DOUGLAS SIQUEIRA FREITAS

HIDDEN DEFICIENCY OF NICKEL IN SOYBEAN: ASSESSMENT AND CONTROL

LAVRAS - MG
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APROVADA em 10 de agosto de 2018.

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2018

Aos meus pais, Daiton e Rosângela, meu irmão, Rafael, amigos e demais familiares.

DEDICO

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“Seja a mudança que você quer ver no mundo.”

Mahatma Gandhi

RESUMO

Os estudos sobre a fertilização com níquel (Ni) em plantas cultivadas são notavelmente novos, embora os seus efeitos positivos venham sendo relatados desde o início dos anos 80. Esse elemento, emergente como um micronutriente, compõe as enzimas urease em plantas e hidrogenase em microrganismos. Nesse cenário, as plantas leguminosas, por sua associação simbiótica com bactérias diazotróficas, podem ser mais severamente afetadas pela deficiência de Ni em solos agrícolas. A soja, além de importante fonte de alimento, é uma leguminosa muito cultivada atualmente no cenário mundial. Assim, práticas que levem a uma produção mais eficiente e sustentável dessa espécie vegetal são visadas para garantir a segurança alimentar atual e futura. Para a confecção do primeiro artigo, associamos as evidências da deficiência de Ni presentes na literatura com os baixos teores naturais desse elemento no solo, o que é comumente encontrado em áreas agrícolas, para verificar uma possível ocorrência da deficiência de Ni em plantas de soja. Para tal, foram cultivados 17 genótipos de soja, comumente adotados pelos agricultores, com e sem o suprimento de Ni, em casa de vegetação e campo, para a avaliação da fisiologia, metabolismo e produção das plantas. Notamos que a fertilização com esse micronutriente maximizou grande parte dos pontos testados embora sem a ocorrência de sintomas foliares, demonstrando, dessa forma, que a deficiência de Ni em plantas de soja ocorre de forma oculta. Para a confecção do segundo artigo, partirmos da seleção genotípica realizada no primeiro estudo, permitindo-nos identificar os genótipos quanto a sua responsividade a fertilização com Ni. Ao genótipo mais responsivo, foram oferecidas seis doses desse micronutriente visando estabelecer as faixas de deficiência, desenvolvimento adequado e toxidez em plantas de soja. Como esperado, o genótipo avaliado demandou maiores concentrações de Ni para atingir seu máximo desenvolvimento, e curiosamente, as bactérias fixadoras de nitrogênio, associadas a esse, também. Para chegar a essa conclusão, avaliou-se tanto como o genótipo se desenvolvia em função das doses de Ni, bem como as concentrações desse micronutriente e seu efeito sobre a atividade dos nódulos radiculares. Com os trabalhos desenvolvidos, fornecemos não só formas de detectar a deficiência oculta de Ni que ocorre nos genótipos de soja cultivados, mas, também como contornar essa limitação pela oferta de doses de Ni adequadas para o máximo desenvolvimento dessas plantas.

Palavras-chave: Dose. Fertilização. Genótipo. *Glycine max*.

ABSTRACT

Studies on nickel (Ni) fertilization in cultivated plants are remarkably new, although their positive effects have been reported since the early 1980s. This element, emerging as a micronutrient, takes place in the enzymes urease, in plants, and hydrogenase, in microorganisms. So, legume plants, due to their symbiotic association with diazotrophic bacteria, may be one of the species most affected by Ni deficiency in agricultural soils. The most important legume species currently cultivated is soybean, an important source of food on the world`s scenario. Thus, practices that lead to a more efficient and sustainable production of this species are aimed to ensuring the current and the future food security. In the first paper, we followed the evidence of Ni deficiency presented in the literature and associate them with the low natural contents of this element commonly found in agricultural soils, and, thus, we confirmed the occurrence of Ni deficiency also in soybean plants. However, in this species, Ni deficiency occurred as a hidden deficiency. To detect this event, we cultivated 17 soybean genotypes, which are commonly cultivated by farmers, with and without the supply of Ni in greenhouse and field, evaluating their physiology, metabolism and production. Therefore, we noticed that fertilization with this micronutrient maximized all evaluated points, even without expressing leaf symptoms of the Ni deficiency. In the second paper, we started from the selection of genotypes previously done, which discriminated them as to their responsiveness to Ni fertilization. To the most responsive genotype, we offered the fertilization with increasing doses of this micronutrient in order to establish the concentrations that would provide the ranges of undernutrition, adequate development and toxicity. As expected, the genotype tested required a higher concentration of Ni to reach its maximum development, and curiously, the nitrogen-fixing bacteria associated with these, too. To reach this conclusion, we evaluated the genotype development following the supply of the Ni doses and how the concentrations of Ni and activity of the root nodules accompanied them. With the developed works, we provide not only ways to detect the hidden deficiency that occurs in the new soybean genotypes, but also how to circumvent this limitation by provision of adequate doses for the maximum development of soybean plants.

Keywords: Dose. Fertilization. Genotype. *Glycine max*.

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PRIMEIRA PARTE

1 INTRODUÇÃO

O níquel (Ni) vem intrigando os pesquisadores desde a sua descoberta. Os primeiros estudos com esse elemento foram voltados a avaliar sua toxicidade às plantas. Contudo, essa visão começou a mudar quando foi observado que as condições de cultivo, usualmente empregadas em estudos de fitotoxidez de Ni, não refletiam aquelas que são verificadas em áreas agrícolas. Logo percebeu-se que um ajuste nas concentrações de Ni aplicadas às plantas resultaram, não somente na ausência de toxidez, mas na descoberta de sua essencialidade as mesmas, por compor o sítio ativo da enzima urease.

A urease é essencial para que o N-amônia possa ser assimilado e, segue, até o presente momento, sendo a única enzima de plantas composta por Ni. Em microrganismos, contudo, esse importante elemento é cofator essencial de muitas outras enzimas, como a hidrogenase, que está presente em bactérias diazotróficas, i.e., que realizam a fixação biológica de nitrogênio (FBN) atmosférico às plantas leguminosas. A hidrogenase se destaca nesse cenário por ser capaz de potencializar ainda mais a FBN. E, dessa forma, visto que plantas leguminosas são diretamente dependentes de ambos os processos nos quais o Ni exerce papel fundamental, essas podem ser mais afetadas por uma nutrição inadequada desse micronutriente se comparada a outras espécies vegetais.

Embora a deficiência de Ni já tenha sido relatada em algumas espécies, os estudos mais atuais estão sendo voltados para a soja, uma importante planta leguminosa que é mundialmente cultivada. Isso porque os grãos produzidos por essas plantas são a maior fonte de proteína usada atualmente e fazem parte da dieta de humanos e animais, dessa forma, atendendo a demanda mundial por alimento.

As estimativas de progressão preveem que será necessário aumentar o rendimento produtivo atual das plantas de soja para que se possa garantir a segurança alimentar das futuras gerações, sem causar maiores impactos ao meio ambiente. Para esse fim, dentre outras estratégias, o melhoramento genético tem sido adotado. Os novos genótipos de soja, embora mais produtivos, demandam mais nutrientes para o seu desenvolvimento adequado, e nesse cenário o Ni assume um grande papel.

Embora até o presente momento não tenha sido verificada a deficiência de Ni em plantas de soja (*Glycine max* [L.] Merrill) cultivadas a campo, estudos atuais indicam que isso possa estar ocorrendo, mesmo que de forma oculta, i.e., sem a presença de sintomas aparentes nas folhas. A ocorrência da deficiência de Ni em soja é corroborada, não somente pelos efeitos benéficos já relatados em outras espécies vegetais, mas também pela baixa concentração disponível de Ni verificada em áreas agrícolas onde essa espécie vegetal é cultivada.

Dessa forma, nesse estudo objetivou-se explorar os efeitos do Ni como um micronutriente para a soja, i.e., em doses benéficas, pela sua aplicação sobre uma gama de genótipos de soja para constatar uma possível deficiência. Para tal, foram testados possíveis efeitos genotípicos e de doses sobre o crescimento, fisiologia, metabolismo e produção de plantas de soja cultivadas tanto em condições de casa de vegetação quanto em campo.

2 REFERENCIAL TEÓRICO

2.1 Níquel: a descoberta de um novo elemento

O uso do níquel (Ni) pelo homem remonta ao século IV a.C. embora os relatos da sua descoberta se tenham início somente no ano de 1694, quando o minerador Hairne encontrou um estranho material que lembrava as cores de minérios de cobre (BARTOW, 1953). Em um primeiro momento, os esforços falhos para produzir ligas de cobre a partir desta substância levaram a sua denominação como "*nikka*", que por uma tradução livre do antigo dialeto alemão para o português significa "diabo" (LANGE, 1923). Levados por essa denominação, a comunidade científica da época não teve interesse em aprofundar os estudos sobre esse material mineral.

No ano de 1726 as arguições sobre esse assunto retornaram, graças a publicação de um artigo na "*Philosophical Transactions*" relatando que o material que fora encontrado continha vários elementos em sua composição já conhecidos, como cobalto e cobre, e, a partir desse momento, alguns nomes da época como John Andreas Cramer, Linneaus e Wallerius, começaram a fazer adendos sobre a constituição do novo material.

Após o relato à Academia Sueca em 1751 feito por Axel Fredrick Cronstedt, estudando um material semelhante encontrado em uma mina de cobalto em Helsingland na Suíça, que esse pesquisador indicou a presença de um novo elemento químico (GUSENIUS, 1969). Mais tarde, Cronstedt denominou esse novo elemento como "Níquel", e embora seu relato tenha sido aceito por muitos químicos, outros se recusaram a concordar. Foi somente a partir dos estudos realizados por Torbern Bergman, a partir de uma forma pura que era atraída por ímã, que a presença de um novo elemento foi comprovada. As dúvidas não foram completamente sanadas até a publicação dos estudos de Proust, Richter e Tupputi

realizados de 1803 a 1811, os quais conseguiram isolar esse elemento e distinguir sais de Ni (ENGHAG, 2004; HENDRYCH; WEDEN, 1934).

2.2 Amigo ou inimigo? Da toxidez de níquel a sua essencialidade

Estudos para tentar compreender os efeitos do Ni sobre a saúde humana foram os primeiros a tomar lugar (DENKHAUS; SALNIKOW, 2002). De acordo com Cempel e Nikel (2006), a exposição direta e recorrente a compostos a base desse elemento poderiam causar efeitos adversos a saúde humana, o que pode justificar o fato das primeiras pesquisas com Ni em plantas terem sido direcionadas a sua toxidez (CHEN; HUANG; LIU, 2009; MUHAMMAD et al., 2013; SEREGIN; KOZHEVNIKOVA, 2006; YUSUF et al., 2011).

Altas concentrações desse elemento podem desde inibir o crescimento das plantas, provocar redução na atividade respiratória e metabólica, até causar mudanças anatômicas e morfológicas, afetando principalmente etapas do processo fotossintético (SENGAR et al., 2008; SEREGIN; KOZHEVNIKOVA, 2006). Contudo, deve-se enfatizar que os estudos avaliando a fitotoxidez causada por Ni têm sido conduzidos expondo plantas a doses elevadas desse elemento, como em aplicações com alguns materiais de lodo de esgoto (MORENO; GARCÍA; HERNÁNDEZ, 2003; ŠČANČAR et al., 2000; WONG et al., 2001) ou em solos contaminados (EVERHART et al., 2006; JAMIL et al., 2014; PARIDA; CHHIBBA; NAYYAR, 2003), o que não representa a concentração usual de Ni em sistemas agrícolas (RODAK et al., 2015; UREN, 1992) nos quais efeitos benéficos do Ni podem realmente ser esperados.

Os primeiros indicativos que os efeitos do Ni às plantas cultivadas eram dependentes da aplicação de doses adequadas foram apresentados por Dixon et al. (1975). Empregando baixa dose, esses pesquisadores, evidenciaram que o Ni não somente deixou de ser tóxico às plantas, mas, que exercia um papel fundamental

no desenvolvimento das plantas por compor o sítio ativo da enzima urease. Apesar disso, a essencialidade do Ni foi evidenciada somente alguns anos depois, quando plantas leguminosas, cultivadas sob depleção de Ni, apresentaram acúmulo tóxico de ureia na ponta dos folíolos (ESKEW; WELCH; NORVELL, 1984; WINKLER et al., 1983). Mais tarde, Brown, Welch e Cary (1987) demonstraram a essencialidade desse elemento também para não leguminosas. Ao cultivar em diferentes fontes nitrogenadas associada a depleção de Ni, os autores observaram que essas não mais produziam sementes viáveis.

Embora esse elemento seja um micronutriente, e dessa forma, essencial as plantas, ainda poucos estudos com a aplicação de Ni em espécies cultivadas têm sido desenvolvidos até o presente momento. Dessa forma, parece razoável afirmar que essa seria uma etapa muito importante para assegurar sua aplicação em sistema de cultivo agrícola, especialmente em leguminosas.

2.3 Urease: a única enzima contendo níquel em plantas

A urease, além de ser a primeira enzima a ser extraída e cristalizada a partir de plantas também é a primeira e única, até o presente momento, a conter um sítio ativo no qual o Ni é essencial (POLACCO; MAZZAFERA; TEZOTTO, 2013). Essa enzima é encontrada em muitas, se não em todas espécies de plantas (WITTE et al., 2001) nas quais, dois tipos de ureases já foram identificadas, uma urease específica do embrião, presente nas sementes, codificada pelo gene *Eu1*, e a outra, a urease ubíqua, encontrada em todos os tecidos, codificada por *Eu4* (HOLLAND; POLACCO, 1992; TORISKY et al., 1994). A urease tem o papel de catalisar a hidrólise da ureia ($[NH_2]_2CO$) em amônia (NH_3) e carbamato (CH_3NO_2), reação que é seguida pelo decaimento não enzimático, espontâneo, do carbamato a amônia, conforme apresentado na Figura 1.

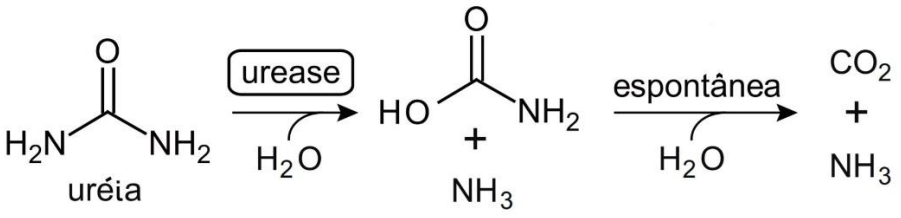

Figura 1 Modelo da reação da enzima urease demonstrando como ocorre a hidrólise da ureia em amônia.
Fonte: adaptado de Witte (2011).

Nessas reações, dois mols de amônia e um mol de dióxido de carbono são gerados (WITTE, 2011). Em plantas, as constantes de K_M das ureases variam de 0,15 a 3 mM, embora a maioria possua um K_M em torno de 0,5 mM, valor semelhante à faixa de concentração da ureia nos tecidos vegetais (0,2 a 0,9 mM) (MENEGASSI et al., 2008). A atividade da urease é fundamental para o desenvolvimento adequado das plantas, uma vez que o N-ureia deve ser convertido a N-amônia para só então ser assimilado (MÉRIGOUT et al., 2008). Curiosamente, a urease específica de sementes não tem papel de assimilação de N, mas propõem-se que esteja envolvida na defesa de plantas (POLACCO; HOLLAND, 1993).

2.4 Níquel além da urease

Em microrganismos, i.e., eubactérias, arqueobactérias e fungos, o Ni, além de compor a urease, é cofator catalítico essencial de outras sete enzimas, nomeadamente monóxido de carbono desidrogenase, glioxalase I, superóxido dismutase, acetil-CoA sintase, metil coenzima M redutase, acireductona dioxigenase e [NiFe]-hidrogenase (LI; ZAMBLE, 2009). Dentre essas, vale ressaltar a última enzima devido sua ocorrência e função desempenhada em bactérias diazotróficas do solo, que são muito importantes no cenário agrícola

mundial (HERRIDGE; PEOPLES; BODDEY, 2008).

Essas bactérias podem desenvolver uma estreita relação simbiótica com as plantas cultivadas, especialmente leguminosas (eudicotiledôneas da família Fabaceae). Nas raízes dessas espécies as bactérias diazotróficas, após a colonização, desenvolvem nódulos – estruturas tuberosas constituídas de células infectadas, e, a partir desses, são capazes de capturar o dinitrogênio (N_2) presente na atmosfera do solo e convertê-lo em amônia, forma assimilável de N para as plantas (DIXON; KAHN, 2004; HOFFMAN et al., 2014). Esse processo de redução é conhecido como fixação biológica de nitrogênio (FBN), e é feito por um complexo de metaloenzimas, dentre as quais estão a nitrogenase, responsável pela quebra da tripla ligação do N_2 (N≡N), nomeadamente etapa de fixação, e a [NiFe]-hidrogenase, que oxida o H_2 produzido colateralmente na etapa de fixação em prótons e elétrons para a nitrogenase, maximizando sua eficiência (Figura 2) (BAGYINKA, 2014; RUIZ-ARGÜESO; PALACIOS; IMPERIAL, 2001).

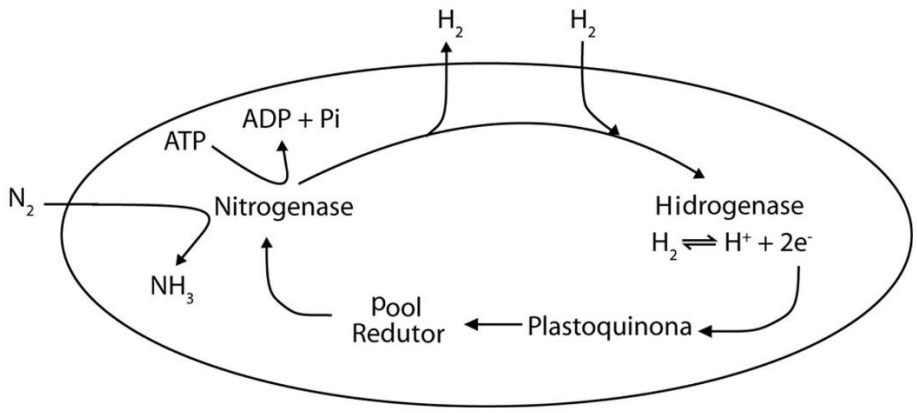

Figura 2 Modelo da redução de N_2 atmosférico a amônia pela atividade da nitrogenase durante a fixação biológica em nódulo radicular, reação na qual H_2 é formado e oxidado pela atividade da hidrogenase, reciclando elétrons para o sistema.

Fonte: adaptado de Wilson et al. (2010).

Detalhando o papel e estrutura do Ni na hidrogenase, observa-se que as [NiFe]-hidrogenases são formadas por uma subunidade pequena (~ 34 kDa) e outra grande (~ 64 kDa) (BAGYINKA, 2014). O Ni e o Fe, elementos que nomeiam essa enzima, estão locados no centro ativo, que se situa na porção medial da subunidade grande. Essa região contém um canal através do qual o H_2 gasoso penetra no centro ativo, onde se liga e é então catalisado. A subunidade pequena é composta apenas por 3 *clusters* de Fe e S (LACASSE; DOUGLAS; ZAMBLE, 2016). Dessa forma, a reciclagem do gás hidrogênio começa com os elétrons sendo transportados do centro de NiFe através dos *clusters* próximos a essa unidade, seguindo para o *cluster* distal composto por FeS, onde são finalmente transferidos para o receptor de elétrons do terminal (SCHILTER et al., 2017).

Dessa forma, as plantas leguminosas, pelo seu vínculo com processo de FBN, podem ser mais dependentes de um fornecimento adequado de Ni que as espécies não fixadoras.

2.5 Deficiência de níquel em plantas cultivadas

Os relatos de deficiência de Ni em plantas cultivadas datam desde o início do século 20 (WOOD; REILLY; NYCZEPIR, 2004a), antes mesmo da descoberta da sua essencialidade. Os primeiros relatos surgiram da observação de uma anormalidade no crescimento e desenvolvimento em pomares de pecã (*Carya illinoinensis* [Wangenh.] K. Koch), espécie arbórea muito cultivada no cinturão de nozes dos Estados Unidos, compreendendo o sul da Geórgia, e grande parte da Planície Costeira da Costa do Golfo. O sintoma foi denominado por Blackmon como "*Mouse-ear*", em português "Orelha de Rato". Essa denominação ocorreu em virtude da deformidade na ponta dos folíolos, dada pelo encurtamento do ápice foliar, formando pequenas dobras, assemelhando-se a uma orelha de rato (NYCZEPIR; WOOD; REILLY, 2006). Em primeira instância, os pesquisadores

da época atribuíram o sintoma a uma doença viral e só mais tarde a distúrbios nutricionais. A aplicação de muitos elementos como Cu, Mn e Ca foi testada para reduzir a sintomatologia, sem nenhum efeito (WOOD; REILLY; NYCZEPIR, 2004b). Logo após a descoberta do Ni como micronutriente, esse sintoma foi então atribuído a deficiência desse elemento, contudo, somente os estudos mais recentes é que efetivamente demonstram que o sintoma "*Mouse-ear*", devido a deficiência de Ni, é resultado da destruição do metabolismo de C e N nas plantas de pecã, levando ao acumulo tóxico de, respectivamente, ácido lático e oxálico (BAI, 2006; BAI; REILLY; WOOD, 2007) e ureia (TEZOTTO et al., 2016).

