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NICKEL ENHANCES FLAVONOIDS BIOSYNTHESIS AND BIOLOGICAL NITROGEN FIXATION IN SOYBEAN PLANTS

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MARCO ANTONIO BOSSE - Nascido em Três Lagoas, Mato Grosso do Sul, no dia 26 de março de 1993. Em 2017, graduou-se em Engenharia Agronômica pela Universidade Tecnológica Federal do Paraná, Câmpus Pato Branco, PR. Durante a graduação participou do programa Ciências sem Fronteiras, realizando intercâmbio acadêmico na University of Minnesota, nos Estados Unidos da América. Em agosto de 2019, ingressou no Mestrado do Programa de Pós-Graduação em Agronomia (Produção Vegetal), da Universidade Estadual Paulista, Câmpus Jaboticabal, sob a orientação do Prof. Dr. André Rodrigues dos Reis.

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SUMÁRIO

NÍQUEL AUMENTA A SÍNTESE DE FLAVONOIDES E FIXAÇÃO BIOLÓGICA DE NITROGÊNIO NA SOJA

RESUMO – Níquel (Ni) é um elemento essencial para o metabolismo de nitrogênio (N) e pode afetar diversos processos fisiológicos desde a germinação até o crescimento vegetativo, visto que é um componente estrutural das enzimas urease e [NiFe]-hidrogenase, possuindo um papel fundamental na fixação biológica de nitrogênio (FBN) em leguminosas. Portanto, o presente estudo teve como objetivo investigar os efeitos da fertilização com Ni na atividade da enzima urease, no perfil de flavonoides, nodulação, metabolismo de ureídeos e antioxidante, e o seu impacto na germinação e produtividade da planta. Nós testamos três doses de Ni (0.0, 1,5, e 3,0 mg kg^{-1}) e dois genótipos de soja: cultivar comercial TMG 2158, e uma *near-isogenic lines* (NILs), urease-nula (*eu3-a).* Ni aumentou a atividade da urease, convertendo ureia em amônia nas folhas. Ni também aumentou os níveis de daidzeína, genisteína, rutina e kaempferol nas raízes e a nodulação, principalmente para o genótipo TMG 2158. Houve correlação entre os teores de daidzeína, nodulação, ureídeos e produtividade em resposta a fertilização com Ni, exceto para os NILs. Esses resultados sugerem que o Ni também desempenha um papel no metabolismo de flavonoides (principalmente daidzeína), contribuindo para a quimiotaxia dos rizóbios, o que resulta em um aumento de nodulação e metabolismo de ureídeos e maior quantidade de nitrogênio orgânico disponível para as plantas de soja, resultando em maior produtividade. Além disso, a fertilização com Ni aumentou o teor de açúcares totais de todos os genótipos de soja. A fertilização com Ni também levou à diminuição da peroxidação lipídica das membranas celulares do genótipo TMG 2159*,* provavelmente devido ao aumento da atividade de APX, que desempenhou um papel importante na eliminação de H_2O_2. Em relação à reserva de Ni nas sementes, Ni melhorou a germinação e o desenvolvimento das plântulas por meio do aumento da atividade da urease e da remobilização do N, embora esses resultados sejam altamente dependentes do genótipo da soja.

Palavras-chave: atividade da urease, daidzeína, fotossíntese, metabolismo antioxidante, ureídeos.

NICKEL ENHANCES FLAVONOIDS BIOSYNTHESIS AND BIOLOGICAL NITROGEN FIXATION IN SOYBEAN PLANTS

ABSTRACT – Nickel (Ni) is an essential element to N metabolism and affects a wide range of physiological processes, from seed germination to vegetative growth, since it is a structural component of urease and [NiFe]-hydrogenase, playing an important role during biological nitrogen fixation (BNF) in legume plants. Therefore, this study aimed to investigate the effects of Ni fertilization on urease activity, flavonoid profile, nodulation, ureide and antioxidant metabolism and its impact on seed germination and plant yield. We tested three Ni doses (0.0, 1.5, and 3.0 mg kg^{-1}) and three soybean genotypes (commercial soybean (TMG 2158), and one soybean near-isogenic lines (NILs), urease-null (*eu3-a*). Ni increased urease activity, breaking down urea into ammonia in the leaves. Ni enhanced daidzein, genistein, rutin and kaempferol in roots and nodulation mainly for TMG 2158 plants. There was a relationship between daidzein, nodulation, ureides and seed yield in response to Ni fertilization, except for NILs. These results suggest that Ni plays a role in flavonoids metabolism (mainly daidzein) inducing chemotaxis between soybean plants and rhizobia, boosting nodulation and ureide metabolism. Enhanced biological nitrogen fixation induced by Ni fertilization promoted higher nodulation generating more organic nitrogen available to soybean plants resulting in a higher number of pods per plant and seed yield. Furthermore, Ni fertilization increased total sugars content of all soybean genotypes. Ni fertilization decreased lipid peroxidation of cell membranes of genotype TMG 2159, probably due to an increase in APX activity, which played an important role in H_2O_2 scavenging. Ni also improved seed germination and seedling development by increasing urease activity and N remobilization, although it is highly dependent on the soybean genotype.

Keywords: antioxidant metabolism, daidzein, photosynthesis, urease activity, ureides.

CHAPTER 1 – General considerations

1. Introduction

Nickel (Ni) is essential for plants, since it is a cofactor that is part of the active site of urease enzyme (Dixon et al., 1975). In addition, Ni is also an essential cofactor of many other enzymes, such as hydrogenase, which is present in diazotrophic bacteria (Bagyinka, 2014) that are directly involved in nitrogen metabolism.

Urease is responsible for catalyzing the hydrolysis of urea, producing ammonia and carbon dioxide (Witte, 2011), while hydrogenase catalyzes the oxidation of molecular hydrogen (H_2), generated during the process of biological nitrogen fixation (BNF) by the enzyme nitrogenase, recovering a certain amount of energy required in this process, as well as reducing the competition between H_2 and nitrogen gas (N_2) from the nitrogenase active site (González-Guerrero et al., 2014), resulting in a more efficient BNF.

Although Ni deficiency in field-grown plants is not observed often, recent studies show latent Ni deficiency in modern varieties of soybean plants, which did not exhibit visual symptoms in leaves. (Freitas et al., 2019). The authors demonstrated that low Ni concentrations benefit N metabolism in soybean plants, resulting in increased seed yield. The authors also found that Ni fertilization caused increased number of nodules; however, the mechanisms involved in this process have not been elucidated.

The first step in nodule formation between plants and diazotrophic bacteria is the formation of a chemical gradient formed by different metabolites exuded by the roots, such as organic acids and amino acid sugars, which attract rhizobia by chemotaxis. Another essential compound for nodulation are flavonoids, which are responsible for inducing the expression of *nod* genes in bacteria, which will result in root infection and nodule formation (Barbour et al., 1991; Kape et al., 1991; Hirsch, 1992). However, there are still few studies investigating the effect of Ni on urease activity in relation to the nodulation process, especially in the metabolism of flavonoids.

Urease is not only involved in the remobilization and assimilation of N in plants, but also in plant defense against pathogens such as fungi and insects. (Carlini and Polacco, 2008; Stanisçuaski and Carlini, 2012; Medeiros-Silva et al., 2014). Furthermore,

recent studies have shown that Ni can also act in the antioxidant metabolism of plants by activating the isoform of glyoxylase I, which plays an important role in the degradation of methylglyoxal (MG), a toxic compound naturally produced by cell metabolism (Fabiano et al., 2015). Thus, further investigations are needed in order to elucidate the effects of Ni in beneficial doses on the antioxidant system of plants, since most studies are focused on the toxicity effects of this micronutrient.

This study aimed to evaluate the effects of Ni on urease enzyme activity and its influence on flavonoid profile, photosynthesis, antioxidant metabolism, as well as the effect of Ni contents in soybean grain on germination using mutant seeds for the urease enzyme.

2. Literature review

2.1 Nickel

Ni was the last element to be included in the list of essential plant nutrients. In 1983, the authors Eskew et al. (1983) showed that soybean plants under Ni depletion accumulated urea at the leaflets tips. In addition, (Brown et al., 1987) observed that barley plants after three successive generations of cultivation under Ni deficiency, no longer produced viable seeds (Brown et al., 1987).

In the soil, Ni is rapidly absorbed by plants in the form of Ni^{2+} and is transported from the roots to the shoots via xylem. It is highly mobile and translocated from old to the new leaves. Furthermore, Ni can also be transported to other parts of plants such as fruits and seeds (Page et al., 2006). Ni deficiency reduces the activity of the urease enzyme, resulting in accumulation of urea in tissues and the formation of necrotic lesions on the leaves known as “mouse ear” (Eskew et al., 1983). Furthermore, Ni deficiency disrupt metabolism of ureides, amino acids and organic acids in plants (Bai et al., 2006).

Most studies regarding Ni in plants are focused on toxicity, which can result in plant growth inhibition, reduced respiratory activity, and anatomical and morphological changes (Seregin and Kozhevnikova, 2006; Reis *et al.*, 2017; Hassan *et al.*, 2019). However, recent studies have increasingly shown Ni beneficial effects for plants, especially in modern soybean cultivars (Barcelos et al., 2018; Freitas et al., 2018; Levy et al., 2019).

2.2 Urease

Ni takes part in the constitution of the active site of the urease enzyme (Dixon et al., 1975) which is involved in nitrogen metabolism. Urease in plants acts on the assimilation of arginine-derived urea (an amino acid considered to be the main N storage compound), through the hydrolysis of urea into two ammonia molecules (Figure 1) (Polacco et al., 2013).

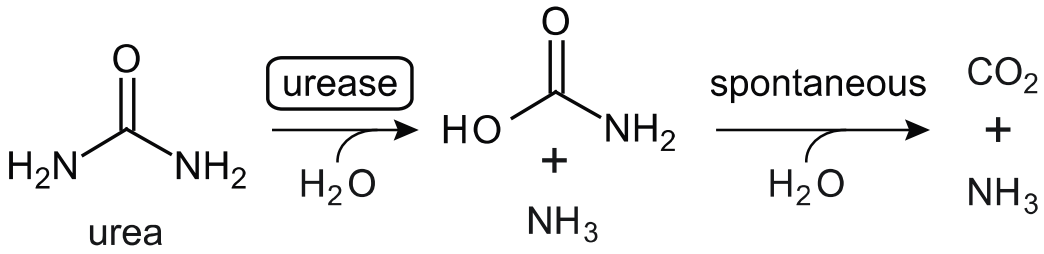

Figure 1. Urea hydrolysis reaction catalyzed by the urease enzyme.
Source: Witte (2011).

The main function of urease is to break down urea into ammonia for N remobilization in plant tissues. Ammonia is incorporated into organic compounds catalized by glutamine synthetase (Sirko & Brodzik, 2000).

In soybean, embryo urease (eSBU), encoded by the *Eu1* gene, is an abundant protein in seeds, while the ubiquitous urease (uSBU), encoded by *Eu4* gene, is found in smaller amounts in all plant organs (Polacco et al., 1982). Recently, a third gene was found and named *Eu5* (Polacco et al., 2013).

For urease to be in its active form, the action of a complex of three accessory proteins is necessary: UreD, UreF and UreG, which are responsible for transporting and binding Ni ions (Figure 2) (Witte, 2011). The *Eu3* gene encodes the accessory protein UreG (Glyma08g08970). eu3-a is a soybean mutant that has zero ureolytic activity and, therefore, is unable to metabolize urea (Freyermuth et al., 2000; Tezotto et al., 2016).

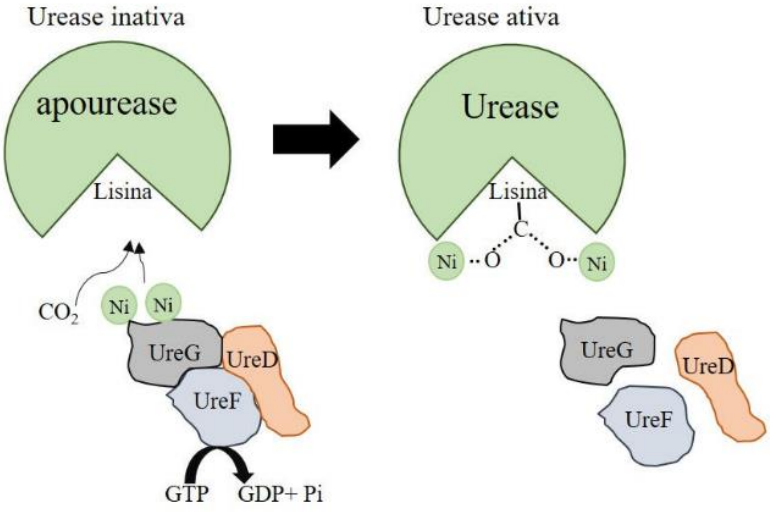

Figure 2. Plant urease activation model involving the binding of three accessory proteins (UreD, UreF and UreG) to apourease. Accessory proteins dissociate from urease after activation.
Source: Witte (2011).

Furthermore, urease appears to play an important role during seed germination and early seedling development. This importance was found in *Arabidopsis thaliana* seeds, where the absence of ureolytic activity promoted an inhibition of germination (Zonia et al., 1995). However, recent studies have shown that both Ni concentration in seeds and urease activity did not interfere on seed germination, although enhanced in seedling development was observed (Kutman et al., 2013; Rechenmacher et al., 2017). The role of Ni in seeds for plant growth and N metabolism is still poorly understood, making further studies necessary.

2.3 Importance of nickel for legumes

In microorganisms, Ni is also an essential catalytic cofactor of other enzymes, such as carbon monoxide dehydrogenase I, superoxide dismutase and [NiFe]-hydrogenase (Li e Zamble, 2009).

Rhizobia bacteria can develop a symbiotic relationship with legume plants allowing the use of dinitrogen gas (N_2) present in the atmosphere. The N_2 is converted into ammonia by nitrogenase activity, which is an enzyme complex responsible for breaking down the triple bond of N_2 (Andrews et al., 2009). Another enzyme that participates in this

process is [NiFe]-hydrogenase, which oxidizes the H_2 produced collaterally in the proton and electron fixation step for nitrogenase (Bagyinka, 2014). Thus, leguminous plants may be more dependent on adequate Ni fertilization due to BNF.

It is important to highlight that flavonoids are essential compounds during early nodulation process and BNF. Flavonoids secreted by roots act in the communication between rhizosphere organisms and plants (Sugiyama et al., 2016; Matsuda et al., 2020; Okutani et al., 2020). For soybean plants, the main flavonoids found are daidzein and genistein, and they are inducers of *nod* genes expression, which are responsible for the production and secretion of compounds known as nodulation factors (Nod Factors) by symbiotic bacteria, which induce a series of physiological changes in plant cells to nodule formation occurs (Subramanian et al., 2007; Panche et al., 2016).

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CHAPTER 2 – Physiological impact of flavonoids on nodulation and ureide metabolism in legume plants[1]

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Abstract

Legume plants from Fabaceae family (phylogenetic group composed by three subfamilies: Caesalpinioideae, Mimosoideae, and Papilionoideae) can fix atmospheric nitrogen (N_2) into ammonia (NH_3) by the symbiotic relationship with rhizobia bacteria. These bacteria respond chemotactically to certain compounds released by plants such as sugars, amino acids and organic acids. Root secretion of isoflavonoids acts as inducers for *nod* genes in rhizobia and ABC transporters and ICHG (isoflavone conjugates hydrolyzing beta-glucosidase) at apoplast are related to the exudation of genistein and daidzein in soybean roots. Biological nitrogen fixation (BNF) occurs inside the nodule by the action of nitrogenase enzyme, which fixes N_2 into NH_3, which is converted into ureides (allantoin and allantoic acid). In this review, we bring together the latest findings on flavonoids biosynthesis and ureide metabolism in several legume plant species. We emphasize how flavonoids induce *nod* genes in rhizobia, affecting chemotaxis, nodulation, ureide production, growth and yield of legume plants. Mainly, isoflavonoids daidzein and genistein are responsible for *nod* genes activation in the rhizobia bacteria. Flavonoids also play an important role during nodule organogenesis by acting as auxin transporter inhibitors in root cells, especially in indeterminate nodules. The ureides are the main N transport form in tropical legumes and they are catabolized in leaves and other sink tissues to produce amino acids and proteins needed for plant growth and yield.

Keywords: allantoic acid, allantoin, nitrogen fixation, rhizobium, chemotaxis.

1. Introduction: flavonoids biosynthesis pathway

Nitrogen (N) is the primary gas in the atmosphere in the form of dinitrogen (N_2) (about 78%) and it is an essential nutrient for the metabolism of plants. The N_2 is not available for all living organisms, including plants, but some prokaryotic organisms known as diazotrophic can use as N source, and they can also develop a symbiotic association with some families of plants (commonly, Fabaceae), converting N_2 into ammonia (NH_3) in a process called biological nitrogen fixation (BNF) (Howard & Rees, 1996; Zehr *et al.*, 2003).

The symbioses between the nitrogen-fixing bacteria, mainly from the family *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium,* and *Sinorhizobium* with legume plants such as *Vigna unguiculata*, *Pisum sativum, Phaseolus vulgaris, Medicago sativa*, *Trifolium* spp. and *Glycine max* has been largely studied (Kumar *et al.*, 2020). From this symbiotic system, a complex chemical signaling process evolved in the rhizosphere allowing the establishment of active microbial communities (Scharf *et al.*, 2016; Sugiyama *et al.*, 2017). According to Badri and Vivanco (2009), a chemical gradient is formed in soil by different metabolites exudated by roots such as, organic acids, amino acids and sugars, which are used to chemotactic attract rhizobia, and phenolic compounds such as flavonoids, which are also responsible for inducing *nod* gene expression in rhizobia, resulting in rood infection and nodule organogenesis (Barbour *et al.*, 1991; Kape *et al.*, 1991; Hirsch, 1992).

In legume plants, flavonoids are the main compounds secreted by roots, which can vary depending on species (Perret *et al.*, 2000). Once the bacteria received these chemical signals, they release *nod* factors, which are responsible to lead chemical and physical modification in the host, resulting and root infection and nodule organogenesis (Peters *et al.*, 1986; Barbour *et al.*, 1991; Liu & Murray, 2016).

The final products of symbiotic N_2 fixation are amides and ureides, depending on the legume species: legume species from temperate regions export fixed N in the form of amides, while species from tropical regions, in the form of ureides, allantoin ($C_4H_6N_4O_3$), and acid allantoic ($C_4H_8N_4O_4$), for example, soybean (*Glycine max)* (Streeter, 1991; Baral *et al.*, 2016).

In this review, we summarize the main physiological mechanisms of flavonoids related to the nodulation process and ureides metabolism in several tropical legume plant species.

2. The role of flavonoids on nodulation of legume plants

Bacteria from *Rhizobium* group such as *Azorhizobium, Bradyrhizobium, Mesorhizobium*, and *Sinorhizobium* can develop symbiotic associations with legume plants and fix N_2 into NH_3 that can be metabolized by plants (Izaguirre-Mayoral *et al.*, 2018). The interaction between bacteria and host plant occurs through chemical signaling exchange, where the bacteria recognize organic compounds of low molecular weight exudated by roots such as flavonoids, which trigger the expression of rhizobia genes required for nodulation (Redmond *et al.*, 1986; Kosslak *et al.*, 1987; Mierziak *et al.*, 2014; Singla & Garg, 2017).

Flavonoids are molecules of low molecular weight formed by a group of approximately 6000 compounds that play different roles in plants, such as the regulation of auxin transport, modulation of reactive oxygen species, and UV protection (Agati *et al.*, 2012; Ferreyra *et al.*, 2012; Saito *et al.*, 2013). The isoflavonoids are a subgroup of flavonoids and are secreted into the rhizosphere by the roots of legume plants, acting in the symbiotic process by inducing *nod* genes expression in rhizobia (Kosslak *et al.*, 1987). These compounds are originated from two different biosynthetic pathways in plants: the acetate pathway, responsible for the generation of two molecules of malonyl-CoA, and the shikimic pathway, which product is p-coumaroyl-CoA produced from phenylalanine. From the condensation of three malonyl-CoA molecules with p-coumaroyl-CoA is generated naringenin chalcone by the enzyme chalcone synthase (CHS). Naringenin chalcone is subsequently isomerized into flavanone by the enzyme chalcone flavanone isomerase (CHI). These compounds are the precursors of other flavonoids such as flavonols, flavones, isoflavones, flavonols, and anthocyanins (Table 1, Figure 1) (Nabavi *et al.*, 2020).

In legume plants, the main flavonoids found are from isoflavonoid group, and they are known as daidzein and genistein (Table 2, Figure 2), which plays a crucial role as signaling molecules, but they seem to have an irrelevant role as chemoattractants (Kosslak *et al.*, 1987; Kape *et al.*, 1991; Compton *et al.*, 2020). According to Barbour *et al*, (1991), the component related to chemotaxis and *nod* gene inducing are chemically separate.

The enzyme isoflavone synthase (IFS) is essential for the synthesis of isoflavonoids (Figure 2). In soybean, for example, the main isoflavone found in the leaves is malonyl daidzin, while in the roots, is malonyl daidzin and daidzin (Table 1). However, the main isoflavones secreted in the rhizosphere are daidzein and genistein (Sugiyama *et al.*, 2017).

Daidzein and genistein are produced via two branches of isoflavone biosynthetic pathway (Figure 2). Chalcone is the precursor of daidzein, isoliquiritigenin is produced by the enzyme chalcone reductase (CHR) and subsequently converted to daidzein by chalcone isomerase (CHI) and IFS. On the other hand, genistein is produced from naringenin chalcone by the action of CHI and IFS. Therefore, CHR is essential for daidzein synthesis, while IFS is essential for the production of both daidzein and genistein (Subramanian *et al.*, 2006; Vadivel *et al.*, 2019; Matsuda *et al.*, 2020).

2.1 Flavonoids and auxin transporters determine nodule organogenesis

Some flavonoids can modulate auxin transport, which main role is the regulation of lateral roots and nodules development by stimulating cell division (Mathesius, 2008). According to Peer & Murphy (2007), the flavonoids can inhibit the auxin transport by acting in the expression and localization of PIN proteins, changing the activity of ABC transporters (ATP-binding cassete), which are the main transporters of auxin.

To evaluate the effects of flavonoids on auxin transport, Wasson et al. (2006) silenced the gene responsible for the expression of CHS and they observed an inhibition on nodule formation and an increase in auxin transport in root cells of *Medicago truncatula*, demonstrating the role of flavonoids not only in chemotaxis but also in endogenous processes in the root cells such as nodule initiation and auxin transport. Similar results were found by Subramanian et al. (2006) in soybean plants by silencing the gene responsible for IFS, which synthesizes daidzein and genistein, leading to a reduction of isoflavone levels and nodulation. However, the silencing did not affect the auxin transport and the levels of this hormone in the cells, showing that auxin transport depends on the nodule type.

Legume plants can develop two types of nodules: determinate and indeterminate nodules. The determinate nodules, formed by tropical plants such as soybean (*Glycine max)* and bean (*Phaseolus vulgaris*), do not retain an active meristem, resulting in limited cellular growth. In indeterminate nodules, such as those formed by species including *Medicago truncatula*, *Medicago sativa*, *Trifolium sp.*, there is a persistent nodule meristem and the cell divisions occur in the inner cortex and pericycle (Hirsch, 1992).

The formation mechanism of determinate and indeterminate nodules differs significantly regarding the role of auxin: high levels of auxin are needed in legumes with indeterminate nodules

due to continuous cell division in the apical meristem (Van Noorden *et al.*, 2006; Wasson *et al.*, 2006). In contrast, determinate nodules expand by radial cell expansion that may be less dependent on modulation of auxin levels, where the flavonoids are more important only as inducers of expression of *nod* genes in rhizobia (Subramanian *et al.*, 2006).

It is also interesting to note that flavonoid levels in plants can vary in the same plant depending on the growth stage. In soybean, higher amounts of daidzein were secreted into the rhizosphere at vegetative stages than reproductive stages, but the main isoflavonoid found in root tissue was malonyldaidzin (Sugiyama *et al.*, 2016). Furthermore, Matsuda *et al.*, (2020) demonstrated a diurnal variation in flavonoid synthesis in soybean roots, where daidzein levels increased during the day.

Recent studies suggest that the secretion of isoflavones is related to ATP-dependent transport of isoflavone aglycones and the apoplast-localized isoflavone conjugate-hydrolyzing beta-glucosidase (ICHG), which can hydrolyze the malonylated form of isoflavone conjugates, allowing the production of isoflavone aglycons (biologically active form), being both mechanisms important for the chemical communication between host and rhizobia (Suzuki *et al.*, 2006; Sugiyama *et al.*, 2016; Matsuda *et al.*, 2020).

White *et al.* (2017) investigated the impact of isoflavonoids exudation on the rhizosphere microbiome. The authors detected changes in the bacterial community diversity of roots depending on isoflavonoid levels. Results found by Okutani *et al.* (2020) indicate that daidzein presents more a repellant than an attractant role on bacterial communities, reducing their diversity. These findings reveal an important mechanism used by plants, where they can modify the bacterial community structure of proximal soils to avoid damage from pathogens and improve plants' nutrition and health (Kwak *et al.*, 2018).

It is noteworthy that plant microbiome is modulated by agricultural management and plant selection, as well as by other biotic and abiotic factors. Understanding how these factors influence the microbiome is essential for the development of new crop management strategies (Compant *et al.*, 2019). For instance, nitrogen fertilization can inhibit nodulation and N_2 fixation in legumes and affect the secretion of flavonoids (Liu *et al.*, 2019).

