direct

binding with zealactones will be necessary to better elucidate ZmWBC33 functions. Besides studying the effect of nitrate and ammonium on SL exudation and biosynthesis in maize root, the research also highlighted the importance of SLs as endogenous signals involved with N in lateral root (LR) development. Accordingly, the results showed that nitrate and ammonium supply markedly stimulate LR development, thus suggesting that this stimulation could be linked to the complete or partial inhibition of SL production observed in response to nitrate and ammonium, respectively.

Since our previous results suggest that SLs and auxin might cooperate to regulate the response of maize primary root to nitrate (Manoli *et al*., 2016), the hypothesis that the negative effect of nitrate on SL biosynthesis/exudation could depend on auxin was further studied (Chapter 3). Given that the modulation of lateral root (LR) development in response to nutrient availability in cereals is highly complex (Bray and Topp, 2018) and only few LR mutants have been described in maize, being generally auxin-related (Yu *et al*., 2018), a number of genes putatively associated with the signalling leading to LR development through auxin signalling were identified and their expression were assessed in root tissues of maize seedlings grown without nitrogen or supplied with nitrate, together with auxin and SL analogues and inhibitors. The gene expressions evidenced peculiar trends in response to nitrate, auxin, SLs and specific inhibitors, allowing to select few of them as good candidates to better characterize and deepen the auxinic action involved in the nitrate signalling. For instance, the Aux/IAA-coding gene *IAA29/RUL1* appeared promising as a good marker to study the interplay between auxin and SL in nitrate signalling.

LR density was also assessed in seedlings treated as for gene expression analysis with N-deprived or nitrate-provided nutrient solution and in the presence of SLs and auxin analogues or inhibitors. Our results show that the LR development in response to nitrate provision involves SLs as an upstream signal to the downstream auxin signalling. Nevertheless, the nitrate-dependent induction of LR development appears to involve an auxin-independent pathway too.

Globally, our results suggest that SLs and auxin share overlapping and divergent pathways to regulate maize lateral root development in response to nitrate availability.

Finally, an untargeted RNA-seq analysis on maize root apex led us to discriminate the different and common elements within nitrate and ammonium-triggered response in all root transcriptome (Chapter 4). Among the specific responses, nitrate predominantly repressed the expression of genes related to transmembrane transport and cell wall, while ammonium strongly perturbs the hormonal balance in favour of a biotic-stress typical response. On the other hand, both the N-sources induce repression in cytoskeleton-related and redox homeostasis genes. Physiologically, a toxic effect of

ammonium on biomass accumulation was also evidenced, together with a strong impact on leaf pigments, whereas nitrate-supplied plants displayed an increased total biomass thanks to a slow but positive effect of nitrate on root architecture. In particular, ammonium-treated plants displayed a negative trend for chlorophyll accumulation, a constant and positive trend in anthocyanins levels and a global non-variation in flavonoids level. On the other hand, -N and $+NO_3^-$ plants showed an increasing trend in the chlorophyll level, a decreasing trend in flavonoids level, and a stable level of anthocyanins. These results show that ammonium particularly affects the pigment status, while nitrate-supplied plants remain similar to the N-deficient ones. Ammonium also increased the total amount of free amino acids at the leaf level, which can in turn result as a toxic accumulation, while nitrate induces a high level of free arginine, glutamine and histidine in roots. On the contrary, ammonium generally induced low level of hydrolysed amino acids in all the maize plant, while a high amount of hydrolysed amino acids was generally the response to N-deprivation. These results provided new insight to better characterize how the early sensing of N-deficiency or nitrate/ammonium provision by root could impact on the overall plant growth and physiology.

However, some open questions still remain to be answered and additional experiments are in progress to better understand the exact involvement of strigolactones with nitric oxide (NO) in maize root development. The interplay existing between SLs and NO has been already reviewed (Kolbert, 2018), and our previous work (Manoli *et al*., 2014; Manoli *et al*., 2016) led to hypothesise that the signalling cascade deriving from nitrate perception by maize root could at least in part depend on the production of nitric oxide via nitrate reductase. In this scenario, the inhibition of SL biosynthesis and exudation observed upon nitrate supply might be regulated by NO early production, so future work will be aimed at better investigate the events directly regulate by nitrate itself from those NO-dependent.

To start achieving that, maize seedlings grown with nitrate (1 mM) were also supplied with a NO scavenger (cPTIO, namely 2-4-Carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) and a nitrate reductase inhibitor (sodium tungstate dihydrate, namely $Na_2WO_4 \bullet 2H_2O$, a NO-producing enzyme identified in plants) to analyse root development. Moreover, the RNA-Seq technique was employed to obtain the transcriptomic profile of maize roots provided with nitrate plus 1 mM cPTIO, to compare it with the dataset of RNA-seq data already obtained for –N and nitrate –supplied roots (chapter 4), each treatment tested in triplicate. The aim of this experiment is to discriminate between nitrate responsive genes and pathways depending on nitrate itself or on NO biosynthesis early occurring immediately after nitrate provision.

Currently, the data set are under analysis, but the results will help in deciphering the complex molecular regulation of the nitrate response in maize root and will provide new insight into the mechanisms underlying plant adaptation to N fluctuations.

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