A partir das observações em pecã, a deficiência de Ni foi constatada em pomares de "*river birch*" (*Betula nigra* L.), e também em condições de cultivo controladas em outras espécies de plantas (GERENDAS; SATTELMACHER, 1997; KUTMAN; KUTMAN; CAKMAK, 2013, 2014; NYCZEPIR; WOOD, 2012; URUÇ PARLAK, 2016). Wood (2013) levantou ainda a hipótese de que a deficiência desse micronutriente estaria ocorrendo também em outras espécies vegetais, embora sem apresentar sintomas claros de sua deficiência, i.e., danos foliares visíveis.

2.6 Níquel em solos

O Ni está presente em quase todos os solos, onde pode derivar do material parental (litosfera), deposição antropogênica ou ambos (KABATA-PENDIAS, 2011). Em áreas nas quais um leito máfico e/ou ultramáfico está presente, altas concentrações desse elemento podem ser encontradas (ECHEVARRIA et al., 2006). A ocorrência de Ni geogênico foi encontrada em diferentes tipos de rochas. Rochas ultramáficas, como peridotito, dunito, e piroxenito têm maior teor desse elemento, seguido por rochas máficas (gabro e basalto) e intermediárias. As rochas ígneas também podem apresentar esse elemento em sua composição,

principalmente aquelas ricas em minerais ferromagnéticos e sulfetos (e.g., olivina e biotita). Nesses minerais, o Ni pode substituir o Fe ou o Mg por causa da similaridade dos raios iônicos desses elementos (McGRATH, 1995).

Durante a formação do solo, a intensidade do intemperismo de rochas ricas em Ni varia muito de acordo com os regimes de clima e umidade. De acordo com a intensidade de intemperismo, aparecem minerais recém-formados que contêm Ni em concentrações semelhantes ao leito de rocha. Eles são principalmente minerais argilosos ricos em Mg ou oxi-hidróxidos de Fe-Mn (McGRATH; LOVELAND, 1992).

Nas áreas de alta ocorrência de Ni, como na Nova Caledônia, por exemplo, desenvolvem-se plantas hiperacumuladoras desse elemento, que têm a capacidade de acumular concentrações muito altas em seus tecidos (JAFFRÉ et al., 2013; VAN DER ENT et al., 2013). Em algumas espécies, a concentração de Ni pode atingir até 16,9% em peso na seiva do floema e existem mais de 400 espécies de plantas já relatadas como hiperacumuladoras de Ni (VAN DER ENT; MULLIGAN, 2015).

Apesar disso, a concentração de Ni na maioria dos solos cultivados raramente excede a 50 mg kg^{-1} (ECHEVARRIA et al., 2006; RODAK et al., 2015), embora poucos levantamentos tenham sido feitos com intuito de definir a concentração de Ni em solos agrícolas.

2.7 A soja ganha um grande aliado

Segundo a Organização das Nações Unidas (ONU, 2013), estima-se que durante as próximas décadas a população mundial irá atingir o equivalente a 9 bilhões de pessoas (LUTZ; KC, 2010; ROBERTS, 2011). Para atender a demanda crescente por alimento e fornecer segurança alimentar a aproximadamente 870 milhões de pessoas que atualmente estão subnutridas, calcula-se que até 2050

seria necessário dobrar a produção mundial e assim, colocar uma maior pressão sobre os ambientes naturais (FAO, 2009; HUBERT et al., 2010; KEATINGE et al., 2011; PELLETIER; TYEDMERS, 2010). Mas porque a soja (*Glycine Max* (L.) Merrill) se enquadraria nesse cenário? Os grãos dessa leguminosa são constituídos por aproximadamente 40% de proteína, sendo assim a maior fonte vegetal disponível desse composto na cadeia alimentícia, estando presente na maioria das dietas de humanos e animais, além do seu emprego como fonte de combustíveis e de produtos do dia-a-dia (HARTMAN; WEST; HERMAN, 2011). O cultivo de soja, junto com o cultivo das culturas de milho, arroz e trigo, é atualmente responsável por produzir quase dois terços das calorias agrícolas globais (RAY et al., 2013). Dessa forma, o cultivo dessa leguminosa atende a uma demanda mundial por alimento atual e para um cenário futuro.

Baseado em estimativas de progressão, apesar de muitos países cultivarem soja, com destaque para os Estados Unidos e Brasil que detêm 33 e 32% da produção mundial, respectivamente, o aumento na produção atualmente verificado não será suficiente para atender a demanda da população por essa *commodity* (USDA, 2017). Esse fato se deve principalmente à dificuldade dos agricultores em associar boas práticas de cultivo, custos de produção, proteção ao meio-ambiente e condições justas de trabalho (WILSON, 2008). Para auxiliar na resolução desse problema, aumentar o rendimento produtivo das plantas de soja, em vez de liberar mais terras para a agricultura, tem sido adotado como solução preferencial (FOLEY et al., 2011; TILMAN et al., 2011). Nesse sentido, vários genótipos de soja têm sido desenvolvidos e colocados no mercado agrícola, visando aumentar a resistência das plantas ao ataque de pragas e para garantir seletividade a herbicidas que são comumente usados no manejo da soja, o que impacta, positivamente, o cenário agrícola mundial (QAIM; ZILBERMAN, 2003). O melhoramento genético da soja também está relacionado à maior demanda nutricional nos genótipos. Visto que uma das formas de elevar a

produtividade máxima das plantas cultivadas é a manutenção adequada de nutrientes dentro do sistema solo-planta (TAMAGNO et al., 2017).

O Ni assume seu papel como grande aliado do cultivo de soja nesse aspecto. Seu fornecimento via fertilização possibilita melhor nutrição das plantas, sanando uma possível deficiência oculta desse micronutriente, i.e., sem a presença de sintomas visíveis. Fato esse, que estaria sendo potencializado em virtude da alta dependência dessa espécie vegetal pela FBN. Portanto, o manejo do Ni no sistema agrícola levaria a uma maior produção por área plantada, reduzindo os custos de produção, dessa forma, afetando positivamente o sistema produtivo como um todo.

2.8 No caminho certo: relatos atuais da deficiência de níquel em soja

Os estudos já realizados com soja trazem consigo fortes indicativos da possibilidade de uma deficiência de Ni nessas plantas em campos agrícolas, embora somente sintomas indiretos e/ou em plantas cultivadas sob condições controladas, tenham sido reportados na literatura até o momento. Conforme Polacco, Mazzafera e Tezotto (2013), mutantes de soja completamente negativos para a expressão da urease ou mesmo tipos selvagens tratados com inibidores dessa enzima apresentam acumulo de ureia nas folhas, simulando o que ocorreria em plantas naturais deficientes em Ni. Recentemente também foi relatada uma maior resistência das plantas a ataque de patógenos (BARCELOS et al., 2018), ganhos fisiológicos e de crescimento (KUTMAN; KUTMAN; CAKMAK, 2014), bem como maior produtividade em plantas de soja em virtude da fertilização com esse micronutriente (KUTMAN; KUTMAN; CAKMAK, 2013; LAVRES; FRANCO; CÂMARA, 2016). Um outro ponto que reforça os indícios da deficiência de Ni em soja é o fato de seu cultivo geralmente ocorrem sobre solos com baixo conteúdo de Ni extraível no Brasil (RODAK et al., 2015).

Em plantas de pecã fertilizadas com Ni, foi constatado aumento na síntese foliar de ureídeos – alantoína e ácido alantóico (BAI; REILLY; WOOD, 2007). Esses compostos são usados pelas plantas para transportar o N_2 assimilado nos nódulos para sua parte aérea. Embora, esses achados sejam em plantas de pecã e não em soja, evidenciam ainda mais que a deficiência de Ni pode estar ocorrendo em plantas leguminosas visto sua estreita relação com FBN.

Em virtude dos relatos acima apresentados, acredita-se que é justificado aprofundar os estudos dos efeitos da aplicação de Ni visando seus efeitos benéficos em plantas de soja.

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SEGUNDA PARTE – ARTIGOS

Artigo redigido conforme a norma do periódico científico *Frontiers in Plant Science* (versão aceita e publicada)

ARTIGO 1 – Hidden nickel deficiency? Nickel fertilization via soil improves nitrogen metabolism and grain yield in soybean genotypes

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Keywords: Ammonia, Biological nitrogen fixation, *Glycine max*, Photosynthesis, Urea, Urease activity, Ureides.

Abstract

Nickel (Ni) - a component of urease and hydrogenase - was the latest nutrient to be recognized as an essential element for plants. However, to date there are no records of Ni deficiency for annual species cultivated under field conditions, possibly because of the non-appearance of obvious and distinctive symptoms, i.e., a hidden (or latent) deficiency. Soybean, a crop cultivated on soils poor in extractable Ni, condition that commonly verified in cultivated soils, has a high dependence on biological nitrogen fixation, in which Ni plays a key role. Thus, we hypothesized that Ni fertilization in soybean genotypes results in a better nitrogen physiological function and in higher grain production due to the hidden deficiency of this micronutrient. To verify this hypothesis, two simultaneous experiments were carried out, under greenhouse and field conditions, with Ni

supply of 0.0 or 0.5 mg of Ni kg^{-1} of soil. For this, we used 15 soybean genotypes and two soybean isogenic lines (urease positive, *Eu3*; urease activity-null, *eu3-a*, formerly *eu3-e1*). Plants were evaluated for yield, Ni and N concentration, photosynthesis, and N metabolism. Nickel fertilization resulted in greater grain yield in some genotypes, indicating the hidden deficiency of Ni in both conditions. Yield gains of up to 2.9 g per plant in greenhouse and up to 1,502 kg ha^{-1} in field conditions were associated with a promoted N metabolism, namely, leaf N concentration, ammonia, ureides, urea, and urease activity, which separated the genotypes into groups of Ni responsiveness. Nickel supply also positively affected photosynthesis in the genotypes, never causing detrimental effects, except for the *eu3-a* mutant, which due to the absence of ureolytic activity accumulated excess urea in leaves and had reduced yield. In summary, the effect of Ni on the plants was positive and the extent of this effect was controlled by genotype-environment interaction. The application of 0.5 mg kg^{-1} of Ni resulted in safe levels of this element in grains for human health consumption. Including Ni applications in fertilization programs may provide significant yield benefits in soybean production on low Ni soil. This might also be the case for other annual crops, especially legumes.

1 Introduction

Nickel (Ni) was the latest element to be included in the list of essential nutrients to plants. The first evidence of its essentiality was verified in soybean plants (*Glycine max* [L.] Merrill) in 1983, under controlled conditions of Ni depletion, when these plants accumulated toxic concentrations of urea in leaflet tips (Eskew et al., 1983). The evidence that Ni is an essential plant micronutrient was confirmed four years later, when after three successive generations of growing barley plants (*Hordeum vulgare* L.) in Ni-depleted controlled conditions plants failed to produce viable grains (Brown et al., 1987).

In plants, Ni constitutes the active site of two metalloenzymes that are directly involved in nitrogen metabolism (N metabolism): urease (Dixon et al., 1975) and hydrogenase (Evans et al., 1987). Urease is responsible for hydrolysis of urea into two molecules of ammonia and one of carbon dioxide (Polacco et al., 2013; Witte, 2011), while legume plants in symbiosis with N_2-fixing bacteria form root nodules, in which hydrogenase catalyzes the oxidation of molecular hydrogen (H_2) into protons and electrons (Bagyinka, 2014; Brazzolotto et al., 2016; Shafaat et al., 2013).

For legume plants that are highly efficient in biological nitrogen fixation (BNF), such as soybean, urease and hydrogenase have a very significant role. When nitrogenase reduces atmospheric N_2, these Ni metalloenzymes acts in two

downstream biological reactions. Most N fixed in root nodules, as ammonia, is converted into ureides (allantoin and allantoic acid), which are the main forms of N exported to aboveground plant parts (Collier and Tegeder, 2012). Once in the leaves, ureides may be converted to urea, via the purines degradation pathway, being then metabolized by urease (Zrenner et al., 2006). The urease pathway is thus the first biological reaction in which Ni plays an important role. Nitrogenase not only reduces N_2 to ammonia, but also produces molecular hydrogen. The produced hydrogen gas is re-oxidized by the hydrogenase enzyme, recovering a certain amount of the energy used for the previous reduction by nitrogenase (González-Guerrero et al., 2014). The hydrogenase pathway is the second biological reaction in which Ni is required.

The importance of Ni as a micronutrient has been demonstrated under greenhouse conditions (Brown et al., 1987; Dixon et al., 1975; Eskew et al., 1983; Evans et al., 1987). Subsequently, it was possible to attribute the 'mouse-ear' symptomology on pecan orchards (*Carya illinoinensis* [Wangenh.] K. Koch) to Ni deficiency (Wood et al., 2004a, 2004b, 2006). Ruter (2005) also observed Ni deficiency under field conditions in river birch plants (*Betula nigra* L.).

Nickel deficiency in these plants occurred in soils poor in extractable Ni. Even though plants usually have a low demand for this micronutrient (Seregin and Kozhevnikova, 2006), it can be expected that Ni-poor soils might also cause a hidden (or latent) deficiency in other plant species (Wood, 2013). Under such circumstances, plants would not express their maximum growth potential even without any deficiency symptoms, as visible lesions are the last step of a series of metabolic problems.

Soybean is a summer crop of a great economic and social importance worldwide, being the major source of vegetable oil (FAO, 2017). Cultivation of this crop is common on soils low in extractable Ni (Dąbkowska-Naskręt et al., 2014; Jaworska et al., 2013; Licht et al., 2006; Morrison et al., 2009; Roca et al., 2008; Rodak et al., 2015). Because of that, a hidden deficiency of this micronutrient can be predicted. In addition, the high dependence of this legume on BNF may further increase its demand for Ni.

Recent studies have demonstrated that fertilization with Ni can increase N assimilation and N metabolite levels in plants (Dalir and Khoshgoftarmanesh, 2015; Hosseini and Khoshgoftarmanesh, 2013; Khoshgoftarmanesh et al., 2011; Tan et al., 2000; Uruç Parlak, 2016). In soybean, this effect in N metabolism (Kutman et al., 2013, 2014) as well as in BNF stimulation (González-Guerrero et al., 2014; Lavres et al., 2016; Macedo et al., 2016) is also observed, yet these results were obtained under artificial growth conditions (greenhouse with soil or

nutrient solution). Furthermore, only a limited number of genotypes were tested. Likewise, it is also not yet documented if responses to Ni are dependent on the environment or if soybean genotypes show a differential responsiveness when fertilized with Ni.

Considering the dependence of soybean on BNF and an often-low content of extractable Ni in soils, the hypothesis of this study was that Ni fertilization in soybean genotypes, under greenhouse and field conditions, promotes both growth and physiological activity, alleviating situations of hidden Ni deficiency.

2 Material and Methods

2.1 Experimental Design

In order to verify Ni-fertilization effects in soybean plants, two simultaneous experiments were performed (from November 2015 to March 2016) with genotypes that are not only important in local farming practices, but also have a wide range of genetic potential for grain yield.

The first experiment - under greenhouse conditions - was a 17 x 2 completely randomized factorial design (soybean genotypes x Ni doses), with four replicates. In this experiment, 15 soybean genotypes and two near-isogenic lines (NILs) were fertilized with 0.0 mg kg^{-1} or 0.5 mg of Ni kg^{-1} (Table 1). Positive urease (*Eu3*) and urease activity-null (*eu3-a*, formerly *eu3-e1*) NILs only differ between each other in the integrity of the *UreG* gene, which codifies an accessory protein necessary to Ni incorporation into urease (Tezotto et al., 2016).

The second experiment - under field conditions - reproduced the treatments adopted in the greenhouse, with 15 x 2 factorial design (soybean genotypes x Ni doses), with four replicates. The NILs (*Eu3* and *eu3-a*) were not cultivated in the field experiment.

2.2 Cultivation Conditions

In the greenhouse experiment, soybean plants were cultivated in 4-L pots filled with soil collected from a native forest. This soil was classified as *Latossolo Vermelho Amarelo distrófico típico* (Embrapa Soils, 2013), corresponding in US Soil Taxonomy (Soil Survey Staff 1999) to the Ustox Ustox Sub-Order of Oxisol. Before sowing, soil pH was adjusted to 6.0 with soil application of 1.75 g of calcium carbonate kg^{-1} and 0.75 g of magnesium carbonate kg^{-1} in each pot. Nickel treatments comprised a control - 0.0 mg of Ni kg^{-1} - and 0.5 mg of Ni kg^{-1} applied to the soil as nickel sulfate ($NiSO_4.6H_2O$). The other macro and micronutrients

were supplied via soil (except N) at the following rates: 200 mg of P kg^{-1} ($Ca[H_2PO_4]_2$), 75 mg of P kg^{-1} + 100 mg of K kg^{-1} (KH_2PO_4), 50 mg of S kg^{-1} ($MgSO_4.7H_2O$), 5.0 mg of Cl kg^{-1} ($MnCl_2.4H_2O$), 5.0 mg of Mn kg^{-1} ($MnSO_4.H_2O$), 3.0 mg of Zn kg^{-1} ($ZnSO_4.7H_2O$), 1.0 mg of B kg^{-1} (H_3BO_3), 1.0 mg of Cu kg^{-1} ($CuSO_4.5H_2O$), 0.5 mg of Mo kg^{-1} ($[NH_4]_6Mo_7O_{24}.4H_2O$), and 0.1 mg of Co kg^{-1} ($CoSO_4.7H_2O$). Soybean plants obtained N through inoculation of seeds with N_2-fixing bacteria (*Bradyrhizobium japonicum*, strain SEMIA 5079 and *Bradyrhizobium elkanii*, strain SEMIA 5019). Soil physical and chemical characteristics after soil fertilization and pH correction are listed on Table 2. Greenhouse temperatures were kept at 28 ± 5°C during the day and 20 ± 5°C at night, by means of an automatic computer-controlled system. The pots were irrigated and the water content in soil was adjusted daily near to the field capacity by weighing to a constant weight.

In the field experiment, soybean plants were cultivated in 15-m^2 plots (6 lines of 6.25 m, equally spaced by 0.4 m) in the city of São Gabriel do Oeste, the largest soybean producer region in Brazil. This region's weather, according to the Köppen-Geiger classification, is described as tropical with mesothermal characteristics (Cwa), with an average temperature of 25°C during the day and 19°C during the night, and an average annual precipitation of 1,625 mm. The experimental site is located at an altitude of 665 m. The soil of this experimental site, classified as *Latossolo Vermelho Amarelo eutrófico típico* (Embrapa Soils, 2013), corresponds also to an Oxisol, according to the Soil Taxonomy (Soil Survey Staff, 1999), and has an agricultural cultivation history of annual species. Nickel fertilization was performed via soil at a rate of 1.0 kg of Ni ha^{-1} (equivalent to 0.5 mg kg^{-1} of Ni) as nickel sulfate ($NiSO_4.6H_2O$). A control treatment, i.e., 0.0 kg Ni ha^{-1}, was used as well. Other macro and micronutrients were supplied as follows: 1) via soil (except N): 80 kg of P ha^{-1} ($Ca[H_2PO_4]_2.H_2O$), 130 kg of K ha^{-1} (KCl), 2.0 kg of Mn ha^{-1} ($MnSO_4.H_2O$), 4.0 kg of Zn ha^{-1} ($ZnSO_4.7H_2O$), 1.5 kg of B ha^{-1} (H_3BO_3), and 1.5 kg of Cu ha^{-1} ($CoSO_4.7H_2O$); and, 2) via seeds: 15 g of Mo ha^{-1} ($[NH_4]_6Mo_7O_{24}.4H_2O$) and 2.0 g of Co ha^{-1} ($CoSO_4.7H_2O$). Soybean plants acquired N through inoculation of seeds with N_2-fixing bacteria (*Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*). Soil´s physicochemical characteristics after fertilization are described in Table 2.

Expanded leaves in the flowering stage, i.e., the R1-R2 phenological stages, according to Fehr and Caviness (1977), were analyzed in both experiments for Ni and N concentration, for N metabolic compounds (urease, urea, ureides, and ammonia), as well as with regards to photosynthesis (SPAD index, electron transport rate [ETR], photochemical quenching [qP], non-photochemical quenching [qN], and maximum fluorescence [F_M]).

Mature grains were harvested in the R8 stage (95 % of the pods below 15 % moisture, presenting mature pod color,) for Ni and N concentration analysis and determination of grain yield.

For analyses in the greenhouse experiment, two plants per pot were collected, while five plants per plot were collected, pooled, and divided into uniform sub-samples for analyses in the field experiment.

2.3 Grain Yield Evaluation

Soybean grains produced in each experiment were harvested and weighed for grain yield determination. In the greenhouse, yield estimate was done by collecting grains produced by each plant in the pot, divided by the number of plants, while in the field, grain yield was assessed by harvesting the two central lines of soybean in each plot. Grain yield was converted to dry weight by the correction of 13 % moisture. The moisture was determined with an automatic measuring device (Gehaka G650i, Brazil).

2.4 Nickel and Nitrogen Determination in Leaf and Grain

Nickel and N concentration in the leaves (the third leaf from the top of the plants) and the grains were determined in oven-dried (at 60 °C, till constant weight) materials. For determination of Ni, 0.25 g of ground-dried plant material was digested in a closed-vessel microwave system (CEM Mars 5, USA), using 30 % hydrogen peroxide and 65 % nitric acid. The final Ni concentration was determined through inductively coupled plasma-optical emission spectrometry (Perkin Elmer Optima 5300, USA). Certified reference materials NIST® SRM® 1573a (tomato leaves) and BCR® 414 (plankton) were used for QA/QC protocols. Readings below 0.2 mg kg^{-1} were considered as not detectable and so not used for calculations. For determination of N, 0.35 g of ground-dried plant material were measured using elementary analyzer (Vario EL, German).