Overall, high levels of inorganic nitrogen, especially nitrate, inhibit BNF in some legume plants because BNF is more biochemically costly than using soil inorganic nitrogen (Hartwig, 1998; Salvagiotti *et al.*, 2008; Menge *et al.*, 2015; Bahulikar *et al.*, 2020). The inhibitory effects of nitrate

on nodulation involve modifications of flavonoid profile and defense metabolism, as well as changes to redox (Van Noorden *et al.*, 2016). High concentrations of nitrate limit root infection nodule development and nitrogenase activity and promotes lateral root growth to explore more soil volume for N uptake. The inhibitory effect by nitrate is may be due to changes in the photoassimilate supply from the nodule to roots (Ohyama *et al.*, 2011; Saito *et al.*, 2014; Dwivedi *et al.*, 2015).

3. Nitrogen metabolism in legume plants

After rhizobia infection inside root cells, they start to differentiate into nitrogen-fixing bacteroids (Gage, 2004). Nitrogenase is the enzyme responsible for fixing N_2 and the enzymatic reaction occurs inside the bacteroids, an environment with low O_2 concentration. The bacteroids are surrounded by the symbiosome space showing low O_2 concentration since nitrogenase is inhibited in the presence of O_2 (Luciński *et al.*, 2002). Nitrogenase is an enzymatic complex formed by two metalloprotein components: Molybdenum-iron (MoFe) protein, which catalysis the N_2 reduction, and iron (Fe) protein that transfers electrons to MoFe protein. The enzyme is active only these two metalloproteins are associated and the final product is NH_3^+ (Andrews *et al.*, 2009).

Following N_2 fixation by nitrogenase, the NH_3^+ generated inside the bacteroids is protonated to NH_4^+ in the peribacteroidal space and then, exported to the cytosol of the nodule-infected cell through the symbiosome membrane (Figure 3). In the cytosol, NH_4^+ is converted to glutamine (Gln) by glutamine synthetase (GS) (Udvardi & Poole, 2013). The amino acids glutamine and asparagine are the most common N transport form in legume species from the temperate origin (*Lupinus perennis, Pisum sativum, Trifolium Pratense*), while ureides (allantoin and allantoic acid) are the main N form in tropical species (*Glycine max, Vigna unguiculata*, *Phaseolus vulgaris*) exported to leaves and other sink tissues through xylem (Tegeder, 2014; Valentine *et al.*, 2017).

4. Metabolism and catabolism of ureides

The NH_4^+ produced during N_2 fixation is incorporated in glutamine for all legume species. However, in legume species from tropical regions, the exportation of N compounds occurs in the form of purines, such as allantoin and allantoic acid (ureides) (Valentine *et al.*, 2017). This process

starts with the conversion of glutamine in xanthine by de *novo* purine synthesis pathway in the nodules (Figure 3) (Werner & Witte, 2011).

In *Arabidopsis thaliana*, the *novo* purine synthesis of xanthine starts by the salvage conversion of guanine into guanosine monophosphate (GMP) by hypoxanthine/guanine phosphoribosyl-transferase, which is converted into guanosine by 5'-nucleotidase, followed by deamination into xanthosine by guanosine deaminase, and finally its conversion into xanthine by purine (xanthosine) nucleosidase (Yin *et al.*, 2014; Baral *et al.*, 2016).

An alternative route for the *de novo* purine synthesis of xanthine starts with the deamination of adenosine monophosphate (AMP) into inosine monophosphate (IMP) by AMP deaminase, which is converted to inosine by 5'-nucleotidase to produce hypoxanthine that is finally oxidized into xanthine by xanthine oxidoreductase (Deng & Ashihara, 2010).

The next step consists of xanthine oxidation to urate that is transported to the cytosol of nodule-uninfected cells and further imported into peroxisomes (Figure 3), where the oxidation of urate to 5-hydroxyisourate (HIU) occurs by flavine adenine dinucleotide dependent urate oxidase (UOH) (Hicks *et al.*, 2013; Kunze & Hartig, 2013).

The unstable substrate HIU is hydrolyzed to produce 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) by 5-hydroxyisourate hydrolase to be finally decarboxylated into (S)-allantoin by 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase with the production of one molecule of CO_2 (Lamberto *et al.*, 2010; Baral *et al.*, 2016). (S)-allantoin can be converted into allantoic acid by the allantoinase enzyme (Werner & Witte, 2011).

Ureides are transported to shoot through the xylem, being translocated to the mature leaves by transpiration flow (Werner & Witte, 2011; Baral *et al.*, 2016), where the allantoic acid is converted into the highly unstable (S)-ureidoglycine and carbamic acid by allantoate amidohydrolase (AAH). The generated carbamic acid undergoes rapid decay to CO_2 and NH_3^+, while (S)-ureidoglycine is hydrolyzed to (S)-ureidoglycolate by (S)-Mn^{2+}-dependent ureidoglycine aminohydrolase (UGlyAH). Finally, (S)-ureidoglycolate undergoes a two-step conversion to glyoxylate by the stereospecific ureidoglycolate amidohydrolase (UAH) (Werner *et al.*, 2013; Shin *et al.*, 2014), with the total release of four molecules of NH_3^+ and two CO_2 molecules (Duran & Todd, 2012).

The ureides can represent over 90% of the total N transported in the xylem in tropical legume plants (Todd *et al.*, 2006; Raso *et al.*, 2007), and they can be stored in high amounts in different plant organs as described in Table 2 (Tan *et al.*, 2008).

5. Ureide synthesis dynamics

Although ureides are considered compounds of complex synthesis, they are more efficient to transport N than asparagine and glutamine by the perspective of the carbon economy, for example, allantoin and allantoic acid have a 4N:4C ratio, while asparagine and glutamine have a 2N:4C and 2N: 5C ratio, respectively (Gaufichon *et al.*, 2010; Ohyama *et al.*, 2017).

Changes in the transport of nitrogen compounds in the xylem sap are indicative of N metabolism in roots and leaves (Thomas *et al.*, 2005). A recent study showed that phosphorus deficiency promoted changes in the transport of N-compounds in legume plants. The transport of ureides conserves more C than the transport of amides, thus, especially in conditions of P deficiency, legumes from temperate climate increase the ratio of ureides: amino acids that are exported from nodules by reducing the synthesis of amino acids (Valentine *et al.*, 2017).

Recent reports have been shown that under water deficit condition, there is an accumulation of ureides in shoots and leaves in soybean and common bean plants, not by an increase in BNF, but by the reduction in the exportation rate and low catabolism of these compounds in leaves, what is associated to a reduced flow of Mn to the ureide-degrading enzymes (Coleto *et al.*, 2014; Cerezini *et al.*, 2017)

Another important aspect regarding BNF is the inoculation practice. A three-fold increase was observed in soybean plants inoculated with *Bradyrhizobium japonicum* in comparison to *B. elkanii*, even under drought stress (Ramos *et al.*, 2005). Similar results were found for peanut plants, where the inoculation with *Bradyrhizobium* sp. and the co-inoculation lead to a higher ureide biosynthesis (Gericó *et al.*, 2020).

Future prospects

In this review, we presented several studies showing the importance of roots exudates, especially flavonoids, in nodulation process and nitrogen metabolism. Even though the studies regarding this topic and its influence in plant microbiota have been conducted for decades, the methodology used presented some limitations, especially regarding the sample collection and

flavonoids identification, which had a huge improvement with the application of modern techniques, such as ultra-performance liquid chromatography (UPLC) (Guo *et al.*, 2011; White et al., 2017; Zhao et al., 2020).

A better understanding about the bioactive molecules and microorganisms are essential to build new insights about BNF, such as microbiota engineering and intercropped cultivation, which can be used to overcome the agriculture challenges agriculture, like increased demand for food, climate changes and sustainability (Liu *et al.*, 2019; Santos *et al.*, 2019; Zhao *et al.*, 2020).

The flavonoids are compounds with different functions in plants and can influence nodulation process acting in the induction of *nod* gene expression in rhizobia (Kape *et al.*, 1991; Hirsch, 1992). In soybean plants, the major isoflavonoids exudate by roots are daidzein and genistein, being essential for BNF. Further studies are needed to understand how different rates and sources of nitrogen affect the biosynthesis of flavonoids and their relation to nodulation, BNF and yield of legume plants.

Regarding BNF, ammonia can be transported as amides or ureides, depending on the legume species, which favor the transport of nitrogen from the nodules to the shoot with less energy cost. Once in the leaves or other sink tissue, the ureides are catabolized in ammonia that is converted into amino acids and proteins needed for plant growth. Therefore, the development of cultivars and management practices that enhance the BNF can contribute to the sustainability of the ecosystems by the use of a renewable nitrogen source. To achieve this goal, is needed more detailed data about the dynamics related to flavonoids biosynthesis, and how it can affect nodulation, ureide metabolism and yield of legume plants.

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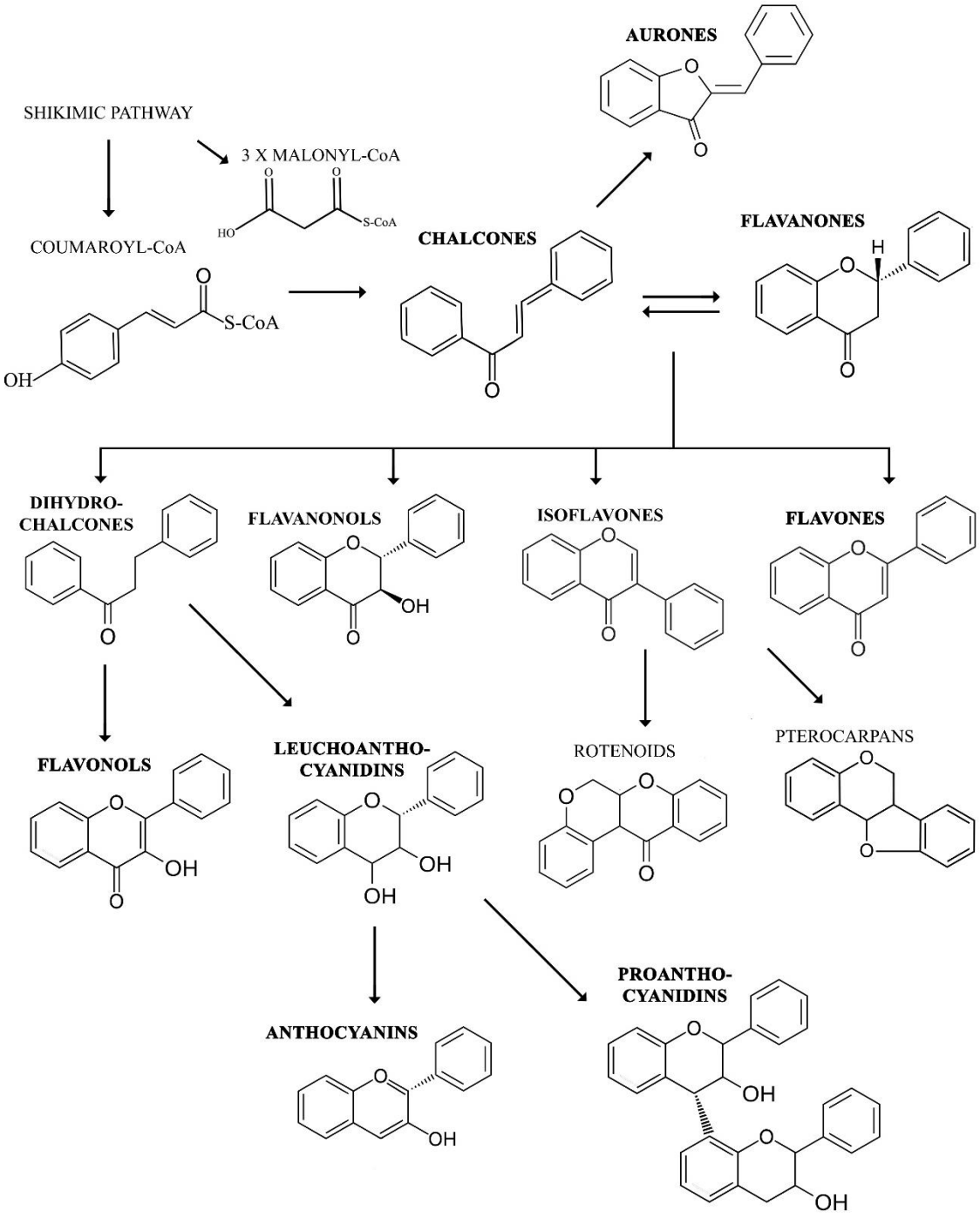

Figure 1. Classification of flavonoids in plants. **Source:** Mierziak et al. (2014)

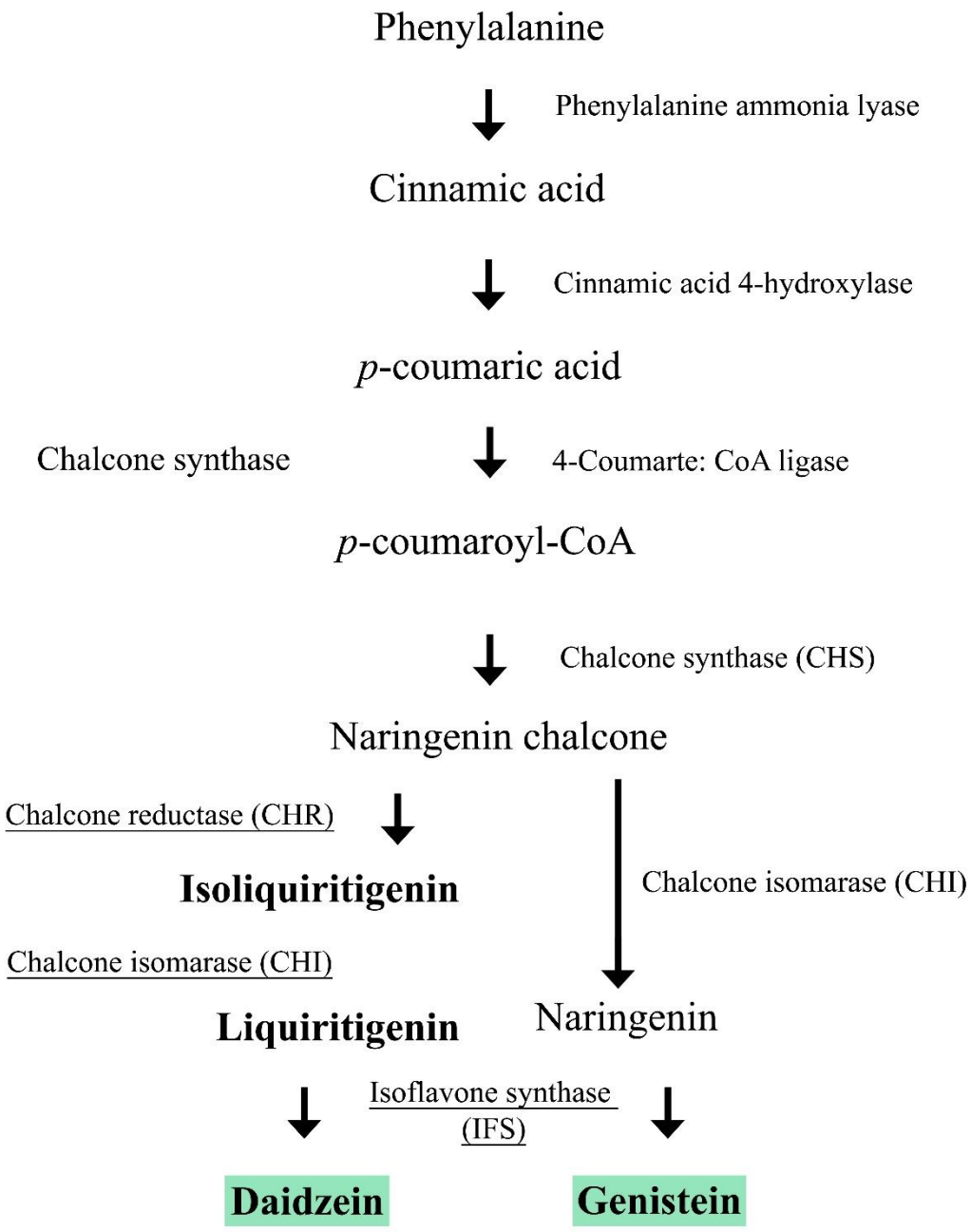

Figure 2. Partial diagram of the phenylpropanoid pathway, showing intermediates and enzymes involved in isoflavone biosynthesis, as well as some branch pathways. Legume-specific enzymes are underlined; legume-specific compounds are shown in bold type.

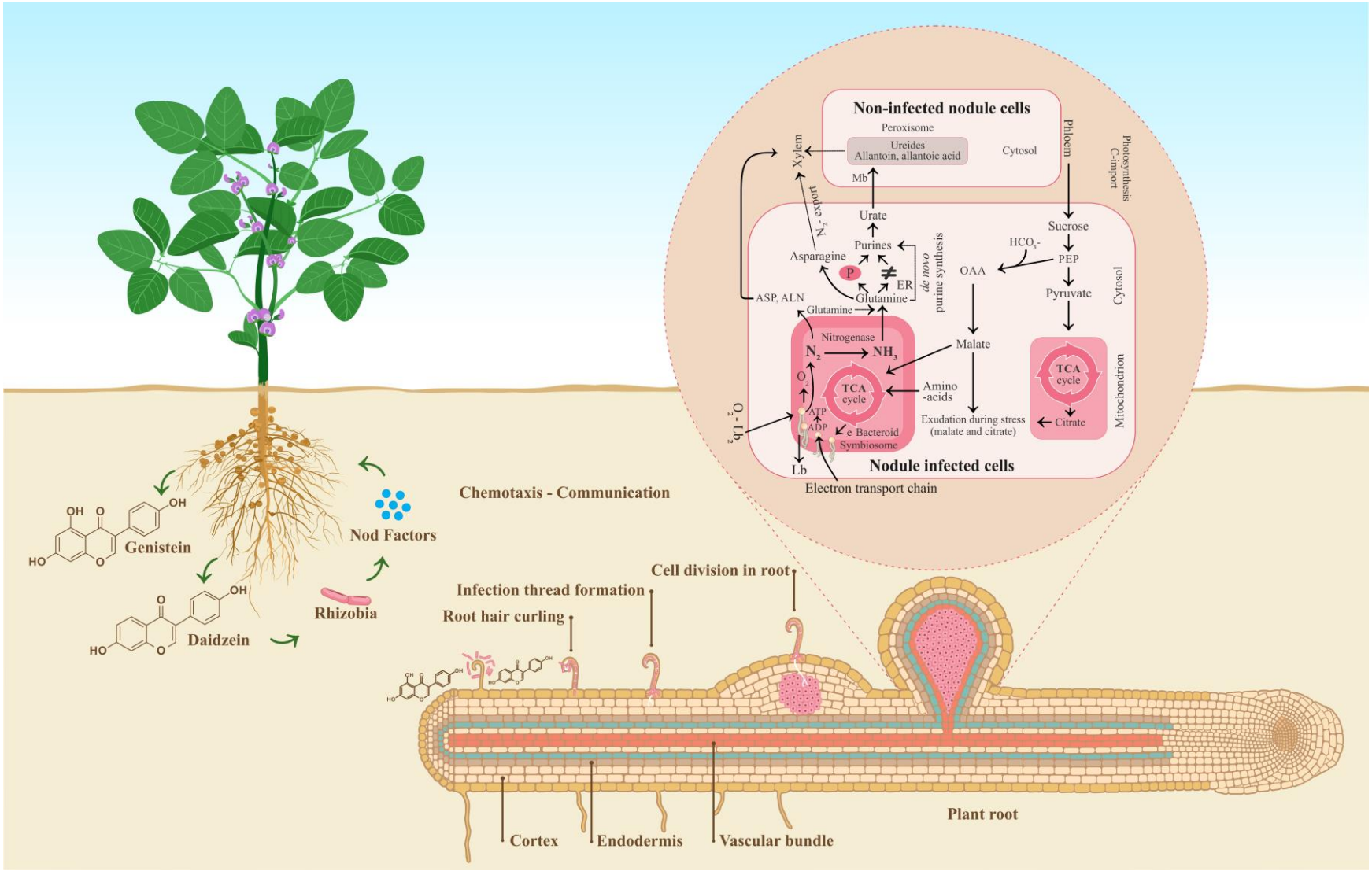

Figure 3 Schematic diagram demonstrating the role of flavonoids not only in chemotaxis but also in endogenous processes in the root cells such as nodule. The flavonoids are compounds with different functions in plants and can influence nodulation process acting in

the chemotaxis between legume plants and rhizobia bacteria (Barbour *et al.*, 1991; Kape *et al.*, 1991; Compton *et al.*, 2020). This scheme shows different stages of ureide synthesis in infected and uninfected cells. ER: endoplasmic reticulum; Lb: leg-hemoglobin; Mb: microbody; OAA: oxaloacetate; P: peroxisome; PEP: phosphoenol pyruvate; infected cell: cell which harbors rhizobial strains; TCA: citric acid cycle (tricarboxylic acid cycle); uninfected cell: cell that does not possess a Rhizobium species. Urates are the ions and salts formed from xanthine catalyzed by xanthine dehydrogenase (XDH; consisting of a flavin nucleotide and sulfurated molybdenum co-factor; EC 1.2.1.37; (Hesberg *et al.*, 2004) during the catabolism of purine nucleotides (Kunze & Hartig, 2013; Tegeder, 2014; Sandalio & Romero-Puertas, 2015).

Table 1. Types and amounts of flavonoids released by roots of various legume plant species.

Species	Cultivar	Flavonoids	Average concentration	Units	Cultivation System	Collection medium	References
Cicer arietinum	nd	Biochanin A	25.00	IA g^{-1} FW	Vermiculite	Phosphate buffer	Armero *et al.* (2001)
	nd	Medicarpin	25.00	IA g^{-1} FW	Vermiculite	Phosphate buffer	Armero *et al.* (2001)
	nd	Formononetin	15.00	IA g^{-1} FW	Vermiculite	Phosphate buffer	Armero *et al.* (2001)
	nd	Maackiain	5.50	IA g^{-1} FW	Vermiculite	Phosphate buffer	Armero *et al.* (2001)
Glycine max	Enrei	Malonyldaidzin	4.20	µmol g^{-1} DW	Hydroponics	Roots	Sugiyama *et al.* (2016)
	Enrei	Daidzin	1.70	µmol g^{-1} DW	Hydroponics	Roots	Sugiyama *et al.* (2016)
	Enrei	Daidzein	1.80	µmol g^{-1} DW	Hydroponics	Roots	Sugiyama *et al.* (2016)
	Enrei	Malonylgenistein	0.80	µmol g^{-1} DW	Hydroponics	Roots	Sugiyama *et al.* (2016)
	Enrei	Genistin	0.80	µmol g^{-1} DW	Hydroponics	Roots	Sugiyama *et al.* (2016)
	Enrei	Genistein	0.40	µmol g^{-1} DW	Hydroponics	Roots	Sugiyama *et al.* (2016)
	Enrei	Daidzein	60.00	pmol $plant^{-1}$	Hydroponics	Roots exudates	Sugiyama *et al.* (2016)
	Enrei	Daidzin	11.00	pmol $plant^{-1}$	Hydroponics	Roots exudates	Sugiyama *et al.* (2016)
	Enrei	Malonyldaidzin	2.20	µmol g^{-1} DW	Hydroponics	Leaves	Sugiyama *et al.* (2016)
	Enrei	Daidzin	3.90	µmol g^{-1} DW	Hydroponics	Leaves	Sugiyama *et al.* (2016)
	Enrei	Daidzein	0.10	µmol g^{-1} DW	Hydroponics	Leaves	Sugiyama *et al.* (2016)
	Enrei	Malonylgenistein	8.00	µmol g^{-1} DW	Hydroponics	Leaves	Sugiyama *et al.* (2016)

Shintambaguro	Malonyldaidzin	2.90	µmol g^{-1} DW	Soil	Roots	Sugiyama *et al.* (2017)
Shintambaguro	Daidzin	1.30	µmol g^{-1} DW	Soil	Roots	Sugiyama *et al.* (2017)
Shintambaguro	Daidzein	1.30	µmol g^{-1} DW	Soil	Roots	Sugiyama *et al.* (2017)
Shintambaguro	Malonylgenistin	0.60	µmol g^{-1} DW	Soil	Roots	Sugiyama *et al.* (2017)
Shintambaguro	Genistin	0.30	µmol g^{-1} DW	Soil	Roots	Sugiyama *et al.* (2017)
Shintambaguro	Genistein	0.30	µmol g^{-1} DW	Soil	Roots	Sugiyama *et al.* (2017)
Shintambaguro	Daidzein	16.00	nmol g^{-1} soil	Soil	Roots exudates	Sugiyama *et al.* (2017)
Shintambaguro	Genistein	2.50	nmol g^{-1} soil	Soil	Roots exudates	Sugiyama *et al.* (2017)
Shintambaguro	Malonyldaidzin	0.80	µmol g^{-1} DW	Soil	Leaves	Sugiyama *et al.* (2017)
Shintambaguro	Daidzin	0.10	µmol g^{-1} DW	Soil	Leaves	Sugiyama *et al.* (2017)
Shintambaguro	Malonylgenistin	0.50	µmol g^{-1} DW	Soil	Leaves	Sugiyama *et al.* (2017)
Shintambaguro	Genistin	0.40	µmol g^{-1} DW	Soil	Leaves	Sugiyama *et al.* (2017)
Enrei	Daidzein	5.00	µmol g^{-1} DW	Hydroponics	Root	Matsuda *et al.* (2020)
Enrei	Daidzin	1.50	µmol g^{-1} DW	Hydroponics	Root	Matsuda *et al.* (2020)
Enrei	Malonyldaidzin	12.50	µmol g^{-1} DW	Hydroponics	Root	Matsuda *et al.*, 2020
Enrei	Genistein	0.30	µmol g^{-1} DW	Hydroponics	Root	Matsuda *et al.* (2020)
Enrei	Genistin	0.10	µmol g^{-1} DW	Hydroponics	Root	Matsuda *et al.* (2020)
Enrei	Malonylgenistein	2.00	µmol g^{-1} DW	Hydroponics	Root	Matsuda *et al.* (2020)
Enrei	Daidzein	3.00	nmol $plant^{-1}$	Hydroponics	Roots exudates	Matsuda *et al.* (2020)
Enrei	Daidzin	0.25	nmol $plant^{-1}$	Hydroponics	Roots exudates	Matsuda *et al.* (2020)
Enrei	Malonyldaidzin	0.20	nmol $plant^{-1}$	Hydroponics	Roots exudates	Matsuda *et al.* (2020)
Enrei	Genistein	0.09	nmol $plant^{-1}$	Hydroponics	Roots exudates	Matsuda *et al.* (2020)