2.5 Analysis of Photosynthesis

Photosynthetic function was determined on the third leaf from the top of the plants. As previously mentioned, soybean plants photosynthesis was evaluated by measuring the SPAD index, as well as ETR, qP, qN, and F_M. Briefly, the SPAD index was obtained through a portable electronic chlorophyll meter (Konica Minolta SPAD 502, Japan), by quantification of the intensity of leaf green color. To calculate the qP, qN, and ETR parameters (White and Critchley, 1999), *a*-chlorophyll fluorescence and light curve were determined. For the determination

of *a*-chlorophyll fluorescence, intact leaves were measured between 8:00 a.m. and 12:00 noon, using a modulated pulse fluorometer (Heinz Walz Mini-PAM, Germany). To obtain the light curves, leaves were exposed to nine pulses of actinic (photosynthetic active) light, with increasing intensities (0 - 6,500 mol m^{-2} s^{-1}) at intervals of 40 s, using the fluorometer. In order to obtain F_M, leaves were kept in darkness for a minimum of 2 h to inactivate the photochemical phase. Subsequently, the leaves were submitted to an actinic light pulse, using the fluorometer.

2.6 Evaluation of N metabolism

Urease activity and the major metabolic compounds involved in N metabolism (urea, ureides, and ammonia) were quantified in the fourth leaf collected from the top of the plants. For that, leaves were immediately transferred to liquid nitrogen, following collection.

For determination of leaf urease activity, a modified method described by Hogan et al. (1983), was used. Extraction was done with 8.0 mL of phosphate buffer at pH 7.4 for each 0.3 g of fresh material, which was incubated during 1 h at 30°C. One 0.5-mL aliquot was collected and added to 2.5 mL of reagent 1 (0.1 M phenol; 170 µM of sodium nitroprusside) and 2.5 mL of reagent 2 (0.125 M sodium hydroxide; 0.15 M dibasic sodium phosphate; sodium hypochlorite - 3% of Cl_2). Samples were then incubated at 37 °C for 35 min. Urease activity was determined by colorimetry (color intensity) in a spectrometer (Shimadzu UV-1280, Japan) at 625 nm absorbance.

Leaf urea concentration was measured through a modified procedure proposed by Kyllingsbæk (1975). Extraction was done with 1.0 mL of 10 mM formic acid for each 0.5 g of fresh material, under agitation. The extract was centrifuged at 13,200 RPM during 5 min, at 4°C. One 150-µL aliquot was collected and added to 3.0 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, under agitation, then kept in dark in an ice-cooled system for 5 min. Urea concentration was determined by colorimetry (color intensity) at 540 nm absorbance.

Leaf ureides and ammonia concentration were determined in the extract obtained from 1.0 g of fresh material in 10 mL of solution (60 % [v/v] methanol; 25 % [v/v] chloroform). The extract was centrifuged at 13,200 RPM during 5 min.

Subsequently, the supernatant was collected to determine these compounds.

Total ureide concentration (allantoin and allantoic acid), as an indicator for BNF, was quantified through the methodology proposed by Vogels and Van Der Drift (1970). One 300-µL extract aliquot was added to 500 µL of solution 1 (50 % [v/v] 0.5 N sodium hydroxide; 50 % [v/v] 0.15 N hydrochloric acid). The mixture was incubated at 100 °C during 5 min. These solutions were then cooled to ambient temperature. Subsequently, the mixture was added to solution 2 (11.5 % [v/v] 0.4 M phosphate buffer at pH 7; 11.5 % [v/v] phenyl hydrazine; 70 % [v/v] 0.65 N hydrochloric acid at -20°C; 7 % [v/v] potassium ferrocyanide). Ureides concentration was determined through colorimetry at 535 nm absorbance.

Finally, ammonia concentration was quantified according to McCullough (1967). For that, one 150-µL extract aliquot was added to 2.0 mL of colorimetric solution. This solution was prepared using a 1:1 proportion of phenol reagent (2.5 g phenol and 12.5 mg sodium nitroprusside in 250 mL) with 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 during 1 h. Ammonia concentration was then determined by colorimetry (color intensity) at 630 nm absorbance.

2.7 Statistical Analysis

Statistical analysis was performed through a two-way analysis of variance (ANOVA) and mean values were compared by the Dunnett's test ($P = 0.05$).

In order to assess the Ni treatment´s overall effect on soybean N metabolism (leaf urea, ureides and ammonia concentration, and urease activity), as well as on leaf N concentration and grain yield, a partial principal component analysis (PCA) was made for each experiment individually (greenhouse and field conditions). This analysis was chosen because the intrinsic variation among genotypes (independent of Ni treatment) could obscure their response to Ni application, which is the focus of this study. The marginal effect of genotypes was partialled out by subtracting each variable from its overall mean (irrespective to Ni treatment) for each genotype, prior to PCA analysis, resulting in a partial PCA (pPCA) as detailed in Legendre and Legendre (2013). This procedure does not change the interaction between genotypes and Ni treatments, but place all genotypes on a common scale, facilitating the visualization of how their responsiveness varies with Ni application.

3 Results

Analysis of variance of the greenhouse experiment revealed that soybean plant response was dependent on genotypes and Ni doses (A x B) for leaf Ni concentration, grain Ni concentration, grain yield, urease activity, ammonia concentration, urea concentration, SPAD index, ETR, and qN (Table 3). For leaf N concentration, grain N concentration and ureides concentration, the effect of Ni fertilization was independent of the genotypes. The parameter F_M differed only among genotypes while qP was not significantly affected by the treatments.

For the field experiment, ANOVA indicated, as observed in greenhouse experiment, a significant interaction between Ni fertilization and genotypes (A x B) for leaf Ni concentration, grain Ni concentration, grain N concentration, grain yield, urease activity, as well as ammonia, urea and ureides concentrations (Table 3). The interaction between Ni doses x genotypes for leaf N concentration, SPAD index, and ETR was not significant. The parameters qP, qN, and F_M differed only among genotypes.

In both cultivation conditions, the genotypes comparison considering each dose separately, i.e., 0.0 mg of Ni kg^{-1} or 0.5 mg of Ni kg^{-1}, is only presented in ANOVA (Table 3).

Soil extractable Ni concentration after soybean cultivation increased with Ni fertilization by approximately 2.6 times in the greenhouse soil (from < 0.20 mg kg^{-1} to 0.52 mg kg^{-1}), and by approximately 1.7 times in the field soil (from 0.40 mg kg^{-1} to 0.69 mg kg^{-1}).

3.1 Grain Yield

Nickel fertilization of greenhouse-grown soybean plants promoted increases in grain yield for 12 out of 15 genotypes evaluated and the *Eu3* isogenic line, with increases of up to 2.9 g per plant (Figure 1). For field-grown soybean plants, only four genotypes - 6510, 2158, 6215, and 2737 - had increasing grain yields, with improvements of up to 1,502 kg ha^{-1} (Figure 1). The *eu3-a* mutant was the only treatment to express toxicity with Ni fertilization, as the addition of Ni reduced grain yield by 1.7 g per plant (Figure 1).

3.2 Nickel and Nitrogen Concentration in Aboveground Plant Tissues

Soil application of Ni resulted in higher leaf Ni concentration in all soybean genotypes in both cultivation conditions, i.e., greenhouse and field (Table 4). Nickel fertilization of soybean in the greenhouse promoted average increases in leaf Ni concentration of 1.5 times in genotypes (mean values without and with Ni ranged from 0.95 to 1.42 mg kg^{-1}), and of 2.6 times on NILs (0.97 to 2.56 mg kg^{-1}). The field-grown plants showed an average increase of 2.2 times in leaf Ni concentration (mean values without and with Ni ranged from 0.62 to 1.34 mg kg^{-1}) (Table 4).

Greenhouse-grown plants generally did not translocate more Ni to grains when fertilized with this micronutrient (Table 4). Among the 17 genotypes evaluated, 10 showed no increase in grain Ni concentration (mean values without and with Ni ranged from 1.56 to 1.70 mg kg^{-1}), two of them - 7379 and *eu3-a* - had a decrease (2.36 to 1.72 mg kg^{-1}), and only five - 7200, 6510, 2158, 6215, and 620 - presented an increase in Ni concentration (1.53 to 2.19 mg kg^{-1}). On the contrary, among the 15 field-grown soybean genotypes, 10 showed an increased in grain Ni concentration (mean values without and with Ni ranged from 1.55 to 2.35 mg kg^{-1}) and five - 7849, 3730, 2158, 6215, and 620 - did not (1.68 to 1.94 mg kg^{-1}).

Nitrogen in leaf and grain presented a behavior similar to that verified for Ni concentration in soybean aboveground tissues (Table 4). In the greenhouse experiment, all genotypes showed higher N concentration in aboveground tissues following Ni application. The average increase was by 1.1 times in soybean leaves (mean values without and with Ni ranged from 34.8 to 39.8 g N kg^{-1} with Ni), and of 1.1 times in grains (57.9 to 62.1 g N kg^{-1}). Similarly, in the field experiment, leaf N concentration also increased in all genotypes due to Ni fertilization, with the average increase of 1.1 times (mean values without and with Ni ranged from 51.6 to 54.4 g N kg^{-1}) (Table 4). However, this improvement on leaf N concentration did not result in higher grain N concentration, which occurred only in four - 7379, 7200, 1378, and 620 - out of the 15 genotypes (mean values without and with Ni ranged from 51.9 to 58.9 g N kg^{-1}) (Table 4).

3.3 Photosynthesis

Nickel fertilization in soybean genotypes affected positively the photosynthetic activity (Figure 2). For these variables, only the mean of Ni-dose effects in the genotypes were presented, since the interaction of genotype x Ni dose was caused by NILs alone (data not shown).

Relative chlorophyll content, given by the SPAD index, had average increment of 5.6 in the greenhouse experiment and of 1.4 in the field experiment with Ni application (Figure 2a and 2f). A higher efficiency of the photosystem II (PSII) was also verified by increases in ETR values in both conditions (greenhouse and field), with average increment of 8.7 μmol e^{-1} m^{-2} s^{-1} in the greenhouse-grown plants and 2.7 μmol e^{-1} m^{-2} s^{-1} in field-grown plants when Ni was applied (Figure 2b and 2g). The parameters qP, qN, and F_M were not affected by Ni fertilization (Figure 2c, 2d, 2e, 2h, 2i, and 2j).

Concerning Ni fertilization in NILs, *Eu3* did not show response in the photosynthesis (Figure 2a to 2e). On the other hand, the Ni-fertilized *eu3-a* plants reduced ETR by 13.2 μmol e^{-1} m^{-2} s^{-1} (Figure 2b) and increased qN value by 0.4 (Figure 2d).

3.4 N metabolism

3.4.1 Leaf Urease Activity

Leaf urease activity was very responsive to Ni fertilization (Table 5). Sixteen out of 17 soybean cultivars grown under greenhouse had higher activity of this enzyme when fertilized with Ni, except for the *eu3-a* mutant, which is unable to codify urease activation protein. Under field conditions, only five genotypes (7200, 2728, 690, 791, and 1378) did not show increases on the activity of this enzyme following Ni fertilization. Average increments of urease activity were up to 1.9 times in the greenhouse and 1.1 times in the field (Table 5).

3.4.2 Leaf Ureide Concentration

Nickel fertilization positively affected the synthesis of total ureides (allantoin and allantoic acid), which are the main way of exporting N fixed by

nodules to other soybean plant tissues (Table 5). Nickel fertilization in the greenhouse-grown soybean promoted increases in ureide concentration for all 17 genotypes, with an average increment of 1.3 times. For field-grown soybean, only four (6510, 2158, 6215, and 2737) out of the 15 genotypes had higher ureide concentration in response to Ni fertilization, with average increments of 1.8 times in leaf ureide concentration (Table 5).

3.4.3 Leaf Ammonia Concentration

As ammonia is a product from urea hydrolysis, its leaf concentration was also very responsive to Ni fertilization, indicating, thus, that this micronutrient improved N assimilation in plants (Table 5). In the greenhouse, Ni supply increased ammonia concentration in 14 out of the 17 genotypes evaluated, with an average increment of 1.9 times. Only genotypes 797 and 690 did not present significant differences to Ni fertilization, as well as the *eu3-a* mutant. Under field conditions, exactly the same genotypes responded to Ni fertilization, with an average increase in ammonia concentration of 1.4 times (Table 5).

3.4.4 Leaf Urea Concentration

A higher urease activity due to Ni fertilization is expected to reduce leaf urea concentration. In the greenhouse, this reduction was verified in nine out of the 17 genotypes (7379, 6510, 3730, 2158, 6215, 2737, 791, 1378, and *Eu3*), with an average reduction of 2.9 times (Table 5). In contrast, the *eu3-a* mutant presented an increase of 1.1 times in urea concentration. Under field-grown conditions, exactly the same genotypes presented reduction in leaf urea concentration in response to Ni fertilization, with an average reduction of 2.7 times (Table 5).

Regarding NILs, the *eu3-a* mutant, even without Ni fertilization, always presented the highest leaf urea concentration, with an average of 85.8 µmol g FW^{-1}, a value that was 1.9 times higher than that verified for *Eu3* (Table 5). When Ni fertilized, *eu3-a* showed an expressive accumulation of urea - 98.2 µmol g FW^{-1} - while *Eu3* was able to hydrolyze this molecule, resulting in only 10.0 µmol g FW^{-1} of urea. In addition, the excessive urea accumulation in *eu3-a* leaves caused visible lesions in the leaflet tips (Figure 3). Such lesions contained a very high level of urea, with an average concentration of 576 µmol g FW^{-1}.

3.4.5 Principal Components Analysis (PCA)

In order to promote a better understanding of the overall Ni fertilization effect on soybean yield, leaf N concentration, leaf ammonia, leaf ureides, leaf urea, and urease activity for each genotype, two pPCA were performed (one for each experiment), with the marginal effect of genotype (overall mean for each genotype, independently of Ni treatment) being partialled out. For the greenhouse experiment, the first two principal components represented 82 % of total variation (Figure 4), whereas, for the field experiment, the first two components represented 70 % of total variation (Figure 5). In both experiments, the first component (horizontal axis) represented most of the total variation and clearly separated treatments with and without Ni fertilization. Grouping of the samples receiving Ni towards the left side of the pPCA biplot indicates increased grain yield, leaf N concentration, leaf ammonia, leaf ureides, and urease activity, associated with decreases in leaf urea, with the opposite for mutant *eu3-a* (Figures 4 and 5).

Soybean genotypes were separated in groups by their responsiveness to Ni fertilization, as follows: 1) In the greenhouse experiment: Group A (high response) - 6510, 2158, 6215, 2737, and *Eu3*; Group B (moderate response) - 7379, 7200, 2728, 7849, 3730, 8015, 791, 1378, and 620; Group C (low response) - 797 and 690; Group D (unresponsive) - *eu3-a* (Figure 4); and, 2) In the field experiment: the same groups were evident, except for NILs, which were not studied under this condition (Figure 5).

4 Discussion

In both greenhouse and field conditions, initial soil Ni concentration (~0.3 mg kg^{-1}) and initial grain Ni concentration (~1.8 mg kg^{-1}) were not high enough to supply soybean plant-specific requirements (Tables 1 and 2), since Ni fertilization via soil led to physiologic enhancements (Figure 2), better N metabolism (Table 5), and higher grain yield (Figure 1). The lack of Ni-deficiency symptoms associated with these results revealed a hidden Ni deficiency. To the best of our knowledge, this is the first study to report a hidden deficiency of this micronutrient in soybean under field conditions. Previous studies, although being carried out on greenhouse-grown soybean plants alone, corroborate the Ni performance verified in this study. Kutman et al. (2013) and Lavres et al. (2016) demonstrated that Ni fertilization induces yield gains, while Kutman et al. (2014) showed that soybean seeds with Ni concentration up to 5.35 mg kg^{-1} did not

express their maximum yield and, thus, an external supply of this micronutrient was required. Thus, these previous results give support to our data, indicating a higher grain yield in soybean plants when fertilized with Ni. Our study also revealed that not all soybean genotypes respond in the same way to Ni fertilization, since despite improvements in the photosynthetic apparatus (Figure 2a, 2b, 2f, and 2g) and a better N metabolism (Table 5), when supplied with Ni, some of the soybean genotypes did not produce higher grain yield (Figure 1).

Based on our data, the genotypes were separated into groups of Ni responsiveness based on the responses of their N metabolism (Table 5 and Figures 1, 4, and 5).

The genotypes classified in Group A (Figures 4 and 5) had an N-assimilation boost, that is, higher leaf ammonia concentration and reduced leaf urea concentration, due to a higher urease activity (Table 5), thus this group was considered as highly responsive to Ni fertilization. To be able to transport N-urea to N-sink tissues, soybean plants produce ammonia, as result of urea hydrolysis by urease activity (Polacco et al., 2013; Wang et al., 2008; Winter et al., 2015; Witte, 2011). According to Mokhele et al. (2012) and Ohyama et al. (2017), when degraded, ammonia produces different amino acids, so that a higher free amino acid pool affect positively several plant-growth process, especially secondary compounds synthesis. Although little is known about Ni influences ammonia metabolism in plants, Bai et al. (2006, 2007) observed that pecan plants under low Ni supply showed an inhibition in the shikimate pathway, disrupting the conversion of free amino acids to other products and, thus, blocking some N pathways. Moreover, the genotypes in this group also had the higher increases in ureides synthesis, products of purine degradation and main form of N transport from nodules, during BNF, to aboveground parts in legume plants (Rentsch et al., 2007). As observed by Lavres et al. (2016), yield increases in soybean plants due to Ni fertilization resulted from a more efficient BNF, probably due to a higher activity of hydrogenase. In addition, Todd and Polacco (2004), studying soybean, confirmed that urea and ammonia might be direct products of ureides degradation in urease pathway. Regardless of the cultivation condition, i.e., greenhouse or field, the genotypes in Group A had increases in grain production.

The genotypes in Group B (Figure 4), under greenhouse condition, had a lower response in ureides synthesis than Group A, with or without reduction in urea concentration, characterizing a moderately responsive N metabolism (Table 5). In this case, usually a higher yield was found due to Ni supply (Figure 1). Field-conditions were more restrictive since the genotypes in this group presented no yield increases (Figures 1 and 5), associated mainly with no increases in ureides (Table 5). Thus, our data revealed that the absence of response to Ni

fertilization in any step of N metabolism might result in lack of yield gains, in which some compounds are more limiting than others. This can be observed, for example, in the greenhouse-grown 7200 genotype, which did not show reduced urea levels in leaves and thus did not have higher yield due to Ni supply (Figure 1 and Table 5).

The genotypes in Group C (Figures 4 and 5), showed low response in N metabolism when Ni fertilized in both conditions. In this group, soybean plants lacked response in leaf ammonia, with this N compound being the key factor that limits productivity gains (Figure 1 and Table 5).

Group D (Figure 4), with no response in N metabolism to Ni supply, comprised the *eu3-a* - urease activity-null. This mutant has a blockage in ammonia synthesis, via urease, and thus, had a significant accumulation of leaf urea with Ni fertilization, which caused toxicity symptoms (Figure 3 and Table 5). The excessive urea accumulation resulted in lower grain yield (Figure 1). This emphasizes the critical role of Ni in N metabolism.

A more efficient N metabolism with Ni supply is corroborated by the higher N concentration in the leaves (Tables 4 and 5). According to Kutman et al. (2013), soybean plants increased N concentration by up to 30 % when fertilized with Ni, indicating that this micronutrient improves internal N utilization efficiency and N remobilization.

With Ni supply, we verified a higher Ni concentration in soybean leaves, as was observed for N concentration. However, higher concentrations of Ni and N in the leaf were not always related to a higher grain concentration (Table 4). Thus, our data indicate that the translocation rate for these nutrients is controlled by phenotype-specific properties. According to Belimov et al. (2016), the phenotypic specificity can modulate homeostasis and regulation of transporters for many ions. Moreover, since Ni absorption by roots of soybean can be via passive diffusion or active transport (Seregin and Kozhevnikova, 2006; Yusuf et al., 2011), the relative Ni concentration may vary among genotypes. The same phenotype-specific effect on grain yield, photosynthesis, and N metabolism evidenced that the cultivation conditions have influenced genotypes response to Ni fertilization (Figures 1 and 2, Tables 3 and 5).

Since many farmers all over the world have used Ni fertilization without clear evidence of its need for crop growth, there are concerns about a possible toxicity of this element in cultivated plants (Kretsinger et al., 2013). Our data revealed that a soil-applied Ni rate of 0.5 mg kg^{-1} resulted in Ni leaf concentrations up to 2.26 mg kg^{-1} and Ni grain concentrations up to 3.07 mg kg^{-1} (Table 4). These

values are well below the levels considered toxic to plants, which are > 10 mg kg^{-1} in sensitive species, > 50 mg kg^{-1} in moderately tolerant species, and > 1,000 mg kg^{-1} in Ni hyperaccumulator plants (Chen et al., 2009; Seregin and Kozhevnikova, 2006; Yusuf et al., 2011).