Enrei	Genistin	0.01	nmol plant^{-1}	Hydroponics	Roots exudates	Matsuda *et al.* (2020)
Enrei	Malonylgenistein	0.05	nmol plant^{-1}	Hydroponics	Roots exudates	Matsuda *et al.* (2020)
Tianlong #1	Daidzein	2.56	µmol g^{-1} FW	Hydroponics	Roots	Ahmad *et al.* (2017)
Tianlong #1	Daidzin	0.70	µmol g^{-1} FW	Hydroponics	Roots	Ahmad *et al.* (2017)
Tianlong #1	Malonyldaidzin	2.99	µmol g^{-1} FW	Hydroponics	Roots	Ahmad *et al.* (2017)
Tianlong #1	Genistein	0.10	µmol g^{-1} FW	Hydroponics	Roots	Ahmad *et al.* (2017)
Tianlong #1	Genistin	0.07	µmol g^{-1} FW	Hydroponics	Roots	Ahmad *et al.* (2017)
Tianlong #1	Malonylgenistin	0.22	µmol g^{-1} FW	Hydroponics	Roots	Ahmad *et al.* (2017)
Williams	Malonyldaidzein	950	nmol seedling^{-1} 24 h^{-1}	AC (water)	Cotton in root contact	Graham (1991)
Williams	Malonylgenistein	650	nmol seedling^{-1} 24 h^{-1}	AC (water)	Cotton in root contact	Graham (1991)
Williams	Daidzein	550	nmol seedling^{-1} 24 h^{-1}	AC (water)	Cotton in root contact	Graham (1991)
Williams	Genistein	700	nmol seedling^{-1} 24 h^{-1}	AC (water)	Cotton in root contact	Graham (1991)
Preston	Daidzein	0.40	nmol plant^{-1} 18 h^{-1}	MES	MES	Schmidt *et al.* (1994)
Preston	Coumestrol	0.05	nmol plant^{-1} 18 h^{-1}	MES	MES	Schmidt *et al.* (1994)
Preston	Genistein	0.03	nmol plant^{-1} 18 h^{-1}	MES	MES	Schmidt *et al.* (1994)
Peking	Daidzein	1.25	nmol root^{-1} 2d^{-1}	MES-$CaCl_2$	MES-$CaCl_2$	Pueppke *et al.* (1998)
Peking	Glycitein	0.35	nmol root^{-1} 2d^{-1}	MES-$CaCl_2$	MES-$CaCl_2$	Pueppke *et al.* (1998)
Peking	Genistein	0.20	nmol root^{-1} 2d^{-1}	MES-$CaCl_2$	MES-$CaCl_2$	Pueppke *et al.* (1998)
Peking	Coumestrol	0.08	nmol root^{-1} 2d^{-1}	MES-$CaCl_2$	MES-$CaCl_2$	Pueppke *et al.* (1998)
McCall	Daidzein	0.75	nmol root^{-1} 2d^{-1}	MES-$CaCl_2$	MES-$CaCl_2$	Pueppke *et al.* (1998)

	McCall	Glycitein	0.20	nmol root^{-1} 2d^{-1}	MES-$CaCl_2$	MES-$CaCl_2$	Pueppke *et al.* (1998)
	Bac	Genistein	11.10	nmol g root^{-1} FW h^{-1}	Hydroponics	Roots Exudates	Píslewska *et al.* (2002)
		Luteone	8.41	nmol g root^{-1} FW h^{-1}	Hydroponics	Roots Exudates	Píslewska *et al.* (2002)
		Wighteone	1.62	nmol g root^{-1} FW h^{-1}	Hydroponics	Roots Exudates	Píslewska *et al.* (2002)
Lupinus luteus	nd	Genistein	20.35	nmol g root^{-1} FW h^{-1}	Hydroponics	Water	Kneer *et al.* (1999)
Medicago sativa	Moapa 69	Echinatin	0.55	nmol g^{-1} FW 8h^{-1}	Hydroponics	Nutrient Solution	Maxwell & Phillips (1990)
	Moapa 69	Liquiritigenin	0.35	nmol g^{-1} FW 8h^{-1}	Hydroponics	Nutrient Solution	Maxwell & Phillips (1990)
Cajanus cajan (L.) Millsp.		Genistein	1.48	µmol g^{-1} DW	Soil	Roots	Zhang *et al.* (2013)
		Apigenin	0.74	µmol g^{-1} DW	Soil	Roots	Zhang *et al.* (2013)
Phaseolus vulgaris	PI 165426CS	Naringenin	0.35	µmol plant^{-1} d^{-1}	Hydroponics	Nutrient Solution	Hungria *et al.* (1991)
	PI 165426CS	Eriodictyol	0.25	µmol plant^{-1} d^{-1}	Hydroponics	Nutrient Solution	Hungria *et al.* (1991)
	PI 165426CS	Genistein (glycoside)	0.04	µmol plant^{-1} d^{-1}	Hydroponics	Nutrient Solution	Hungria *et al.* (1991)
	Rab39	Cumestrol	1.10	nmol seedling^{-1} h^{-1}	Hydroponics	MES	Bolaños-Vásquez & Werner (1997)
	Rab39	Daidzein	0.75	nmol seedling^{-1} h^{-1}	Hydroponics	MES	Bolaños-Vásquez & Werner (1997)
	Rab39	Naringenin	0.55	nmol seedling^{-1} h^{-1}	Hydroponics	MES	Bolaños-Vásquez & Werner (1997)
	Rab39	Liquiritigenin	0.45	nmol seedling^{-1} h^{-1}	Hydroponics	MES	Bolaños-Vásquez & Werner (1997)

	Rab39	Isoliquiritigenin	0.25	nmol seedling^{-1} h^{-1}	Hydroponics	MES	Bolaños-Vásquez & Werner (1997)
	Rab39	Genistein	nd	nmol seedling^{-1} h^{-1}	Hydroponics	MES	Bolaños-Vásquez & Werner (1997)
	Celina	Daidzein	6.61	nmol g^{-1} DW	Soil	Roots	Isobe *et al.* (2001)
	Celina	Genistein	2.18	nmol g^{-1} DW	Soil	Roots	Isobe *et al.* (2001)
	Celina	Coumestrol	0.93	nmol g^{-1} DW	Soil	Roots	Isobe *et al.* (2001)
	Hilds Maxi	Coumestrol	75.00	µmol g^{-1} DW	Filter papers	Roots exudates	Haase *et al.* (2007)
	Hilds Maxi	Genistein	3.00	µmol g^{-1} DW	Filter papers	Roots exudates	Haase *et al.* (2007)
	Hilds Maxi	Daidzein	5.50	µmol g^{-1} DW	Filter papers	Roots exudates	Haase *et al.* (2007)
	Hilds Maxi	Isoliquiritigenin	5.50	µmol g^{-1} DW	Filter papers	Roots exudates	Haase *et al.* (2007)
	Bianco di Bagnasco	Daidzein	0.06	nmol g^{-1} FW	Hydroponics	Nutrient Solution	Malusà *et al.* (2006)
		Naringenin	0.05	nmol g^{-1} FW	Hydroponics	Nutrient Solution	Malusà *et al.* (2006)
Trifolium repens L.	Milo	Medicarpin	130.98	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.* (2008)
	Milo	Genistein	44.78	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.* (2008)
	Milo	Biochanin A	15.48	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.* (2008)
	Milo	Daidzein	9.05	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.* (2008)
	Milo	Formononetin	3.99	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.* (2008)
	Sonja	Medicarpin	234.58	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.* (2008)
	Sonja	Genistein	24.05	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.* (2008)
	Sonja	Biochanin A	15.13	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.* (2008)

	Sonja	Daidzein	169.53	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.* (2008)
	Sonja	Formononetin	4.40	nmol g^{-1} DW	Soil	Roots	Carlsen *et al.*(2008)
	Sonja	Coumestrol	201.70	nmol g^{-1} DW	Soil	Roots	Carlsen *et al*. (2008)
Arachis hypogeae L.	Ganhua-5	Quercetin	0.12	nmol g^{-1} FW	Hydroponics	Roots exudates	Zhang *et al.* (2016)
	Ganhua-5	Luteolin	0.16	nmol g^{-1} FW	Hydroponics	Roots exudates	Zhang *et al.* (2016)
Vigna sinensis	Kintok	Daidzein	6.10	nmol g^{-1} DW	Soil	Roots	Isobe *et al.* (2001)
	Kintok	Genistein	1.81	nmol g^{-1} DW	Soil	Roots	Isobe *et al.* (2001)
	Kintok	Coumestrol	3.02	nmol g^{-1} DW	Soil	Roots	Isobe *et al.* (2001)

nd: not determined; IA: integration area; HC: hydroponic culture; AC: aeroponic culture; MES 2-(N-morpholino) ethanesulfonic acid

Table 2. Updated review of estimated ureides concentration in nodulated legume species.

Crop	Cultivar	Plant Organ	Age	Ureides	Trial	References
Arachis hypogaea L.	Granoleico	Leaves (µmol g^{-1} FW)	35	2.3	Field	Gericó *et al.* (2019)
Inga indulis		Leaves (µmol g^{-1} FW)	120	7.2	Greenhouse	Justino *et al.* (2017)
		Roots (µmol g^{-1} FW)	120	4.4	Greenhouse	Justino *et al.* (2017)
		Nodules (µmol g^{-1} FW)	120	11.1	Greenhouse	Justino *et al.* (2017)
Glycine max L. Merr.	7379	Leaves (µmol g^{-1} FW)	R1-R2	26.4	Field	Freitas *et al.* (2018)
	7200	Leaves (µmol g^{-1} FW)	R1-R2	26.4	Field	Freitas *et al.* (2018)
	6510	Leaves (µmol g^{-1} FW)	R1-R2	22.8	Field	Freitas *et al.* (2018)
	2728	Leaves (µmol g^{-1} FW)	R1-R2	18.8	Field	Freitas *et al. (*2018)
	7849	Leaves (µmol g^{-1} FW)	R1-R2	21.5	Field	Freitas *et al.* (2018)
	3730	Leaves (µmol g^{-1} FW)	R1-R2	18.3	Field	Freitas *et al.* (2018)
	2158	Leaves (µmol g^{-1} FW)	R1-R2	16.3	Field	Freitas *et al.* (2018)
	797	Leaves (µmol g^{-1} FW)	R1-R2	26.7	Field	Freitas *et al.* (2018)
	6215	Leaves (µmol g^{-1} FW)	R1-R2	22.6	Field	Freitas *et al.* (2018)
	690	Leaves (µmol g^{-1} FW)	R1-R2	25.7	Field	Freitas *et al.* (2018)
	2737	Leaves (µmol g^{-1} FW)	R1-R2	24.2	Field	Freitas *et al.* (2018)
	8015	Leaves (µmol g^{-1} FW)	R1-R2	11.8	Field	Freitas *et al.* (2018)
	791	Leaves (µmol g^{-1} FW)	R1-R2	15.7	Field	Freitas *et al.* (2018)
	1378	Leaves (µmol g^{-1} FW)	R1-R2	17	Field	Freitas *et al.* (2018)
	620	Leaves (µmol g^{-1} FW)	R1-R2	17	Field	Freitas *et al.* (2018)

Glycine max L. Merr.	2158	Leaves (µmol g^{-1} FW)	R1-R2	19	Greenhouse	Freitas *et al.* (2019)
Glycine max L. Merr.	Zodiac	Leaves (µmol g^{-1} DW)	42	7-12	Greenhouse	Rotaru (2016)
	Zodiac	Nodules (µmol g^{-1} DW)	42	19-55	Greenhouse	Rotaru (2016)
Glycine max L. Merr.		Nodules (µmol g^{-1} DW)	37	30	Greenhouse	Carter & Tegeder (2016)
Glycine max L. Merr.	IAC-17	Xylem Sap (µmol N mL^{-1})	R2	40	Greenhouse	Amarante *et al.* (2006)
Glycine max L. Merr.	R01-581F	Petiole (µmol g^{-1} DW)	45-55	7	Greenhouse	Cerezini *et al.* (2017)
	R01-416F	Petiole (µmol g^{-1} DW)	45-55	9	Greenhouse	Cerezini *et al.* (2017)
	R02-1325	Petiole (µmol g^{-1} DW)	45-55	5.5	Greenhouse	Cerezini *et al.* (2017)
	CD 215	Petiole (µmol g^{-1} DW)	45-55	2.2	Greenhouse	Cerezini *et al.* (2017)
	BRS 317	Petiole (µmol g^{-1} FW)	45-55	2.9	Greenhouse	Cerezini *et al.* (2017)
Glycine max L. Merr.	R01-581F	Nodules (µmol g^{-1} DW)	45-55	7.5	Greenhouse	Cerezini *et al.* (2017)
	R01-416F	Nodules (µmol g^{-1} DW)	45-55	7.4	Greenhouse	Cerezini *et al.* (2017)
	R02-1325	Nodules (µmol g^{-1} DW)	45-55	11	Greenhouse	Cerezini *et al.* (2017)
	CD 215	Nodules (µmol g^{-1} DW)	45-55	6	Greenhouse	Cerezini *et al.* (2017)
	BRS 317	Nodules (µmol g^{-1} DW)	45-55	4.9	Greenhouse	Cerezini *et al.* (2017)
Glycine max L. Merr.	R01-581F	Leaves (µmol g^{-1} DW)	45-55	3.5	Greenhouse	Cerezini *et al.* (2017)
	R01-416F	Leaves (µmol g^{-1} DW)	45-55	3.6	Greenhouse	Cerezini *et al.* (2017)
	R02-1325	Leaves (µmol g^{-1} DW)	45-55	3.1	Greenhouse	Cerezini *et al.* (2017)

	CD 215	Leaves (µmol g^{-1} DW)	45-55	2.9	Greenhouse	Cerezini *et al.* (2017)
	BRS 317	Leaves (µmol g^{-1} DW)	45-55	4	Greenhouse	Cerezini *et al.* (2017)
Glycine max L. Merr.	Biloxi	Leaves (mmol g^{-1} DW)	30	25	Greenhouse	Izaguirre-Mayoral & Sinclair (2005)
	Biloxi	Nodules (µmol g^{-1} DW)	30	3.9	Greenhouse	Izaguirre-Mayoral & Sinclair (2005)
	PI227557	Leaves (mmol g^{-1} DW)	30	30	Greenhouse	Izaguirre-Mayoral & Sinclair (2005)
	PI227557	Nodules (µmol g^{-1} DW)	30	3	Greenhouse	Izaguirre-Mayoral & Sinclair (2005)
Vigna unguiculata L. Walp	Tuniziesi	Xylem Sap (µg mL^{-1})	48-50	26.9	Field	Mohammed *et al.* (2020)
	Botanyiriga	Xylem Sap (µg mL^{-1})	48-50	19	Field	Mohammed *et al.* (2020)
	Moatuya	Xylem Sap (µg mL^{-1})	48-50	8.4	Field	Mohammed *et al.* (2020)
	Alan Cash	Xylem Sap (µg mL^{-1})	48-50	9	Field	Mohammed *et al.* (2020)
	Namuziesi	Xylem Sap (µg mL^{-1})	48-50	38.1	Field	Mohammed *et al.* (2020)
	Apagbaala	Xylem Sap (µg mL^{-1})	48-50	108.1	Field	Mohammed *et al.* (2020)
	Songotra	Xylem Sap (µg mL^{-1})	48-50	44.8	Field	Mohammed *et al.* (2020)
	Padi-tuya	Xylem Sap (µg mL^{-1})	48-50	57.7	Field	Mohammed *et al.* (2020)
	Baawutawuta	Xylem Sap (µg mL^{-1})	48-50	10.6	Field	Mohammed *et al.* (2020)
	Bakumasali	Xylem Sap (µg mL^{-1})	48-50	70	Field	Mohammed *et al.* (2020)
	Biunaa	Xylem Sap (µg mL^{-1})	48-50	23	Field	Mohammed *et al.* (2020)
	Nandanbaaya	Xylem Sap (µg mL^{-1})	48-50	87.4	Field	Mohammed *et al.* (2020)
	Tinguri-1	Xylem Sap (µg mL^{-1})	48-50	13.4	Field	Mohammed *et al.* (2020)
	Tinguri-2	Xylem Sap (µg mL^{-1})	48-50	50.4	Field	Mohammed *et al.* (2020)
	SARVx-09-001	Xylem Sap (µg mL^{-1})	48-50	45.4	Field	Mohammed *et al.* (2020)
	SARVx-09-002	Xylem Sap (µg mL^{-1})	48-50	69.4	Field	Mohammed *et al.* (2020)
	SARVx-09-003	Xylem Sap (µg mL^{-1})	48-50	14.6	Field	Mohammed *et al.* (2020)

SARVx-09-004	Xylem Sap (µg mL^{-1})	48-50	44.8	Field	Mohammed *et al.* (2020)
Chaparipie	Xylem Sap (µg mL^{-1})	48-50	38.1	Field	Mohammed *et al.* (2020)
Lawra-Bengsogla	Xylem Sap (µg mL^{-1})	48-50	15.7	Field	Mohammed *et al.* (2020)
Baabili-Toboonaa	Xylem Sap (µg mL^{-1})	48-50	19.6	Field	Mohammed *et al.* (2020)
Lawra-Ormondow	Xylem Sap (µg mL^{-1})	48-50	25.8	Field	Mohammed *et al.* (2020)
Dobezi-Bengsogla	Xylem Sap (µg mL^{-1})	48-50	35.3	Field	Mohammed *et al.* (2020)
Bengberizie	Xylem Sap (µg mL^{-1})	48-50	14.6	Field	Mohammed *et al.* (2020)
Jolirayiri-Bengsogla	Xylem Sap (µg mL^{-1})	48-50	15.1	Field	Mohammed *et al.* (2020)
Nyagli-Bengjie	Xylem Sap (µg mL^{-1})	48-50	45.9	Field	Mohammed *et al.* (2020)
UWC-13-04	Xylem Sap (µg mL^{-1})	48-50	26.9	Field	Mohammed *et al.* (2020)
Tamayiri	Xylem Sap (µg mL^{-1})	48-50	10.1	Field	Mohammed *et al.* (2020)
Bengberipiela	Xylem Sap (µg mL^{-1})	48-50	141.7	Field	Mohammed *et al.* (2020)
Lawra-Bengpiela	Xylem Sap (µg mL^{-1})	48-50	31.9	Field	Mohammed *et al.* (2020)
Bengjie	Xylem Sap (µg mL^{-1})	48-50	19	Field	Mohammed *et al.* (2020)
Bengberisogla	Xylem Sap (µg mL^{-1})	48-50	29.1	Field	Mohammed *et al.* (2020)
Sombo-Wululu	Xylem Sap (µg mL^{-1})	48-50	9	Field	Mohammed *et al.* (2020)
Goohi-Ormonsireh	Xylem Sap (µg mL^{-1})	48-50	42.6	Field	Mohammed *et al.* (2020)
UWC-13-01	Xylem Sap (µg mL^{-1})	48-50	15.7	Field	Mohammed *et al.* (2020)
Goohi-Bengsogla	Xylem Sap (µg mL^{-1})	48-50	174.2	Field	Mohammed *et al.* (2020)
Kumbo-Bengpiela	Xylem Sap (µg mL^{-1})	48-50	19	Field	Mohammed *et al.* (2020)
Lawra-Lekou	Xylem Sap (µg mL^{-1})	48-50	29.7	Field	Mohammed *et al.* (2020)
Lamuro	Xylem Sap (µg mL^{-1})	48-50	14.6	Field	Mohammed *et al.* (2020)
Tigboro	Xylem Sap (µg mL^{-1})	48-50	11.2	Field	Mohammed *et al.* (2020)
Bengber	Xylem Sap (µg mL^{-1})	48-50	121	Field	Mohammed *et al.* (2020)

	Doggoh	Xylem Sap (µg mL^{-1})	48-50	227.4	Field	Mohammed *et al.* (2020)
	Kaleo-Ormondow	Xylem Sap (µg mL^{-1})	48-50	40.3	Field	Mohammed *et al.* (2020)
	Daffiama-Ormondow	Xylem Sap (µg mL^{-1})	48-50	18.5	Field	Mohammed *et al.* (2020)
	Zambogu-Dapiela	Xylem Sap (µg mL^{-1})	48-50	29.1	Field	Mohammed *et al.* (2020)
	Sombo-Bengsogla	Xylem Sap (µg mL^{-1})	48-50	32.5	Field	Mohammed *et al.* (2020)
	Bamahu-Ormondow	Xylem Sap (µg mL^{-1})	48-50	17.9	Field	Mohammed *et al.* (2020)
	Asontem	Xylem Sap (µg mL^{-1})	48-50	21.8	Field	Mohammed *et al.* (2020)
	Nhyira	Xylem Sap (µg mL^{-1})	48-50	28	Field	Mohammed *et al.* (2020)
Vigna unguiculata L. Walp	IPA 206	Nodules (mmol g^{-1} DW)	37	14	Greenhouse	Santos *et al.* (2018)
Vigna unguiculata L. Walp	Vita 7	Xylem Sap (µmol N ml^{-1})	Flowering	37	Greenhouse	Amarante *et al.* (2006)
Phaseolus vulgaris L.	Carioca	Xylem Sap (µmol N ml^{-1})	Flowering	27	Greenhouse	Amarante *et al.* (2006)
Phaseolus vulgaris L.	Tenderlake	Xylem Sap (µmol ml^{-1})	45	4	Greenhouse	Figueiredo *et al.* (2008)
Phaseolus vulgaris L.	Magdaleno	Leaves (mmol g^{-1} DW)	30	4	Greenhouse	Izaguirre-Mayoral *et al.* (2015)
Phaseolus vulgaris L.	Magdaleno	Nodules (mmol g^{-1} DW)	30	10	Greenhouse	Izaguirre-Mayoral *et al.* (2015)

CHAPTER 3 - Nickel boosts daidzein biosynthesis in roots and increases nodulation, biological nitrogen fixation and seed productivity of soybean plants

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Abstract

Nickel (Ni) plays an important role in nitrogen metabolism and biological nitrogen fixation in legume plants. Recent studies have demonstrated the beneficial effects of Ni fertilization on soybean nodulation and crop yield; however, the physiological mechanism regulating Rhizobia chemotaxis is not understood. This study aimed to investigate the effects of Ni fertilization on urease activity, flavonoid profile, nodulation, ureide metabolism and its impact on plant growth and yield. We tested fertilization with three Ni doses (0.0, 1.5, and 3.0 mg kg^{-1}) and two soybean genotypes (commercial soybean (TMG 2158) and urease-null (*eu3*-a), which is a near-isogenic line (NIL) growing under greenhouse conditions. Ni fertilization decreased nitrate reductase activity of both NILs and increased the activity for TMG 2158 plants. Ni increased urease activity, breaking down urea into ammonia in the leaves. Ni enhanced daidzein, genistein, rutin and kaempferol in roots and nodulation mainly for TMG 2158 plants. There was a positive correlation between daidzein, nodulation, ureides and seed yield in response to Ni fertilization, only to the genotype TMG 2158. These results suggest that Ni plays a role in flavonoids metabolism (mainly daidzein) inducing chemotaxis between soybean plants and rhizobia boosting nodulation and ureide metabolism. Enhanced biological nitrogen fixation induced by Ni fertilization promoted higher nodulation generating more organic nitrogen available to soybean plants resulting in a higher number of pods per plant and seed yield. This study showed important information on how Ni modulates nodulation to increase the yield of commercial soybean plants.

Keywords: Biological nitrogen fixation, nodules, flavonoids, ureides.

1. Introduction

Nitrogen (N) is a key nutrient to achieve high productivities in soybean [*Glycine max* (L.) Merr.] crop due to high seed protein content (Diers *et al.*, 1992). Different from cereal crops, N fertilization is not usual for soybean, which mainly relies on biological nitrogen fixation (BNF) and N uptake from soil (Cafaro La Menza *et al.*, 2020).

BNF consists of atmospheric nitrogen (N_2) reduction into ammonia (NH_3), catalyzed by nitrogenase enzyme complex present in some group of bacteria that develop a symbiotic relationship with plant hosts (Mantilla-Paredes *et al.*, 2009). Ureides are the

final product of BNF in legume plants from tropical regions, such as soybean, and they represent the main N form transported in the xylem sap (Todd *et al.*, 2006).

Hydrogenase and urease are two Ni-dependent metalloenzymes, which are directly involved in nitrogen metabolism. Hydrogenase is present in nitrogen-fixing organisms and is responsible for recycling H_2 produced by a side reaction of nitrogenase, increasing N fixation efficiency (Dixon, 1972; Bagyinka, 2014). Urease is responsible for urea hydrolysis into two molecules of ammonia and one of carbon dioxide (Dixon *et al.*, 1975; Witte, 2011). Furthermore, it has been reported that urease also may interfere with chemotactic signals triggered by soybean plants in the rhizosphere, demonstrating the multifunctionality of this enzyme (Medeiros-Silva *et al.*, 2014).

Urease activity depends on attachment with Ni by two accessory proteins encoded by the genes *Eu2* and *Eu3* in soybean plants. A null mutant for the *Eu3* gene has been characterized (Freyermuth *et al.*, 2000) resulting in a *eu3-a* mutant with no urease activity (Tezotto *et al.*, 2016). Ni deficiency and low urease activity can affect N metabolism and lead to an accumulation of toxic levels of urea in shoots disrupting plant growth and yield. Recent findings demonstrated that modern soybean cultivars can show latent Ni deficiency under field conditions (Freitas *et al.*, 2018). In addition, Freitas *et al*, (2019) showed that Ni fertilization increases urease activity, photosynthetic rates, nodulation, ureide concentration and yield of soybean plants. The authors concluded that Ni fertilization with 3 mg dm^{-3} was an optimal concentration to improve seed yield and nodulation. However, it was not clear how Ni fertilization promoted an increase in nodule number per plant, which may be related with chemotaxis.

Chemotaxis between legume roots and bacteria depends on the exudation of sugars, amino acids and organic acids by plant roots in the rhizosphere. Root secretion of isoflavonoids acts as inducers for *nod* genes in rhizobia (Matsuda *et al.*, 2020). In legume plants, the main flavonoids found are from the isoflavonoid group, and they are known as daidzein and genistein, which trigger *Nod* genes expression in rhizobia that are responsible for the synthesis and exudation of Nod factors, which are lipo-oligosaccharides responsible for inducing a series of physiological changes in plant cells, resulting in root infection and nodule organogenesis (Liu & Murray, 2016).

This study hypothesizes that Ni fertilization enhances root isoflavonoids (daidzein and genistein), controlling chemotaxis and nodulation of soybean plants. We aimed to evaluate the effect of Ni fertilization on urease activity, root and nodule flavonoid profile and its relation with nodulation, ureide metabolism and seed yield. In addition, NIL

genotype (*eu3-a* mutant) and its functional allele were included to evaluate the effect of Ni on urease activity and its impacts on seed yield.

2. Material and Methods

2.1 Plant growth and experiment design

The experiment was carried out under greenhouse conditions at São Paulo State University (UNESP), Tupã, São Paulo State, Brazil. In this experiment, one commercial soybean genotype (TMG 2158) and one near-isogenic line (NIL) (*eu3-a* mutant) were fertilized with 0.0 mg kg^{-1}, 1.5 mg kg^{-1} and 3.0 mg kg^{-1}, using a nickel sulfate solution ($NiSO_4.6H_2O$). Ni doses range was based on a previous study (Freitas *et al.*, 2019) as optimal concentration to enhance soybean nodulation.

The experimental design was a 3 × 3 completely randomized factorial design (soybean genotypes × Ni doses), with eight replications for each genotype, totaling 48 pots. The pots of 4 L were filled with a commercial growing substrate Carolina Soil ®. Growing substrate samples were submitted to an acid digestion and the heavy metal contents were determined using an ICP-OES. According to the analysis, the following levels were found: 2.8 mg of As kg^{-1}; 0.8 mg of Cd kg^{-1}; 2.8 mg of Pb kg^{-1}; 1060.1 mg of Cr kg^{-1}; 13.1 mg of Hg kg^{-1}; 435.0 mg of Ni kg^{-1} and 14.9 mg of Se kg^{-1}.

Macro and micronutrients were also supplied (except N) at the following doses: 200 mg of P kg^{-1} (P_2O_5) + 50.3 mg of K kg^{-1} (KCl), 9.1 mg of Mn kg^{-1} ($Cl_2Mn.4H_2O$), 11.1 mg of Zn kg^{-1} ($ZnSO_4.7H_2O$), 2.9 mg of B kg^{-1} (H_3BO_3), 2.6 mg of Cu kg^{-1} ($CuSO_4.5H_2O$), 2.3 mg of Mo kg^{-1} ($[NH_4]6Mo_7O_{24}.4H_2O$) and 1.4 mg of Co kg^{-1} ($CoCl_2.6H_2O$). The soybean seed inoculations were performed at sowing using a liquid *Bradyrhizobium elkanii* inoculant (SEMIA 587 and SEMIA 5019). The recommend dose was 360 ml of inoculant ha^{-1}.

During the R1-R2 phenological stages (Fehr & Caviness, 1977) four replications were harvested, where the third trifoliolate leaves (fully expanded) were collected to analyze N metabolic compounds (nitrate reductase, urease, urea, ureides, nitrate, ammonia, amino acids and isoflavonoids content). The nodule number (>3.0 mm) and the dry weight of nodules were also evaluated at this stage. At the end of the experiment, the last four replicates were collected to evaluate the number of pods and seeds per plant.

2.2 Biochemical analyses

To determine nitrate reductase (NR) activity (Radin, 1973), fresh leaf samples (0.2 g) were incubated in a potassium phosphate buffer KH_2PO_4 100 mM + KNO_3 200 mmol L^{-1} (pH 7.5) during 1 h at 30 ˚C. After incubation, 50 µL from solution described above was used to determine NO_2^- by adding 500 µL of sulfanilic acid 1% in HCl 1.5 N, followed by the addition of 500 µL of naphthyl ethylenediamine 0.02%. Absorbance was determined at 560 nm and compared to a sodium nitrite standard curve.

The determination of leaf urease activity was performed according to Hogan *et al.* (1983). Foliar discs (0.2 g) were transferred to 5 mL of phosphate buffer (pH 7.5), containing N-propanol 2% and 6.5 g of urea in 500 mL of distilled water, which was incubated for 3 h at 30 ˚C. One 5 µL aliquot was collected and added to 1.65 mL of distilled H_2O, followed by 100 µL of reagent 1 (0.74 M phenol; 1.14 mM of sodium nitroprusside) and 200 µL of reagent 2 (0.37 M sodium hydroxide; 0.41 M dibasic sodium phosphate; sodium hypochlorite - 3% of Cl_2). Samples were then incubated at 50 ˚C for 20 min. Urease activity was determined by colorimetry at 625 nm absorbance.

Leaf urea concentration was measured according to Kyllingsbaek, (1975). Extraction was done with 3.0 mL of 10 mM formic acid for each 0.6 g of fresh material. The extract was centrifuged at 13,200 RPM during 5 min, at 4 ˚C. One 250 µL aliquot was collected and added to 1.0 mL of color developing reagent. Such reagent was prepared using a 1:1 proportion of the colorimetric reagent (7% [v/v] 0.2 M diacetylmonoxime; 7% [v/v] 0.05 M thiosemicarbazide) with the acid reagent (20% [v/v] sulphuric acid; 0.06% [v/v] 74 mM ferric chloride hexahydrate; 9% [v/v] ortho-phosphoric acid). Samples were incubated during 15 min at 99 ˚C, then kept in dark and cooled in ice for 5 min. Urea concentration was measured at 540 nm. It was used urea as a standard curve.

About 1.0 g of fresh leaves were extracted into 10 mL of MCW solution (methanol, chloroform, water, 12/5/3 v/v/v), according to Bieleski and Turner (1966). Subsequently, the supernatant was collected to determine the ureides, nitrate, ammonia, and amino acids.

Ureides were determined according to Vogels and Van Der Drift (1970). For allantoin, 25 µL of hydrophilic portion of MCW extract was added to 250 µL of NaOH 0.5 M and 20 µL of 0.33% phenylhydrazine. The mixture was incubated at 100 ˚C during 8 min. This solution was then cooled to ambient temperature and it was added 250 µL of HCl 0.65 N, being incubated one more time at 100 ˚C for 4 min. When the solution

reached room temperature, it was added 250 µL of phosphate buffer solution 0.4 M, pH 7.0, and 250 µL of 0.33% phenylhydrazine. The mixture was homogenized and kept at room temperature by 5 min, then cooled in ice for 5 more minutes. Then, it was added 1.25 mL of 37% HCl and 250 µL of 1.65% $K_3Fe(CN)_6$. After 15 min at room temperature, ureides were determined by reading the absorbance in a spectrophotometer (SP-220, bioespectroTM) at 535 nm. For allantoic acid determination, the same procedure was adopted except by the addition of 250 µL of NaOH 0.5 M, 20 µL of 0.33% phenylhydrazine as well as incubation at 100 °C during 8 min. The results were expressed as µmol g^{-1} DW. To quantify ureides, a standard allantoin curve was used.

The nitrate analysis was performed according to Cataldo *et al.* (1975), using 50 µL of hydrophilic portion of MCW and 40 µL of 5% salicylic acid in concentrated H_2SO_4. After 20 minutes at room temperature, 1 mL of NaOH 2N was added. The samples were cooled to room temperature and absorbance was determined at 410 nm. To quantify nitrate, a standard calcium nitrate curve was used.

Ammonia concentration was quantified according to McCullough, (1967), where 25 µL of hydrophilic portion of MCW was added to 500 µL of colorimetric solution (2.5 g phenol and 12.5 mg sodium nitroprusside in 250 mL) and with 500 µL the phosphate reagent (1.25 g sodium hydroxide, 13.4 g monobasic sodium phosphate, and 2.5 ml 5% sodium hypochlorite in 250 mL). Samples were incubated at 37 °C for 1 h. Ammonia concentration was then determined by colorimetry at 630 nm absorbance. To quantify ammonia, a standard ammonium sulfate curve was used.

Finally, the amino acids were quantified according to Yemm *et al.* (1955), using 70 µL of hydrophilic portion of MCW, 500 µL 0.2 M sodium citrate, 200 µL ninhydrin 5% in ethylene glycol, and 1 mL 0.0002 M KCN. Samples were incubated at 100 °C during 15 min. Then, the samples were cooled with tap water for 10 min, and 1 mL of 60% ethanol was added. The absorbance was read at 570 nm. A methionine standard curve was used to calculate the concentration of amino acids.

2.3 Flavonoids determination

The root and nodule samples were harvested in the R1 – R2 stage, where they were oven-dried at 50 °C for 3 days. For the flavonoids extraction, 0.05 g of roots and nodules were added to 2 ml of methanol 70%, followed by sonication for 30 min. The samples were filtered through a nylon filter 13 mm in diameter and pore size of 0.22 µm. The flavonoids concentration was analyzed using an HPLC (LC-20AT, Shimadzu, Kyoto,

Japan). The detection of the mobile phase consisted of 0.1% (v/v) acetic acid in filtered distilled water (solvent A) and 0.1% (v/v) formic acid in methanol (solvent B), and the detection wavelength was 270 nm. The flavonoids analysis was run with a linear gradient elution of solvent B, from 60 to 70% in 20 min. The injection volume used was 20 µL.

2.4 Seed yield

Mature grains were harvested in the R8 stage (95% of the pods below 15% moisture, presenting mature pod color). The yield estimation was done by counting the number of pods per plant, the number of seeds per plant, and the weight of seeds per plant. Grain yield was converted to dry weight by the correction of 13% moisture.

2.5. Chemical analysis of plant tissue

Leaf and seed samples were identified, packaged in paper bags, and dried in an oven at ± 65 °C for 48 hours. Nitric-perchloric digestion was performed, and the identity and amount of nutrients were determined using radial visualization on an inductively coupled plasma optical emission spectrometer (ICP-OES) equipped with a nebulization chamber as detailed described by Reis *et al*, (2017).

2.6 Statistical analysis

For statistical analysis, the R software (R Development Core Team) was used. The significance of the effects of all variables evaluated as well as their interactions on the reported traits were evaluated by analysis of variance (ANOVA) at 5% significance. Significant differences between means were then determined using the Tukey test at 5 % significance. To evaluate the overall effects of Ni fertilization on soybean genotypes, a Pearson's correlation analysis ($p < 0.05$) was made and represented as a heatmap for each genotype, as well as a principal component analysis (PCA).

3. Results

Ni fertilization affects differently the soybean genotypes tested. The application of 1.5 mg kg^{-1} of Ni was enough to increase the nitrate reductase activity in genotype TMG 2158, while for NIL, the activity decreased. Remarkably, TMG 2158 plants presented the lowest nitrate reductase activity value for all Ni treatments.

The higher doses of Ni increased the urease activity for TMG 2158, which was 1.55-fold higher than TMG 2158 at 3.0 mg kg^{-1}. As expected, the urease activity for *eu3-a* was null, since this mutant is unable to express urease activation protein (Figure 1b). Urea concentration was higher in *eu3-a* plants, followed by TMG 2158. Ni fertilization decreased urea concentration in TMG 2158. The dose of 3.0 mg kg^{-1} resulted in the lowest urea concentration for TMG 2158 and *eu3-a* (Figure 1c).

Both genotypes and Ni fertilization resulted in differences in the isoflavonoids profile. In roots, the highest dose of Ni (3.0 mg Ni kg^{-1}) increased the concentration of daidzein, rutin, and kaempferol in TMG 2158 plants. For *eu3-a* plants, there is a significant decrease in daidzein levels according to Ni supply, which may indicate a toxicity effect of Ni. Only genistein and rutin showed better results than control when 1.5 mg Ni kg^{-1} was applied.

In nodules, smaller amounts of isoflavonoids were observed in comparison to roots. Kaempferol was not detected in the nodule of all genotypes tested, and rutin was not detected only in *eu3-a* plants. Daidzein increased with Ni supply only in TMG 2158 plants and decreased for *eu3-a* plants. Regarding genistein, only *eu3-a* plants demonstrate a significant decrease comparing the highest Ni dose and control. (Figure 2)

Fertilization of Ni with 3.0 mg kg^{-1} promoted nodulation only to TMG 2158 plants, corresponding to an increase of 20.80% in comparison to untreated plants. Even though Ni supply did not affect nodule number in *eu3-a* mutants, we observed a drastic reduction in the number of nodules in *eu3-a* plants in comparison to TMG 2158 plants, demonstrating the role of urease on nodulation (Figure 3a). For nodule dry weight, Ni application did not affect the genotypes significantly (Figure 3b).

Regarding the N metabolism, ureides concentration increased in response to Ni supply for TMG 2158. However, Ni negatively affected *Eu3-a* plants ureides concentration (Figure 4a). The highest values were obtained when 1.5 mg Ni kg^{-1} was applied in TMG 2158. Overall, we observed an increase of red coloration intensity inside the nodules in response to Ni supply (Figure 4f). This effect was more notable in the genotype TMG 2158.

Nitrate concentration in leaves of TMG 2158 and *Eu3-a* decreased in response to Ni supply. These results were similar to those found for NR activity (Figure 1a), except for *Eu3-a* mutant (Figure 1a). There was a negative effect of Ni on ammonia levels for TMG 2158 and *Eu3-a* plants. For amino acids, all genotypes showed a drastic reduction in response to Ni supply. Fertilization with 3.0 mg Ni kg^{-1} reduced amino acid

concentration in TMG 2158 and *Eu3-a* in 19.83-fold and 2.73-fold compared with untreated plants, respectively (Figure 4d).

Regarding seed yield, TMG 2158 was more responsive to Ni application than NIL. Application of 1.5 mg Ni kg^{-1} increased 19% more pods than control and increase seed weight by 15% (Figure 5a and c). Fertilization with 3.0 mg Ni kg^{-1} increased the number of seeds per plant by 51% in comparison to untreated plants (Figure 5b). Overall, *eu3-a* mutantes had lower seed yield, presenting the lowest yield for all parameters evaluated and no response to Ni fertilization.

PCA analysis was used to summarize the major patterns of variation of each genotype. The first two components explained 76.6% and 72.7% of the variables of the genotypes TMG 2158 and *eu3-a*, respectively (Figure 7a and b).

4. Discussion

Nitrogen assimilation starts with the reduction of nitrate to nitrite by nitrate reductase enzyme, which is followed by the conversion of nitrite to ammonium, which is converted to glutamine by glutamine synthetase (GS) (Fan *et al.*, 2002). The effects of Ni on nitrate reductase activity are not completely elucidated, but several studies demonstrated that high concentration of Ni can inhibit nitrate reductase activity, which seemed to be the case of both NILs mutants (Figure 1a).

Kevresan et al. (2001) reported a reduction of nitrate reductase activity in pea plants (*Pisum sativum* L. 'NS Lim') at all Ni concentrations tested (1×10^{-7}, 1×10^{-5}, and 1×10^{-3} mol dm^{-3}). This happens probably due to the toxicity effect of Ni reducing the energy supply for other physiological functions (Yusuf *et al.*, 2011; Hassan *et al.*, 2019).

However, this inhibitory effect was not observed in TMG 2158 plants, which had an increase in nitrate reductase activity in response to Ni application. Ni responsiveness varies between plants of genus and species (Seregin & Kozhevnikova, 2006; Freitas *et al.*, 2018) (Fig 1a).

Interestingly, nitrate concentration in leaves of TMG 2158 plants was negatively correlated with parameters such as urease activity, daidzein concentration in roots and nodules, nodule number, ureides, and seed yield (Figure 7d). These results may contributed to a better performance of TMG 2158 genotypes since it is well known that high nitrate levels can inhibit BNF in some legume plants and it can limit root infection, nodule development, and nitrogenase activity (Ohyama *et al.*, 2011).

As expected, Ni increased urease activity because it is a structural component of the enzyme, which is responsible for the hydrolysis of urea into ammonia (Dixon *et al.*, 1980; Polacco *et al.*, 2013), except by urease-negative soybean mutant, that is not able to metabolize urea since urease is not active in these plants (Tezotto *et al.*, 2016).

Several reports related similar results regarding the positive effect of Ni on urease activity in soybean plants (Barcelos *et al.*, 2017; Freitas *et al.*, 2019). Our data revealed that Ni supply promoted a reduction of urea concentration in *eu3-a* plants, even though this genotype did not present urease activity, suggesting Ni toxicity in the arginine metabolism, especially in the arginase, which is responsible to convert arginine into urea and ornithine (Polacco *et al.*, 2013).

As we hypothesized, Ni fertilization affected isoflavonoids profile, specially daidzein (Figure 2). The isoflavonoids play an important role during nodulation processes since it is responsible to trigger the expression of rhizobia genes (Figure 6) (Singla & Garg, 2017). Subramanian *et al.* (2006), demonstrated that endogenous isoflavones, such as daidzein and genistein, are essential for the establishment of symbiosis in soybean plants by silencing the expression of isoflavone synthase (IFS), a key enzyme in the biosynthesis of isoflavone. To the best of our knowledge, this is the first study to relate Ni fertilization to the synthesis of flavonoids in soybean plants.

In this study, TMG 2158 was the most responsive genotype to Ni fertilization. The increase in nodule number (Figure 3a) and the constant nodule dry weight (Figure 3b) suggests that the nodule size reduced according to Ni fertilization.

The PCA analysis (Figure 7a) showed that the majority of positively correlated variables are grouped next to the individual treated with Ni. The heatmap revealed that urease activity is positively correlated with Ni concentration in leaf, daidzein concentration in roots and nodules, and the number of nodules (Figure 7d). The number of nodules also showed a positive correlation with daidzein concentration in both roots and nodules, which may indicate a possible new role of urease as a modulator of isoflavonoid synthesis.

According to Sugiyama et al., (2016), the mechanisms that regulate isoflavones during soybean growth are not very well elucidated, but they observed a higher secretion of daidzein and genistein under nitrogen-deficient conditions. It may reveal that an impaired N metabolism could affect the isoflavonoids pathway and its secretion, which may be occurring with *eu3-a* plants.

The *eu3-a* plants showed no increase in nodule number in response to Ni fertilization (Figure 3a) and Ni application resulted in toxicity effects on isoflavonoids pathway by reducing the levels of daidzein in roots and nodule and genistein in nodules (Figure 2a, e, and c), and by the high levels of urea and nitrate in leaves (Figure 1c and Figure 4b, respectively). This Ni toxicity effect can also be observed in the PCA of *eu3-a* plants, where most of the variables were concentrated around the control treatment (0.0 mg Ni kg^{-1}) (Figure 7b).

Despite the higher isoflavonoids concentration observed in *eu3-a* plants, the nodule number also did not increase. The heatmap (Figure 7f) revealed no significant correlation between daidzein concentration in roots and nodule number for the NIL. It can be explained by the reduction of the isoflavonoids levels, associated with the null urease activity, which may have harmed the chemotaxis and nodulation since these isoflavonoids are crucial for nodulation and nodule organogenesis (Liu & Murray, 2016).

There were effects of urease activity on the nodule number since the urease negative mutants showed the lowest nodulation (Figure 3). Medeiros-Silva et al., (2014), demonstrated that soybean ureases alter the physiology of nodules and interfere with chemotactic signals triggered by soybean plants in the rhizosphere, even though the authors did not elucidate this mechanism. Our data suggested that urease activity may regulate isoflavonoids synthesis, which can be enhanced by Ni fertilization.

In this study, ureides concentration increased for TMG 2158 genotype whereas it decreased for *eu3-a* mutants, (Figure 4a and b), probably due to Ni toxicity as described previously. In TMG 2158 plants, we observed a positive correlation between Ni concentration in leaves and urease activity, which showed a positive correlation with the parameters related to N metabolisms such as daidzein, number of nodules, and ureides (Figure 7d).

Freitas et al. (2019), demonstrated the positive effects of Ni on BNF by an increase in nitrogenase activity and ureide concentration. The ureides are the final products of the N_2 fixed in legumes and can represent over 90% of the total N transported in the xylem sap in tropical legume plants such as soybean (Raso *et al.*, 2007; Baral *et al.*, 2016). Furthermore, it is known that low doses of Ni can enhance BNF, once Ni acts as a structural component of hydrogenase present in the bacteroid, an enzyme responsible for the oxidation of H_2, which process results in ATP required by nitrogenase to reduce N_2 into ammonia (Ureta *et al.*, 2005; Lavres *et al.*, 2016).

The reduction in the levels of ammonia and amino acids under Ni fertilization of both genotypes (Figure 4c) can be associate with the increase of protein levels in leaves and grain filling. The NH_3/NH_4^+ is assimilated into glutamine and other amino acids, which are used for protein synthesis (Ohyama *et al.*, 2017). However, this effect can also be associate with the reducing level of Mn in leaves (Supplementary Table 1), reducing the activity of ureide-degrading enzymes, which are dependent on this micronutrient (Baral *et al.*, 2016).

Finally, seed yield results of *eu3-a* plants (Figure 5) made clear the negative effect of lack urease activity on soybean productivity. Furthermore, the results obtained with TMG 2158 demonstrated not only the benefits on N metabolism but also the different responsiveness to Ni fertilization according to soybean genotypes. These results showed that new high-yielding genotypes may present a hidden deficiency for Ni, once the application of this fertilization enhances nitrogen metabolism, especially the ureides synthesis, resulting in higher seed yield (Lavres *et al.*, 2016; Freitas *et al.*, 2018)

5. Conclusion

Fertilization with Ni increased nitrate reductase activity in commercial soybean plants. Urease activity in leaves, flavonoid profile (daidzein, rutin and kaempferol) in roots of soybean plants was enhanced by Ni fertilization, except for the NIL. These results suggest that Ni plays a role in flavonoid metabolism (mainly in daidzein) inducing chemotaxis between soybean plants and rhizobia boosting nodulation and ureide metabolism. Higher nodulation regulated by Ni fertilization generated more available organic nitrogen to soybean plants resulting in higher seed yield for commercial soybean variety.

New experiments are needed to investigate the exact mechanisms of action of urease on isoflavonoids pathway as well as the genes expression to identify possible genes controlling daidzein biosynthesis to select more responsive genotypes to Ni fertilization, resulting in a better BNF and yield of soybean plants.

Author contribution

ARR designed the experiment, MAB performed the experiments, biochemical analysis wrote the main text and performed the statistical analysis, NACM performed the biochemical analysis, ARR and NACM revised the manuscript.

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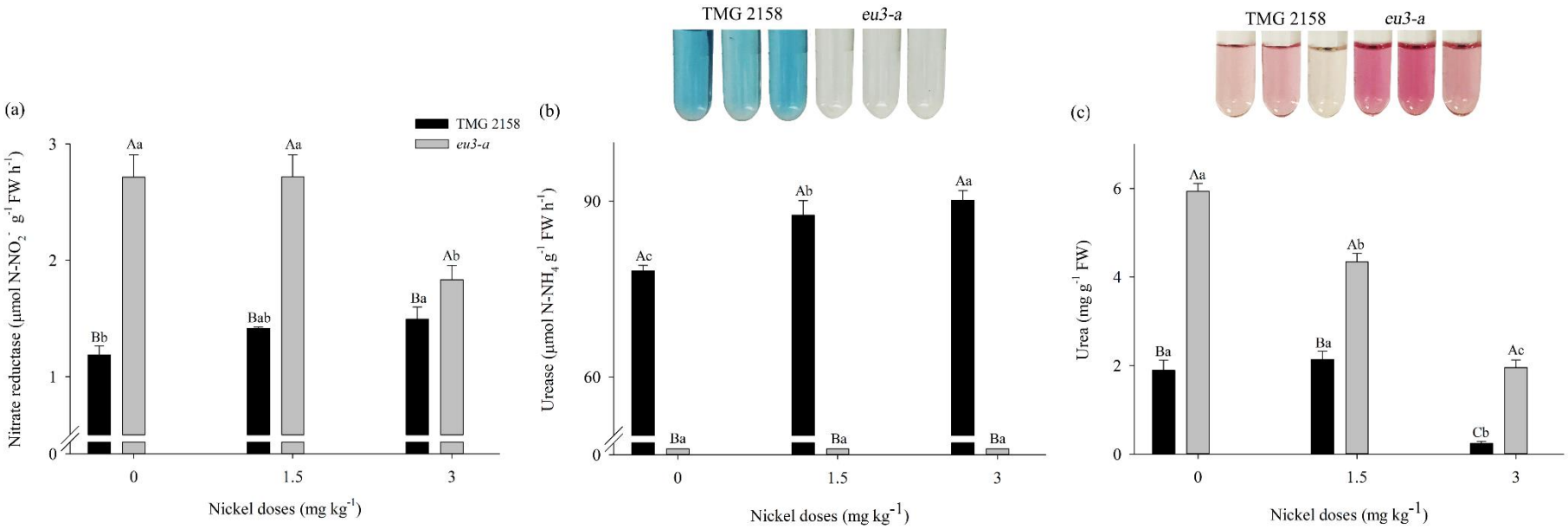

Figure 1. Nitrate reductase activity (a), urease activity (b), and urea concentration (c) in soybean leaves in response to Ni supply. The standard error of the mean (n = 4) is indicated by errors bars. Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 6.99 (a); 2.99 (b); 6.47 (c).