Some photosynthetic parameters considered as stress indicators also confirmed the absence of Ni toxicity in the soybean genotypes. The quenchings, qP and qN, are protective mechanisms that plants employ to dissipate energy from photochemical processes and should only be accessed by plants in case of light stress (Ashraf and Harris, 2013; Dall'Osto et al., 2017). Therefore, the lack of responses of qP and qN with Ni fertilization indicates that plants did not experience oxidative damage in PSII reaction centers (Figure 2c, 2d, 2h, and 2i). Moreover, according to Baker (2008), healthy leaves have F_M values of approximately 0.8, which is similar to the value found in the genotypes, even when Ni fertilized (Figure 2e and 2j). Positive photosynthetic responses, ETR and SPAD index, increased in Ni-fertilized plants (Figure 2a, 2b, 2f, and 2g), indicating a more efficient photosynthetic apparatus in the soybean genotypes.

The *eu3-a* mutant accumulated toxic levels of urea in leaves, even without Ni supply (Table 5). With addition of 0.5 mg kg^{-1} of Ni via soil, urea toxicity symptoms were intensified, being also associated with Ni-toxicity symptoms (Figure 3). The toxic level of Ni (Table 4) was high enough to reduce the mutant´s growth (data not show) and ETR (Figure 2b), and increase the stress indicator qN (Figure 2d). Aiming to obtain the Ni-toxicity symptoms in soybean plants, Reis et al. (2017) observed formation of brown color on leaves induced by the presence of Ni inside cells, similarly to what was observed in the *eu3-a*.

Finally, concerning food safety of Ni fertilization in soybean plants, we first need to set the maximum allowable daily intake (ADI) of Ni for humans, which is expected to be 1.33 mg Ni per day for an adult and 0.31 mg Ni per day for a child. Such ADIs are based on a reference dose (RfD) for Ni of 0.02 mg kg^{-1} per day (IRIS, 1991), which was calculated from a no-observed-adverse-effect level (NOAEL) of 5.0 mg kg^{-1} per day (Ambrose et al., 1976; Institute of Medicine US and Panel on Micronutrients, 2002), and a body mass of 66.6 kg for an adult and 15.4 kg for a child (Cole et al., 2007; Guilherme et al., 2015).

Next, assuming that a grain containing ~3 mg kg^{-1} of Ni in dry weight - the highest concentration of Ni in grains in this study - is used for assessing the risk of Ni ingestion via food chain, then a child needs to ingest >100 g of soybean grains (dry weight) per day in order to overcome a risk coefficient of 1. Such daily consumption of soybean is far beyond the recommended ingestion standards of *in natura* grains and soybean products. According to Do et al. (2007), the daily

intake of *in natura* soybean grains is 2.5 ± 4.9 g (*n = 708*). In Asian countries - the largest consumers of soybean - the daily intake of soybean and soy-related foods is 23.0 ± 18.2 g (Do et al., 2007; Katsuyama et al., 2009; Toyomura and Kono, 2002). Thus, the amount of Ni in soybean grains found in this study is considered safe and does not pose a threat to human health if direct consumption of grain is taking into account.

5 Conclusions

Fertilization with a 0.5 mg Ni kg^{-1} dose via soil resulted in higher grain yield in 12 greenhouse-grown genotypes and 4 field-grown genotypes, revealing a hidden Ni deficiency under both cultivation conditions. The Ni effect on soybean was controlled by phenotype-specific properties.

Yield increases resulted from a more efficient N metabolism, especially the N compound ureide. The higher ureides synthesis, possibly originated from a higher N_2-fixation, and their catalysis by urease activity must result in higher ammonia concentration, so that increases in grain yield can be realized. The genotypes were separated into groups of Ni responsiveness based on the responses of their N metabolism: high response (with enhanced N metabolism), moderate response (limited by low ureides synthesis and/or urea synthesis), low response (limited by ammonia synthesis), and unresponsive (limited by urease activity).

Nickel fertilization resulted also in photosynthetic enhancements in soybean plants - especially in the photochemical phase - except for the *eu3-a*. Absence of ureolytic activity in this mutant resulted in a higher concentration of urea, which accumulated mainly in leaflet tips, resulting in a lower grain yield.

Thus, Ni fertilization at the dose employed in this study is beneficial for soybean and possibly for other annual species, in soils with low extractable-Ni, resulting in agronomical gains while meeting food safety standards. However, more studies are required to set an accurate Ni rate and to verify residual effects of Ni in the soil, especially for oxidic conditions prevalent in tropical agroecosystems. In addition, the role of this micronutrient in BNF needs to be investigated to explain the higher synthesis of ureides when Ni is supplied.

6 Conflict of Interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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8 Author Contributions

DS and BW were in-charge for development of hypothesis, experiment conduction, data analysis, and writing of this manuscript. AR and FdB are experts in plant physiology, contributing mainly in the field experiment, and in review of this manuscript. TS is expert in statistical analysis and soil microbiology, contributing mainly in data analysis and in review of this manuscript. JS is expert in BNF, contributing in the understanding of how nickel affect N_2-fixation process and in review of this manuscript. LG and MC are co-advisors and the coordinators of our research group. Their contributions extend to all steps of the research that led to this manuscript.

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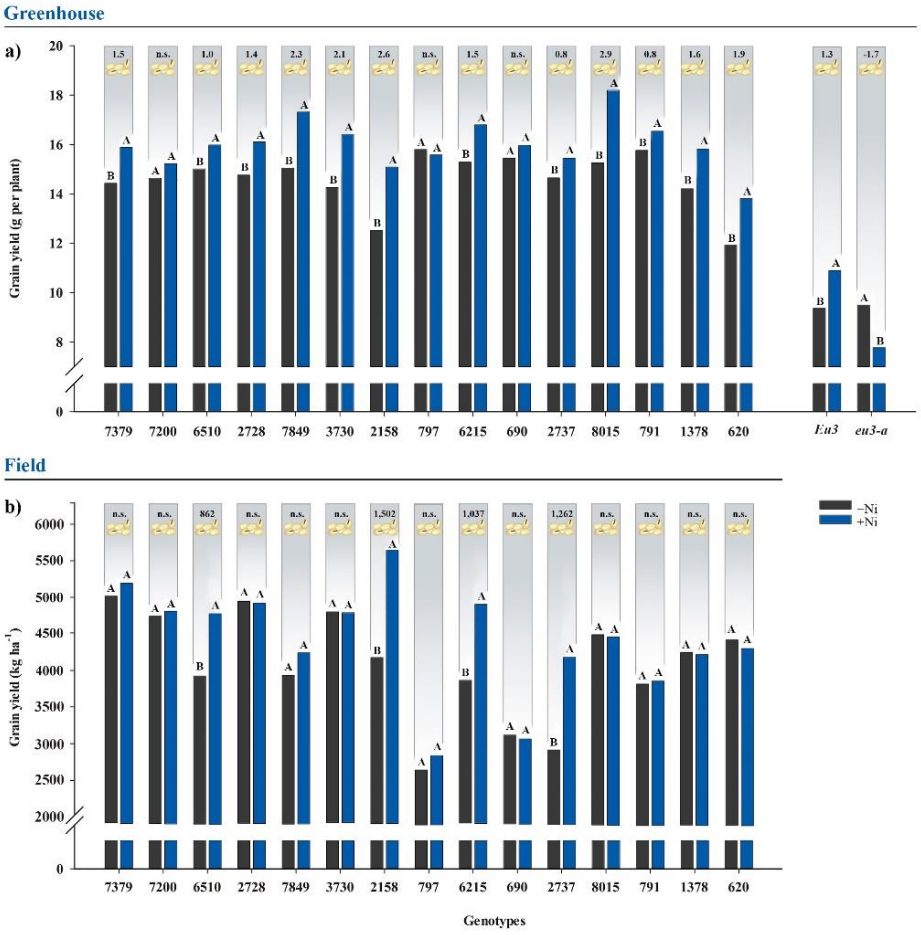

FIGURE 1 | Effects on grain yield due to fertilization with 0.0 mg of Ni kg^{-1} (–Ni) and 0.5 mg of Ni kg^{-1} (+Ni) in 15 soybean genotypes and two near-isogenic lines (NILs, *Eu3* and *eu3-a*) cultivated in a) greenhouse and b) field conditions. *Means were compared by the effect of the Ni doses in each genotype by Dunnett's test at P < 0.05, and those followed by the same letter do not differ. Values indicated in the upper part of the figure correspond to the amplitude of difference between Ni doses in grain yield. Grain yield was corrected to 13 % of moisture. n.s., not significant. The NILs were not tested in the field experiment.*

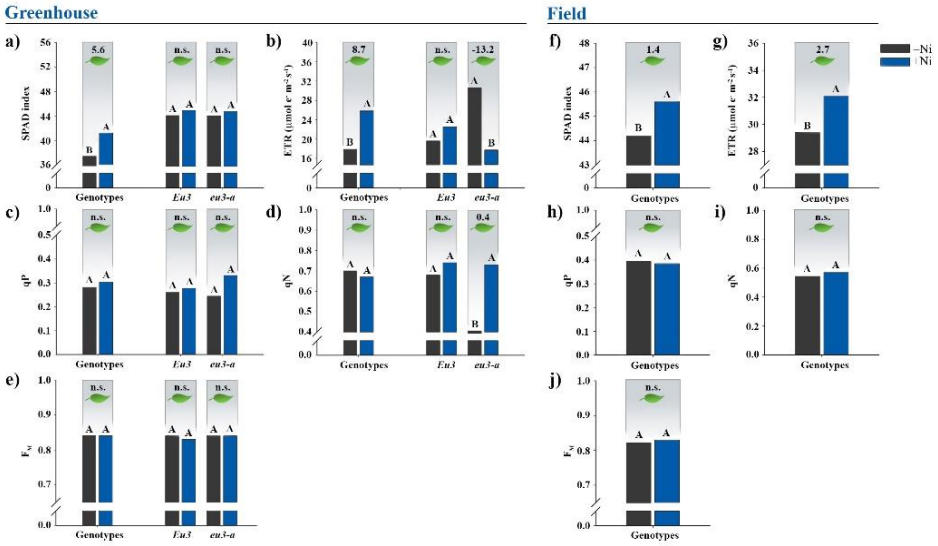

FIGURE 2 | Effects on leaf photosynthesis due to fertilization with 0.0 mg of Ni kg^{-1} (–Ni) and 0.5 mg of Ni kg^{-1} (+Ni) in 15 soybean genotypes and two near-isogenic lines (NILs), *Eu3* and *eu3-a*, cultivated in a) to e) greenhouse condition and f) to j) field condition. *Means were compared by the effect of the Ni doses in each genotype by Dunnett's test at P < 0.05, and those followed by the same letter do not differ. In greenhouse, only the mean of Ni-dose effects in the genotypes were presented since interaction genotype x Ni dose was caused by NILs alone. Values indicated in the upper part of the figure correspond to the amplitude of difference between Ni doses in photosynthesis. n.s., not significant. ETR, electron transport rate. qP, photochemical quenching. qN, non-photochemical quenching. F_M, maximum fluorescence. The NILs were not tested in the field experiment.*

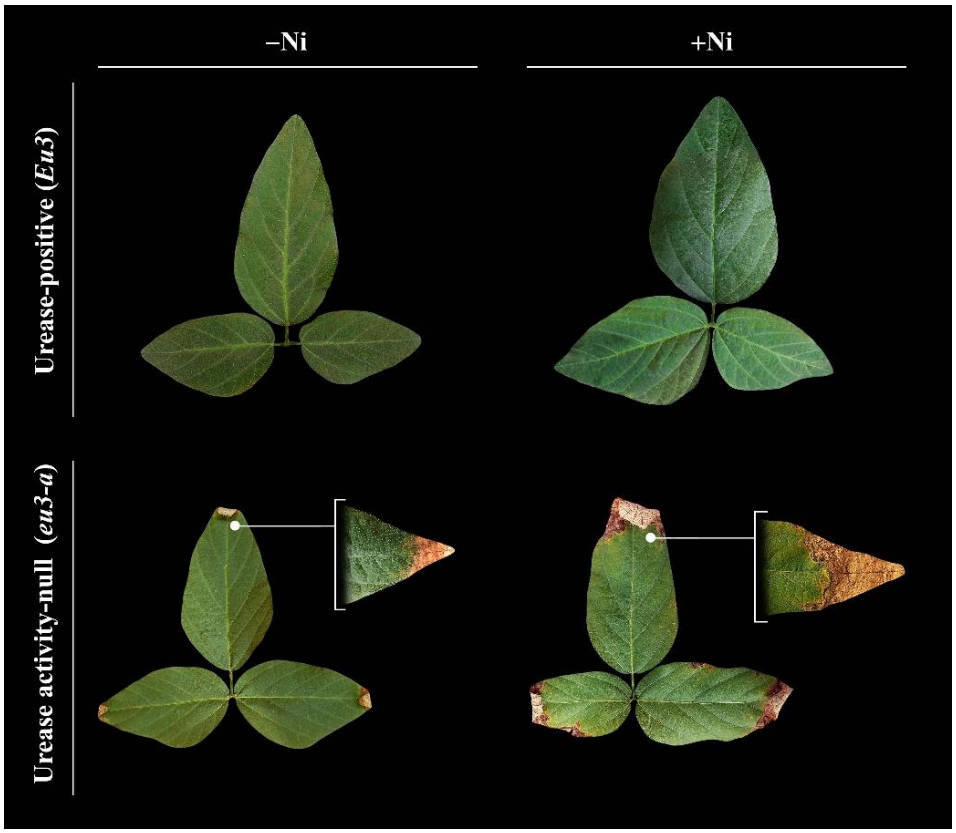

FIGURE 3 | Contrast of leaves of two near-isogenic soybean lines at flowering stage, urease-positive (*Eu3*) and urease activity-null (*eu3-a*), fertilized with 0.0 mg of Ni kg^{-1} (–Ni) and 0.5 mg of Ni kg^{-1} (+Ni). Independently of Ni dose, *Eu3* line developed normally while *eu3-a* line presented symptoms of hyponasty and initial necrosis lesions on leaflet tips. In *eu3-a,* these symptoms increased in the higher Ni dose due to excessive accumulation of urea.

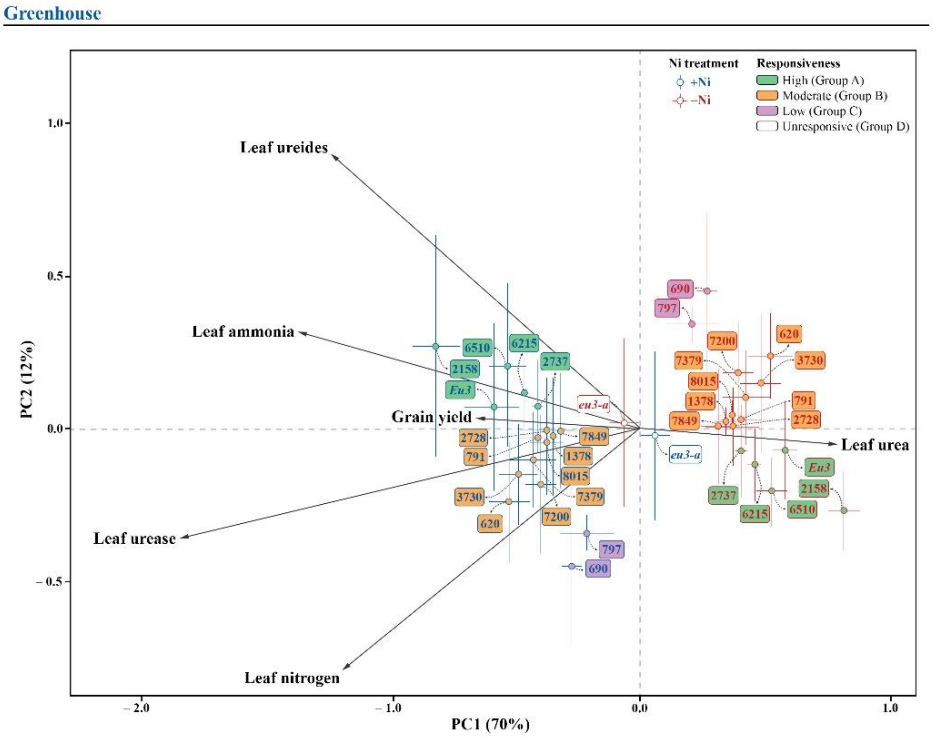

FIGURE 4 | Biplot of partial principal components analysis of the variables related to N metabolism, leaf N concentration and grain yield for 15 soybean genotypes and two near-isogenic lines (NILs, *Eu3* and *eu3-a)*, fertilized with 0.0 mg of Ni kg^{-1} (–Ni) and 0.5 mg of Ni kg^{-1} (+Ni), cultivated in greenhouse condition. In the figure, genotypes are divided into four groups according to responsiveness of N metabolism to Ni fertilization: Group A, high; B, moderate; C, low; and D, unresponsive.

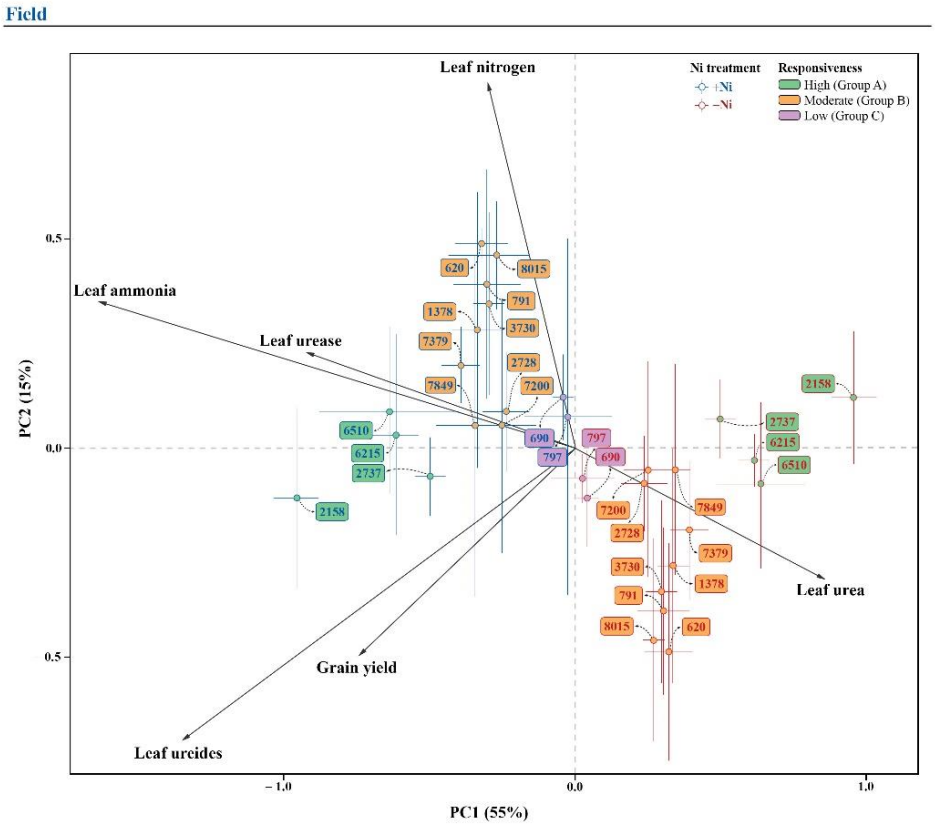

FIGURE 5 | Biplot of the partial principal components analysis of variables related to N metabolism, leaf N concentration and grain yield for 15 soybean genotypes, fertilized with 0.0 mg of Ni kg^{-1} (+Ni) and 0.5 mg of Ni kg^{-1} (–Ni), cultivated in field condition. In the figure, genotypes are divided into three groups according to responsiveness of N metabolism to Ni fertilization: Group A, high; B, moderate; and C, low.

TABLE 1 | Summary of characteristics for 15 soybean genotypes and two near-isogenic lines with urease-positive (*Eu3*) and urease activity-null (*eu3-a*).

Genotype	Company[a]	Patent[b]	Transgenic event	Maturity[c]	Growth habit	Grain initial Ni concentration (mg kg^{-1})
7379	GDM	31763	MON87701 x MON89788	7.4	Indeterminate	1.26
7200	NIDERA	28708	GTS-40-3-2	6.4	Indeterminate	1.47
6510	GDM	30256	GTS-40-3-2	6.5	Indeterminate	2.84
2728	MONSOY	28121	MON87701 x MON89788	7.2	Indeterminate	1.83
7849	BAYER	29661	MON87701 x MON89788	7.8	Indeterminate	1.32
3730	MONSOY	28124	MON87701 x MON89788	7.3	Indeterminate	1.90
2158	TMG	31291	MON87701 x MON89788	5.8	Indeterminate	2.53
797	MONSOY	31211	MON87701 x MON89788	7.9	Indeterminate	1.38
6215	TMG	33040	MON87701 x MON89788	6.4	Indeterminate	2.25
690	GENEZE	30151	GTS-40-3-2	6.9	Indeterminate	1.94
2737	COODETEC	28992	GTS-40-3-2	7.3	Indeterminate	2.33
8015	COODETEC	33191	MON87701 x MON89788	8.0	Determinate	1.50
791	BAYER	25931	GTS-40-3-2	7.9	Indeterminate	1.75
1378	SYNGENTA	31435	MON87701 x MON89788	8.0	Determinate	1.47
620	TMG	33097	MON87701 x MON89788	6.2	Indeterminate	1.64
Eu3[d]	-	-	*eu3-a/eu3-a* x *Eu3/Eu3*	-	Determinate	1.57
eu3-a[d]	-	-	*eu3-a/eu3-a* x *Eu3/Eu3*	-	Determinate	1.58

[a]*Maintainer of genotype.*
[b]*Details about patent register can be found at Brazil (2016).*
[c]*Maturity groups defined by Alliprandini et al. (2009).*
[d]*Isogenic lines described in Tezotto et al. (2016).*

TABLE 2 | Chemical characterization and particle size distribution before sowing of the native forest soil *Latossolo Vermelho Amarelo distrófico típico* (Oxisol) used in the greenhouse experiment and the cultivated soil *Latossolo Vermelho Amarelo eutrófico típico* (Oxisol) used in the field experiment.