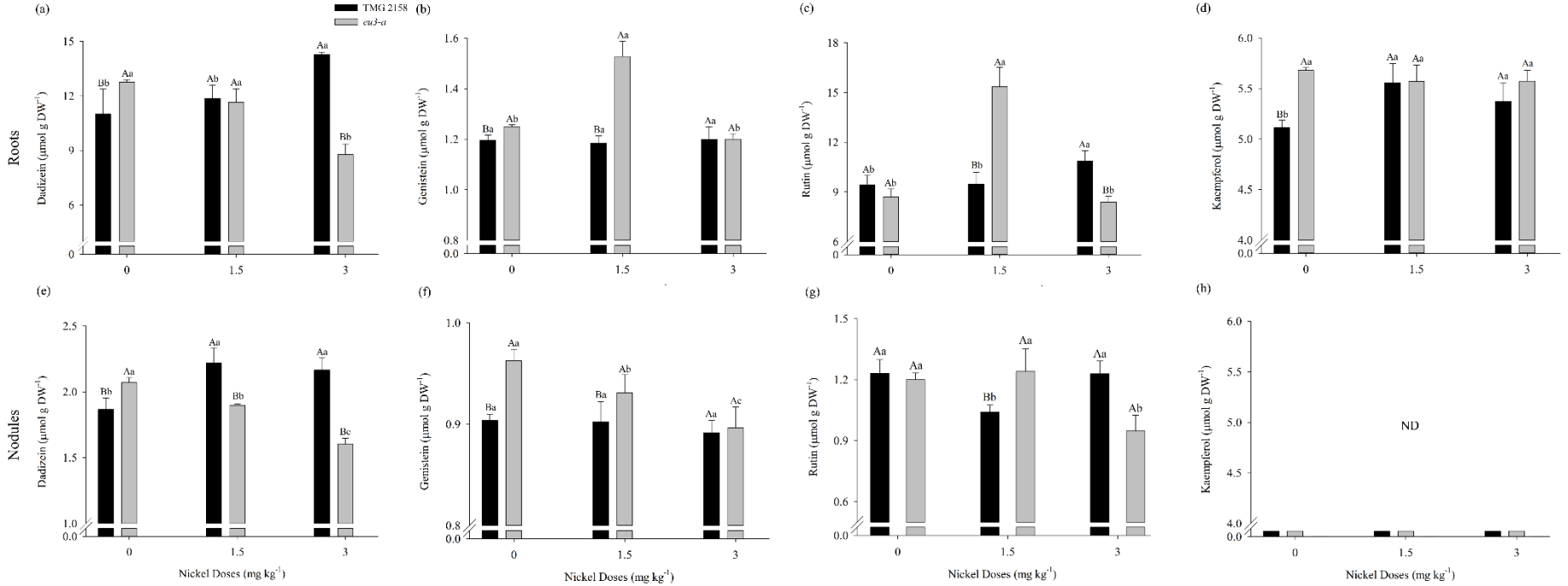

Figure 2. Daidzein, genistein, rutin and kaempferol in soybean roots and nodules in response to Ni supply. Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 6.34 (a); 2.91 (b); 6.73 (c); 2.49 (d); 3.79 (e); 1.70 (f); 6.06 (g). ND = no detected (h).

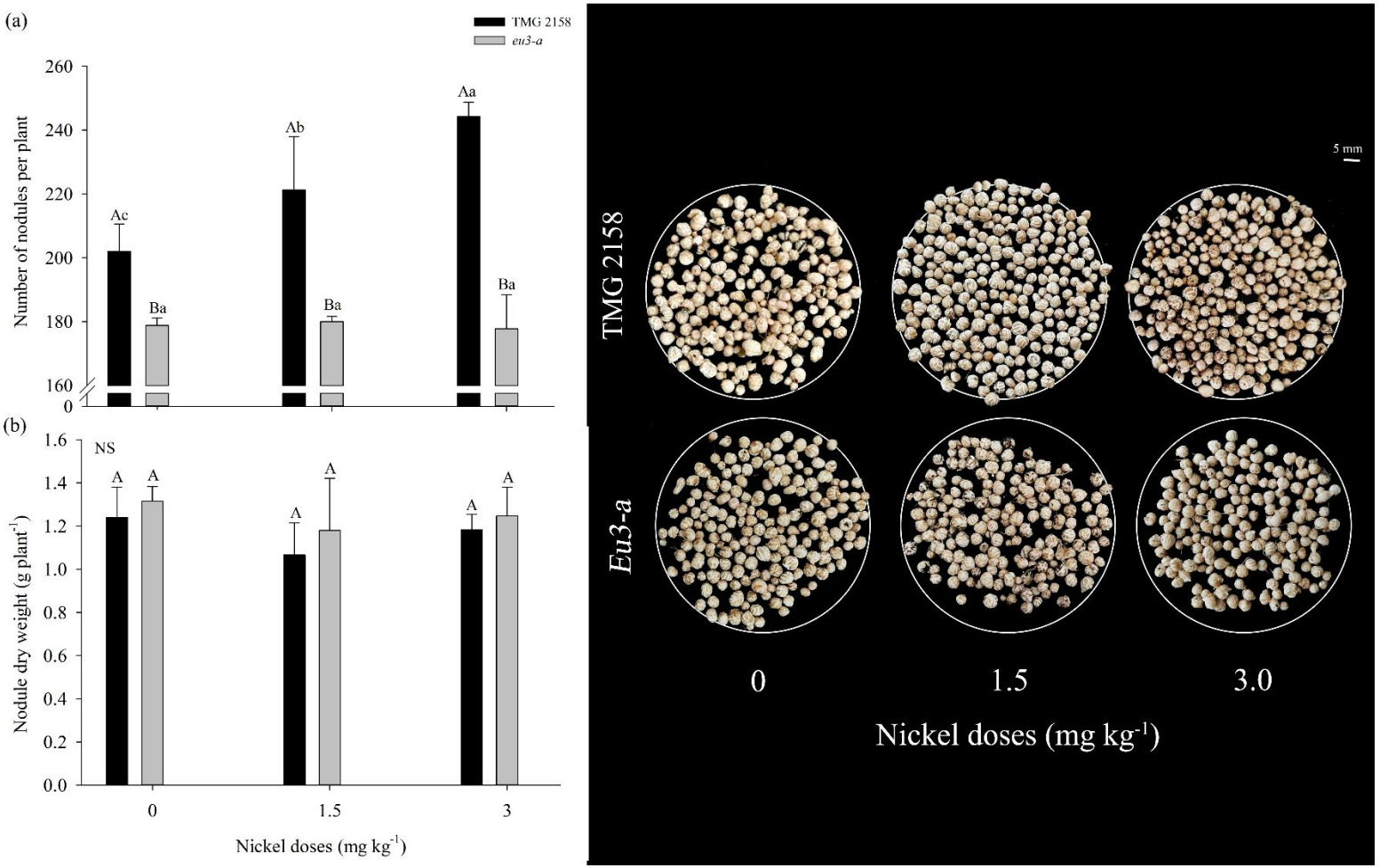

Figure 3. Number of nodules per plant (a) and nodule dry weight (g plant^{-1}) in response to Ni supply. Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 4.52 (a) and 12.70.

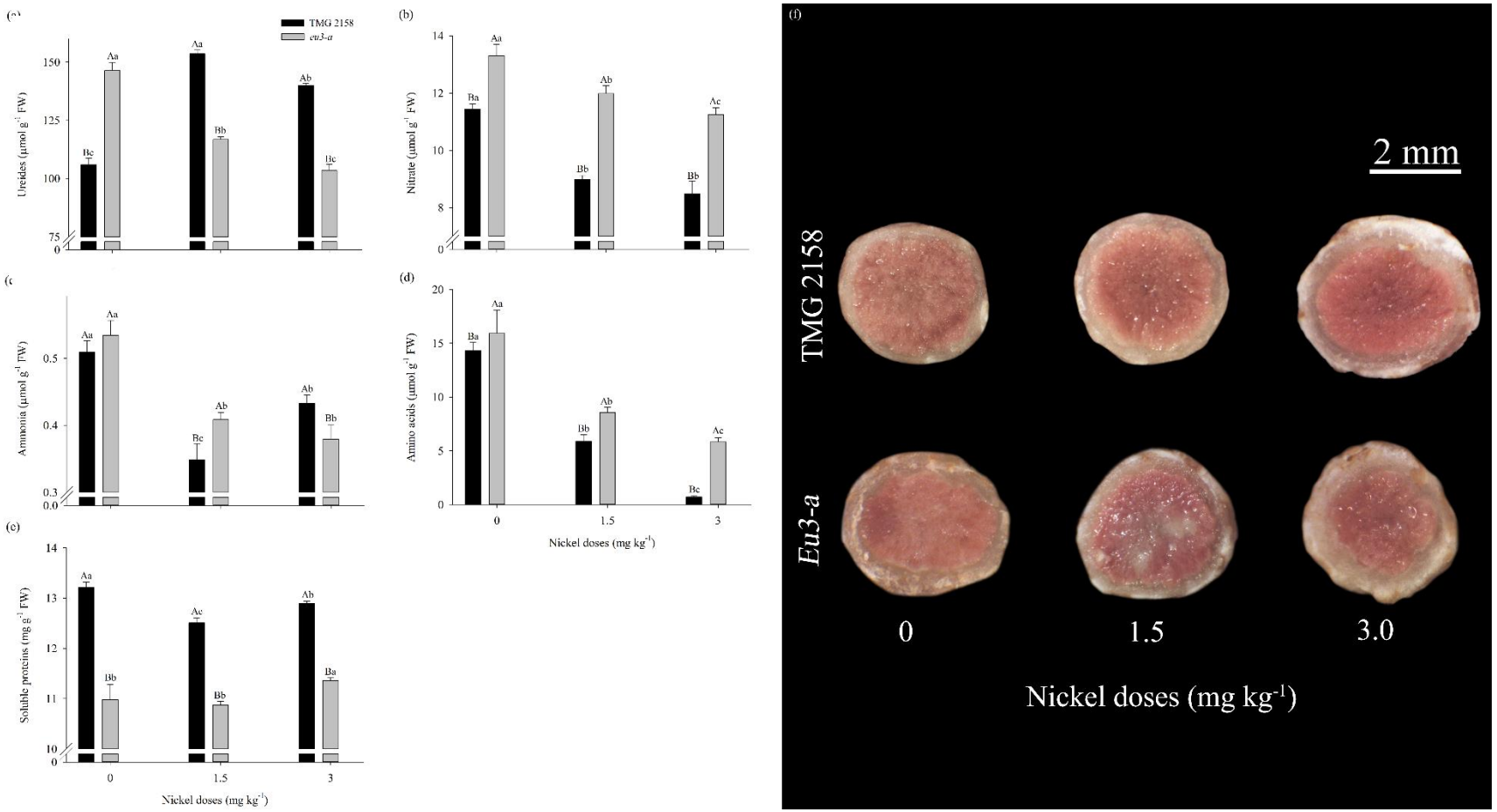

Figure 4. Ureides (a), nitrate (b), ammonia (c), amino acids (d) and soluble proteins (e) in soybean leaves in response to Ni supply and longitudinal section of the root nodules (f). Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 1.78 (a); 2.77 (b); 4.29 (c); 11.76 (d); 1.20 (e).

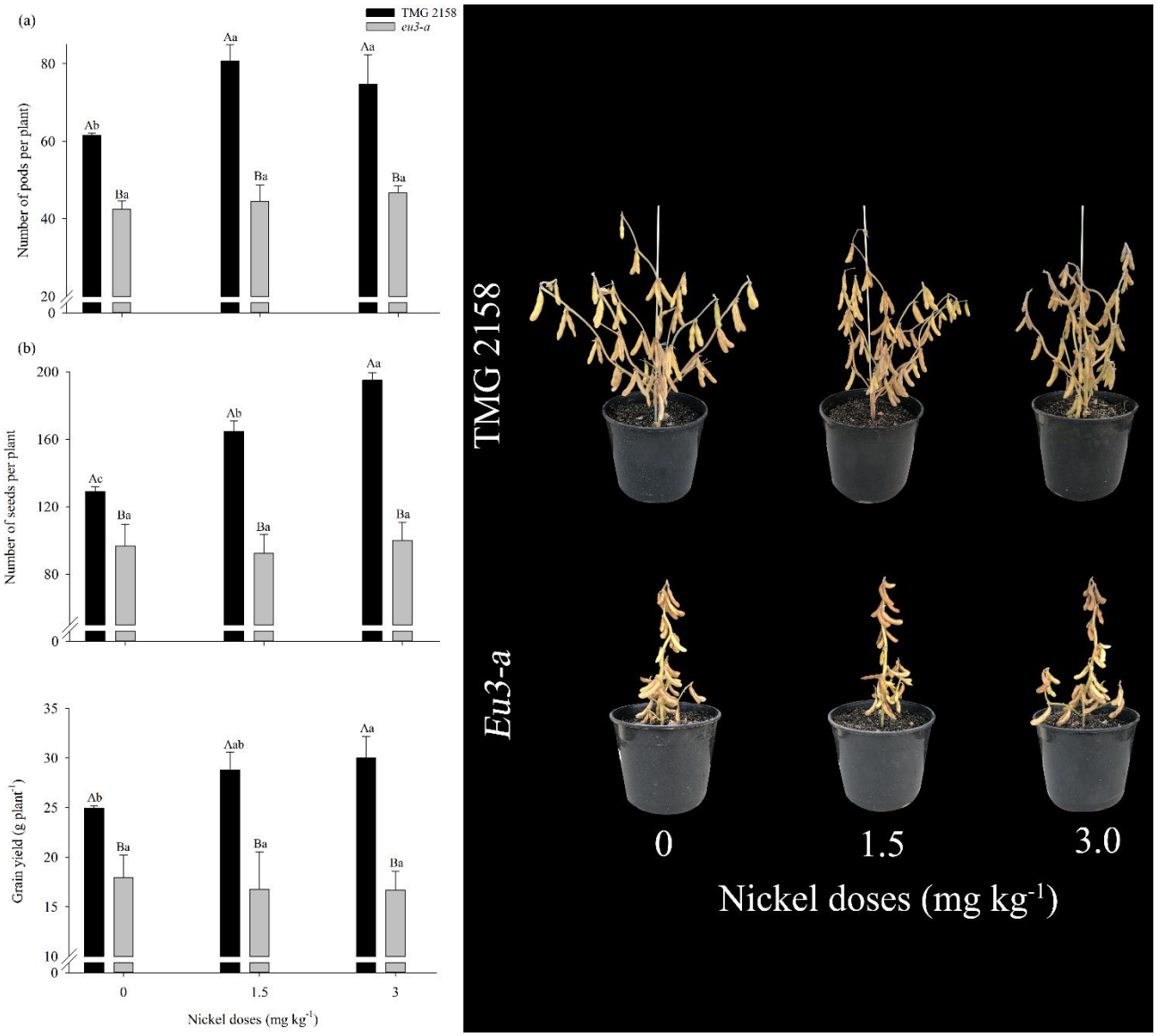

Figure 5. Number of pods (a), number of seeds (b) and grain yield (c) of soybean plants in response to Ni supply. Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 6.93 (a); 6.8 (b); 10.06 (c).

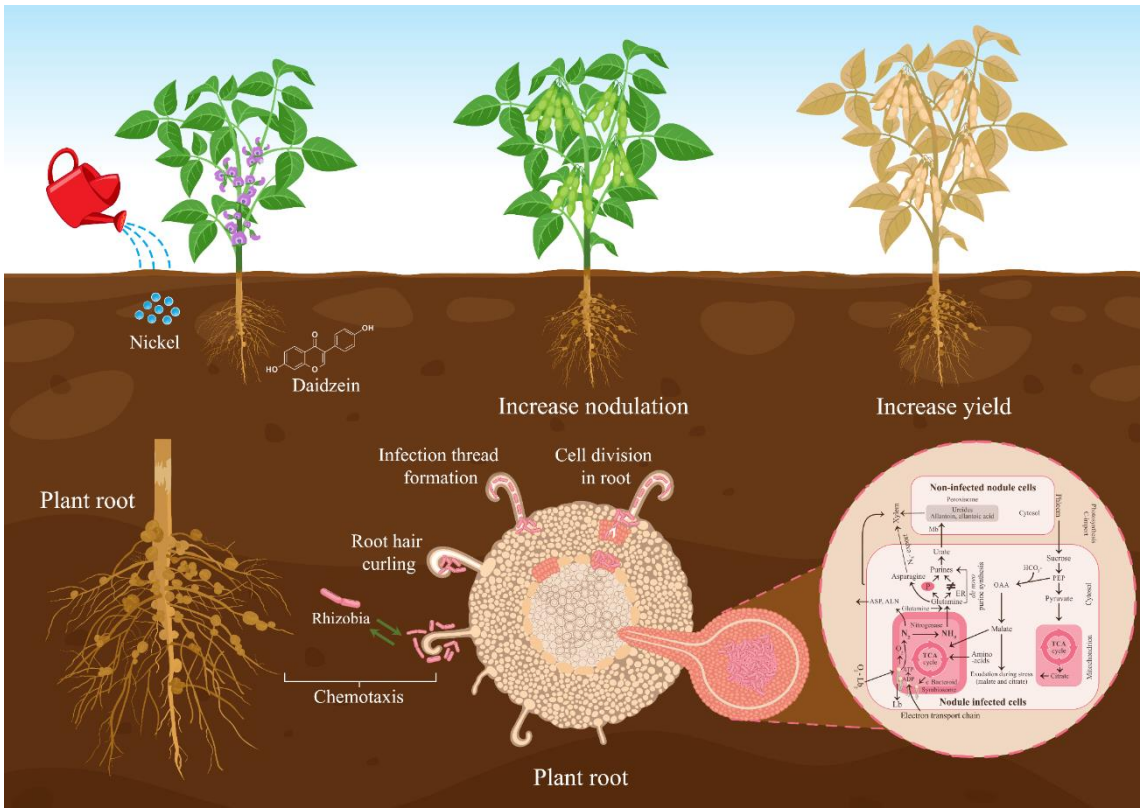

Figure 6. Proposal mechanisms of Ni effects on flavonoid profile increasing nodulation and ureides metabolism in soybean plants.

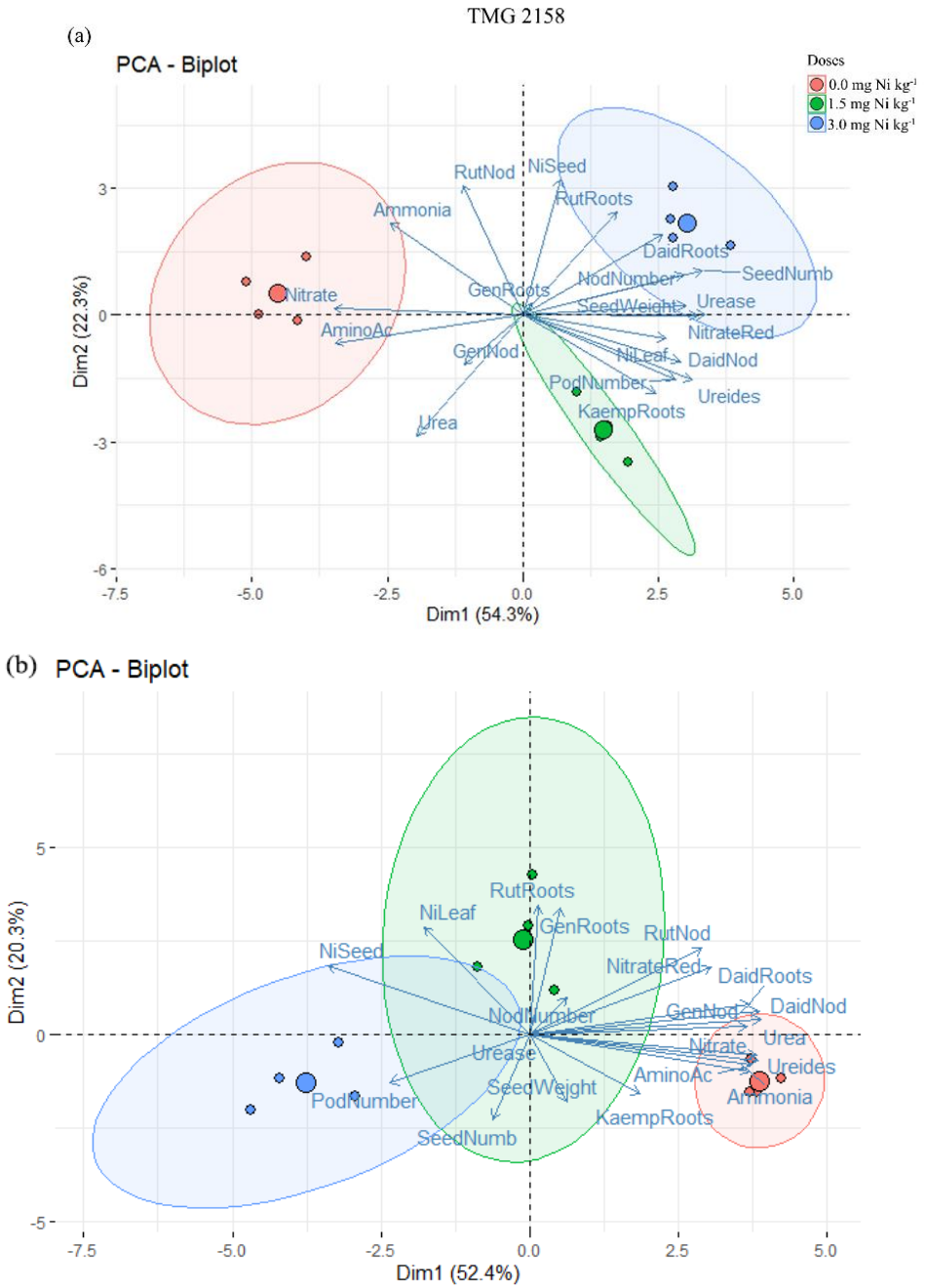

Figure 7. Principal component analysis of soybean genotypes in response to Ni fertilization. Abbreviations: NitrateRed - nitrate reductase, DaidRoots - daidzein concentration in roots, GenRoots - genistein concentration in roots, RutRoots - rutin concentration in roots, KaempRoots - kaempferol concentration in roots, DaidNod - daidzein concentration in nodules, GenNod - genistein concentration in nodules, RutNod - rutin concentration in nodules, Nodules - nodule number, AminoAc - amino acids concentraion in leaves, GrainNumb – grain number, NiLeaf - nickel concentration in leaves, NiGrain - nickel concentration in grains.

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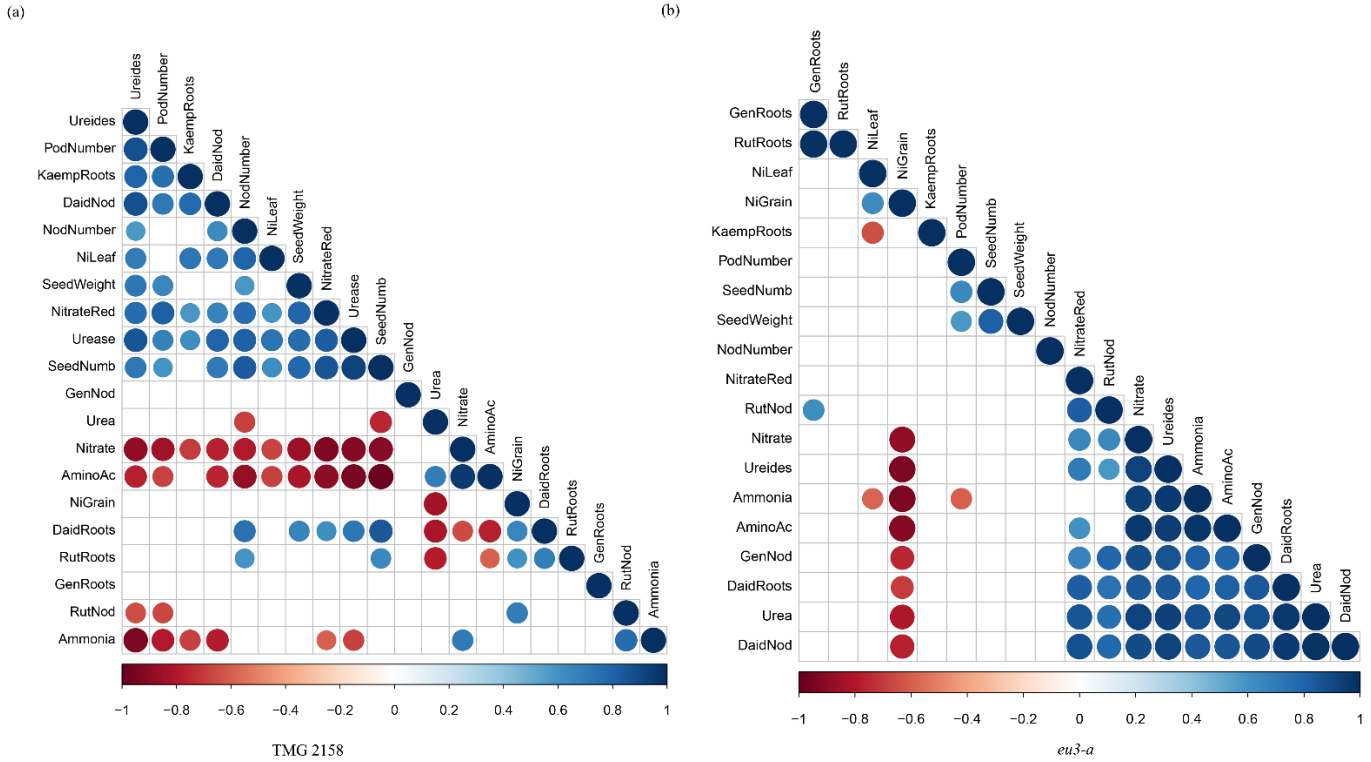

Figure 8. Heatmap indicating the correlations of Person ($p < 0.005$) obtained from parameters measured from soybean genotypes in response to Ni fertilization. Abbreviations: NitrateRed - nitrate reductase, DaidRoots - daidzein concentration in roots, GenRoots - genistein concentration in roots, RutRoots - rutin concentration in roots, KaempRoots - kaempferol concentration in roots, DaidNod - daidzein concentration in nodules, GenNod - genistein concentration in nodules, RutNod - rutin concentration in nodules, Nodules - nodule number, AminoAc - amino acids concentraion in leaves, GrainNumb – grain number, NiLeaf - nickel concentration in leaves, NiGrain - nickel concentration in grains.

Supplementary material Table 1. Element concentration in leaves and seeds in response to Ni supply.

Leaf nutrient content

Genotypes	Ni Doses (mg kg^{-1})	P	K	Ca	Mg	S	B	Co	Cu	Fe	Mn	Mo	Ni	Zn
		(g kg^{-1})						(mg kg^{-1})						
TMG 2158	0.0	2.96 ± 0.04 B	21.14 ± 0.27 B	9.75 ± 0.16 B	3.37 ± 0.04 AB	2.21 ± 0.03 B	38.33 ± 0.59 B	0.75 ± 0.16 B	6.46 ± 0.32 B	539.83 ± 10.67 A	134.02 ± 6.73 A	117.59 ± 2.87 A	40.58 ± 0.75 B	35.65 ± 1.21 A
TMG 2158	1.5	3.14 ± 0.07 A	21.57 ± 0.27 B	9.48 ± 0.20 B	3.30 ± 0.15 B	2.29 ± 0.05 B	37.19 ± 0.78 B	1.31 ± 0.07 A	7.40 ± 0.49 A	533.71 ± 16.81 AB	96.34 ± 3.10 B	107.46 ± 0.36 B	41.73 ± 0.94 A	36.51 ± 0.91 A
TMG 2158	3.0	3.17 ± 0.05 A	23.98 ± 0.65 A	10.39 ± 0.15 A	3.55 ± 0.01 A	2.53 ± 0.02 A	44.58 ± 1.16 A	1.42 ± 0.22 A	6.07 ± 0.15 B	532.00 ± 3.33 B	70.55 ± 0.53 C	105.92 ± 1.82 B	41.73 ± 0.55 AB	35.46 ± 1.31 A
eu3-a	0.0	3.03 ± 0.01 B	17.81 ± 0.29 A	12.18 ± 0.05 A	3.89 ± 0.13 A	2.38 ± 0.11 A	33.79 ± 0.19 B	0.76 ± 0.07 B	6.01 ± 0.22 B	520.67 ± 11.27 A	193.14 ± 0.65 A	126.93 ± 1.64 A	41.58 ± 0.36 B	40.91 ± 0.7 A
eu3-a	1.5	3.23 ± 0.09 A	18.29 ± 0.54 A	9.74 ± 0.31 B	3.39 ± 0.10 B	2.43 ± 0.03 A	41.55 ± 0.14 A	1.40 ± 0.09 A	6.53 ± 0.22 A	516.08 ± 16.97 AB	167.68 ± 6.92 B	101.68 ± 1.64 C	43.36 ± 1.07 A	36.34 ± 1.04 B
eu3-a	3.0	2.92 ± 0.07 B	17.56 ± 0.64 A	8.58 ± 0.08 C	2.96 ± 0.10 C	2.06 ± 0.07 B	32.77 ± 2.17 B	1.05 ± 0.14 B	5.58 ± 0.44 B	480.85 ± 14.11 B	120.12 ± 3.82 C	116.91 ± 1.40 B	42.45 ± 1.04 AB	27.43 ± 1.01 C
	CV (%)	1.96	2.36	1.78	2.95	2.55	2.85	11.96	5.18	2.51	3.41	1.59	1.97	2.96

Seed nutrient content

Genotypes	Ni Doses (mg kg^{-1})	P	K	Ca	Mg	S	B	Co	Cu	Fe	Mn	Mo	Ni	Zn
		(g kg^{-1})						(mg kg^{-1})						
TMG 2158	0.0	3.49 ± 0.06 A	15.67 ± 0.31 A	2.54 ± 0.62 A	1.88 ± 0.51 A	2.53 ± 0.01 AB	28.13 ± 0.15 B	1.53 ± 0.20 A	6.15 ± 1.13 B	120.62 ± 5.95 B	34.75 ± 1.29 A	99.08 ± 2.51 A	46.61 ± 0.56 B	36.92 ± 0.66 AB
TMG 2158	1.5	3.10 ± 0.03 A	14.18 ± 0.32 B	2.46 ± 0.42 A	2.31 ± 0.13 A	2.43 ± 0.06 B	41.72 ± 0.65 A	1.54 ± 0.10 A	7.69 ± 0.52 B	160.69 ± 12.56 A	27.08 ± 0.81 C	97.20 ± 0.59 AB	43.60 ± 2.81 B	33.85 ± 2.55 B
TMG 2158	3.0	3.30 ± 0.16 A	15.24 ± 0.47 A	3.22 ± 0.10 A	2.61 ± 0.03 A	2.63 ± 0.04 A	47.76 ± 7.71 A	1.66 ± 0.33 A	8.14 ± 1.04 A	107.62 ± 12.21 B	31.27 ± 0.76 B	91.55 ± 6.50 B	50.09 ± 0.76 A	39.45 ± 0.31 A
eu3-a	0.0	4.80 ± 0.43 B	12.49 ± 0.27 B	2.37 ± 0.07 A	2.00 ± 0.32 A	2.28 ± 0.10 C	44.33 ± 0.40 A	2.51 ± 0.46 A	8.35 ± 0.26 B	22.23 ± 7.22 B	25.11 ± 2.03 B	53.20 ± 2.41 B	37.36 ± 2.11 B	51.52 ± 1.60 AB
eu3-a	1.5	4.77 ± 0.09 B	16.24 ± 0.21 A	2.48 ± 0.02 A	2.22 ± 0.06 A	2.88 ± 0.09 B	39.10 ± 1.86 A	1.85 ± 0.24 A	10.25 ± 0.18 A	21.39 ± 1.73 B	31.52 ± 1.05 A	56.88 ± 1.71B	53.57 ± 0.42 A	48.81 ± 0.25 B
eu3-a	3.0	5.41 ± 0.04 A	16.66 ± 0.37 A	2.43 ± 0.06 A	2.06 ± 0.07 A	3.15 ± 0.06 A	42.33 ± 10.03 A	2.42 ± 0.14 A	8.53 ± 0.78 B	40.28 ± 1.82 A	31.66 ± 1.28 A	85.37 ± 1.05 A	52.78 ± 1.15 A	53.66 ± 0.42 A
	CV (%)	4.66	2.21	11.96	11.16	11.71	12.9	14.22	9.11	10.37	4.22	3.88	3.32	2.9

CHAPTER 4 - Nickel enhances photosynthesis, ROS scavenging system and seed germination of soybean plants

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Abstract

Nickel (Ni) is a micronutrient that plays an important role in nitrogen metabolism, especially in legume plants, which may influence other important physiological processes such as photosynthesis and seed germination. This study aimed to investigate the effect of Ni doses on photosynthesis and antioxidant metabolism as well as the effect of Ni on seed germination. Three Ni doses (0; 1.5; 3.0 mg kg^{-1}) were applied to two soybean genotypes: commercial soybean (TMG 2158) and urease-null (*eu3-a*) soybean near-isogenic lines (NILs). Gas exchange parameters, total sugars, lipid peroxidation measured by malondialdehyde (MDA) concentration, hydrogen peroxide concentration, activity of superoxide dismutase (SOD, EC: 1.15.1.1), catalase (CAT, EC:1.11.1.6), ascorbate peroxidase (APX, EC:1.11.1.11) in leaves were evaluated. Furthermore, a germination test was performed. Ni fertilization increased net photosynthesis and total sugars concentration of both genotypes .Ni fertilization also led to a decrease in lipid peroxidation of cell membranes of genotype TMG 2159, due to an increase in APX activity, which played an important role in reactive oxygen species scavenging system. Ni fertilization increased number of pods per plant, number of seeds per plant and seed yield. Also, Ni improved seed germination and seedling development of TMG 2158 by increasing urease activity and N remobilization in comparison with *eu*3-a. Therefore, we demonstrate that Ni plays critical roles on soybean antioxidant system, seed yield and physiological quality and vigour of soybean seeds.

Keywords: antioxidant metabolism, seed germination, oxidative stress, seed nickel, ureides.

1. Introduction

Nitrogen (N) is the most required mineral nutrient by plants and its deprivation can be a limitation factor for plant development. Overall, nitrogen can be remobilized from senescing leaves to expanding leaves in the vegetative stage, as well as to developing seeds in the reproductive stage (Islam *et al.*, 2016; de Souza *et al.*, 2020). This N-cycling pathway in plants can occurs through arginine (Arg) catabolism, producing ornithine (Orn) and urea (Polacco *et al.*, 2013).

Urea is hydrolyzed by urease, which is a Ni-dependent metalloenzyme (Dixon *et al.*, 1975). In soybean plants, three urease isoforms have been described: the ubiquitous urease, encoded by the *Eu4* gene, which is expressed at low levels in all tissues (Polacco *et al.*, 1985); the embryo-specific urease, encoded by *Eu1*, highly expressed in developing embryos and accumulating in mature seeds (Polacco & Havir, 1979; Polacco & Winkler, 1984); and a third gene called *Eu5* that was discovered in the soybean genome (Polacco *et al.*, 2013), which is expressed in the first stages of root development and during seed maturation (Wiebke-Strohm *et al.*, 2016). Urease activity depends on the attachment with Ni by two accessory proteins encoded by the genes *Eu2* and *Eu3* in soybean plants. A null mutant for the *Eu3* gene has been characterized (Freyermuth *et al.*, 2000) and the deletion of this gene resulted in a *eu3-a* mutant with no urease activity (Polacco *et al.*, 2013; Tezotto *et al.*, 2016; de Souza *et al.*, 2020).

It is known that during the photosynthetic metabolism of plants, reactive oxygen species (ROS) are generated and it is increased under stress conditions. Therefore, the activation of enzymatic and nonenzymatic antioxidant mechanisms is important to keep the physiological state of the plant (Foyer *et al.*, 2017; Farooq *et al.*, 2019). Ni may also interfere with a number of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Sachan & Lal, 2017; Barcelos *et al.*, 2018). However, most of the literature is focused on the toxic effects of Ni on the antioxidant enzymes, which may not represent most physiologically relevant conditions and need for this micronutrient (Reis *et al.*, 2017).

Bilogical nitrogen fixation (BNF) in legumes is also enhanced by Ni fertilization, specially due to its role in the activation of hydrogenase enzyme that recycles H_2, a by-product of N_2 reduction in nitrogen fixation by rhizobia (Bagyinka, 2014; Lavres *et al.*, 2016). Freitas *et al.* (2019), demonstrated that Ni fertilization positively affected N metabolism in soybean by increasing nodules number and consequently, ureides synthesis. Moreveover, according to the same authors, Ni application also improved plant

physiology such as chlorophyll content and photosynthesis, leading to an increase in plant growth and productivity, as observed in different studies (Lavres *et al.*, 2016; Rodak *et al.*, 2021).

In addition, optimal concentrations of micronutrients in seeds can improve germination and seedling development. Seeds with low Ni content demonstrated an impaired endogenous urea metabolism, which is produced by plants through arginine (Arg) catabolism, and is a crucial process for N remobilization during seed germination (Rainbird et al., 1984; Zonia et al., 1995). Brown et al. (1987), demonstrated that barley seeds with negligible Ni were inviable, proving the importance of this micronutrient for seed germination and the essentiality of Ni for cereals and possibly all higher plants.

Therefore, this study hypothesizes that Ni activates different antioxidant enzymatic pathways to scavenge ROS in soybean plants, and Ni content in seeds increase seed germination. We aimed to evaluate the effects of Ni fertilization on photosynthesis and antioxidant enzymes as well as the effect of Ni content in seeds on seed germination and soybean development using a commercial soybean genotype (TMG 2158) and mutant soybean seeds for urease: *eu3-a* (urease-null).

2. Material and Methods

2.1 Plant growth and experiment design

The experiment was carried out under greenhouse conditions at São Paulo State University (UNESP), Tupã, São Paulo State, Brazil. In this experiment, one commercial soybean genotype (TMG 2158) and one near-isogenic line (NIL) (*eu3-a* mutant) were fertilized with 0.0 mg kg^{-1}, 1.5 mg kg^{-1} and 3.0 mg kg^{-1}, using a nickel sulfate solution ($NiSO_4.6H_2O$). Ni doses range was based on a previous study (Freitas *et al.*, 2019) as optimal concentration to enhance soybean nodulation.

The experimental design was a 3 × 3 completely randomized factorial design (soybean genotypes × Ni doses), with eight replications for each genotype, totaling 48 pots. The pots of 4 L were filled with a commercial growing substrate Carolina Soil ®. Growing substrate samples were submitted to an acid digestion and the heavy metal contents were determined using an ICP-OES. According to the analysis, the following levels were found: 2.8 mg of As kg^{-1}; 0.8 mg of Cd kg^{-1}; 2.8 mg of Pb kg^{-1}; 1060.1 mg of Cr kg^{-1}; 13.1 mg of Hg kg^{-1}; 435.0 mg of Ni kg^{-1} and 14.9 mg of Se kg^{-1}.

Macro and micronutrients were also supplied (except by N) at the following doses: 200 mg of P kg^{-1} (P_2O_5) + 50.3 mg of K kg^{-1} (KCl), 9.1 mg of Mn kg^{-1} ($Cl_2Mn.4H_2O$), 11.1 mg of Zn kg^{-1} ($ZnSO_4.7H_2O$), 2.9 mg of B kg^{-1} (H_3BO_3), 2.6 mg of Cu kg^{-1} ($CuSO_4.5H_2O$), 2.3 mg of Mo kg^{-1} ($[NH_4]6Mo_7O_{24}.4H_2O$) and 1.4 mg of Co kg^{-1} ($CoCl_2.6H_2O$). The soybean seed inoculation were performed at sowing using a liquid *Bradyrhizobium elkanii* inoculant (SEMIA 587 and SEMIA 5019). The recommend dose was 360 ml of inoculant ha^{-1}.

The analysis of gas exchange and the antioxidant metabolism were performed during the R1-R2 phenological stages (Fehr & Caviness, 1977), between 8:00 and 12:00 a.m, using the first and second fully developed trifoliate of 4 replicates. At the end of the experiment, the other 4 replicates were harvested and the seeds were used for a germination test.

2.2. Gas exchange parameters

Determination of gas exchange was carried out using a portable gas exchange device (Infra-Red Gas Analyzer - IRGA, brand LICOR®-6400). The following parameters were determined: net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), stomatal conductance (mol H_2O m^{-2} s^{-1}), internal CO_2 concentration in the sub-stomatal chamber (μmol CO_2 mol^{-1}) and transpiration rate (mmol H_2O m^{-2} s^{-1}). For measurements, the standard conditions used were: 1200 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR), provided by LED lamps, 380 ppm of CO_2, and a chamber temperature of 28 °C

2.3. Antioxidant metabolism

Leaf material was macerated in liquid N and stored in a freezer at −80 °C until it was processed for analyses of hydrogen peroxide concentration, lipid peroxidation, superoxide dismutase activity, catalase activity, ascorbate peroxidase activity.

Lipid peroxidation was estimated based on the concentration of malondialdehyde (MDA) according to Heath and Packer (1968). For extraction, 0.5 g of leaf material was macerated in 4 mL of 0.1% (w/v) trichloroacetic acid solution, then centrifuged at 4000 rpm for 15 min at 4 °C. Then, 1.0 ml of 0.5% thiobarbituric acid (TBA) was added to 200 μL of supernatant. The mixture was agitated vigorously and incubated at 95 °C for 30 min. The material was kept accommodated on ice inside a closed polystyrene box for 10 min and centrifuged at 10,000 rpm for 1 min. The absorbance was then recorded at 535

nm, and a non-specific absorbance at 600 nm using a spectrophotometer. To calculate the content of MDA, the molar extinction coefficient 155 mM^{-1} cm^{-1} was used, and the results were expressed in nmol MDA g^{-1} fresh weight (FW).

Hydrogen peroxide concentration (H_2O_2) measurement was carried out as described by Alexieva *et al.* (2001). It was used 200 µL of supernatant, which was added to 200 µL of 0.1 M potassium phosphate buffer (pH 7.5) and 800 µL of 0.1 M KI solution. The microtubes rest for 1 h in the dark accommodated on ice inside a closed polystyrene box. The absorbance was then recorded at 390 nm and the results were calculated based on a calibration curve and expressed in µmol H_2O_2 g^{-1} FW.

For the determination of protein and activity of the enzymes of superoxide dismutase (SOD, EC:1.15.1.1), catalase (CAT, EC:1.11.1.6) and ascorbate peroxidase (APX, EC:1.11.1.11), 1.5 g of foliar material was macerated in 4 mL of 0.1 M potassium phosphate buffer (pH 7.5). Protein determination was performed according to Bradford (1976), using a calibration curve of bovine serum albumin and the absorbance was recorded at 595 nm using a spectrophotometer. The protein concentration was expressed as mg g^{-1} FW.

APX activity was measured according to Gratão *et al.* (2008), using a solution containing 500 µL of 80 mM potassium phosphate buffer (pH 7.0) + 100 µL of 5 mM ascorbic acid, and 100 µL of 1 mM EDTA was made. The solution was kept in a water bath at 30 °C for 10 min. Then, 100 µL of 0.1 mM H_2O_2 and 10 µL of protein extract was added. The extract was rapidly shaken and then immediately analyzed using a spectrophotometer at 290 nm for 1 min. The results were expressed as nmol min^{-1} mg $protein^{-1}$.

CAT activity was determined using the methodology of Azevedo *et al.* (1998). In Eppendorf tubes, 1 mL of 0.1 M potassium phosphate buffer (pH 7.5), and 2.5 µL H_2O_2 30% were added, followed by 20 µL of protein extract. Immediately after the addition of the protein extract, the tubes were quickly mixed by vortexing. Enzyme activity was determined by monitoring the absorbance recording at 240 nm over a 1 min interval using a spectrophotometer. The results were expressed as µmoL min^{-1} mg $protein^{-1}$.

SOD activity was determined according to Giannopolitis and Ries (1977). The extracted sample of 50 µL was added in a glass test tube, containing 2 mL of 50 mM sodium phosphate buffer, pH 7.8, 200 µL of 75 µM nitroblue tetrazolium, 200 µL of 0.1 mM EDTA, 250 µL of 13 mM methionine, and 250 µL of 2 µM riboflavin. The preparation of controls followed the same methodology replacing the plant extract with

sodium phosphate buffer. The tubes were placed inside the reaction chamber under artificial lighting of a 15 W fluorescent lamp at 25 ºC for 20 min to form blue formazan compound. The reading was recorded on a spectrophotometer at a wavelength of 560 nm. The results were expressed in Unit SOD mg^{-1} protein.

2.4. Germination test

Before germination, the seeds were treated with a fungicide and insecticide solution (1.5 mL kg^{-1} of thiamethoxam + 1.0 mL kg^{-1} of metalaxyl-M and fludioxonil + 3 ml of carboxin and thiram). The experimental design was a 2 × 3 completely randomized factorial design (soybean genotypes × Ni doses), with 4 replications for both genotype and with 20 seeds for each replication, distributed between three sheets of "germitest" that were moistened with distilled water at 2.5 times the dry sheet weight, and kept in a germinator at a constant temperature of 25 °C. The experiment evaluation was performed eight days after initiation, where the percentage of germinated seeds and the length of hypocotyl and roots were evaluated.

2.5. Biochemical analysis

To determine the nitrogen metabolism compounds (ureides, ammonia, amino acids) and total sugar from seedlings, about 1.0 g of macerated plant material were extracted into 10 ml of MCW solution (methanol, chloroform, water, 12/5/3 v/v/v), according to Bieleski and Turner (1966).

To determine nitrate reductase (NR) activity (Radin, 1973), fresh leaf samples (0.2 g) were incubated in a potassium phosphate buffer KH_2PO_4 100 mM + KNO_3 200 mmol L^{-1} (pH 7.5) for 1 h at 30 ˚C. After incubation, 50 µL from solution described above was used to determine NO^{-2} by adding 500 µL of sulfanilic acid 1% in HCl 1.5 N, followed by the addition of 500 µL of naphthyl ethylenediamine 0.02%. Absorbance was determined at 560 nm and compared to a sodium nitrite standard curve.

Ureides were determined according to Vogels and Van Der Drift (1970). For allantoin, 25 µL of hydrophilic portion of MCW extract was added to 250 µL of NaOH 0.5 M and 20 µL of 0.33% phenylhydrazine. The mixture was incubated at 100 ˚C for 8 min. This solution was then cooled to ambient temperature and it was added 250 µL of HCl 0.65 N, being incubated one more time at 100 ˚C for 4 min. When the solution reached room temperature, it was added 250 µL of phosphate buffer solution 0.4 M (pH

7.0), and 250 µL of 0.33% phenylhydrazine. The mixture was homogenized and kept at room temperature for 5 min, then cooled in ice for 5 more minutes. Then, it was added 1.25 mL of 37% HCl and 250 µL of 1.65% $K_3Fe(CN)_6$. After 15 min at room temperature, ureides were determined by reading the absorbance in a spectrophotometer (SP-220, bioespectroTM) at 535 nm. For allantoic acid determination, the same procedure was adopted except by the addition of 250 µL of NaOH 0.5 M, 20 µL of 0.33% phenylhydrazine as well as incubation at 100 ˚C during 8 min. The results were expressed as µmol g^{-1} DW. To quantify both allantoin and allantoic acid, a standard allantoin curve was used.

Ammonia concentration was quantified according to McCullough, (1967), where 25 µL of hydrophilic portion of MCW was added to 500 µL of colorimetric solution (2.5 g phenol and 12.5 mg sodium nitroprusside in 250 mL) and with 500 µL the phosphate reagent (1.25 g sodium hydroxide, 13.4 g monobasic sodium phosphate, and 2.5 ml 5% sodium hypochlorite in 250 mL). Samples were incubated at 37 ˚C for 1 h. Ammonia concentration was then determined by colorimetry at 630 nm absorbance. To quantify ammonia, a standard ammonium sulfate curve was used.

Amino acid content was quantified according to Yemm *et al.* (1955), using 70 µL of hydrophilic portion of MCW, 500 µL 0.2 M sodium citrate, 200 µL ninhydrin 5% in ethylene glycol, and 1 mL 0.0002 M KCN. Samples were incubated at 100 ˚C for 15 min. Then, the samples were cooled with tap water for 10 min, and 1 mL of 60% ethanol was added. The absorbance was read at 570 nm. A methionine standard curve was used to calculate the concentration of amino acids.

The determination of leaf urease activity was performed according to Hogan *et al.* (1983). Foliar discs (0.2 g) were transferred to 5 mL of phosphate buffer (pH 7.5), containing N-propanol 2% and 6.5 g of urea in 500 mL of distilled water, which was incubated for 3 h at 30 ◦C. One 5 µL aliquot was collected and added to 1.65 mL of distilled H2O, followed by 100 µL of reagent 1 (0.74 M phenol; 1.14 mM of sodium nitroprusside) and 200 µL of reagent 2 (0.37 M sodium hydroxide; 0.41 M dibasic sodium phosphate; sodium hypochlorite 3%). Samples were then incubated at 50 ◦C for 20 min. Urease activity was determined by colorimetry at 625 nm absorbance.

Total sugar concentration was determined according to the methodology of Dubois *et al. (* 1956). The water-soluble extract of 15 µL was diluted in 500 µL of deionized H_2O, 500 µL of 5% phenol and 2 mL of concentrated sulfuric acid added slowly

in glass tubes. This mixture was homogenized in a vortex and cool to room temperature and subsequently, the absorbance spectrophotometer was read at 490 nm.

2.5 Statistical analysis

For statistical analysis, the R software (R Development Core Team) was used. The significance of the effects of all variables evaluated as well as their interactions on the reported traits were evaluated by analysis of variance (ANOVA) at 5 % significance. Significant differences between means were then determined using Tukey test at 5 % significance. To evaluate the overall effects of Ni fertilization on soybean genotypes, a Pearson's correlation analysis ($p < 0.05$) was made and represented as a heatmap for each genotype, as well as a principal component analysis (PCA) in order to determine the relationship between the variables evaluated.

3. Results

3.1. Gas exchange parameters and total sugar concentration

Interactions ($p \leq 0.01$) were observed between Ni fertilization and soybean genotypes for net photosynthesis, stomatal conductance, intercellular CO_2 concentration, transpiration and total sugar in soybean leaves.

Ni application increased net photosynthetic rate by 45 % for genotype TMG 2158 and 21% for *eu3-a* at 3.0 mg Ni kg^{-1} compared to untreated plants. Considering stomatal conductance in comparison to control plants, Ni provided an increase of 100% for TMG 2158 at 3.0 mg Ni kg^{-1}. No significant result was found for *eu3-a* plants (Figure 1b). Internal CO_2 concentration was also affected by Ni application. There was an average increase of 27 % for TMG 2158 plants whereas. Ni application showed no effect for internal CO_2 concentration in *eu3-a* plants (Figure 1c). Transpiration was also affected by Ni application, which increased the transpiration rates for TMG 2158 and *eu3-a* by 80% and 24%, respectively (Figure 1d).