Properties	Units	Method/Extractant	Greenhouse	Field
Sand	g kg^{-1}	Hydrometer	740	656
Silt	g kg^{-1}	Hydrometer	30	154
Clay	g kg^{-1}	Hydrometer	230	190
SOM	g kg^{-1}	Colorimetric	16.0	39.0
pH	-	Water	6.0	6.5
Al	$cmol_c$ kg^{-1}	Potassium chloride	0.0	0.0
Al + H	$cmol_c$ kg^{-1}	Calcium acetate, pH 7.0	0.7	2.3
N	g kg^{-1}	Kjeldahl	1.3	2.2
P	mg kg^{-1}	Mehlich-1	27.8	34.4
K	mg kg^{-1}	Mehlich-1	47.0	170
Ca	$cmol_c$ kg^{-1}	Potassium chloride	2.7	5.2
Mg	$cmol_c$ kg^{-1}	Potassium chloride	1.7	2.1
S	mg kg^{-1}	Dicalcium phosphate	18.1	7.5
B	mg kg^{-1}	Hot water	0.5	1.3
Cu	mg kg^{-1}	Mehlich-1	1.0	2.3
Fe	mg kg^{-1}	Mehlich-1	30.6	17.4
Mn	mg kg^{-1}	Mehlich-1	7.2	78.0
Zn	mg kg^{-1}	Mehlich-1	2.4	9.3
Ni	mg kg^{-1}	Mehlich-1	$< 0.2^a$ - 0.6^b	0.4^a - 0.7^b

[a]After fertilization with 0.0 mg of Ni kg^{-1}.
[b]After fertilization with 0.5 mg of Ni kg^{-1}.
Soil classification according to Embrapa Soils (2013).
SOM, soil organic matter.

TABLE 3 | Two-way analysis of variance of 15 soybean genotypes and two near-isogenic lines (NILs) cultivated in greenhouse and field fertilized with 0.0 mg of Ni kg^{-1} and 0.5 mg of Ni kg^{-1}.

	Source of variation – Greenhouse				
	Ni leaf	***N leaf***	***Ni grain***	***N grain***	***Grain yield***
Genotype (A)	**	**	**	**	**
Ni dose (B)	**	**	**	**	**
A x B	**	*n.s.*	**	*n.s.*	**
CV (%)	*6.2*	*7.2*	*15.7*	*5.1*	*3.2*
	Leaf ammonia	***Leaf urea***	***Leaf urease***	***Leaf ureides***	***SPAD index***
Genotype (A)	**	**	**	**	**
Ni dose (B)	**	**	**	**	**
A x B	**	**	**	*n.s.*	*
CV (%)	*14.7*	*26.7*	*12.9*	*18.3*	*5.5*
	ETR	***qP***	***qN***	***F_M***	
Genotype (A)	**	*n.s.*	**	*	
Ni dose (B)	**	*n.s.*	*n.s.*	*n.s.*	
A x B	**	*n.s.*	*	*n.s.*	
CV (%)	*12.5*	*24.9*	*16.2*	*1.1*	
	Source of variation – Field				
	Ni leaf	***N leaf***	***Ni grain***	***N grain***	***Grain yield***
Genotype (A)	**	**	**	**	**
Ni dose (B)	**	**	**	**	**
A x B	**	*n.s.*	**	*	*
CV (%)	*13.6*	*6.5*	*16.3*	*6.0*	*13.3*
	Leaf ammonia	***Leaf urea***	***Leaf urease***	***Leaf ureides***	***SPAD index***
Genotype (A)	**	**	**	**	**
Ni dose (B)	**	**	**	**	**
A x B	*	**	**	*	*n.s.*
CV (%)	*12.2*	*14.7*	*1.8*	*28.7*	*4.2*
	ETR	***qP***	***qN***	***F_M***	
Genotype (A)	**	**	**	**	
Ni dose (B)	**	*n.s.*	*n.s.*	*n.s.*	
A x B	*n.s.*	*n.s.*	*n.s.*	*n.s.*	
CV (%)	*13.9*	*20.6*	*15.7*	*9.8*	

n.s., not significant by F-test.
**, significant by F-test at P < 0.05.*
***, significant by F-test at P < 0.01.*
qP, photochemical quenching.
qN, non-photochemical quenching.
F_M, maximum fluorescence.
ETR, electron transport rate.
CV, coefficient of variation.
The NILs were not tested in the field experiment.

TABLE 4 | Effects in leaf Ni and N concentration and grain Ni and N concentration due to fertilization with 0.0 mg of Ni kg^{-1} (–Ni) and 0.5 mg of Ni kg^{-1} (+Ni) in 15 soybean genotypes and two near-isogenic lines (NILs, *Eu3* and *eu3-a*) cultivated in greenhouse and field conditions.

Genotype	Leaf Ni (mg kg^{-1})		Leaf N (g kg^{-1})		Grain Ni (mg kg^{-1})		Grain N (g kg^{-1})	
	–Ni	+Ni	–Ni	+Ni	–Ni	+Ni	–Ni	+Ni
				Greenhouse				
7379	1.18 B	1.51 A	35.7 B	40.7 A	2.45 A	1.70 B	55.1 B	62.2 A
7200	1.03 B	1.57 A	32.1 B	37.6 A	1.39 B	1.85 A	56.0 B	62.9 A
6510	0.83 B	1.57 A	40.7 B	45.9 A	1.38 B	1.89 A	56.7 B	65.5 A
2728	1.22 B	1.54 A	36.4 B	41.3 A	1.82 A	1.45 A	54.0 B	61.1 A
7849	1.00 B	1.22 A	34.3 B	39.5 A	1.48 A	1.55 A	56.5 B	61.3 A
3730	1.02 B	1.35 A	34.4 B	39.4 A	1.52 A	1.66 A	62.5 B	65.9 A
2158	0.99 B	1.90 A	36.6 B	41.5 A	1.86 B	2.68 A	56.3 B	59.1 A
797	1.27 B	1.65 A	37.8 B	42.8 A	1.45 A	1.47 A	61.6 B	62.9 A
6215	1.00 B	1.70 A	36.6 B	41.7 A	1.20 B	2.02 A	53.7 B	62.3 A
690	1.06 B	1.62 A	34.1 B	41.2 A	1.86 A	1.81 A	58.3 B	63.9 A
2737	0.75 B	1.17 A	33.3 B	39.3 A	1.36 A	1.67 A	63.6 B	67.8 A
8015	0.99 B	1.31 A	30.4 B	36.0 A	1.53 A	1.94 A	54.0 B	61.3 A
791	0.80 B	1.30 A	39.7 B	44.6 A	1.40 A	1.60 A	59.0 B	61.1 A
1378	0.76 B	1.01 A	30.3 B	35.4 A	1.56 A	1.88 A	60.0 B	61.3 A
620	0.40 B	0.88 A	26.5 B	32.5 A	1.82 B	2.52 A	54.0 B	56.9 A
Eu3	0.84 B	2.33 A	35.2 B	40.6 A	1.64 A	2.00 A	59.9 B	61.3 A
eu3-a	1.09 B	2.78 A	37.8 B	37.2 A	2.26 A	1.73 B	62.3 B	59.2 A
				Field				
7379	0.45 B	1.57 A	53.5 B	54.9 A	1.22 B	2.66 A	54.2 B	61.5 A
7200	1.30 B	2.01 A	52.6 B	54.1 A	1.40 B	2.04 A	47.1 B	56.3 A
6510	0.81 B	1.28 A	57.8 B	60.2 A	2.29 B	3.07 A	53.6 A	53.7 A
2728	0.54 B	1.55 A	54.7 B	56.5 A	1.60 B	2.27 A	57.2 A	56.3 A
7849	0.85 B	1.79 A	50.7 B	52.5 A	1.30 A	1.65 A	58.0 A	57.6 A
3730	0.39 B	0.93 A	50.4 B	53.0 A	1.86 A	2.13 A	56.0 A	56.6 A
2158	0.31 B	0.65 A	59.5 B	61.3 A	1.91 A	2.20 A	56.7 A	57.6 A
797	0.35 B	0.92 A	42.3 B	44.9 A	1.39 B	1.89 A	59.1 A	58.2 A
6215	0.41 B	1.86 A	56.3 B	59.0 A	1.58 A	1.99 A	58.2 A	57.3 A
690	0.34 B	1.36 A	40.6 B	43.5 A	1.66 B	2.19 A	56.7 A	57.7 A
2737	1.51 B	2.26 A	55.7 B	57.6 A	1.59 B	2.34 A	58.8 A	58.8 A
8015	0.63 B	1.15 A	45.9 B	52.8 A	1.44 B	2.49 A	54.8 A	55.8 A
791	0.39 B	0.74 A	51.6 B	56.3 A	1.53 B	2.37 A	56.9 A	57.8 A
1378	0.56 B	1.01 A	51.8 B	53.8 A	1.34 B	2.20 A	54.2 B	60.1 A
620	0.51 B	0.97 A	50.9 B	55.6 A	1.75 A	1.71 A	52.0 B	57.6 A

Means were compared by the effect of the Ni doses in each genotype by Dunnett's test at $P < 0.05$, and those followed by the same letter do not differ.
The NILs were not tested in the field experiment.

TABLE 5 | Effects on the leaf N metabolism due to fertilization with 0.0 mg of Ni kg^{-1} (–Ni) and 0.5 mg of Ni kg^{-1} (+Ni) in 15 soybean genotypes and two near-isogenic lines (NILs, *Eu3* and *eu3-a*) cultivated in greenhouse and field conditions.

Genotype	Urease activity (µmol g FW^{-1} h^{-1})		Ureides (µmol g FW^{-1})		Ammonia (µmol g FW^{-1})		Urea (µmol g FW^{-1})	
	–Ni	+Ni	–Ni	+Ni	–Ni	+Ni	–Ni	+Ni
				Greenhouse				
7379	8.0 B	16.5 A	13.4 B	18.3 A	5.0 B	8.0 A	27.8 A	11.2 B
7200	8.6 B	16.5 A	16.1 B	18.7 A	4.7 B	9.4 A	19.2 A	14.4 A
6510	8.0 B	14.6 A	17.7 B	26.2 A	3.5 B	9.4 A	42.5 A	7.4 B
2728	9.7 B	15.1 A	18.1 B	23.5 A	7.5 B	10.5 A	25.2 A	23.0 A
7849	9.0 B	13.0 A	19.1 B	24.3 A	7.1 B	10.1 A	14.2 A	13.0 A
3730	8.2 B	19.4 A	16.7 B	21.8 A	6.1 B	9.1 A	44.2 A	31.2 B
2158	8.4 B	22.4 A	22.2 B	32.6 A	2.5 B	12.6 A	44.0 A	12.2 B
797	8.6 B	15.2 A	11.8 B	12.9 A	6.1 A	6.3 A	22.5 A	24.8 A
6215	9.9 B	17.3 A	11.7 B	20.4 A	1.3 B	5.0 A	26.7 A	12.3 B
690	9.2 B	16.2 A	17.0 B	17.7 A	5.6 A	6.2 A	12.7 A	10.3 A
2737	10.3 B	15.7 A	12.9 B	21.1 A	4.7 B	8.0 A	22.4 A	7.5 B
8015	10.5 B	16.0 A	15.2 B	20.8 A	8.9 B	11.6 A	15.9 A	8.4 A
791	8.1 B	15.4 A	14.6 B	19.8 A	7.6 B	11.5 A	34.2 A	17.8 B
1378	9.3 B	14.9 A	19.6 B	24.8 A	6.6 B	9.5 A	45.2 A	28.2 B
620	8.1 B	21.4 A	20.8 B	26.1 A	6.7 B	9.8 A	34.3 A	32.9 A
Eu3	9.3 B	20.5 A	20.0 B	30.1 A	11.3 B	14.3 A	45.8 A	10.0 B
eu3-a	6.8 A	6.9 A	15.1 B	14.6 A	10.0 A	10.0 A	85.8 B	98.2 A
				Field				
7379	11.1 B	11.9 A	26.4 A	33.3 A	11.5 B	15.1 A	37.5 A	15.2 B
7200	13.5 A	13.5 A	26.4 A	33.6 A	11.9 B	15.1 A	25.9 A	19.5 A
6510	13.4 B	14.1 A	22.8 B	37.1 A	12.6 B	18.7 A	32.7 A	5.7 B
2728	11.1 A	11.2 A	18.8 A	25.2 A	12.9 B	16.3 A	34.0 A	31.0 A
7849	10.6 B	11.3 A	21.5 A	29.6 A	9.5 B	13.7 A	19.1 A	17.6 A
3730	11.0 B	11.7 A	18.3 A	19.7 A	11.6 B	15.1 A	59.7 A	42.1 B
2158	10.5 B	13.8 A	16.3 B	41.6 A	12.3 B	18.7 A	33.9 A	9.4 B
797	11.0 B	11.6 A	26.7 A	26.9 A	12.2 A	11.8 A	30.4 A	33.5 A
6215	12.7 B	14.2 A	22.6 B	36.2 A	13.2 B	19.0 A	20.6 A	9.4 B
690	11.8 A	11.9 A	25.7 A	27.0 A	13.3 A	13.4 A	17.1 A	14.0 A
2737	12.6 B	12.9 A	24.2 B	35.0 A	12.2 B	17.5 A	17.2 A	5.8 B
8015	11.9 B	12.7 A	11.8 A	13.2 A	11.1 B	14.9 A	21.5 A	18.1 A
791	11.5 A	11.8 A	15.7 A	18.5 A	11.0 B	14.2 A	46.2 A	24.1 B
1378	10.8 A	11.1 A	17.0 A	21.3 A	9.7 B	13.6 A	61.0 A	38.0 B
620	10.6 B	13.0 A	17.0 A	17.0 A	10.1 B	13.7 A	46.3 A	44.5 A

Means were compared by the effect of the Ni doses in each genotype by Dunnett's test at $P < 0.05$, and those followed by the same letter do not differ.
The NILs were not tested in the field experiment.
FW, fresh weight.

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ARTIGO 2 – Nickel fertilization rates for a responsive soybean genotype

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Keywords: *Glycine max*, Urease activity, N_2-fixation, Nickel deficiency, Nickel toxicity, hydrogenase, critical levels, Ni-fertilization.

Abstract

In crop plants, studies of Ni fertilization are relatively new, although its positive effects have been reported since the early 1980s. Nickel affects a wide range of physiological processes, from seed germination to vegetative growth, because it is a structural component of urease in plants and [NiFe]-hydrogenase in some symbiotic bacteria. One of the most cultivated legumes in the world, soybean, besides being known to have high urease activity during N assimilation, can create a strong symbiotic interaction with N_2-fixing bacteria, which may further increase the dependence of this plant species on accurate Ni fertilization. Although dose studies are already well advanced for some cultivated species, for soybeans there is a lack of published information on this topic. To this end, a greenhouse experiment was performed, to test the effect of applying six Ni doses (0.00, 0.25, 0.50, 1.00, 3.00, and 9.00 mg kg^{-1}) in soil on a soybean genotype known to be highly responsive to fertilization with this micronutrient. Plants were evaluated for physiology, nutrition, N metabolism, and growth. The tested genotype was positively affected by Ni fertilization; this indicated a deficiency of the micronutrient, although no visual symptoms were observed in leaves. Nickel application led to increased N metabolism; with the most prominent effect occurring on the N_2 fixation process. Nodules had a higher Ni concentration than other plant tissues, which was associated with a higher nitrogenase activity. In the

nodules, Ni was concentrated in the areas of highest metabolic activity. The greatest beneficial effects were observed following an application dose of 3.35 mg of Ni kg^{-1}. Adequate values in plant tissues ranged from 1.8 to 2.2 mg of Ni kg^{-1}, while in soil, the extractable Ni ranged from 1.9 to 2.4 mg kg^{-1}. After fertilizer application, the concentration of Ni in soybean grains was considered safe for human consumption. Ni doses higher than 4.25 mg kg^{-1} were toxic to soybean plants, reducing their overall development. The supply of Ni had a direct effect on Fe concentration in soybean plants. We conclude that the adequate range obtained in our study for plants and soil is reliable and may be used as a basis for the definition of Ni fertilization doses in agriculture.

1 Introduction

Most studies on Ni in plants have been conducted in the context of the Ni toxicity problem (Chen et al., 2009; Muhammad et al., 2013; Seregin and Kozhevnikova, 2006; Yusuf et al., 2011) rather than its deficiency, probably due to the recent addition of this element to the list of plant nutrients in the agricultural legislation of many countries (Brazil, 2018a; Wood et al., 2006; Wood and Reilly, 2007). Excessive amounts of Ni are commonly found in studies involving the application of sewage sludge (Moreno et al., 2003; Ščančar et al., 2000; Wong et al., 2001) or Ni-contaminated soils (Jamil et al., 2014; Kucharski et al., 2009; Everhart et al., 2006; Parida et al., 2003), which can have concentrations higher than 650 and 1,000 mg of Ni kg^{-1}, respectively. These scenarios do not represent the natural Ni concentration in most soil-plant agricultural systems (Rodak et al., 2015; Ureta et al., 2005), in which beneficial effects of Ni may actually be expected.

Positive effects of Ni on plants have been reported since the early 1980s (Brown et al., 1987; Eskew et al., 1983, 1984; Malavolta et al., 1962), with Ni being demonstrated as playing a vital role in a wide range of physiological processes, from seed germination to vegetative growth. Plants cannot complete their life cycle without an adequate supply of this metal (Brown et al., 1987) because Ni is a structural component of urease (Dixon et al., 1975), the enzyme which is responsible for hydrolysis of urea into ammonia, a plant N assimilative form (Polacco et al., 2013; Witte, 2011). Nickel is also an essential catalytic cofactor of seven other enzymes found in microorganisms (Li and Zamble, 2009). Noteworthy of these is [NiFe]-hydrogenase, an enzyme found in some symbiotic bacteria that are able to capture atmospheric N_2 and convert it into ammonia for plants by nitrogenase activity (Ruiz-Argüeso et al., 2001); the hydrogenase recycles the H_2 produced by a side reaction of the nitrogenase in root nodules formed by the plant-bacteria association, and thus maximizes the efficiency of this

reaction (Bagyinka, 2014). In this way, legumes that are dependent on N_2-fixation may be particularly susceptible to an inadequate Ni supply.

Positive effects of Ni in soybean (*Glycine max* [L.] Merrill), one of the most important cultivated legumes in the world, date from the discovery of its essentiality (Eskew et al., 1983). However, the first publications documenting yield gains in soybean due to Ni fertilization were relatively recent under greenhouse conditions (Kutman et al., 2013; Lavres et al., 2016), and even more recent under field conditions (Freitas et al., 2018). According to Freitas et al. (2018), Ni deficiency in field-grown soybean plants presents with no leaf symptoms, i.e., it is a hidden deficiency, which is difficult to detect. This last study, focusing on soybean genotypes cultivated under the commonly occurring low Ni concentrations in soil, reported that soybean plants have a genotype-specific response to Ni fertilization. After the genotypic effect is identified in soybean plants, the next step should be to calibrate adequate doses of this micronutrient for fertilization with the Ni-responsive genotypes.

Studies aimed at setting an adequate dose for cultivated plants started in pecan plants (*Carya illinoinensis* [Wangenh.] K. Koch) (Bai, 2006; Bai et al., 2007) and river birch (*Betula nigra* L.) (Ruter, 2005), probably because with these plants Ni deficiency results in "mouse-ear", a key morphological symptom linked to the toxic accumulation of urea in tips and margins of leaflets (Wood et al., 2004a, 2004b). To overcome Ni deficiency in pecan orchards and river birch woodlands, annual doses of 10–100 mg of Ni L^{-1} (Wood et al., 2006; Wood and Reilly, 2007) and 394–789 mg of Ni L^{-1} (Ruter, 2005), respectively, are applied as spray directly on leaves. We hypothesized, however, that the demand for this micronutrient is considerably lower for the adequate development of soybean plants, since they do not exhibit Ni deficiency symptoms. Although studies with Ni doses are still incipient, the doses that have been applied to soybean cultivated in soil vary from 0.5 to 1.0 mg of Ni kg^{-1} (Freitas et al., 2018; Macedo et al., 2016; Rodak et al., 2015, 2018).

Thus, we explored the effects of Ni fertilization on physiology, nutrition, N metabolism, and growth of a Ni-responsive soybean genotype, by application of six Ni doses to the soil. We believe that our findings will provide a basis for adequate Ni concentrations in soil and in soybean plant tissues and will contribute to the academic and practical knowledge on efficient use of agronomic resources.

2 Materials and Methods

2.1 Plant Growth and Experimental Design

To assess how doses of Ni affect a Ni-responsive soybean genotype, a greenhouse experiment was performed from May to September 2016. The high responsiveness of the soybean genotype TMG2158 to Ni fertilization was previously tested by Freitas et al. (2018) in a large screening of soybean genotypes. Detailed information about TMG2158 can be found in Brazil (2018b).

Treatments constituted of the application of six Ni doses (0.00, 0.25, 0.50, 1.00, 3.00, and 9.00 mg kg^{-1}) into the soil using a nickel sulfate ($NiSO_4.6H_2O$) solution, which is a readily available Ni salt. Pots (4 L) were filled with pre-fertilized soil, in which the soybean plants were cultivated. Treatments were distributed in a completely randomized design, with four replicates.

The soil used in this experiment was classified as a typical dystrophic yellow-red Latosol (Embrapa Soils, 2013), similar to the Ustox suborder of Oxisol (Soil Survey Staff, 1999). Soil pH was adjusted to 6.0 with the application of 1.75 g of $CaCO_3$ kg^{-1} and 0.75 g of $MgCO_3$ kg^{-1} to the soil in each pot. Macro- and micronutrients were also supplied to the soil (except for N) at the following rates: 125 mg of P kg^{-1} ($Ca[H_2PO_4]_2$), 75 mg of P kg^{-1} + 100 mg of K kg^{-1} (KH_2PO_4), 50 mg of S kg^{-1} ($MgSO_4.7H_2O$), 5.0 mg of Cl kg^{-1} ($MnCl_2.4H_2O$), 5.0 mg of Mn kg^{-1} ($MnSO_4.H_2O$), 3.0 mg of Zn kg^{-1} ($ZnSO_4.7H_2O$), 1.0 mg of B kg^{-1} (H_3BO_3), 1.0 mg of Cu kg^{-1} ($CuSO_4.5H_2O$), 0.5 mg of Mo kg^{-1} ($[NH_4]_6Mo_7O_{24}.4H_2O$), and 0.1 mg of Co kg^{-1} ($CoSO_4.7H_2O$). Soil physical and chemical characteristics, after pH adjustment and fertilization, are listed in Table 1. Soybean plants obtained N through inoculation of the seeds with N_2-fixing bacteria (*Bradyrhizobium japonicum*, strain SEMIA 5079 and *Bradyrhizobium elkanii*, strain SEMIA 5019).

Greenhouse temperature was kept at 28 ± 5 °C during the day and 20 ± 5 °C at night, using an automatic computer-controlled system. The pots were irrigated and the water content in the soil was adjusted daily to near field capacity by weighing to a constant weight.

Soybean plants were analyzed during the onset of flowering, at phenological stage R1-R2 (Fehr and Caviness, 1977). In this stage, the third (from top) fully expanded leaf and medium-large size nodules (>3.5 mm^2) were collected for analysis. At the end of the soybean production cycle, at phenological stage R8, in which 95% of the pods were below 15% moisture, healthy grains and root material were collected, followed by soil sampling.

2.2 Mineral Analysis

To assess the nutritional status of the plants, the concentrations of macronutrients (N, P, K, Ca, Mg, and S) and micronutrients (B, Zn, Fe, Mn, Cu, and Ni) were determined in leaves, roots, and grains. Nickel concentration was also determined in the nodules. Before the analyses, samples were dried at 60 °C for 48 h.

Concentrations of macro- and micronutrients, except for N, were quantified using 0.25 g of dried plant/nodule material. Samples were digested in acid solution (65% HNO_3 and 30% H_2O_2), inside a closed-vessel microwave (CEM Mars 5, US). Concentrations of the nutrients were determined using an inductively coupled plasma-optical emission spectrometer (ICP-OES) (Spectro Cirus, Germany). The concentration of N was directly quantified from 0.35 g of ground dried plant material using an elementary analyzer (Vario EL, Germany).

After soybean cultivation, soil samples were collected for the determination of extractable Ni concentration. For this, 5 g samples of ground dried soil were added to vessels containing Mehlich-1 solution (0.05 M HCl and 0.0125 M H_2SO_4). The samples were shaken for 10 min at 200 rpm, and after standing for 16 h, the extract was collected (Mehlich, 1953). Nickel concentration was measured using the ICP-OES.

Certified reference materials for Ni in plant tissues (Tomato leaves, NIST1573a, US) and in soil (Montana II Soil, NIST 2711a, US) were used for quality assurance (QA) and quality control (QC) protocols. Readings below 0.1 mg kg^{-1} of Ni were considered as not detectable and were therefore not used.

2.3 Physiological Analysis

Physiological parameters of soybean leaves were determined by measuring the SPAD index, photosynthesis, quantum yield, and electron transport rate (ETR).

The SPAD index was obtained by quantification of the green-color intensity of the leaves using a portable electronic chlorophyll meter (Konica Minolta SPAD 502, Japan). Photosynthesis was calculated by measuring changes in the CO_2 concentration over time in a closed chamber, using a portable infrared gas analyzer (IRGA) (Li-820, Li-Cor, US). The measurements were carried out between 08 h and 12 h, in most cases within 5 min. Once a steady state was attained, data were logged every 15 s for 2 min, and CO_2 fluxes were calculated from a linear regression. Quantum yield and ETR parameters were calculated by

measuring *a*-chlorophyll fluorescence (White and Critchley, 1999). For the determination of chlorophyll-*a* fluorescence, intact leaves were exposed to nine pulses of actinic light (photosynthetically active radiation), in increasing intensities (0–6,500 mol m^{-2} s^{-1}) at intervals of 40 s, using a modulated pulse fluorometer (Heinz Walz Mini-PAM, Germany).

2.4 Plant Growth Analysis

The soybean plant growth parameters were based on grain yield, and dry weight of shoots and roots. For grain yield determination, the soybean grains were harvested and weighed, then converted to dry weight by the correction of moisture to 13%. The moisture was determined with a semi-portable grain moisture tester (Gehaka G650i, Brazil). Root and shoot dry weights were obtained by collecting the below- and aboveground plant material, respectively. Harvested material was placed in a drying cabinet with air circulation, operating at 38 ± 2 °C, for 48 h (until water loss had stabilized), then was weighed.

2.5 N-urea Metabolism Analysis

In order to access the main steps of N-urea metabolism, the urease activity, urea concentration, and ammonia concentration were evaluated in soybean leaves.

Urease activity was estimated using the modified method by Hogan et al. (1983). Extraction was done using 8.0 mL of phosphate buffer at pH 7.4 for each 0.3 g of fresh material, incubated during 1 h at 30 °C. A 0.5 mL aliquot of the solution was collected and added to a new tube. To this was added 2.5 mL of reagent 1 (0.1 M C_6H_5OH; 170 µM of $Na_2Fe(CN)_5NO.2H_2O$) and 2.5 mL of reagent 2 (0.125 M NaOH; 0.15 M Na_2HPO_4; 1 M NaClO - 3% of Cl_2). Samples were then incubated at 37 °C for 35 min. Urease activity was determined by colorimetry (color intensity) in a spectrometer (Shimadzu UV-1280, Japan) at 625 nm absorbance.

Leaf urea concentration was measured by the method proposed by Kyllingsbæk (1975). The extraction was done with 1.0 mL of 10 mM formic acid for each 0.5 g of fresh material, under agitation. The extract was centrifuged at 13,200 rpm for 5 min, at 4 °C. One aliquot of 150 µL was collected and added to 3.0 mL of a colorimetric reagent (7% [v/v] 0.2 M $CH_3C(NOH)COCH_3$; 7% [v/v] 0.05 M $NH_2CSNHNH_2$). Next, the acid reagent (20% [v/v] H_2SO_4; 0.06% [v/v] 74 mM $Cl_3FeH_{12}O_6$; 9% [v/v] H_3PO_4) was added to the mix. Samples were incubated for 15 min at 99 °C, under agitation, then kept in the dark in an ice-

cooled system for 5 min. Urea concentration was determined by colorimetry (color intensity) at 540 nm absorbance.

Leaf ammonia concentration was determined in an extract containing 1.0 g of fresh material in 10 mL of solution (60% [v/v] CH_3OH; 25% [v/v] $CHCl_3$). The extract was centrifuged at 13,200 rpm for 5 min, and the supernatant was collected. The ammonia concentration was quantified, according to the method of McCullough (1967). For this, a 150 µL aliquot of the extract was added to 2.0 mL of a colorimetric solution. This solution was prepared using a 1:1 ratio of phenol reagent (2.5 g C_6H_5OH and 12.5 mg $Na_2Fe(CN)_5NO$ in 250 mL) to phosphate reagent (1.25 g NaOH, 13.4 g NaH_2PO_4, and 2.5 mL 5% NaClO in 250 mL). Samples were incubated at 37 °C for 1 h. Ammonia concentration was then determined by colorimetry (color intensity) at 630 nm absorbance.

2.6 Biological N_2 fixation Analysis

The effects of Ni doses on the N_2 fixation process were studied by quantifying the total concentration of ureides (allantoin and allantoic acid) in the leaves, and by assessing the nodules directly for nitrogenase activity, Ni concentration and distribution, and the number and dry weight of nodules.

Leaf ureides concentration was determined in a solution containing 1.0 g of fresh material and 10 mL of extractant solution (60% [v/v] CH_3OH; 25% [v/v] $CHCl_3$). The extract was centrifuged at 13,200 rpm for 5 min. Subsequently, the supernatant was collected and analyzed according to the methodology proposed by Vogels and Van Der Drift (1970). To this, 300 µL aliquot was added to 500 µL of ultrapure water and 500 µL solution 1 (50% [v/v] 0.5 N NaOH; 50% [v/v] 0.15 N HCl). The mixture was incubated at 100 °C for 5 min, then was cooled to room temperature. Subsequently, 2250 µL solution 2 (11.5% [v/v] 0.4 M phosphate buffer at pH 7; 11.5% [v/v] $C_6H_5NHNH_2$; 70% [v/v] 0.65 N HCl at -20 °C; 7% [v/v] $K_4Fe(CN)_6.3H_2O$) was added to the mixture. Ureides concentration was determined using colorimetry (color intensity) at 535 nm absorbance.

Nitrogenase activity was determined by the acetylene reduction assay of Hardy et al. (1968). For this, 15 nodules were removed from the plant roots and immediately transferred to a 10-mL vial with a rubber stopper. Into the nodule-containing vial, 1 mL of acetylene was injected. After 45 min, 1 mL of headspace gas was removed and transferred to a Vacutainer (BD Medical, US). Production of ethylene by nodules was measured using a gas chromatograph system (Shimadzu GC-2010 Plus, Japan), equipped with 80/100 Porapak N (Supelco, US) to calculate nitrogenase activity.

The quantification method for Ni concentration in the nodules was detailed previously under 'Mineral Analysis'.

To map Ni distribution in nodule tissues, synchrotron micro X-ray fluorescence (μ-SXRF) imaging was carried out at the XRF beamline of the Brazilian Synchrotron Light Laboratory (LNLS). Immediately before starting the analysis, the nodules were frozen in liquid nitrogen and the internal tissues were exposed by freeze-fracture. The X-rays produced by the undulator were monochromatized to 10 keV by Si(111) double crystals, focused to a 20 μm diameter spot size. The energy level was calibrated using a reference Cu foil. Two-dimensional Ni maps were constructed by scanning the samples, fixed on a controlled-motor table, with a cryojet system to keep them frozen. The spectrum was measured by a silicon drift detector (SDD; AXAS-A, KETEK GmbH, Germany), and the Ni and Ca intensities were normalized by the incident X-ray intensity. Calcium assessment was adopted only to limit the nodule epidermis. Nickel distribution was measured for the whole nodule cross-section using 300 cps (counts per second), and on a strip (0.4 mm wide x nodule length) from the middle portion of nodules using 1,500 cps. The dead time threshold was 20% counts lost.

The nodules assessed using μ-SXRF were subsequently submitted to scanning electron microscopy (SEM) (LEO EVOI 40 HV, Carl Zeiss, Germany) for ultrastructural imaging. For this, the nodules were fixed in a modified Karnovsky's solution (2.5% $OHC(CH_2)_3CHO$ and 2.5% HCHO in 0.05 M sodium cacodylate buffer at pH 7.2), then were submitted to negative staining (1% OsO_4 in 0.05 M sodium cacodylate buffer pH 7.2) for 1 h. To avoid preparation artefacts, samples were dehydrated and internal water replaced with acetone, by placing in an acetone gradient of 25, 50, 75, 90, and 100% for 10 min in each solution, then drying to the critical point (CPD 030, BAL-TEC, Germany). Samples were set on a metal stub. As a final step before SEM analysis, samples were subjected to 40 to 60 nm gold coating (CED 020, Balzer, Germany). For imaging, a 25 kV beam was centered on the nodule cross-section with 45x magnification.

The number of nodules was obtained by collecting and counting all of the nodules formed in the root system of the soybean plants. For nodule dry weight, this material was placed in a drying cabinet at 38 ± 2 °C with air circulation, for 48 h (until water loss had stabilized), then weighed.

2.7 Statistical Analysis

Data analysis was performed through a one-way analysis of variance

(ANOVA) and mean values were compared by the least significant difference (LSD) test ($P < 0.05$).

Pearson's product moment correlation (PPMC) analysis was used to examine the relationship between the Ni concentration in soybean plant tissues and the concentrations of the other macro- and micronutrients.

The grain yield, extractable Ni in soil, leaf Ni concentration, and grain Ni concentration variables were fitted by the simplest equation that provided a near-maximal fit to the data, according to the Ni doses.

3 Results

3.1 Soil Ni Concentration and Plant Nutritional Status

The Ni dose of 0.25 mg kg^{-1} was not high enough to increase the extractable Ni concentration in the soil nor in the plant tissues, while the application of 0.50 mg of Ni kg^{-1} resulted in a higher Ni concentration in only some of the soybean plant tissues. In comparison with the control (0.00 mg of Ni kg^{-1}), this last dose raised Ni concentration by 4.3-fold in root, 12.5-fold in nodule, and 3.0-fold in leave (Table 2).

The higher Ni doses, namely 1.00, 3.00, and 9.00 mg of Ni kg^{-1}, raised the extractable Ni concentration in the soil by 6, 17, and 53-fold, respectively (Table 2). This greater Ni concentration in the soil led to its absorption by the roots and translocation to the other plant tissues. When fertilized with 1.0 mg of Ni kg^{-1}, the plants had an increase in Ni concentration of 7.3-fold in roots, 16.8-fold in nodules, 3.3-fold in leaves, and 5.9-fold in grain, while for the dose of 3.0 mg of Ni kg^{-1}, there was an increase of 25.8-fold in roots, 88.3-fold in nodules, 6.0-fold in leaves, and 10.1-fold in grain. The highest Ni concentrations in the soybean plants were observed at the dose of 9.0 mg of Ni kg^{-1}, in which increases of 71.7-fold in roots, 136.8-fold in nodules, 14.3-fold in leaves, and 31.1-fold in grain were recorded.

When the Ni concentration in soybean tissues was compared with the concentrations of other macro- and micronutrients (Table 3), a higher Fe and K absorption by roots was observed following Ni fertilization, and these nutrients were directly translocated to the grains. Nickel fertilization led to an increased N concentration in leaves, but a reduced Fe concentration. The concentrations of N, Ca, and Mg increased in soybean grains following Ni application. Accumulation effect, i.e., considering the gain of mass induced by the treatments, was not observed for any plant tissue analyzed (data not shown).

3.2 Physiological Changes Caused by Ni Fertilization

The physiology of the soybean plants was affected by Ni fertilization (Fig. 1). The SPAD index revealed that the leaves became greener with the application of 0.5 mg of Ni kg^{-1}, reaching their maximum with the application of 3.0 mg of Ni kg^{-1} (Fig. 1a). In the plants treated with 9.0 mg of Ni kg^{-1}, symptoms of Ni toxicity were observed, characterized by the development of small black spots that become necrotic, surrounded by a yellow halo, especially on the margins of the leaves.

Following the SPAD index responses, the photosynthesis and ETR analyses revealed that the leaves not only became greener but were more photosynthetically active (Fig. 1b and 1d). By the quantum yield analysis, Ni supply, even at lower doses, had a positive effect on the photosystem of the soybean plants, indicating healthier and non-photoinhibited leaves, although at the highest Ni dose (9.0 mg of Ni kg^{-1}) this value had significantly decreased (Fig. 1c).

3.3 Plant Growth Changes Caused by Ni Fertilization

Growth increased with the addition of Ni, with maximum values observed in soybean plants fertilized with 3.0 mg of Ni kg^{-1}; this trend did not extend to the higher dose of 9.0 mg of Ni kg^{-1}, in which a decline in plant development was observed (Fig. 2).

The Ni effect started with the dose applications of 0.25 mg kg^{-1} for grain yield (Fig. 2a) and 0.50 mg kg^{-1} for shoot dry weight (Fig. 2b). Gains in root development were more limited, starting only after application of 1.00 mg of Ni kg^{-1} (Fig. 2c).

Following Ni application until the dose of 3.0 mg of Ni kg^{-1}, grain yield increased up to 2.6-fold, which corresponded to gains of up to 12.8 g per plant (Fig. 2a), up to 2.3-fold for shoot dry weight (Fig. 2b), and up to 2.6-fold for root dry weight (Fig. 2c), when compared with the control.

3.4 Metabolic Changes in the N-urea Pathway Caused by Ni Fertilization

The most efficient N-urea metabolism was recorded on the soybean plants fertilized with 3.0 mg of Ni kg^{-1} (Fig. 3). With all doses of Ni application, the soybean plants showed higher urease activity (Fig. 3a), with a reduction in urea concentration in leaves, except for plants treated with the highest Ni dose (Fig. 3b). Ammonia, the product of the urease pathway, had more restricted behavior,

increasing in concentration only in the treatment doses of 0.50–3.00 mg of Ni kg^{-1} (Fig. 3c).

3.5 Metabolic Changes in the Biological N_2 fixation Process Caused by Ni Fertilization

The maximum nitrogenase activity was recorded in plants treated with 3.0 mg of Ni kg^{-1}, with increased activity starting with the application of 0.25 mg of Ni kg^{-1}, and a reduction when the plants were treated with 9.0 mg of Ni kg^{-1} (Fig. 4a). A red color was observed inside nodules following nitrogenase activity.

For the other variables used to estimate biological N_2 fixation, namely ureides concentration, and the number and dry weight of nodules, the maximum response was recorded in the soybean plants treated with 1.00 and 3.0 mg of Ni kg^{-1} (Fig. 4b, 4c, and 4d). For nodule dry weight, the responses to Ni fertilization started with the application of 0.25 mg of kg^{-1}, while for the other two variables, responses were only observed after the application of 0.50 mg of Ni kg^{-1}.

When Ni distribution was considered, the nodule image overview (300 cps), and the more detailed higher count images (1,500 cps), revealed that Ni was concentrated in the central part of the nodules, namely the infection region, and, as the Ni doses increased, it extended to the nodule margins (Fig. 5a to 5f).

3.6 Critical Ni Levels in Soil and in Plant Tissues

The critical Ni level in soil was determined by plotting the grain yield as a function of the concentration of extractable Ni in soil (Fig. 6). Data were best fitted by the Lorentzian equation (3 parameters). Following the proposal by Römheld (2012), four distinct areas of responsiveness to Ni fertilization were established for the tested genotype. First, the deficiency range, in which the Ni doses applied to the soil had a significant effect on plants, increasing soybean yield even when there was no sign of Ni deficiency symptoms. Second, the adequate range, in which the plants reached their maximum yield; the lower threshold was set at 90% of yield, and upper threshold set at 100% of yield. For the soybean plants in this study, the adequate range was from 1.9 to 2.4 mg of Ni kg^{-1} in the soil, corresponding to application doses of 3.2 to 4.2 mg of Ni kg^{-1}, respectively. Within this range, the ideal Ni concentration in soil was identified as 2.1 mg kg^{-1} (95% of yield), corresponding to an application of 3.55 mg of Ni kg^{-1}. Nickel concentrations higher than this were considered to be a luxury consumption (third area), in which the higher Ni supply no longer resulted in gains of yield, up to the final area, the toxicity range. This fourth area was delimited by

the concentration of Ni that caused reduction in grain yield, i.e., values higher than 2.6 mg of Ni kg^{-1} in the soil.

The critical Ni level in the soybean plant tissues was determined in the same way as presented for the soil, by plotting the grain yield as a function of the concentration of Ni in the leaves (Fig. 7). The adequate Ni concentration in leaves was slightly lower than in the soil, ranging from 1.8 to 2.2 mg kg^{-1}, corresponding to the doses of 3.0 to 4.0 mg of Ni kg^{-1}, respectively. The ideal concentration of this micronutrient for the soybean plants was 1.9 mg kg^{-1}, corresponding to the application dose of 3.35 mg of Ni kg^{-1} to the soil. The toxicity range was set at Ni concentrations higher than 2.3 mg of Ni kg^{-1} in the leaf.

The intersection of the plant/soil adequate ranges (corresponding to the doses of 3.2 to 4.0 mg kg^{-1}) was used to discover the Ni concentration in grains (Fig. 8a). For this, the overlap of this intersection with the plot of the Ni concentration in grains × Ni doses was identified, with the data being best fitted by a linear regression. The optimal Ni concentration in grains, i.e., the range resulting in the maximum development of soybean plants, was 12.9 to 15.9 mg of Ni kg^{-1}.