The dose of 3.0 mg Ni kg^{-1} increased total sugar concentration in leaves of genotypes TMG 2158 and *eu3-a* by 10% and 13 %, respectively.

3.2. Oxidative stress and antioxidant metabolism

Interactions ($p \leq 0.01$) between Ni doses and soybean genotypes were observed for lipid peroxidation, H_2O_2 concentration, the activity of SOD and CAT as well as

protein concentration. Ni fertilization led to a decrease in lipid peroxidation of cell membranes for genotype TMG 2159. No significant result was found for *eu3-a* mutants. Lipid peroxidation rates were lower in TMG 2158 thank *eu3-a* plants (Figure 2a).

Regarding the H_2O_2 concentration, Ni application promoted a reduction in the genotypes TMG 2158 and *eu3-a* at the dose 3.0 mg Ni kg^{-1}. The TMG 2158 plants presented the highest H_2O_2 concentration at all Ni doses evaluated in comparison to the NIL (Figure 2b). Ni also reduced SOD activity for both soybean genotypes. The lowest value was found when 3.0 mg Ni kg^{-1} was applied. Overall, the genotype *eu3-a* exhibited the lowest SOD activity (Figure 2c).

Ni fertilization with 1.5 mg kg^{-1} was enough to reduce CAT activity in TMG 2158 by 23%. However, this dose increased the enzyme activity for *eu3-a* plants by 31%. The TMG 2158 plants showed the lowest CAT activity at all Ni doses (Figure 2d). There was not significant interaction between Ni doses and soybean genotypes for APX activity.

For total soluble proteins in leaves, Ni fertilization promoted an increase in *eu3-a* plants but an inverse relationship was observed for the genotype TMG 2158, which also presented the highest content of soluble proteins at all Ni doses (Figure 2f).

3.3. Germination test

Interactions ($p \leq 0.01$), between Ni doses and soybean genotypes, were observed for root length root weight, hypocotyl length, hypocotyl weight, and seed germination. Ni fertilization at 3.0 mg kg^{-1} increased the root length of TMG 2158 by 17%, whereas for *eu3-a*, no significant difference between the Ni rates was observed (Figure 3a). Regarding the root weight, the highest Ni dose did not affect TMG 2158 and *eu3-a* seedlings (Figure 3b).

For hypocotyl length, Ni fertilization promoted an increase (12%) only to TMG 2158 genotype. For the NIL, no significant difference was observed (Figure 3c). The application of 1.5 mg Ni kg^{-1} increased the hypocotyl weight of both genotypes whereas the dose of 3.0 mg Ni kg^{-1} led to a reduction (Figure 3d).

Ni application at 3.0 mg kg^{-1} increased the germination of soybean seeds of TMG 2158 and *eu3-a* by 24% and 14%, respectively.

3.3. Biochemical analysis

Interactions ($p \leq 0.01$), between Ni doses and soybean genotypes, were observed for ureides, ammonia, amino acids, and total sugar. The rate of 3.0 mg Ni kg^{-1} increased ureide concentration by 6% for TMG 2158 and 7% for *eu3-a* plants (Figure 5a). Similarly, the rate of 3.0 mg Ni kg^{-1} increased the ammonia levels in seedlings of both genotypes, especially for the genotype *eu3-a* (112%) (Figure 5b).

Ni fertilization led to a decrease in amino acid levels of genotypes TMG 2158. The genotype *eu3-a* recorded the highest levels of amino acids, but no significant result was found for the *eu3-a* mutants regarding Ni application (Figure 5c).

Ni application increased the urease activity in the genotypes TMG 2158, which recorded an increase of 32% in comparison to untreated plants. No urease activity was observed for the genotype *eu3-a* (Figure 5d), resulting in a urea accumulation and necrosis on leaflet tips (Figure 6).

The principal compoment analysis (PCA) was used to summarize the major patterns of variation of each genotype. The first two components explained 79% and 51.2% of the variation of the genotypes TMG 2158 and *eu3-a*, respectively (Figure 7a and b).

4. Discussion

In this study, the results demonstrated that Ni application at low doses showed positive effects on the photosynthetic apparatus of soybean plants (Figure 1). Similar results were found by (Freitas *et al.*, 2019), where the authors demonstrated that leaves became greener and photosynthetically more active in response to Ni fertilization.

Although the genotypes TMG 2158 and *eu3-a* plants showed an increase in net photosynthesis (Figure 1a).

Both soybean genotypes increased total sugar content in response to Ni fertilization (Figure 1f). For TMG 2158, this increase was expected since Ni application also increased net photosynthesis (Figure 1a), and sucrose is the primary product of photosynthetic leaves and also the main sugar transported from the source to sink tissues (Ruan, 2014).

The increase of sugar concentration in TMG 2158 plants associated with the decrease in MDA might indicate that sugar accumulation also could be acting as an osmotic regulator in vacuoles and cytoplasm, maintaining optimal cellular metabolism and protecting cellular structures from ROS damage (Hussein *et al.*, 2019; Silva *et al.*,

2020). The photosynthetic metabolism of plants commonly generates ROS, which can increase lipid peroxidation. In this study, it is possible to observe that Ni fertilization did not promote cell damage caused by ROS activity, since the MDA concentration did not increase (Figure 2a) (Farooq *et al.*, 2019).

The reduction of SOD in both genotypes (Figure 2c) can be associated with the displacement of Fe, Cu/Zn, or Mn in the metalloenzyme SOD, depending on enzyme type since Ni can decrease Fe uptake (Piccini & Malavolta, 1992; Lešková *et al.*, 2017), Cu and Zn by plants (Parida *et al.*, 2003) (Supplementary Figure 1). The heatmap showed a negative correlation between Ni concentration in seeds and SOD activity for TMG 2158 plants, whereas no significant correlation was observed for *eu3-a* plants (Figure 8a, b and c).

Ni application increased APX activity of both genotypes evaluated (Figure 2e), which could lead to the reduction of H_2O_2 levels once APX is a hydrogen peroxide-scavenging enzyme (Asada, 1992). Gajewska *et al.* (2006), also found high values for APX activity in plants treated with Ni, suggesting that APX played an important role in H_2O_2 scavenging. Interestingly, a negative correlation was observed between APX and MDA and H_2O_2 levels for TMG 2158 plants (Figure 8a).

Another enzyme that could be involved in the reduction of H_2O_2 is glutathione peroxidase (GSH). Fabiano *et al.* (2015), reported that Ni can also activate glyoxalase I, which catalyzes the degradation pathway of methylglyoxal (MG) by reduced glutathione (GSH). Therefore, Ni may be a key element for combating lipid peroxidation by decreasing the level of MG through the activity of glyoxalase as well as participating in the regulation of the GSH pool.

Besides the beneficial effects on gas exchange parameters and antioxidant metabolism, Ni improved seed germination and seedling development. The seed micronutrient reserves are very important for seed quality and mineral nutrition of crops (Kutman *et al.*, 2014). Our study demonstrated that Ni levels in soybean seeds improved seed germination (Figure 3e) and seedling development, especially the root length (Figure 4), probably due to an increase of both embryo-specific and ubiquitous urease activities (Figure 5d).

Kutman *et al.* (2013) assumed that both the ubiquitous and embryo-specific urease activities responded to seed Ni during germination. Rechenmacher *et al.* (2017) investigated the contribution of soybean ureases to seed germination and plant development, and they also did not find differences in germination rates of transgenic and

mutant soybean seeds, but they observed an association between urease activity and developmental pattern. In their experiment, the mutants *eu3-a* showed a delay in development and also recorded the smallest root system.

According to Lavres *et al.* (2016), Ni levels in seeds promote urease activity, which contributes to the production of seeds with better physiological quality. Similar results were found by Barcelos *et al.* (2017). The authors concluded that soybean fertilized with Ni showed higher seed germination and physiological quality and vigour. Urease is an abundant seed protein and plays an important role during germination by recycling arginine nitrogen and avoiding urea accumulation in the tissues (Zonia *et al.*, 1995). Therefore, any disruption during this process, such as Ni deprivation, might result in impairment of the utilization of seed N reserves (Kutman *et al.*, 2013; Polacco *et al.*, 2013).

PCA analysis showed for genotype TMG 2158 (Figure 7a) that variables related to N metabolism and seedling development such as urease, ammonia, ureides, and root length are grouped next to the individuals which were treated with Ni. A similar pattern was observed for the genotype *eu3-a* (Figure 7b), except by urease activity, revealing the Ni effects over these parameters.

Our results also demonstrated that soybean plants that received Ni application resulted in seedlings with higher ureides, ammonia concentration, and urease activity (Figure 6a, b, and c). It is well known the beneficial effects of Ni on biological nitrogen fixation (BNF), once it is required by the hydrogenase enzyme of symbiotic bacteria (*Rhizobium* and *Bradyrhizobium* spp.) (Bagyinka, 2014; Lavres *et al.*, 2016; Freitas *et al.*, 2018).

Ureides are the principal N source for embryo growth and they are metabolized into amino acids in the pod or seed coat, with arginine and glutamine being the main N-containing solutes in the embryo (Rainbird *et al.*, 1984; Tegeder, 2014; Baral *et al.*, 2016; Bosse *et al.*, 2021). Therefore, the observed reduction in amino acid content can be associated with seedling development, since the genotypes, which had higher seedling length, (Figure 4), showed a decrease in amino acid levels according to Ni application (Figure 5c). This increment in seedling length might also be associated with the increase in urease activity by Ni application, improving N recycling by breaking down urea into ammonia (Dixon *et al.*, 1975; Witte, 2011). According to the heatmap (Figure 8a and b), it was observed a positive correlation between urease activity in seedlings and root length for the genotype TMG 2158.

An unexpected result was the increase in seed germination of *eu3-a* mutants, since these seedlings showed null urease activity. This result may suggest that the product of this gene is not involved in plant development. Further investigations are needed to better understand the role of Ni during germination using NILs.

5. Conclusion

Ni fertilization enhance photosynthetic apparaturus, sugar concentration and ROS scavenging systems in soybean plants. Oxidative stress was mitigated by Ni fertilization by decreasing lipid peroxidation governed by boosted APX activity, which played an important role in H_2O_2 scavenging.

Soybean plants fertilized with Ni showed higher number of pods, seed per plants and higher seed yield. Plants treated with Ni showed an enhanced seed germination and seedling development by increasing urease activity and N remobilization, although it is highly dependent on the soybean genotype. These results show important information demonstrating that Ni plays critical roles on soybean photosynthetic and antioxidant system, seed yield and physiological quality and vigour of soybean seeds.

Author contribution

ARR designed the experiment, MAB performed the experiments, biochemical analysis wrote the main text and performed the statistical analysis, NACM performed the biochemical analysis, ARR and NACM revised the manuscript.

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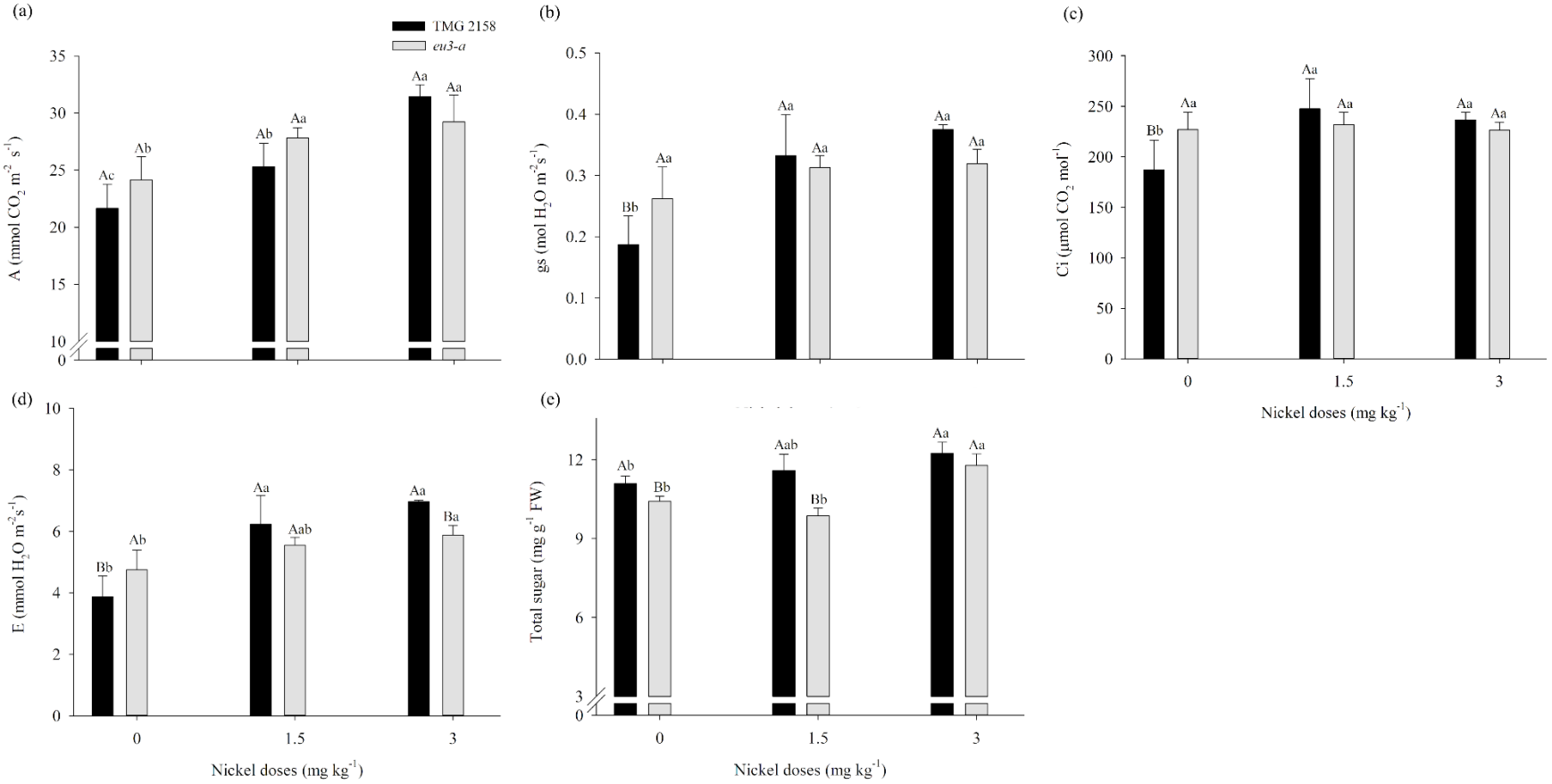

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676 **Figure 1.** Nickel effects on net photosynthesis (a), stomatal conductance (b), intercellular CO_2 concentration (c), transpiration (d) and total sugar
677 (e) in soybean leaves. The standard error of the mean (n = 4) is indicated by errors bars. Different letters indicate difference between means
678 according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) =
679 6.92 (a); 13.94 (b); 8.70 (c); 10.18 (d); 3.59 (e);.

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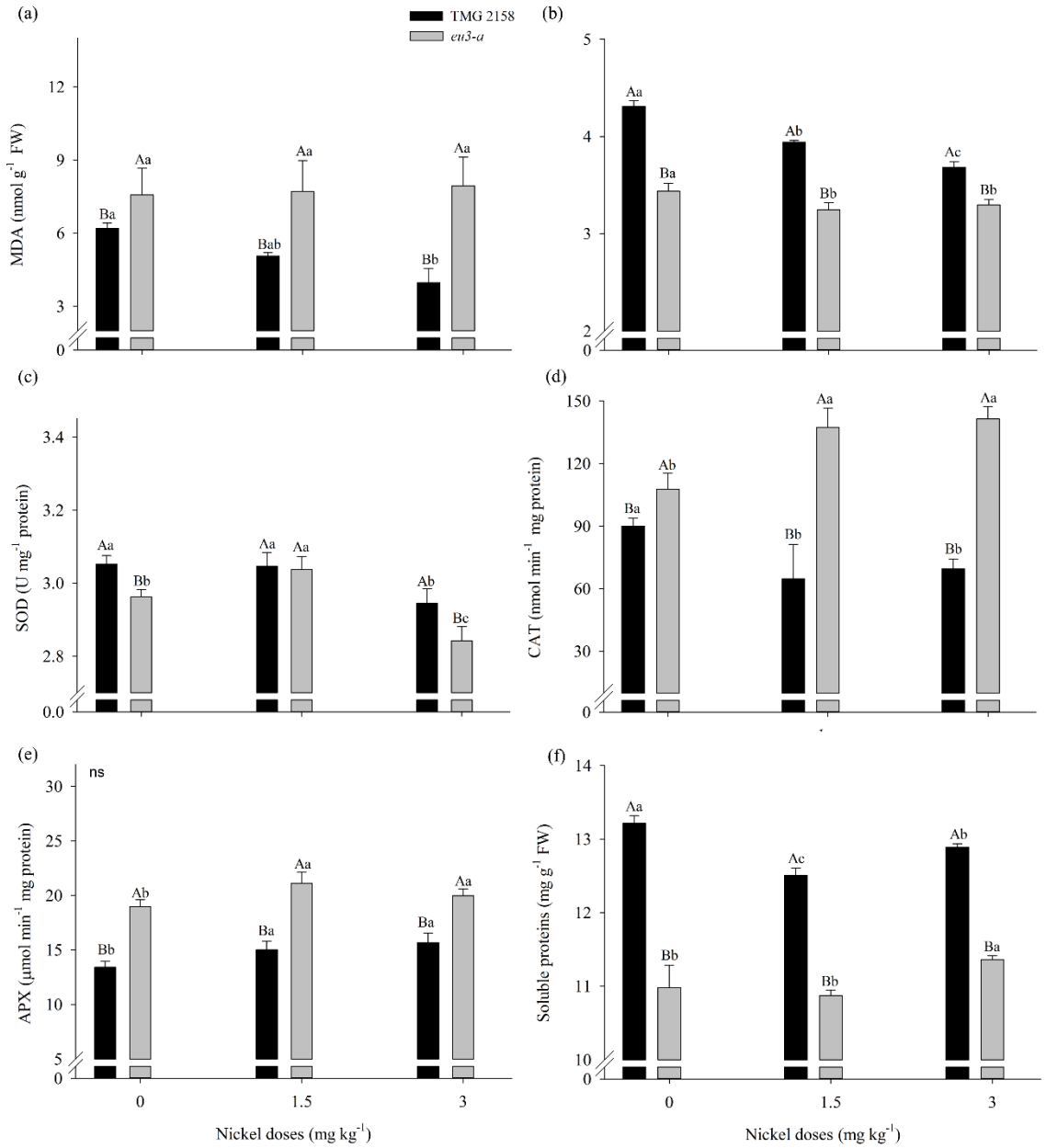


Figure 2. Nickel effects on lipid peroxidation essayed by MDA concentration (a), hydrogen peroxide concentration (b), SOD (c), CAT (d), APX (e) and soluble proteins (f) in soybean leaves. The standard error of the mean (n = 4) is indicated by errors bars. Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 13.68 (a); 1.65 (b); 1.12 (c); 8.90 (d); 4.41 (e); 1.21 (f).

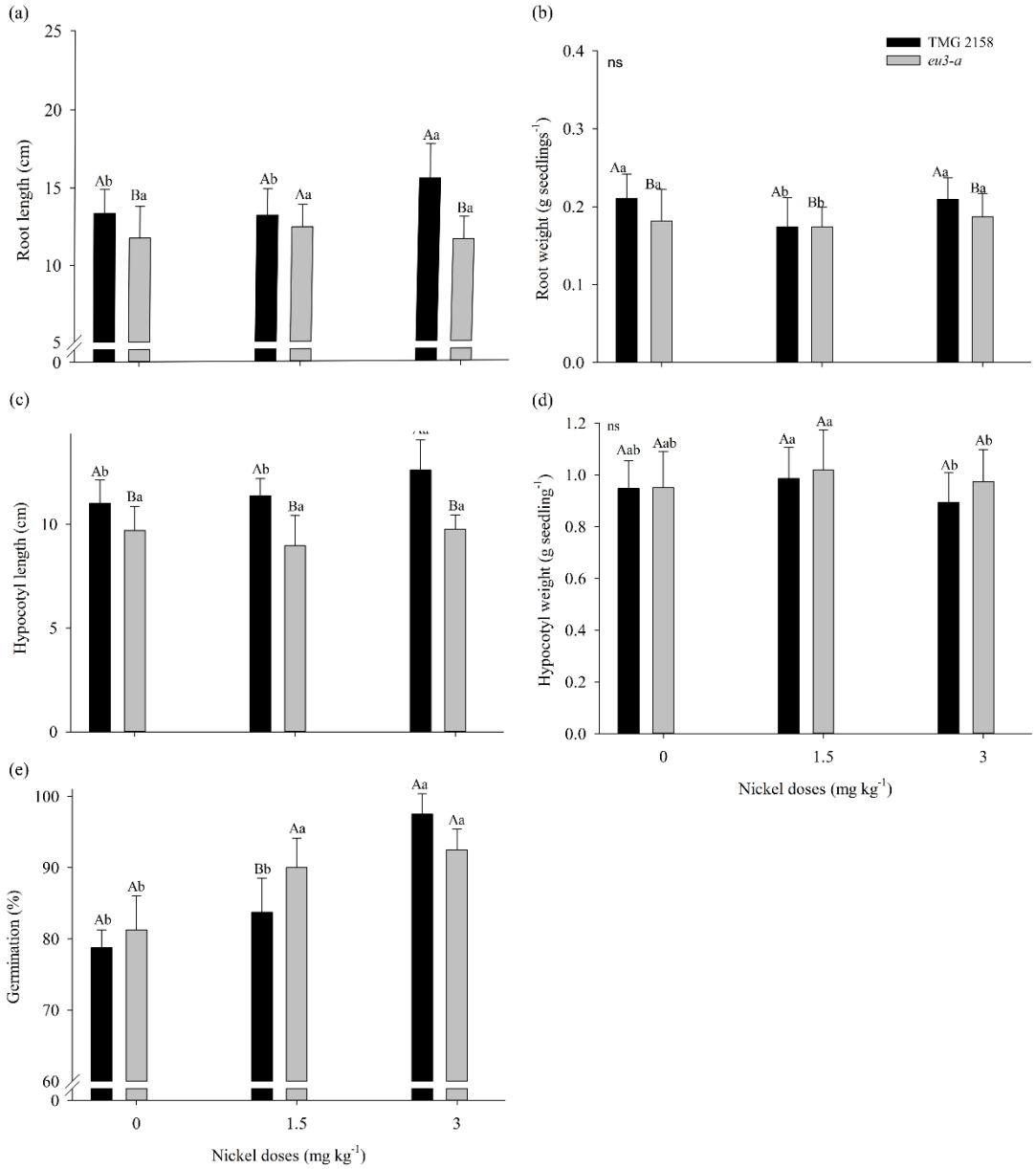


Figure 3. Nickel effects on root length (a), root weight (b), hypocotyl length (c), hypocotyl weight (d) and germination in soybean seedlings. The standard error of the mean (n = 4) is indicated by errors bars. Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 13.59 (a); 17.14 (b); 9.17 (c); 13.27 (d); 4.32 (e).

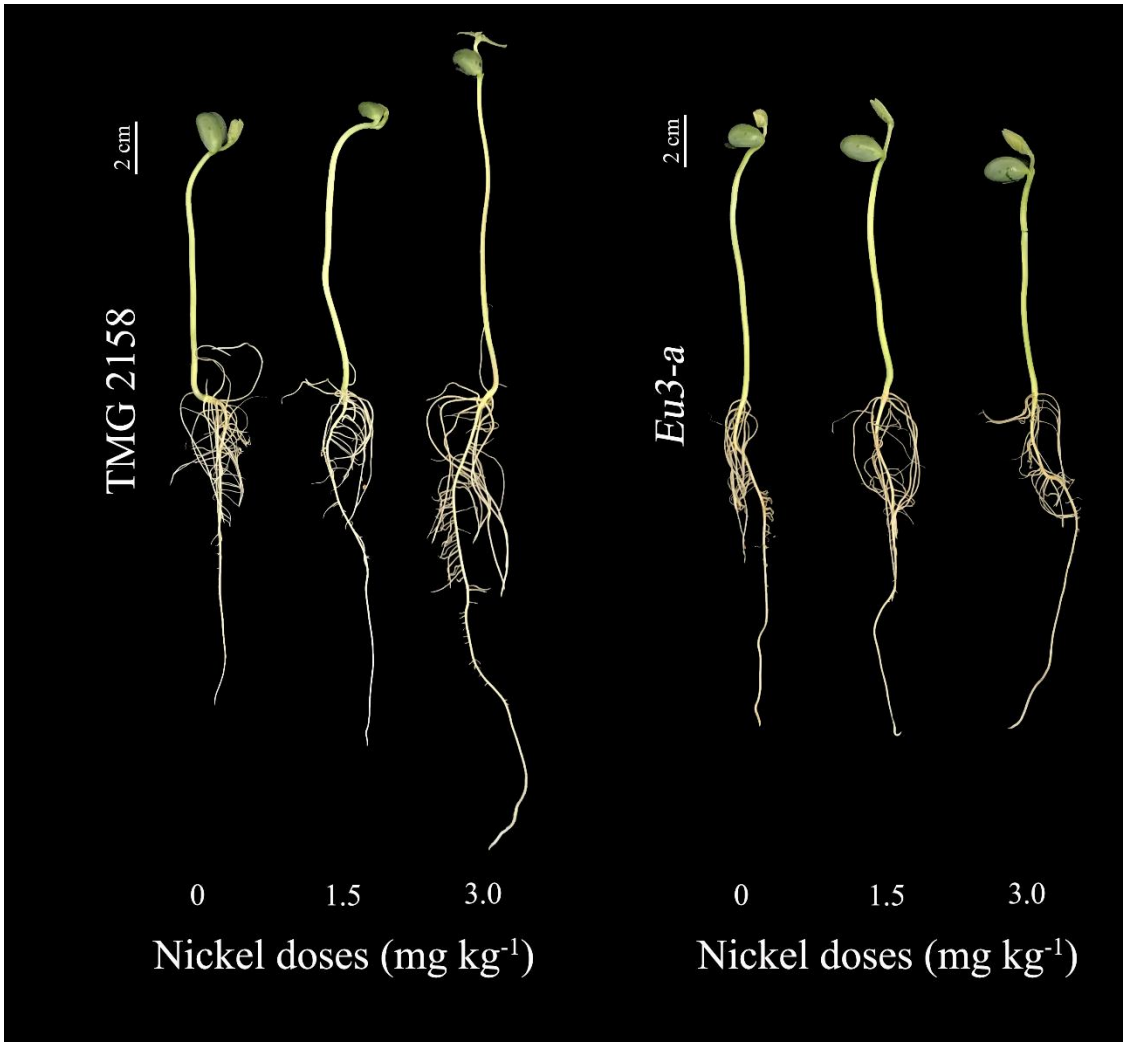


Figure 4. Soybean seed germination in response to Ni supply.