4 Discussion

It is known that the Ni demand of cultivated plants is very low and most soils will contain this micronutrient in sufficient amounts for normal plant growth (Liu et al., 2011; Fageria, 2009; Mengel, 2009); however, the results of the present study suggest that this may not be true for Ni-responsive soybean genotypes. We observed that, under natural soil Ni concentrations, and even when low Ni doses were applied, i.e., 0.25 and 0.50 mg of Ni kg^{-1}, the development of the tested genotype was limited by a hidden Ni deficiency (no symptoms in the leaves), indicating the necessity of its inclusion in the fertilization practice (Fig. 2). This view is also supported by studies on other soybean genotypes which were fertilized with beneficial Ni doses (Vesper and Weidensaul, 1977; Cataldo et al., 1978; Eskew et al., 1983; Winkler et al., 1983; Dalton et al., 1985; Holland and Polacco, 1992; Gerendas and Sattelmacher, 1997; Gerendas et al., 1998; Zobiole et al., 2010; Kutman et al., 2013, 2014; Rodak et al., 2015, Lavres et al., 2016; Macedo et al., 2016; 2018; Freitas et al., 2018; Rodak et al., 2018). A compilation of published data on 33 soybean genotypes fertilized with this micronutrient (Table 4) shows that the concentration of Ni increases by 0.02 to 3.67 mg kg^{-1} in leaves and ≤0.01 to 38.0 mg kg^{-1} in grains. When soil was used as a substrate, the extractable Ni concentration varied from <0.01 to 3.24 mg kg^{-1}. The adequate range for the tested genotype in our study fits within the scope of this literature, with Ni concentrations varying from 1.8 to 2.2 mg kg^{-1} in the leaves, 12.9 to 15.9

mg kg^{-1} in the grains, and 1.9 to 2.4 mg kg^{-1} in the soil (Table 2 and Fig. 6, 7, and 8a). This indicates that these values are reliable and may be adopted as reference values for soybean plant fertilization programs.

Both soybean organs in direct contact with the soil, i.e., roots and nodules, had the highest Ni concentrations when compared with other tissues, being 50% and 68% higher on average, respectively (Table 2). According to Yusuf et al. (2011), Ni uptake occurs both via passive diffusion and active transport, which may justify the higher concentrations of this micronutrient in underground organs. Nickel accumulation in root and nodule tissues has also been verified in other plant species, including nodulated legumes (Seregin and Kozhevnikova, 2006). The requirements for synthesis and activation of hydrogenase may also explain the accumulation of Ni in the nodular tissues (Dalton et al., 1985; Klucas et al., 1983; Stults et al., 1986; Ureta et al., 2005; Yusuf et al., 2011). The presence of high-affinity Ni uptake mechanisms in symbiotic bacteria, such as ATP-binding cassette (ABC)-type transporters and single permeases (Albareda et al., 2015; Brito et al., 2010), may further increasing Ni absorption.

Ni accumulation in nodules (Table 2) indicates a high demand for this micronutrient by bacteria for processing atmospheric N_2 into assimilative forms for plants. In some legumes, Ni is required for root nodule growth (Sengar et al., 2008) and optimal functioning of hydrogenase (Yusuf et al., 2011); therefore, is essential for symbiotic N_2 fixation (Welch, 1981). The efficiency of this process depends largely on hydrogenase activity because the oxidation of H_2 provides the ATP required for N_2 reduction to ammonia by nitrogenase. Moreover, Mishra and Kar (1974) showed that Ni is translocated along the plant conducting system, moving to the areas of greatest metabolic activity, where it becomes concentrated. This was verified in our study by observing the Ni distribution in nodular tissues (Fig. 5). Nickel was mostly concentrated in the central part of the nodule (Fig. 5), matching the region where both hydrogenase and nitrogenase, and so active fixation takes place. Following the increase in Ni doses, this metal occupied increasingly larger areas within the nodules, suggesting an expansion of active fixation sites. This point was further confirmed by the higher nitrogenase activity associated with the increasing internal red color (Fig. 4a), and the number (Fig. 4c) and weight (Fig. 4d) of nodules. This view is also supported by Lavres et al. (2016) and Zobiole et al. (2010), who observed that in low Ni availability conditions, the formation of few nodules and a lower dry mass of nodules, were both associated with lower nitrogenase activity. Therefore, the findings of the present study reiterate that the overall N_2 fixation process is limited in agricultural soils due to low availability of Ni; this supports observations by Lavres et al. (2016) and Ureta et al. (2005), and may be especially true for Ni-responsive

soybean genotypes, in which the genotype × bacteria interaction may be a key factor in any response to fertilization with this micronutrient.

The importance of Ni as a micronutrient also extends to the subsequent steps of N assimilation. We observed that nodules had a higher synthesis of ureides when soybean plants were fertilized with Ni, until the dose of 3 mg of Ni kg^{-1}, by measuring its accumulation in the leaves (Fig. 4b). Utilization of ureides by soybean plants is involved with the breakdown of urea (Ohyama et al., 2017; Todd et al., 2006; Witte, 2011), because Ni fertilization leads to a higher synthesis/activity of urease (Polacco et al., 2013), and so, may increase the amount of ammonia in leaves (Bai, 2006; Bai et al., 2007). Overall N-urea metabolism increased in the tested genotype (Fig. 3), corroborating the results of Freitas et al. (2018). According to these authors, a lack of Ni affects the N assimilation process, particularly regarding ureides and ammonia synthesis.

In the present study, the boost in N metabolism due to Ni fertilization had positive effect on the yield, growth, and physiology of soybean plants (Fig. 1 and 2). In the first studies on Ni fertilization in soybean (Cataldo et al., 1978; Dalton et al., 1985; Eskew et al., 1983), the genotypes tested showed higher dry weight production, increased urease activity, and more prominent N_2 fixation, but no differences in final grain yield, as a result of Ni treatments were detected. The latter result seems no longer to hold true for the new high-yielding genotypes, since the application of 3.0 mg of Ni kg^{-1} resulted in a 2.6-fold higher production of grains in the genotype tested in the present study (Fig. 2a). Freitas et al. (2018), Kutman et al. (2013), and Lavres et al. (2016), studying several other new soybean varieties, observed higher grain production when Ni was supplied in adequate doses, with yield gains of up to 35%. This behavior indicates that soybean breeding, which aims for higher yields, drought resistance, and resistance to biotic and abiotic stresses, is also related to a higher nutrient uptake (Tamagno et al., 2017), which must surely include Ni. Increased soybean productivity may also be traced to a higher photosynthetic rate. We observed an increased intensity in the green color of soybean leaves following the increase in applied Ni (Fig. 1a), which was associated with enhanced activity of the photosynthetic apparatus (Fig. 1b), especially photosystem II, the reaction center of oxygenic photosynthesis (Fig. 1c and 1d). The review by Sengar et al. (2008) corroborates our findings, since treatment with Ni salts has been shown to increase the photosynthetic rate in many other cultivated plant species due to increased levels of chlorophylls and other accessory pigments. In addition, also according to these authors, there are a number of reports that Ni has a protective effect on chlorophyll-rich compounds, preventing their destruction in plants.

Besides the effects of this micronutrient on the metabolism and growth of soil-grown soybean plants, Ni supply affected the mineral nutrition of the tested genotype. The Ni-supplied plants absorbed more Fe and K via their roots following the Ni application (Table 3). A similar result was reported by Rahman et al. (2005) under hydroponic conditions. According to these authors, the supply of 10 μM of Ni increased the Fe concentration in roots. Piccini and Malavolta (1992) also showed an increased concentration of these elements in the aboveground tissues when Ni was given to plants. In addition, both studies reported a reduced concentration of Fe in plant leaves, which is very similar to the findings of the present in a highly Ni-responsive soybean genotype. According to Nishida et al. (2012), this phenomenon may be explained because Ni is able to suppress specific Fe signaling in plant cells, which decreases the transport of Fe to leaf tissues. We believe that the reduction of Fe concentration in soybean leaves is not a detrimental effect of Ni fertilization, but an indirect symptom of Ni deficiency. Where there is an insufficient Ni concentration in the soil, soybean plants may absorb more Fe than is required for normal metabolism, thanks to the physicochemical similarity of these two elements, which causes the apparent toxic effect on Fe concentration. Our study supports this, since reducing Fe and increasing Ni, in adequate doses, always brought benefits to plant development (Table 3 and Fig. 1 to 4). Moreover, Wood (2013), studying pecan - a model plant for Ni deficiency studies - concluded that the application of high Fe doses can directly induce Ni deficiency. Our data also indicate a positive relationship between Ni fertilization and N, K, Ca, Mg, and Fe concentrations in soybean grains (Table 3). To the best of our knowledge, the present study is the first to report the beneficial effect of Ni on the mineral composition of soybean grains, although a recent study by Kutman et al. (2013) reported no effect of Ni on the concentrations of other plant nutrients.

The benefits discussed above were no longer observed from the dose of 4.25 mg of Ni kg^{-1}, which corresponded to 2.3 mg of Ni kg^{-1} in plant leaves (defined as the critical level of Ni) (Fig. 7). Thus, even an Ni-responsive genotype displayed physiological damage (Fig. 1), reduced growth (Fig. 2), lower N-urea metabolism (Fig. 3), and decreased N2 fixation (Fig. 4) when treated with and excessive amount of this metal (Fig. 4). Similar results of Ni toxicity effects in the development of plants has also been reported in other plant species (Nagajyoti et al., 2010; Prasad et al., 2005). In our study, the reduction in these parameters was followed by the occurrence of Ni toxicity symptoms in the leaves, similar to Fe deficiency (Fig. 1a). Chen et al. (2009) reported that high Ni doses can induce a severe reduction in Fe concentration, causing chlorosis as a symptom. According to Ghasemi et al. (2009), Ni-induced Fe deficiency is caused not only by inhibition of root-to-shoot translocation of Fe, but also by the displacement of Fe in FeSOD in plants. The detrimental effects of excessive Ni in soil may also be related to a

decreased photosynthetic rate (Fig. 1). Muhammad et al. (2013) demonstrated that an excess of Ni in tissues directly inhibits chlorophyll synthesis in plants, in particular because Fe uptake is inhibited, and this is an essential element for the biosynthesis of chlorophyll (Rahman et al., 2005).

Nickel was readily mobile in the soybean plants, and accumulated in the grains (Table 2), possibly in association with urease, which is also abundant in soybean grains (Rodríguez-Jiménez et al., 2016). With regard to this, there is a concern that Ni fertilization may result in Ni in soybean grains reaching toxic levels for human consumption. To assess the Ni intake by humans eating the Ni-treated soybean grains (*in natura*, i.e., unprocessed), we first defined the optimal Ni concentration in grains, i.e., the concentration that caused the highest plant development, as 12.9 to 15.9 mg of Ni kg^{-1} in grains (Fig. 8a). To calculate the maximum allowable daily intake (ADI), we used a reference dose (RfD) for Ni, calculated from a no-observed-adverse-effect level (NOAEL), and an average body mass for adults; these reference values were described by Freitas et al. (2018). In the present study, an adult may ingest between 83.7 and 103.1 g of soybean grains (dry weight) per day without presenting adverse symptoms (Fig. 8b). Compared with the current average global consumption (2.5 to 7.4 g per day), and with the greatest soybean consumption, seen in some Asian countries, of 23.0 to 41.2 g per day, these values are considered to be safe. Thus, the average global consumption may increase by around 95%, and Asian consumption by around 66%, and Ni fertilization based on our recommended doses, should not pose a threat to human health.

5 Conclusions

The Ni-responsive soybean genotype, as expected, was positively affected by Ni fertilization, both in its physiology (a higher synthesis of chlorophyll and photosynthesis), growth (yield and weight), and N metabolism (N-urea and N_2 fixation), supporting the essentiality of Ni as a micronutrient for plants. Due to the superior development of soybean plants in the absence of visual symptoms of Ni deficiency in leaves, except perhaps by a reduction in leaf Fe concentration, we confirmed that a hidden deficiency of this micronutrient may occur. The application of Ni doses to soil led also to a higher Ni concentration in nodule tissues and to more active nodules, following Ni application, which may indicate a demand also by bacteria for this micronutrient. Supporting the previous statement, Ni was found in the nodule fixation region, where it occupied increasingly larger areas inside the nodules, suggesting an expansion of active sites for N_2 fixation. The critical levels in plant tissues (1.8 to 2.2 mg of Ni kg^{-1}) were lower than in soil (1.9 to 2.4 mg of Ni kg^{-1}), and the ideal Ni application dose, considered to result in the best development of an Ni-responsive soybean,

was 3.35 mg kg^{-1}. Using the plant/soil dataset, we verified that the optimal Ni concentration in grains ranged from 12.9 to 15.9 mg kg^{-1}, values that are far below the known toxic levels for human intake. Based on our data and their association with the published literature, it can be concluded that the new high-yielding genotypes have a strong symbiotic relationship with N_2-fixating bacteria, and this interaction may be key to understanding the response of soybean to Ni fertilization. Even in an Ni-responsive genotype, application of doses higher than 4.25 mg kg^{-1} proved toxic to the plants, with leaf Ni concentrations higher than 2.3 mg kg^{-1}. The observed toxicity symptoms in leaves were closely related with Fe deficiency. We conclude that this experiment provides an important guide to the adequate concentrations of Ni in soil and in soybean plant tissues, although field experiments are required to fine-tune Ni doses.

6 Conflict of Interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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8 Author Contributions

DSF and BWR contributed equally to this work. JS, MACC, and LRGG are coordinators of our research group.

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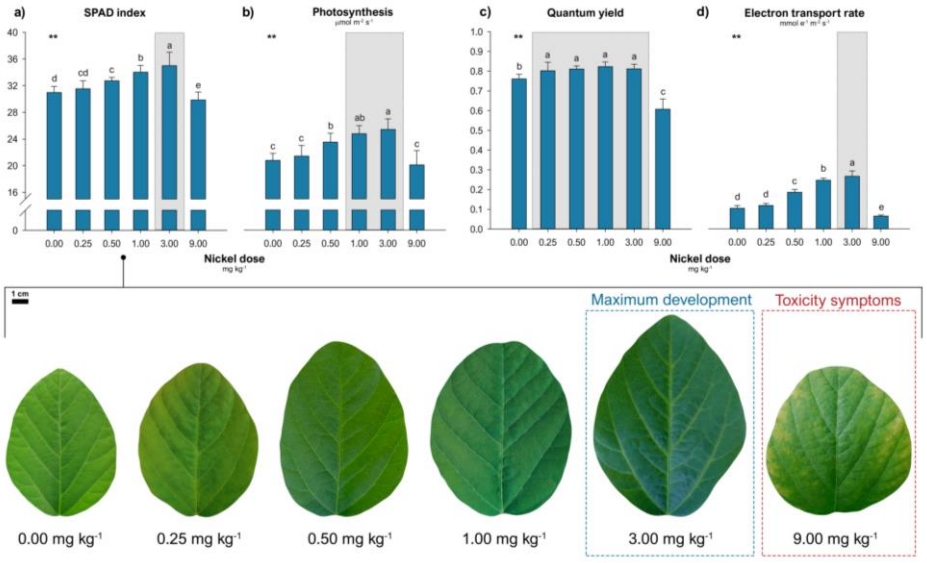

FIGURE 1 | Effect of six Ni doses applied via soil on physiological parameters: a) SPAD index b) photosynthesis, c) quantum yield, and d) electron transport rate of soybean leaves (R1-R2). An increasing intensity in the green color of the leaves was observed with the increase in Ni doses, until a maximum was reached at a dose of 3.00 mg of Ni kg^{-1}, after which symptoms of Ni toxicity were seen. *** significant by F-test at $P < 0.01$. Values are means and standard deviations of 4 replicates. Different letters indicate significant differences according to the least significant difference (LSD) test at $P < 0.05$. The grey rectangles mark the Ni dose(s) which correspond to the most effective plant physiology.*

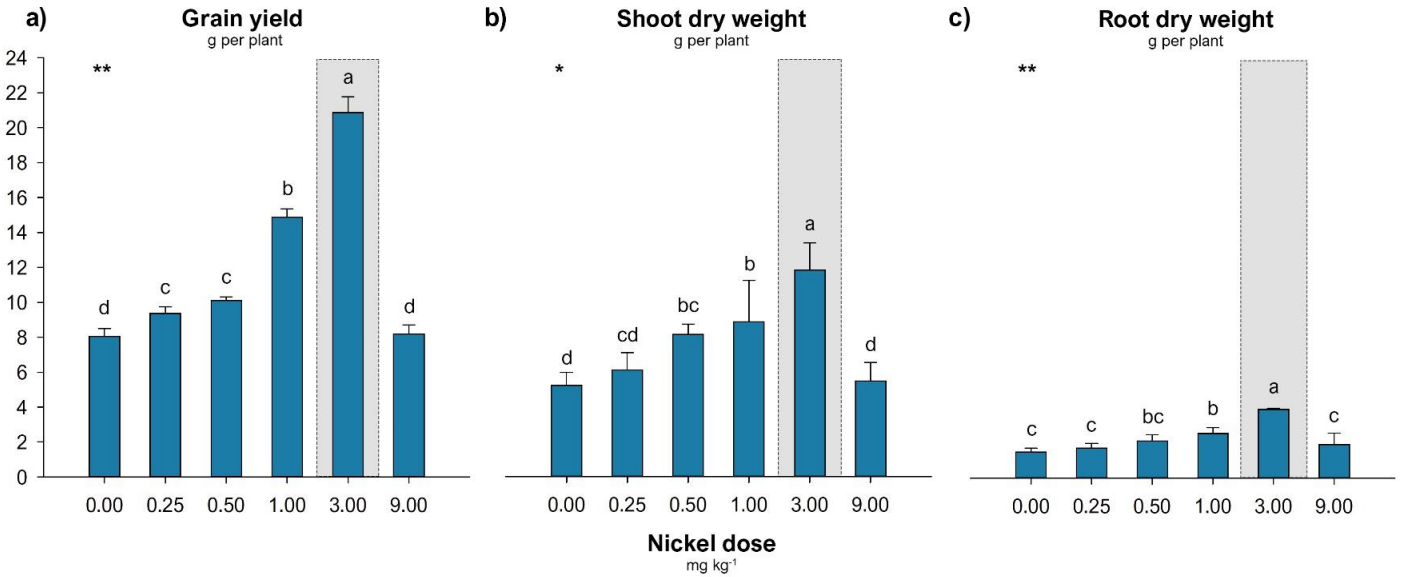

FIGURE 2 | Effect of six Ni doses applied via soil on growth parameters: a) grain yield, b) shoot dry weight, and c) root dry weight of soybean plants (R8). ** significant by F-test at P < 0.05; ** significant at P < 0.01. Values are means and standard deviations of 4 replicates. Different letters indicate significant differences according to the least significant difference (LSD) test at P < 0.05. The grey rectangles mark the Ni dose(s) which correspond to the most productive plant growth.*

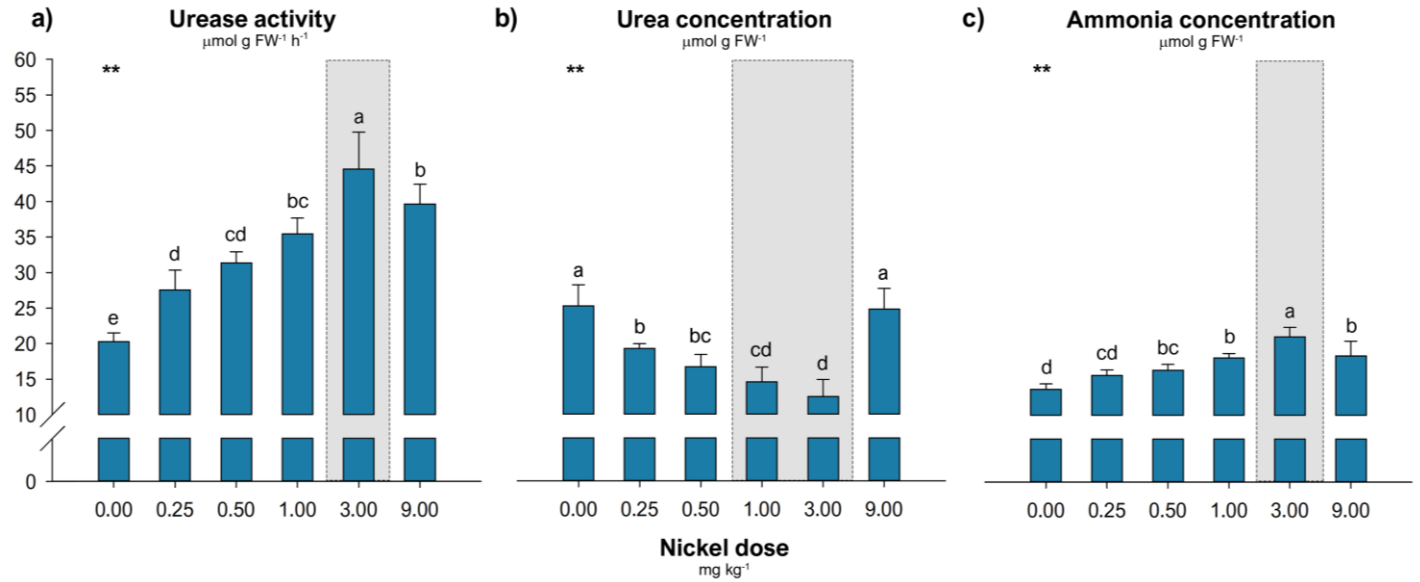

FIGURE 3 | Effect of six Ni doses applied via soil on N-urea metabolism: a) urease activity, b) urea concentration, and c) ammonia concentration of soybean leaves (R1-R2). *** significant by F-test at P < 0.01. Values are means and standard deviations of 4 replicates. Different letters indicate significant differences according to the least significant difference (LSD) test at P < 0.05. The grey rectangles mark the Ni dose(s) which correspond to the most effective plant N-urea metabolism.*

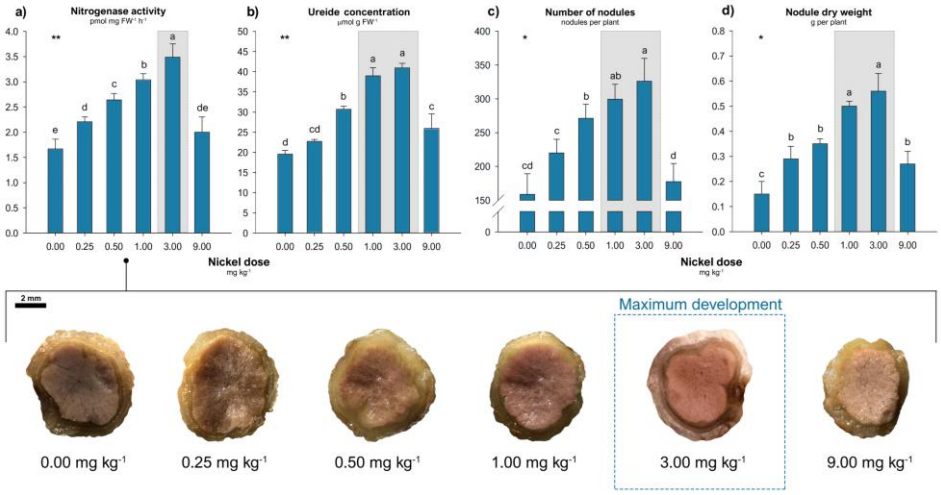

FIGURE 4 | Effect of six Ni doses applied via soil on biological N_2 fixation parameters: a) nitrogenase activity, b) leaf ureide concentration, c) number of nodules, and d) nodule dry weight of soybean plants (R1-R2). A longitudinal section of the root nodules revealed an increasing intensity of red color following the increase in Ni doses up to a maximum at the dose of 3.00 mg of Ni kg^{-1}. ** significant by F-test at $P < 0.05$; ** significant at $P < 0.01$. Values are means and standard deviations of 4 replicates. Different letters indicate significant differences according to the least significant difference (LSD) test at $P < 0.05$. The grey rectangles mark the Ni dose(s) which corresponded to the most effective biological N_2 fixation.*

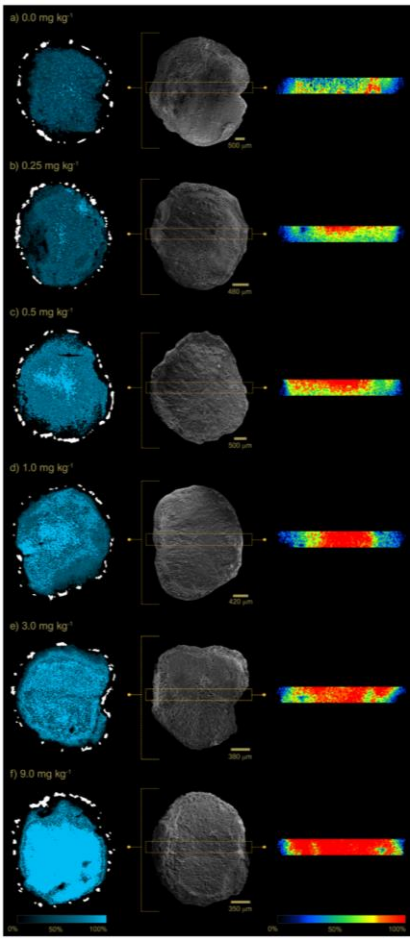

FIGURE 5 | Nickel mapping on cross-sections of soybean root nodules fertilized via soil with six Ni doses (a-f) using synchrotron micro-X-ray fluorescence (μ-SXRF) and scanning electron microscopy (SEM). a) shows the Ni mapping by μ-SXRF using 300 counts per second (cps), in which the linear color scale reflects the intensity of Ni concentration and distribution, ranging from the minimum (black) to the maximum (cyan). Calcium (white) assessment was used only to limit the nodule epidermis; b) shows the nodule structures using SEM (45× magnification); c) shows the high counting rate Ni mapping (1,500 cps), detailing the Ni distribution on a strip across the central nodule region, ranging from the minimum (black) to the maximum (red).

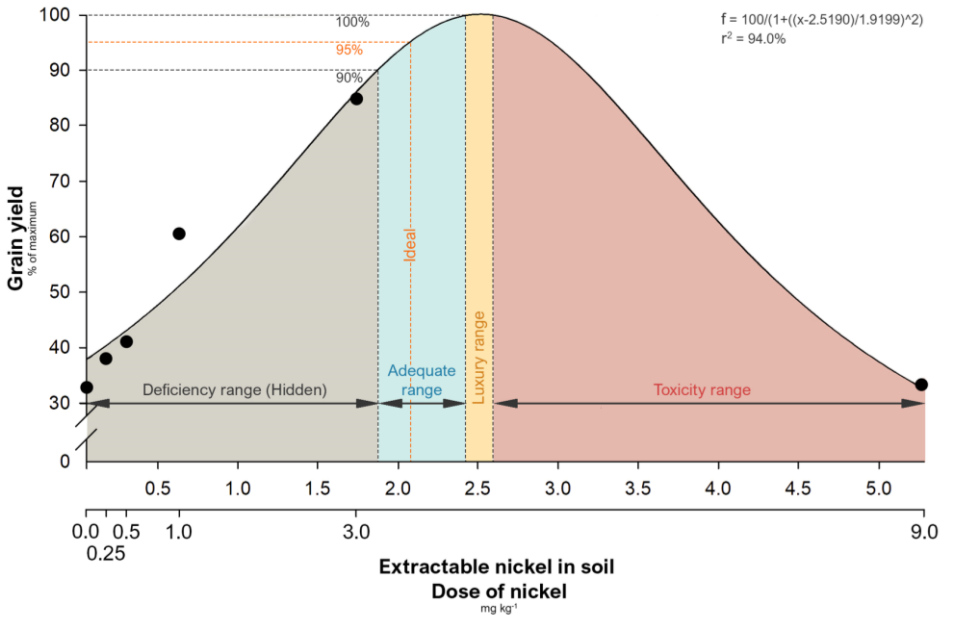

FIGURE 6 | Relationship between the concentration of extractable soil Ni and grain yield in soybean plants fertilized with six Ni doses (0.0, 0.25, 0.5, 1.0, 3.0, and 9.0 mg of Ni kg^{-1}) for definition of the critical soil Ni level. According to the Lorentzian equation (3 parameters) four zones were identified for the tested genotype: deficiency range (without symptoms), adequate range, luxury range, and toxicity range. In the adequate range, in which the grain yield was at a maximum (90% to 100%), Ni concentration in soil ranged from 1.85 to 2.40 mg kg^{-1}, corresponding to the fertilization with doses of 3.2 to 4.2 mg of Ni kg^{-1}. The ideal Ni concentration in soil (95% of yield) was 2.1 mg kg^{-1}, corresponding to 3.55 mg of Ni kg^{-1}. *Zones were defined according to Römheld (2012)*

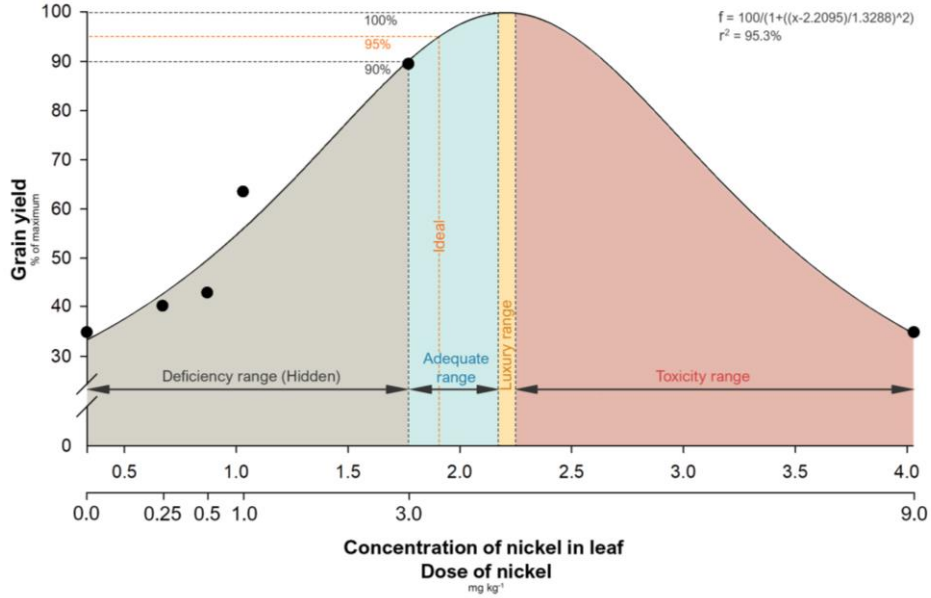

FIGURE 7 | Relationship between the concentration of Ni in soybean leaves and the grain yield of plants fertilized with six Ni doses (0.0, 0.25, 0.5, 1.0, 3.0, and 9.0 mg of Ni kg^{-1}) for definition of the critical plant Ni level. According to the Lorentzian equation (3 parameters) four zones were identified for the tested genotype: deficiency range (without symptoms), adequate range, luxury range, and toxicity range. In the adequate range, in which the grain yield was at a maximum (90% to 100%), Ni concentration in leaves ranged from 1.80 to 2.15 mg kg^{-1}, corresponding to the fertilization with doses of 3.0 to 4.0 mg of Ni kg^{-1}. The ideal leaf Ni concentration (95% of yield) was 1.9 mg kg^{-1}, corresponding to 3.35 mg of Ni kg^{-1}. *Zones were defined according to Römheld (2012)*

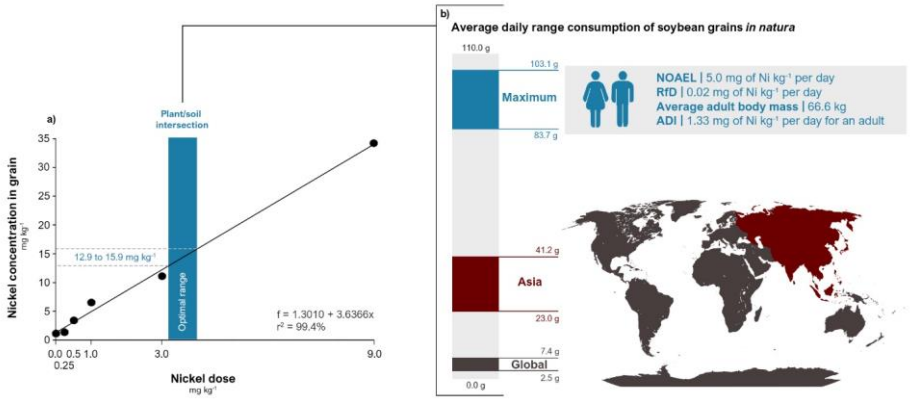

FIGURE 8 | Assessment of Ni intake by humans eating soybean grains (*in natura*, i.e., unprocessed) produced by plants fertilized with this micronutrient; food safety is based on the concentration of Ni in the grains. a) Ni concentration in soybean grains along the Ni doses was explained by a linear adjustment. The Ni concentration in grains causing the best plant development, namely optimal range (12.9 to 15.9 mg of Ni kg^{-1}), was found by plotting, over the adjustment, the intersection of the plant/soil adequate range (3.2 to 4.0 mg kg^{-1}). Assuming this range for b) assessing the risk of Ni ingestion via food chain, an adult (66.6 kg) may ingest from 83.7 to 103.1 g of soybean grains (dry weight) per day. In comparison with the global consumption, and with Asian countries consumption - the largest consumers of soybean - these values are considered safe and does not pose a threat to human health. *For calculation of the maximum allowable daily intake (ADI) of Ni for humans, were used a reference dose (RfD) for Ni, calculated from a no-observed-adverse-effect level (NOAEL), and a body mass for an adult. Reference values listed in Freitas et al. (2018).*

TABLE 1 | Chemical characterization and particle size distribution of the soil *Latossolo Vermelho Amarelo distrófico típico* (Oxisol) used in this experiment, after soil pH adjustment and fertilization.

Properties	Units	Method/Extractant	Greenhouse
Sand	g kg^{-1}	Hydrometer	740
Silt	g kg^{-1}	Hydrometer	30
Clay	g kg^{-1}	Hydrometer	230
Soil organic matter	g kg^{-1}	Colorimetric	4.1
pH	-	Water	6.0
Al	$cmol_c$ kg^{-1}	Potassium chloride	< 0.1
Al + H	$cmol_c$ kg^{-1}	Calcium acetate, pH 7.0	2.4
P	mg kg^{-1}	Mehlich-1	27.2
K	mg kg^{-1}	Mehlich-1	70.0
Ca	$cmol_c$ kg^{-1}	Potassium chloride	3.0
Mg	$cmol_c$ kg^{-1}	Potassium chloride	1.2
S	mg kg^{-1}	Dicalcium phosphate	27.5
B	mg kg^{-1}	Hot water	0.9
Cu	mg kg^{-1}	Mehlich-1	1.0
Fe	mg kg^{-1}	Mehlich-1	25.4
Mn	mg kg^{-1}	Mehlich-1	6.2
Zn	mg kg^{-1}	Mehlich-1	2.2
Ni	mg kg^{-1}	Mehlich-1	< 0.1[a]

[a] *Value before addition of the Ni doses.*

Soil classification according to Embrapa Soils (2013).

TABLE 2 | Effect of six Ni doses applied via soil on the concentration of soil Ni extractable after soybean plants cultivation and Ni concentration in soybean plant tissues and Ni concentration in grain.

Ni dose (mg kg^{-1})	Ni concentration (mg kg^{-1})				
	Soil	Plant tissues			
		Root	Nodule	Leaf	Grain
0.00	0.1 ± 0.1 d	0.6 ± 0.1 e	0.4 ± 0.1 d	0.3 ± 0.1 e	1.1 ± 0.1 d
0.25	0.2 ± 0.1 d	1.1 ± 0.2 de	1.1 ± 0.1 d	0.8 ± 0.1 de	1.4 ± 0.3 d
0.50	0.3 ± 0.1 d	2.6 ± 0.4 cd	5.0 ± 0.6 c	0.9 ± 0.1 cd	3.4 ± 0.4 d
1.00	0.6 ± 0.1 c	4.4 ± 0.1 c	6.7 ± 0.9 c	1.0 ± 0.1 c	6.5 ± 0.4 c
3.00	1.7 ± 0.1 b	15.5 ± 0.6 b	35.3 ± 1.6 b	1.8 ± 0.1 b	11.1 ± 0.8 b
9.00	5.3 ± 0.3 a	43.0 ± 2.0 a	54.7 ± 3.9 a	4.3 ± 0.4 a	34.2 ± 3.9 a
F-test	**	**	**	**	**

n.s., not significant by F-test.
**, significant by F-test at P < 0.05.*
***, significant by F-test at P < 0.01.*
Values are means and standard deviations of 4 replicates.
Different letters indicate significant differences according to least significant difference (LSD) test P < 0.05.
Soil Ni extractable, Mehlich-1 method.
Ni in plant tissues, nitric-perchloric method.

TABLE 3 | Inter-nutrient correlation with the Ni concentration in soybean plant tissues.

Nutrients	Root Ni	Leaf Ni	Grain Ni
N	n.s.	56.5% *	48.6% *
P	n.s.	n.s.	n.s.
K	67.0% **	n.s.	46.0% *
Ca	n.s.	n.s.	63.9% **
Mg	n.s.	n.s.	53.8% *
S	n.s.	n.s.	n.s.
B	n.s.	n.s.	n.s.
Zn	n.s.	n.s.	n.s.
Fe	84.5% ***	-68.3% **	47.0% *
Mn	n.s.	n.s.	n.s.
Cu	n.s.	n.s.	n.s.

n.s., not significant by Pearson Product Moment Correlation (PPMC).
**, significant by PPMC at P < 0.05.*
***, significant by PPMC at P < 0.01.*
****, significant by PPMC at P < 0.001.*

TABLE 4 | Compilation of adequate Ni concentration in plant tissues and soil due to the application of beneficial Ni doses in wide range soybean genotypes cultivated under different substrates.

Ni-fertilization		Ni-plant (mg kg^{-1})			Ni-substrate (mg kg^{-1})			Reference
Dose	Unit	Soybean genotype	Leaf	Grain	Substrate	Extractable	Extractor	
Natural [a]	-	-	-	-	10 subtropical soils	0.08 - 0.50	DTPA	Dalton et al. (1985)
0.00 - 0.80	mg kg^{-1}	M6210	0.10 - 1.80	-	2 Oxisols	0.07 - 0.56	DTPA	Rodak et al. (2018)
0.00 - 5.00	mg kg^{-1}	BRS360	0.08 - 3.02	0.28 - 14.3	14 tropical soils [d]	< 0.01 - 1.87	DTPA	Rodak et al. (2015)
0.00 - 10.0	mg kg^{-1}	COODETEC202	0.31 - 3.67	0.70 - 38.0	Alfisol	0.09 - 2.93	DTPA	Macedo et al. (2016)
0.00 - 0.50	mg kg^{-1}	15 tropical cultivars [b]	0.40 - 1.90	1.36 - 2.68	2 Oxisols	< 0.20 - 0.69	Mehlich-1	Freitas et al. (2018)
0.00 - 0.80	mg kg^{-1}	M6210	0.10 - 1.80	-	2 Oxisols	0.15 - 1.13	Mehlich-1	Rodak et al. (2018)
0.00 - 5.00	mg kg^{-1}	BRS360	0.08 - 3.02	0.28 - 14.3	14 tropical soils [d]	< 0.07 - 3.24	Mehlich-1	Rodak et al. (2015)
Natural [a]	-	6 tropical cultivars [c]	0.05 - 0.14	-	2 Oxisols	-	-	Zobiole et al. (2010)
0.00 - 0.02	mg kg^{-1}	Potencia	0.32 - 1.02	1.26 - 4.60	Oxisol	-	-	Lavres et al. (2016)
0.00 - 5.00	mg kg^{-1}	Wayne	0.98 - 1.40	-	Sand	-	-	Vesper and Weidensaul (1977)
0.00 - 10.0	µg L^{-1}	Maple Presto	-	0.01 - 0.64	Nutrient solution	-	-	Eskew et al. (1983)
0.00 - 10.0	µg L^{-1}	Maple Presto	-	≤ 0.01 - 0.81	Nutrient solution	-	-	Winkler et al. (1983)
0.00 - 0.09	µM	Kalmit	0.12 - 0.45	-	Nutrient solution	-	-	Gerendas and Sattelmacher (1997)
0.00 - 10.0	µM	Williams mutants	0.22 - 0.35	-	Nutrient solution	-	-	Holland and Polacco (1992)
0.00 - 0.05	mM	Williams 82	0.02 - 0.16	0.01 - 1.36	Nutrient solution	-	-	Gerendas et al. (1998)
0.00 - 0.50	mM	Williams	0.29 - 1.36	-	Nutrient solution	-	-	Cataldo et al. (1978)
0.00 - 2*10^{-7}	M	Nova	0.50 - 0.66	-	Nutrient solution	-	-	Kutman et al. (2014)
0.00 - 2*10^{-7}	M	Nova	0.06 - 1.42	0.04 - 8.32	Nutrient solution	-	-	Kutman et al. (2013)
0.00 - 10^{-5}	M	Williams	0.56 - 2.12	-	Nutrient solution	-	-	Dalton et al. (1985)

[a] *Without Ni fertilization;*

[b] *GDM7379, NIDERA7200, GDM6510, MONSOY2728, BAYER7849, MONSOY3730, TMG2158, MONSOY797, TMG6215, GENEZE690, COODETC2737, COODETEC8015, BAYER791, SYNGENTA1378, TMG620;*

[c] *BRS242, Embrapa58, BRS245, BRS133, BRS247, BRS134;*

[d] *2 Oxisols, 10 Oxisols, 1 Inceptisol, 2 Ultisols, 1 Entisol.*

CONSIDERAÇÕES FINAIS

A condução dessa tese permitiu-nos revelar a deficiência oculta de Ni em campos de soja, ressaltando a importância desse micronutriente para as plantas cultivadas. Demonstramos que a deficiência oculta de Ni ocorre em genótipos atualmente cultivados, embora não detectada graças a ausência de sintomas foliares típicos. A clarificação desse fato traz consigo a possibilidade de elevar a produtividade de plantas de soja – uma cultura chave no cenário agrícola mundial – pela sua fertilização com Ni, o que reflete em uma maior segurança alimentar, por elevar a produção por área cultivada, e na redução dos impactos ambientais, devido a menor necessidade de abertura de novas áreas de cultivo. Ademais, demonstramos que os grãos produzidos pelas plantas tratadas com esse micronutriente são seguros para a ingestão humana.

A aplicação de doses benéficas de Ni via fertilização do solo pode contornar esse problema, visto que a dose de 3,35 mg de Ni kg^{-1} levou ao máximo desenvolvimento das plantas de soja. Ressalta-se que não há uma recomendação oficial dos níveis críticos desse micronutriente no sistema solo-planta ou mesmo como manejar a sua adubação em plantas cultivadas, e dessa forma, espera-se que os resultados obtidos possam ser usados como um guia para a fertilização de plantas de soja, auxiliando na adequação de Ni no solo e nos tecidos de plantas de soja. Além disso, visamos promover a inclusão do Ni em programas de fertilização.

Existe muito a ser pesquisado sobre esse micronutriente, principalmente quanto ao manejo da sua adubação, marcha de absorção, explorando as formas e épocas de sua aplicação, o comportamento desse elemento em diferentes solos bem como o efeito residual da aplicação de Ni, além de pesquisas mais básicas visando entender os efeitos do Ni sobre as plantas cultivadas a níveis genômico e proteômico.