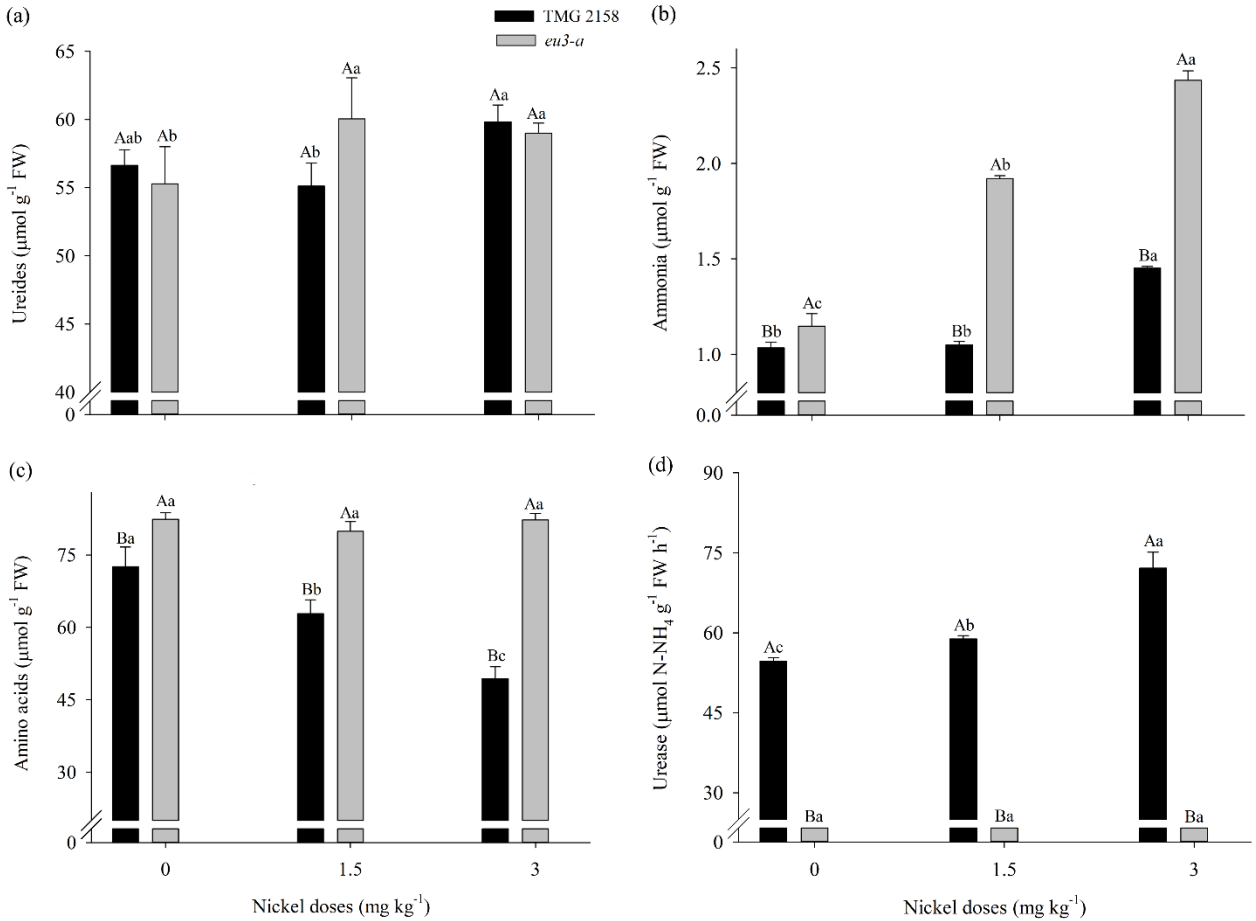


Figure 5. Nickel effects on ureides (b), ammonia (b), amino acids (c) and urease activity (d) in soybean seedlings. Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 3.37 (a); 2.45 (b); 3.51 (c); 4.01 (d).

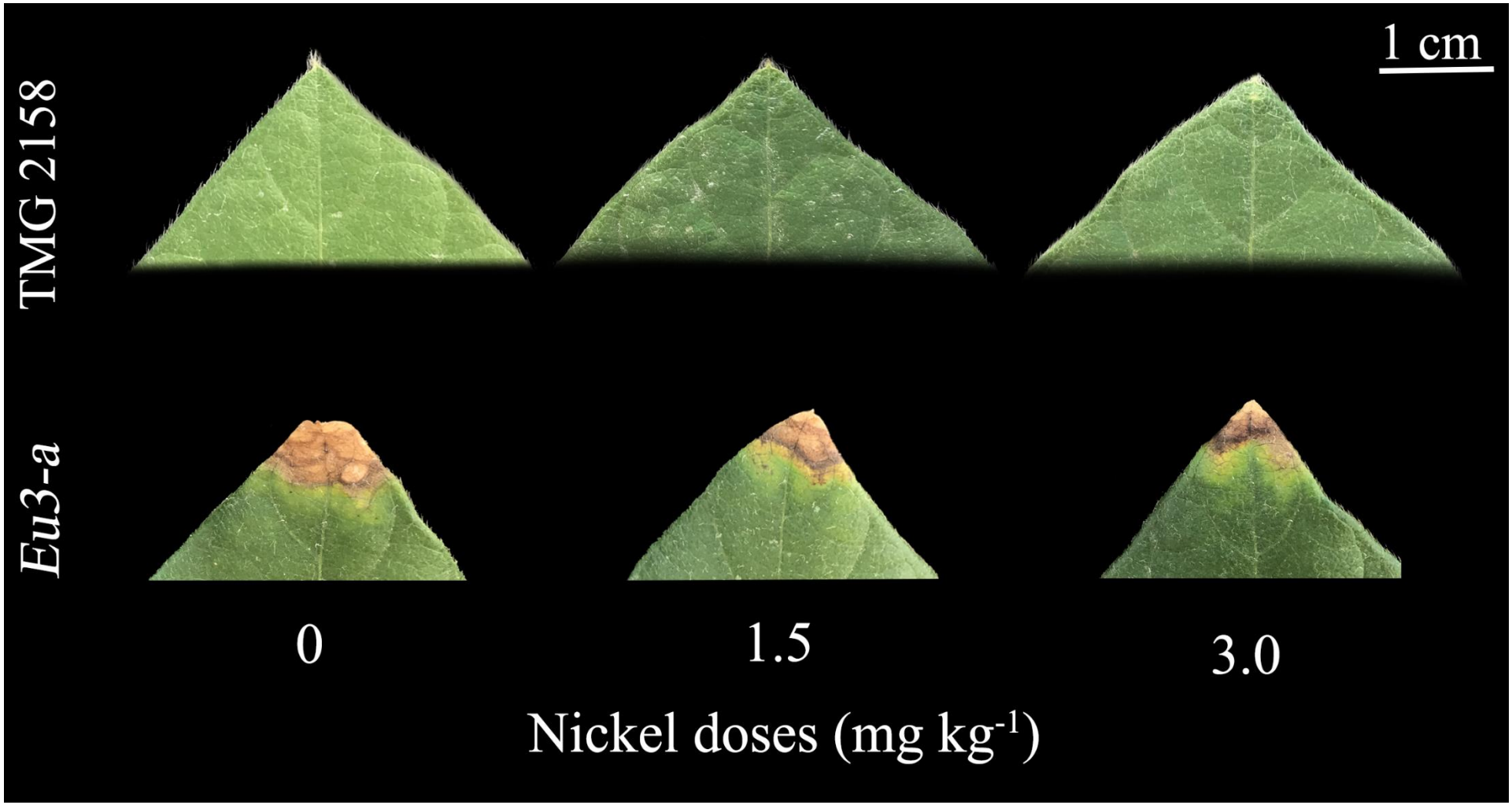


Figure 6. Nickel effects on visual characterization of Ni deficiency symptoms in soybean mutants.

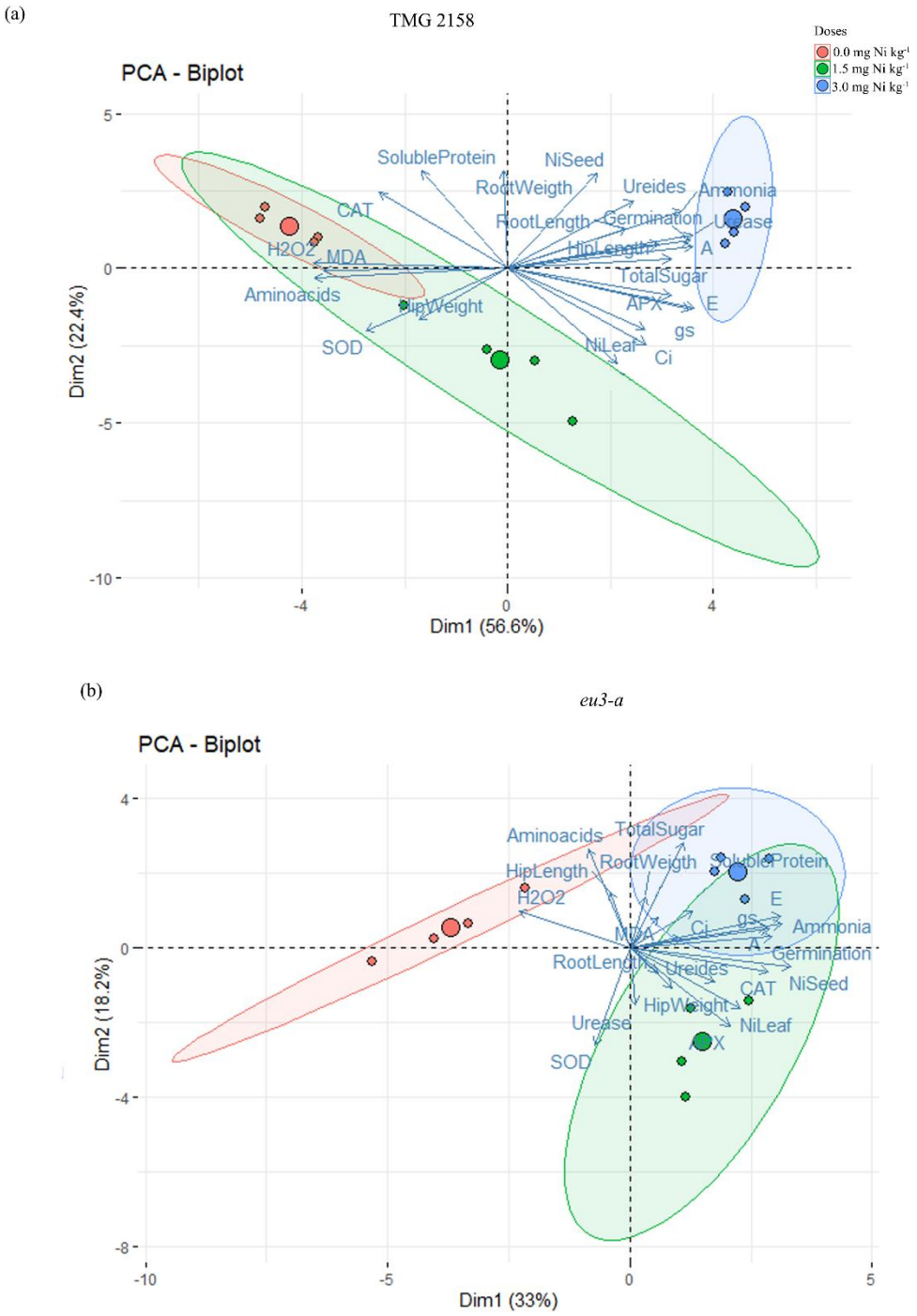


Figure 7. Principal component analysis of soybean genotypes in response to Ni fertilization. Abbreviations: A - net photosynthesis, gs - stomatal conductance, Ci - intercellular CO_2 concentration, E – transpiration, MDA – malondialdehyde, H_2O_2 - hydrogen peroxide, SOD - superoxide dismutase, CAT – catalase, APX - ascorbate peroxide, HipLength - hypocotyl length, HipWeight - hypocotyl weight, NiLeaf - Ni concentration in leaves, NiSeed - Ni concentration in grains

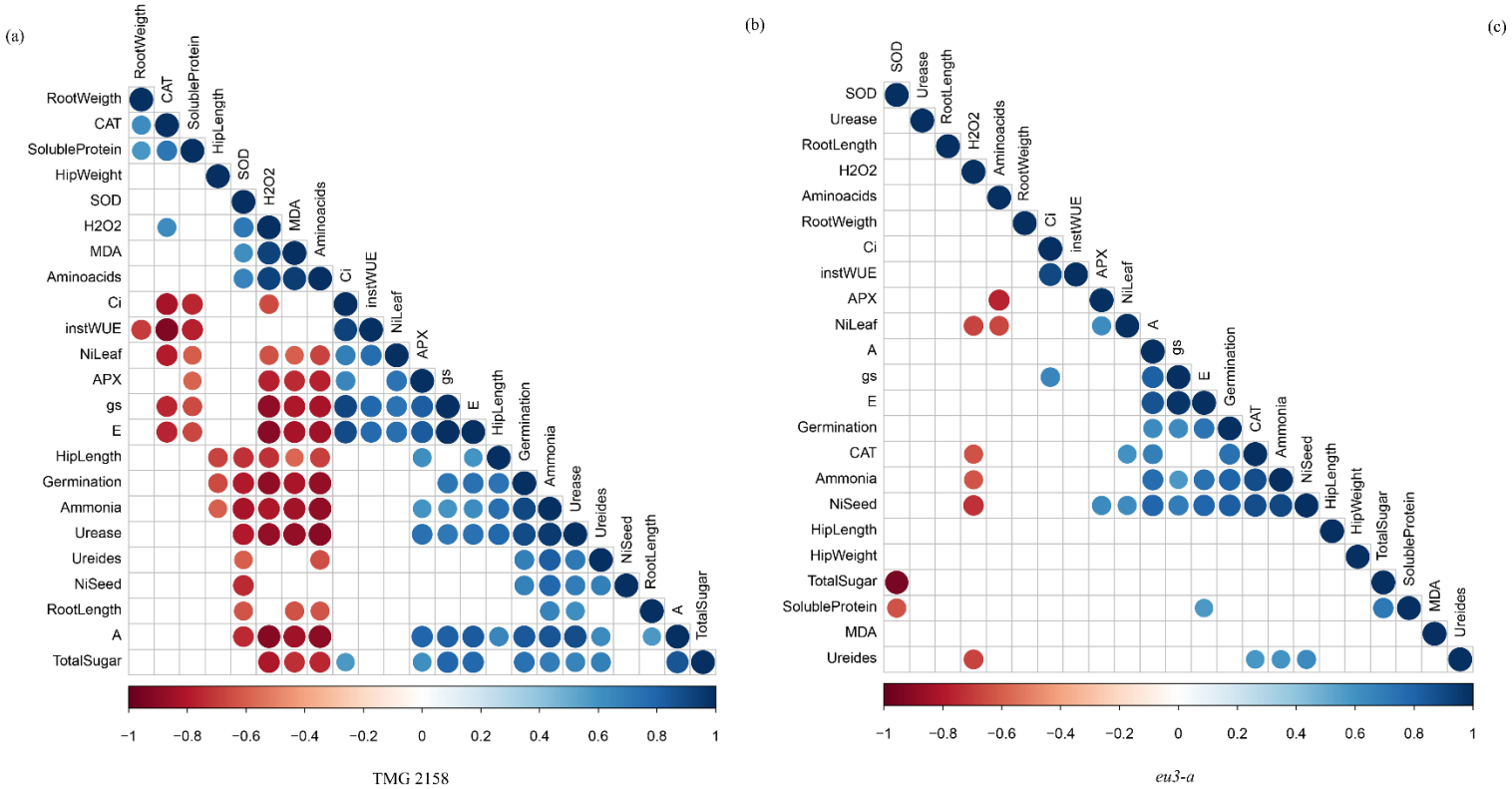
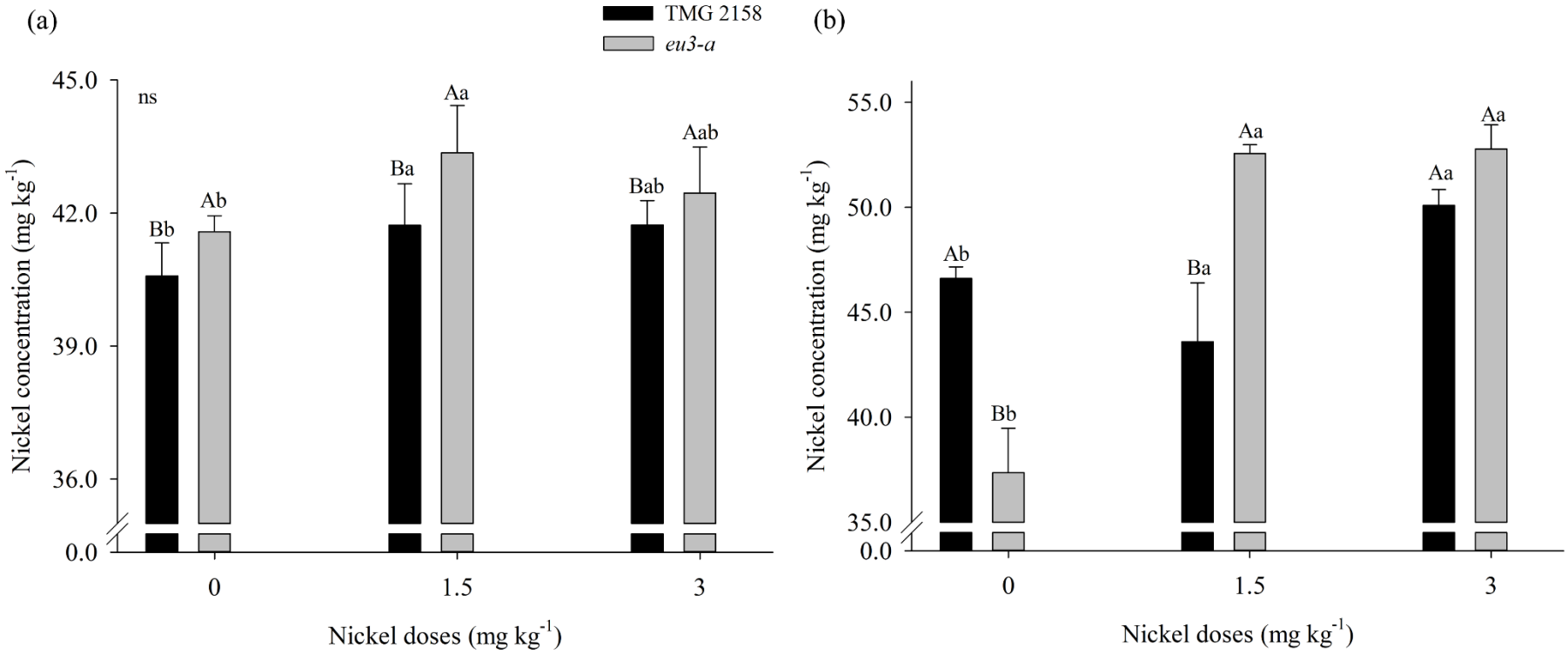


Figure 8. Heatmap showing the Pearson correlation among the parameters in response to Ni fertilization. Abbreviations: A - net photosynthesis, gs - stomatal conductance, Ci - intercellular CO_2 concentration, E – transpiration, MDA – malondialdehyde, H_2O_2 - hydrogen peroxide, SOD - superoxide dismutase, CAT – catalase, APX - ascorbate peroxide, HipLength -hypocotyl length, HipWeight - hypocotyl weight, NiLeaf - Ni concentration in leaves, NiSeed - Ni concentration in grains.

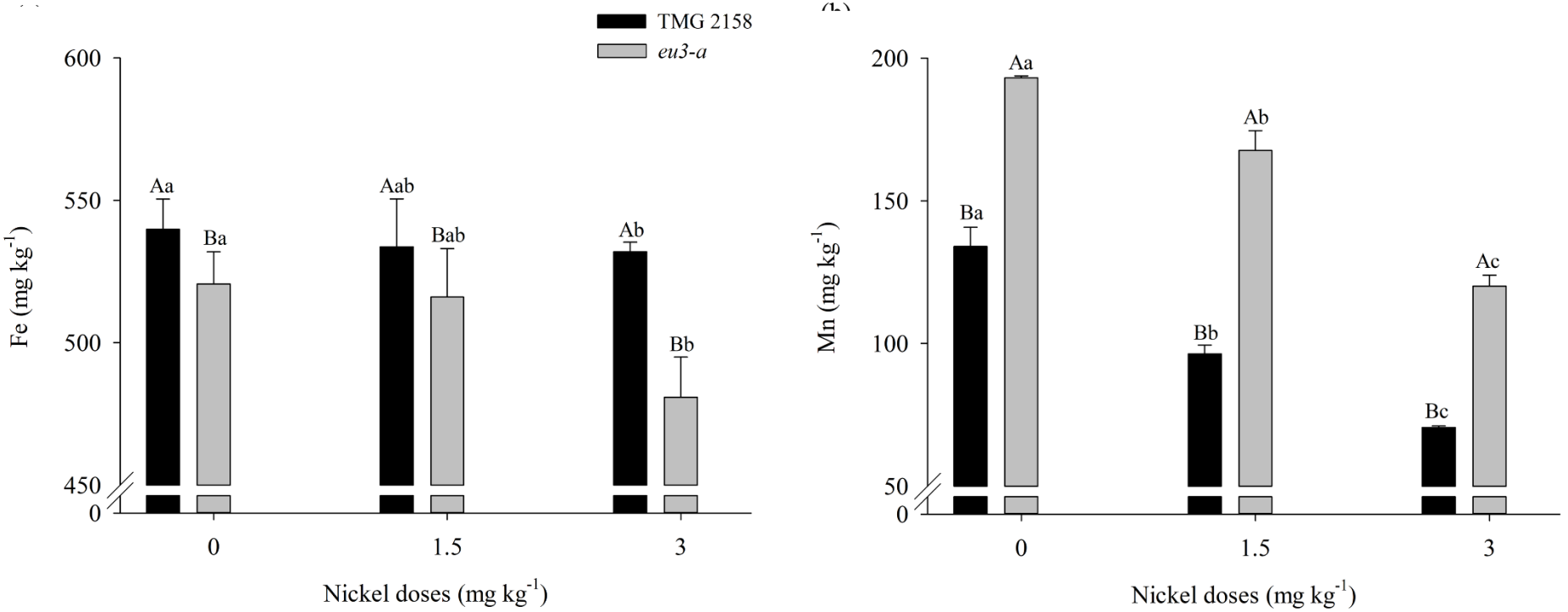
Supplementary material Table 1. Urease activity and grain yield of soybean plants in response to Ni supply.

Genotypes	Ni doses	Urease activity	Grain yield		
		(μmol N-NH_4 g^{-1} FW h^{-1})	(Pods per plant)	(Seeds per plant)	(g $plant^{-1}$)
TMG 2158	0.0	78.1 ± 0.99 C	61.5 ± 0.58 B	129 ± 2.94 C	24.94 ± 0.25 B
TMG 2158	1.5	87.65 ± 2.48 B	80.75 ± 4.11 A	164.75 ± 6.13 B	28.79 ± 1.79 AB
TMG 2158	3.0	90.2 ± 1.62 A	74.75 ± 7.5 A	195 ± 4.55 A	30.01 ± 2.15 A
eu3-a	0.0	0 ± 0.0 A	42.5 ± 2.08 A	96.75 ± 12.66 A	17.95 ± 2.24 A
eu3-a	1.5	0 ± 0.0 A	44.5 ± 4.20 A	92.25 ± 11.24 A	16.77 ± 3.76 A
eu3-a	3.0	0 ± 0.0 A	46.75 ± 1.71 A	100 ± 10.71 A	16.66 ± 1.93 A
	CV (%)	2.99	6.93	6.81	10.06

Means were compared by Tukey test ($p \leq 0.05$), and those followed by the same letter do not differ.



Supplementary material Figure 1. Nickel concentration in leaves (a) and seeds (b) of soybean plants in response to Ni supply. The standard error of the mean (n = 4) is indicated by errors bars. Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 1.97 (a); 3.32 (b).



Supplementary material Figure 2. Fe (a) and Mn (b) concentration in leaves of soybean plants in response to Ni supply. The standard error of the mean (n = 3) is indicated by errors bars. Different letters indicate difference between means according to Tukey test ($p \leq 0.05$). Uppercase letters correspond to soybean genotypes, and lowercase letters correspond to Ni doses. CV (%) = 2.51 (a); 3.40 (b).